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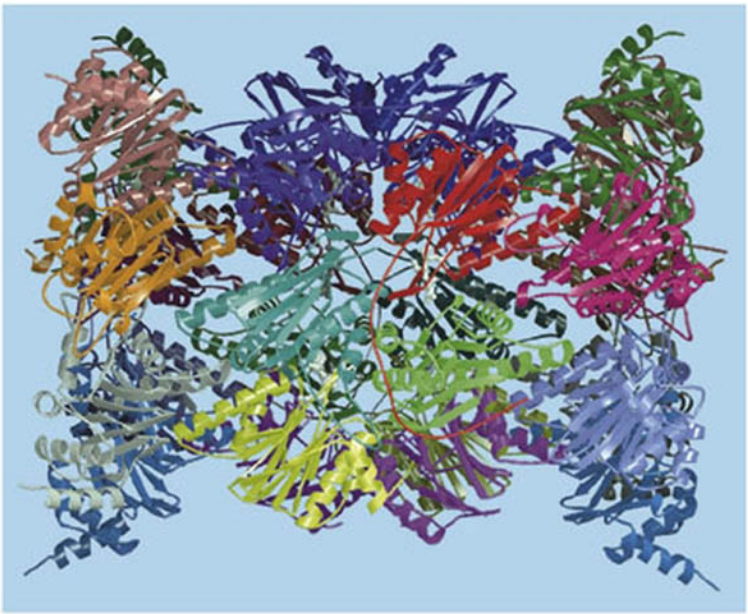
Protein Degradation

Ubiquitin and the Chemistry of Life

Volume 1



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Preface

There is an incredible amount of current global research activity devoted to understanding the chemistry of life. The genomic revolution means that we now have the basic genetic information in order to understand in full the molecular basis of the life process. However, we are still in the early stages of trying to understand the specific mechanisms and pathways that regulate cellular activities. Occasionally discoveries are made that radically change the way in which we view cellular activities. One of the best examples would be the finding that reversible phosphorylation of proteins is a key regulatory mechanism with a plethora of downstream consequences. Now the seminal discovery of another post-translational modification, protein ubiquitylation, is leading to a radical revision of our understanding of cell physiology. It is becoming ever more clear that protein ubiquitylation is as important as protein phosphorylation in regulating cellular activities. One consequence of protein ubiquitylation is protein degradation by the 26S proteasome. However, we are just beginning to understand the full physiological consequences of covalent modification of proteins, not only by ubiquitin, but also by ubiquitin-related proteins.

Because the Ubiquitin Proteasome System (UPS) is a relatively young field of study, there is ample room to speculate on possible future developments. Today a handful of diseases, particularly neurodegenerative ones, are known to be caused by malfunction of the UPS. With perhaps as many as 1000 human genes encoding components of ubiquitin and ubiquitin-related modification pathways, it is almost certain that many more diseases will be found to arise from genetic errors in the UPS or by pathogen subversion of the system. This opens several avenues for the development of new therapies. Already the proteasome inhibitor Velcade is producing clinical success in the fight against multiple myeloma. Other therapies based on the inhibition or activation of specific ubiquitin ligases, the substrate recognition components of the UPS, are likely to be forthcoming. At the fundamental research level there are a number of possible discoveries especially given the surprising range of biochemical reactions involving ubiquitin and its cousins. Who would have guessed that the small highly conserved protein would be involved in endocytosis or that its relative Atg8 would form covalent bonds to a phospholipid during autophagy? We suspect that few students of ubiquitin will be surprised if it or a

ubiquitin-like protein is one day found to be covalently attached to a nucleic acid for some biological purpose.

We are regularly informed by the ubiquitin community that the initiation of this series of books on the UPS is extremely timely. Even though the field is young, it has now reached the point at which the biomedical scientific community at large needs reference works in which contributing authors indicate the fundamental roles of the ubiquitin proteasome system in all cellular processes. We have attempted to draw together contributions from experts in the field to illustrate the comprehensive manner in which the ubiquitin proteasome system regulates cell physiology. There is no doubt then when the full implications of protein modification by ubiquitin and ubiquitin-like molecules are fully understood we will have gained fundamental new insights into the life process. We will also have come to understand those pathological processes resulting from UPS malfunction. The medical implications should have considerable impact on the pharmaceutical industry and should open new avenues for therapeutic intervention in human and animal diseases. The extensive physiological ramifications of the ubiquitin proteasome system warrant a series of books of which this is the first one.

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1
Brief History of Protein Degradation and the Ubiquitin System

Avram Hershko

1.1
Introductory Remarks

The reader of this book may be impressed (and possibly overwhelmed) by the enormous recent progress in this field. The ubiquitin system is now known to be involved in basic biological processes, such as the control of cell division, signal transduction, regulation of transcription, DNA repair, quality control in the endoplasmic reticulum, stress response, induction of immune response and inflammation, apoptosis, embryonic development, and circadian clocks, to mention but a few. It has been implicated in diseases such as many types of cancer, neurodegenerative diseases (such as certain types of Parkinson's, Alzheimer's and Huntington's diseases), retroviral infections, certain types of hypertension, mental retardation, and cachexia associated with cancer, renal failure, or sepsis. New functions of ubiquitin and of ubiquitin-like proteins are being reported almost every month, and the number of publications in this field is increasing at an exponential (and bewildering!) rate. It may be therefore instructive to consider briefly the humble beginnings of this field, how significant progress was achieved, and also how at times progress was impeded by wrong dogmas. Important lessons can be learned from both achievements and failures in science.

1.2
Protein Degradation – Does It Exist?

In the first three decades of the twentieth century, a generally accepted theory of protein metabolism was that proposed by Folin [1]. Based on studies on the chemical composition of urine in humans fed protein-rich or protein-free diets, Folin proposed that there are two separate pathways of protein catabolism, which he called ''endogenous'' and ''exogenous'' types of protein catabolism. According to this concept, ''exogenous'' protein catabolism originates from dietary proteins, accounts for the major part of urea excreted under normal conditions, and shows wide variations according to dietary protein intake. By contrast, ''endogenous'' pro-

tein catabolism was thought to originate from tissue proteins, to be mainly represented by excreted creatinine, not to be affected by the amount of dietary protein intake, and to account for a minor part of nitrogenous compounds excreted in the urine. Because of the minor proportion of "endogenous" protein catabolism, it was thought that cellular proteins are predominantly stable, and only a small fraction resulting from "wear and tear" of tissue proteins is subject to catabolism [1].

In spite of its obviously wrong assumptions (such as that creatinine is the end product of protein catabolism), Folin's theory was widely accepted and cited in textbooks of biochemistry until the late thirties. At that time, a breakthrough in the field was achieved by the pioneering studies of Schoenheimer and co-workers, who introduced the extensive use of isotopically labeled compounds in biological studies. In a typical experiment [2], ^{15}N-labeled L-leucine was administered to well-fed rats, and the distribution of the isotope in excreta and in body tissues was examined. According to the concept of Folin, most exogenously administered leucine should have appeared in urinary waste products. This was not the case: less than one-third of the isotope was excreted in the urine, and most of it was found to be incorporated into tissue proteins [2]. Since the weight of the animals did not change during the experiment, it could be assumed that the mass and composition of body proteins also did not change. It was concluded, therefore, that newly incorporated amino acids must have replaced those in tissue proteins in a process of protein turnover. From these studies a new concept has emerged according to which cellular proteins, and some other body constituents, are in a dynamic state of constant and extensive renewal [3].

Schoenheimer's concept of the dynamic state of body proteins did not remain unchallenged. In 1955, Monod and co-workers studied the origin of amino acids utilized for the synthesis of newly induced β-galactosidase in growing *E. coli* [4]. Bacteria were first labeled with $^{35}SO_4^{2-}$ and then were transferred to unlabeled medium containing the inducer methyl-β-D-thiogalactoside. Newly synthesized β-galactosidase was isolated and was found not to contain significant amounts of radioactivity. This result suggested that in growing *E. coli*, the degradation of most cellular proteins is negligibly slow, otherwise newly synthesized β-galactosidase would have contained ^{35}S-labeled amino acids originating from the degradation of pre-existing proteins. Instead of restricting these conclusions to the case of growing *E. coli*, the authors went on to generalize and proposed that cellular proteins are also stable in mammalian tissues. They furthermore suggested that in Schoenheimer's experiments, incorporation of amino acids into tissue proteins might be due to the replacement of cells lost by cell lysis or the replacement of secreted proteins [4]. This was, in effect, a return to Folin's dogma of static cellular proteins.

So great was the authority of Monod at that time that the dynamic state concept of Schoenheimer fell into disfavor, as judged by contemporary review articles [5]. Gradually, however, experimental evidence accumulated which refuted Monod's hypothesis. Using mammalian cells in culture, Eagle and co-workers carefully examined the problem of cellular protein turnover *vs.* cell turnover or protein secretion [6]. In a variety of growing or resting cells in culture, cellular proteins were replaced at a high rate of approx. 1% h^{-1}. This was due to true protein turnover

and not to the replacement of secreted proteins or lysed cells, as indicated by the lack of significant amounts of labeled proteins in the culture medium. Further work in several laboratories has shown that protein degradation in animal cells is extensive and is highly selective. Thus, for example, abnormal proteins produced by the incorporation of some amino acid analogues or by certain mutations are selectively recognized and are rapidly degraded in cells [7]. However, it is not correct to state (as is written in some current articles) that, until recently, protein degradation was thought to be mainly a "garbage disposal" system to get rid of abnormal proteins. In the late sixties, it was already evident that normal proteins are also degraded in a highly selective mode. The half-lifetimes of different proteins range from several minutes to many days, and rapidly degraded normal proteins usually have important regulatory functions. These properties of intracellular protein degradation and the importance of this process in the control of the levels of specific proteins were summarized by Schimke and Doyle in 1970 [8].

In retrospect, one can only speculate why the concept of intracellular protein degradation was resisted for such a long time. It is possible that one reason was the difficulty in accepting the idea that cells carry out such a wasteful process. A substantial amount of energy is invested in the formation of peptide bonds in the process of protein synthesis, and all this energy is dissipated when the protein is degraded. A possible explanation is that energy expenditure is used to achieve regulation. Our current knowledge of some of the functions of the ubiquitin system is consistent with this notion.

1.3 Discovery of the Role of Ubiquitin in Protein Degradation

Although the basically important cellular functions of selective protein degradation became evident in the late sixties, the molecular mechanisms involved in this process remained unknown. I became interested in the problem of how proteins are degraded in cells when I was a postdoctoral fellow in the laboratory of Gordon Tomkins in 1969–71. Gordon was mainly interested at that time in the mechanisms by which steroid hormones induce the synthesis of specific proteins. His model system for this purpose was the regulation of the enzyme tyrosine aminotransferase (TAT) in cultured hepatoma cells. Like other regulatory proteins, TAT has a rapid degradation rate. I found at that time, quite by accident, that the degradation of TAT is blocked by inhibitors of cellular ATP production, such as fluoride or dinitrophenol [9]. These results confirmed and extended earlier findings of Simpson [10] on the energy-dependence of the liberation of amino acids from proteins in liver slices. Since ATP depletion also prevented the inactivation of the enzymatic activity of TAT, it was concluded that energy is required at an early step in the process of protein degradation [9].

I was very much impressed by the energy-dependence of intracellular protein degradation because it suggested the involvement of a novel mechanism, different from that of known proteolytic enzymes. One attractive possibility that I consid-

ered was that proteins may be modified by some energy-dependent reaction prior to their degradation, and that such modification renders them susceptible to the action of some proteolytic enzyme [11]. To examine the existence of such (or any other) mechanism, a cell-free system was required, which faithfully reproduced energy-dependent protein degradation in the test tube, and which could be subjected to biochemical analysis. A cell-free ATP-dependent proteolytic system from reticulocyte lysates was first established by Etlinger and Goldberg [12]. Subsequently, my laboratory subjected this system to biochemical fractionation, with the aim of isolating its components and characterizing their mode of action. In this work, I was greatly helped by Aaron Ciechanover, who was my graduate student at that time. I have also received a lot of support, help, and great advice from Irwin Rose, in whose laboratory at Fox Chase Cancer Center I worked in a sabbatical year in 1978–79 and for many summers afterwards.

In the initial experiments, we resolved reticulocyte lysates on DEAE-cellulose into two crude fractions: Fraction 1, which contained proteins not adsorbed to the resin, and Fraction 2, which contained all proteins adsorbed to the resin and eluted with high salt. The original aim of this fractionation was to get rid of hemoglobin, which was known to be in Fraction 1, while most non-hemoglobin proteins of reticulocytes were known to be in Fraction 2. We found that neither fraction was active by itself, but ATP-dependent protein degradation could be reconstituted by combination of the two fractions [13]. The active component in Fraction 1 was a small, heat-stable protein; we have exploited its stability to heat treatment for its purification to near homogeneity. We termed this protein at that time APF-1, for **A**TP-dependent **P**roteolysis **F**actor 1 [13]. The identity of APF-1 with ubiquitin was established later by Wilkinson et al. [14], subsequent to the discovery in my laboratory of its covalent ligation to protein substrates, as described below.

The next question was what is the role of this small protein in ATP-dependent protein degradation. It looked smaller than most enzymes, so at first I thought that it might be a regulatory subunit of some enzyme (such as a protein kinase or an ATP-dependent protease) present in Fraction 2. To test this notion, we looked for the association of APF-1/ubiquitin with some protein in Fraction 2. For this purpose, purified radiolabeled APF-1/ubiquitin was incubated with Fraction 2 in the presence or absence of ATP, and subjected to gel filtration chromatography. A marked ATP-dependent association of APF-1/ubiquitin with high molecular weight material was observed [15]. It was very surprising to find that binding was covalent, as indicated by the resistance of the high molecular weight derivative to a variety of denaturing agents [15]. Subsequent work showed that proteins to which ubiquitin is bound are substrates of the ATP-dependent proteolytic system [16]. Based on these findings, we proposed in 1980 that proteins are targeted for degradation by covalent ligation to APF-1/ubiquitin and hypothesized that a protease exists that specifically degrades proteins ligated to ubiquitin [16]. Shortly afterwards, the identity of APF-1 with ubiquitin was established by Wilkinson et al. [14]. Ubiquitin was originally isolated by Goldstein and co-workers in a search for hormones from the thymus, but was subsequently found to be present in all tissues and eukaryotic organisms, hence its name [17]. The functions of ubiquitin were not

known, though it was discovered by Goldknopf, Busch, and co-workers that ubiquitin is conjugated to histone 2A in an isopeptide linkage [18].

1.4 Identification of Enzymes of the Ubiquitin-mediated Proteolytic System

In subsequent work in my laboratory, we tried to isolate and characterize enzymes of the ubiquitin-mediated proteolytic system from Fraction 2 of reticulocytes, using a similar biochemical fractionation–reconstitution approach. Over a period of about ten years (1980–1990), we have identified eight different components in Fraction 2, all of which were required for ubiquitin-ATP-dependent protein degradation. Three of these are involved in the conjugation of ubiquitin to protein substrates. These are the ubiquitin-activating enzyme E1 [19], ubiquitin-carrier protein E2 [20] and ubiquitin-protein ligase E3 [20]. We found that E1 carries out the ATP-dependent activation of the carboxy-terminal glycine residue of ubiquitin [21] by the formation of ubiquitin adenylate, followed by the transfer of activated ubiquitin to a thiol site of E1 with the formation of a thiolester linkage [19, 20]. Activated ubiquitin is transferred to a thiol site of E2 by transacylation, and is then further transferred to an amino group of the protein substrate in a reaction that requires E3 [20]. All three types of enzyme were purified by affinity chromatography on ubiquitin-Sepharose [20]. The terms E1, E2, and E3 were suggested by Ernie Rose; ''E'' stood for **e**nzyme, and not **e**luate, as stated in some articles. We found that the role of E3 is to specifically bind specific protein substrates [22]. Building on this observation, it was proposed that the selectivity of ubiquitin-mediated protein degradation is mainly determined by the substrate specificity of different E3 enzymes [23]. This notion was verified by subsequent work in many laboratories on the selective action of a large number of different E3 enzymes on their specific protein substrates.

Three other components that my laboratory has identified and partially purified from Fraction 2 of reticulocytes, termed CF1–CF3, are involved in the degradation of proteins ligated to ubiquitin [24]. These are apparently subcomplexes of the 26S proteasome, a large ATP-dependent protease complex first described by Rechsteiner and co-workers [25]. CF3 is identical to the 20S proteasome core particle [26], while CF1 and CF2 may be similar to the ''base'' and ''lid'' subcomplexes of the 19S regulatory particle of the 26S proteasome, described more recently by the Finley laboratory [27]. In hindsight, the reason for finding subcomplexes, rather than the complete 26S complex in Fraction 2 was technical: we have routinely prepared Fraction 2 from ATP-depleted reticulocytes [20], under which conditions the 26S proteasome dissociates to its subcomplexes. We found that incubation of the three subcomplexes in the presence of ATP promotes their assembly to the 26S proteasome [24, 26]. The role of ATP in the assembly of the 26S proteasome complex remains unknown.

The last two enzymatic activities that we have described in reticulocytes are ubiquitin C-terminal isopeptidases, which act at the final stages of the ubiquitin proteo-

lytic pathway to release free and reusable ubiquitin from intermediary degradation products. One is an enzyme called isopeptidase T, which preferentially cleaves ubiquitin-Lys^{48}-ubiquitin linkages in polyubiquitin chains [28]. Its main function appears to be the disassembly of polyubiquitin chain remnants following proteolysis of the protein substrate moiety of ubiquitin–protein conjugates by the 26S proteasome complex. Another is a ubiquitin-C-terminal hydrolase that is an integral part of 26S proteasome complex [29]. Its role appears to be to release ubiquitin from linkage to the protein substrate at the final stages of the action of the 26S proteasome. Unlike most ubiquitin-C-terminal hydrolases, this isopeptidase is not inhibited by ubiquitin aldehyde, but is inhibited by the heavy metal chelator *o*-phenanthroline [29]. It appears to be similar to the Rpn11 metalloprotease subunit of the lid subcomplex, which has been recently identified by the Deshaies laboratory and shown to be essential for substrate deubiquitination and degradation [30].

1.5 Discovery of Some Basic Cellular Functions of the Ubiquitin System

The discovery of the basic biochemistry of ubiquitin-mediated protein degradation opened up the way for significant further progress in the elucidation of the roles of this system in a large variety of biological processes. Such further progress required the additional approaches of molecular genetics and cell biology. Thus, the first indication of the role of the ubiquitin system in cell cycle control was the discovery by Varshavsky and co-workers that the ts85 mammalian cell line, which fails to enter mitosis at the restrictive temperature, is defective in the ubiquitin-activating enzyme E1 [31]. The cloning of various genes of the ubiquitin system in yeast by the same laboratory led to insights into the roles of the polyubiquitin gene in stress response [32] and to the identification of the product of the DNA repair gene *RAD6* as an E2 protein [33]. Shortly afterwards, another E2 protein was identified as the product of the *CDC34* gene, known to be involved in the G1 → S transition in the cell cycle [34]. These early studies on the molecular genetics of the ubiquitin system initiated an avalanche of rapid progress in this field by many laboratories.

The entry of molecular genetics into the ubiquitin field did not signal the end of the usefulness of biochemical approaches. A good example of the power of the combination of biochemistry with genetics is the discovery of the Anaphase Promoting Complex/Cyclosome (APC/C), a large multisubunit ubiquitin ligase essential for exit from mitosis by the degradation of mitotic regulators such as cyclin B. In 1983, Hunt and co-workers discovered cyclin B, the first cyclin, as a protein that is destroyed at the end of each cell cycle in early embryos of marine invertebrates [35]. This discovery not only opened up a new era in cell cycle research, but also kindled interest in the problems of what is the machinery that targets cyclin B for degradation, and why does it act only at the end of mitosis? Though researchers of the cell cycle were at that time searching for a putative ''cyclin protease'', I thought that a specific ubiquitin ligase might exist that acts on cyclin B only at the end of

mitosis. In 1991, independent work from the laboratory of Kirschner [36] and from my laboratory [37] showed that cyclin B is degraded by the ubiquitin system. Both laboratories employed biochemical approaches, using cell-free systems from early embryos of frogs [36] and clams [37]. Initial fractionation of the system in my laboratory [38] showed that in addition to E1, two novel components were required to reconstitute cyclin–ubiquitin ligation. These were a specific E2, termed E2-C, and an E3-like activity, which, in clam extracts, was associated with particulate material. In 1995, rapid progress in this system took place due to the convergence of information from biochemical experiments with genetic analysis in yeast. In work done in collaboration with Joan Ruderman, we solubilized the E3-like activity and partially purified and characterized it [39]. It was found to be a large (~1500 kDa) complex, which has cyclin–ubiquitin ligase activity. The activity of this enzyme is regulated in the cell cycle: it is inactive in the interphase and becomes active at the end of mitosis by phosphorylation. We called this complex the cyclosome, to denote its large size and important roles in cell-cycle regulation [39]. A similar complex was isolated from frog extracts at the same time by the Kirschner lab, and was called the Anaphase Promoting Complex [40]. The identification of subunits of the APC/C was made possible by work from the Nasmyth laboratory, who used an elegant genetic screen to identify yeast genes required for the proteolysis of cyclin B [41]. The products of some of these genes, *CDC16*, *CDC23* and *CDC27*, had been previously shown to be required for the onset of anaphase in budding and fission yeasts. Thus, the genetic work also proved the relevance of the biochemical results on APC/C to its role in exit from mitosis in cells. Subsequent work by several groups showed that APC/C is also involved in the degradation of some other important mitotic regulators, such as securin, an inhibitor of anaphase onset (reviewed in Ref. [42]). In addition, the APC/C is the target of the spindle assembly checkpoint system, a surveillance mechanism that allows sister chromatid separation only after all chromatids have been properly attached to the mitotic spindle [43].

1.6 Concluding Remarks

Several lessons can be learned from our story. One is not to accept authority in science. Monod's statement that there is no protein turnover in animal cells should not have been accepted without examination of the assumptions on which the statement was based. A second is that if you believe that you have a biologically important problem to study, you should pursue it, even if very few other researchers are interested in it. At the beginning, very few scientists were interested in the ubiquitin system (and some of the few who knew about it thought it was all wrong), but being obstinate was rewarding in the long run. If everyone only worked on subjects that are in the current mainstream of science, very few new fields would be discovered. The third lesson, which I keep reiterating with the hope of convincing a few young scientists, is the continued importance of bio-

chemistry in biomedical research. The ubiquitin system could not have been discovered without the use of biochemical approaches, and biochemistry continues to be essential, in combination with molecular genetics and cell biology, in unraveling the myriad cellular functions of this system and their underlying molecular mechanisms.

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2
N-terminal Ubiquitination: No Longer Such a Rare Modification

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Abstract

The ubiquitin–proteasome system (UPS) is involved in selective targeting of innumerable cellular proteins via a complex pathway that plays important roles in a broad array of processes. An important step in the proteolytic cascade is specific recognition of the substrate by one of many ubiquitin ligases, E3s, that is followed by generation of the polyubiquitin degradation signal. For most substrates, it is believed, though it has not been shown directly, that the first ubiquitin moiety is conjugated, via its C-terminal Gly^{76} residue, to an ε-NH_2 group of an internal lysine residue. Recent findings indicate that for an increasing number of proteins, the first ubiquitin moiety is fused linearly to the α-NH_2 group of the N-terminal residue. An important biological question relates to the evolutionary requirement for an alternative mode of ubiquitination.

2.1
Background

Two distinct structural elements play a role in the ubiquitination of a target protein: (i) the E3 recognition site and (ii) the anchoring residue of the polyubiquitin chain. In most cases, it is believed, though it has been shown for only a few proteins, that the first ubiquitin moiety is transferred to an ε-NH_2 group of an internal lysine residue in the substrate. The N-terminal domain of the target protein has attracted attention both as an E3 recognition domain and, recently, as a ubiquitination site.

As for specific recognition, in certain rare cases, the stability of a protein is a direct function of its N-terminal residue, which serves as a binding site for the ubiquitin ligase E3α (Ubr1 in yeast; 'N-end-rule'; [1, 2]). Accordingly, two types of N-terminal residues have been defined, ''stabilizing'' and ''destabilizing''. For the Mos protein, it was found that its stability is governed primarily by the penultimate proline residue and by a phosphorylation/dephosphorylation cycle

of serine3 [3]. A mechanistic explanation for the role of the Pro and Ser residues is still missing.

As for the lysine residue targeted, there is no consensus as to its specificity. In some cases distinct lysines are required, while in others there is little or no specificity. Thus signal-induced degradation of IκBα involves two particular lysine residues, 21 and 22 [4]. In the case of Gcn4, lysine residues in the vicinity of a specific PEST degradation signal serve as ubiquitin attachment sites [5]. Mapping of ubiquitination sites of the yeast iso-2-cytochrome *c* has revealed that the polyubiquitin chain is synthesized almost exclusively on a single lysine [6]. In two other examples, that of Mos (see above; [3]) and the model "N-end rule" substrate X-β-gal (where X is a short fused peptide not encoded by the native molecule [7]), one and two lysines, respectively, that reside in proximity to the degradation signal are required for ubiquitination. In striking contrast, ubiquitination of the ζ chain of the T-cell receptor is independent of any particular lysine residue and proceeds as long as one residue is present in the cytosolic tail of the molecule [8]. Similarly, no single specific lysine residue is required for ubiquitination of either c-Jun [9] or cyclin B [10]: any single lysine residue, even artificially inserted, can serve as a ubiquitin acceptor. Important in this context is that only in a handful of cases it has been shown directly, via chromatographic or mass spectrometric analyses, that ubiquitin is indeed anchored to a lysine residue (see for example Refs. [11, 12]). In most cases studied, and there are not too many, the assumption that an internal lysine serves as the polyubiquitin chain anchor is indirect and based on mutational analyses.

One interesting case involves the artificial fusion protein ubiquitin-Pro-X-β-galactosidase. In this chimera, the ubiquitin moiety was fused to the N-terminal Pro residue of the protein. Unlike other ubiquitin-B-X-β-galactosidase species (where *B* is any of the remaining 19 amino acid residues), here ubiquitin is not removed by isopeptidases and serves as a degradation signal following generation of a polyubiquitin chain that is anchored to Lys48 of the artificially fused ubiquitin moiety [13]. However as noted, in this case the ubiquitin moiety was fused to the N-terminal residue artificially.

The first substrate that was identified in which the N-terminal residue serves as a ubiquitination target was MyoD. The basic helix–loop–helix (bHLH) protein MyoD is a tissue-specific transcriptional activator that acts as a master switch for muscle development. MyoD forms heterodimers with other proteins belonging to the bHLH group, such as the ubiquitously expressed E2A, E12 and E47. These dimers are probably the transcriptionally active forms of the factor. Association of MyoD with HLH proteins of the Id family (inhibitors of differentiation that lack the basic domain) inhibits its DNA-binding and biological activities. MyoD is a short-lived protein with a half-life of ∼45 min [14, 15]. Degradation of MyoD is mediated by the ubiquitin system both *in vitro* and *in vivo*. Furthermore, the process is inhibited by its consensus DNA-binding site. In contrast, addition of Id1 destabilizes the MyoD–E47–DNA complex and renders the protein susceptible to degradation [15].

2.2 Results

To analyze specific ubiquitination sites in MyoD, we used site-directed mutagenesis to substitute systematically all the lysine residues with arginines [16]. The protein contains nine lysine residues, most of them located within the N-terminal domain of the molecule. The nine residues are in positions 58, 99, 102, 104, 112, 124, 133, 146 and 241. The various proteins were generated either by expression in bacteria followed by purification, or by *in vitro* translation in reticulocyte lysate in the presence of [^{35}S]methionine. Conjugation and degradation of the proteins were monitored in a reconstituted cell-free system or in cells. Proteins were detected by either Western blot analysis or PhosphorImaging. Surprisingly, even a MyoD species that lacked all lysine residues was still degraded efficiently in an ATP-dependent manner *in vitro*. To demonstrate involvement of the ubiquitin system in the process, we followed the degradation of wild-type (WT) and lysine-less (LL) MyoD in the absence and presence of ubiquitin. Similar to the degradation of the WT protein, degradation of the LL MyoD was completely dependent upon the addition of exogenous ubiquitin to an extract that does not contain it (Fraction II). Furthermore, addition of methylated ubiquitin, which cannot form polyubiquitin chains and serves as a chain terminator [17], inhibited the degradation of LL MyoD. The inhibition could be alleviated by the addition of excess of free ubiquitin. These results strongly suggested that polyubiquitination of LL MyoD is necessary for degradation of the protein. Furthermore, they implied that the polyubiquitin chain is synthesized on internal lysine residues of ubiquitin. To demonstrate directly polyubiquitinated LL MyoD, we used *in-vitro*-translated ^{35}S-labeled protein in a partially reconstituted system. We demonstrated that LL MyoD generates high molecular mass ubiquitinated adducts. It should be noted, however, that these conjugates are of somewhat lower molecular mass than those of the WT MyoD. This can be attributed to the role that the internal lysine residues also play in the process (see also below).

To investigate the physiological relevance of the observations in the cell-free system, we followed the fate of the different MyoD lysine-mutated proteins *in vivo*, using pulse-chase labeling experiments in COS-7 cells that were transiently transfected with the different MyoD cDNAs. In agreement with our *in vitro* data, the lysine-less MyoD protein is degraded efficiently in cells as well. However, we could observe a progressive increase in the half-life of the proteins of up to ~2-fold with the gradual substitution of the lysine residues. While the half-life of WT MyoD was ~50 min, that of LL MyoD was ~2 h. Interestingly, we found that the stability of MyoD is not affected by the substitution of any specific lysine residue, and it is the total number of these residues that determines the half-life of the protein. To identify the system involved in the destruction of LL MyoD *in vivo*, transfected cells were incubated in the presence of inhibitors of proteasomal and lysosomal degradation. Chloroquine, a general inhibitor of lysosomal proteolysis, and E-64, a cysteine protease inhibitor that affects lysosomal, but also certain cytosolic proteases, had no effect on the stability of the LL MyoD. In striking contrast, the

proteasomal inhibitors MG132 and lactacystin blocked degradation of the LL protein significantly. To demonstrate the intermediacy of ubiquitin conjugates in the degradation of LL MyoD, we incubated COS-7 cells, transiently transfected with either WT or LL MyoD cDNAs, with MG132, and followed generation of ubiquitin-MyoD adducts. Immunoprecipitation with anti-MyoD antibody followed by Western blot analysis with anti-ubiquitin antibody revealed accumulation of high molecular mass compounds in cells transfected with either WT or LL MyoD. A similar analysis of mock-transfected cells clearly demonstrated the specificity of both the anti-MyoD and anti-ubiquitin antibodies.

Based on these results, it was clear that polyubiquitination is essential for targeting MyoD for degradation. The lack of internal lysine residues, the only known targets for ubiquitin modification, made it important to identify the functional group that can serve as an attachment site for ubiquitin. Chemically, several groups can generate covalent bonds with ubiquitin. Ser and Thr can participate in ester bond formation, while Cys can generate a thiol ester bond. However, these bonds are unstable and are hydrolyzed in either high pH (Ser and Thr) or high concentration of –SH groups (Cys). The stability of the MyoD-ubiquitin adducts under these conditions made it highly unlikely that any of these modifications is the one we observed.

A likely candidate, however, was the free amino group of the N-terminal residue of the protein, which can generate a stable peptide bond with the C-terminal Gly residue of ubiquitin. Edman degradation of the N-terminal residue of bacterially expressed, *in-vitro*-translated and cellularly expressed MyoDs, has revealed that the ubiquitin attachment site can be the free, unmodified initiator methionine: the proteins were not modified and the N-terminal residue was not acetylated. To demonstrate a role for the free N-terminal amino group in the degradation of MyoD, we chemically modified this group. Initially, we blocked this group in the LL MyoD protein by reductive methylation. While this procedure blocks all amino groups in a protein in a non-discriminatory manner, in this case, it could have been only the α-NH_2 group, which is the only free amino group left in the MyoD molecule. The modification stabilized the protein completely. Whereas a free α-NH_2 appears to be sufficient for degradation (probably following ubiquitination) of LL MyoD, it is not clear whether it also plays a physiological role in targeting the WT molecule, which has nine available lysine residues. In order to investigate the role and biological relevance of the free α-NH_2 group in the targeting of WT MyoD, we selectively blocked it by carbamoylation with potassium cyanate at low pH. This procedure does not modify ε-NH_2 groups of internal lysine residues. Automated Edman degradation along with fuorescamine determination of the extent of remaining free NH_2 groups confirmed that the modification affected only the N-terminal group. The modified protein was subjected to *in vitro* degradation and conjugation in cell extract. In contrast to LL MyoD, the N-terminally carbamoylated protein could not be ubiquitinated and was stable. Thus, a free and exposed NH_2 terminus of MyoD appears to be an essential site for degradation, most probably because it serves as an attachment site for the first ubiquitin moiety. As an additional control, we selectively modified the internal lysine residues of WT MyoD by guanidination with *O*-

methylisourea. The modification, which does not affect the N-terminal group, generates a protein that is essentially the chemically modified counterpart of the LL MyoD that was generated by site-directed mutagenesis. Similar to the LL protein, this MyoD derivative is degraded efficiently in the cell-free system in a ubiquitin- and an ATP-dependent mode.

To analyze the role of the N-terminal residue of MyoD as a ubiquitination site, we fused to WT MyoD, upstream to the N-terminal residue, a 6 × Myc tag, and monitored the stability of the tagged protein. We showed that it is stable both *in vitro* and *in vivo*. It should be noted that the two first N-terminal residues of the Myc tag, methionine and glutamate, are identical to the first two N-terminal residues in MyoD. In addition, the Myc tag also contains a lysine residue. Thus, altogether, six additional lysine residues were added to WT MyoD in addition to its own nine native residues. Nevertheless, the tag stabilizes it, probably by blocking access to a specific N-terminal residue, and, as became clear later (see below), to its neighboring domain.

Taken together, these findings strongly suggested that MyoD is first ubiquitinated at its N-terminal residue, and the polyubiquitin chain is synthesized on this first conjugated ubiquitin moiety. Internal lysine residues also play a role, probably by serving as additional anchoring sites, whose ubiquitination accelerates degradation. Yet they are not essential for proteolysis to occur. In contrast, ubiquitination of the N-terminal residue plays a critical role in governing the protein's stability [16] (Figure 2.1).

Using a similar, though not a complete, set of experiments, 12 additional proteins have been identified recently that appear to undergo N-terminal ubiquitination: (i) the ***h***uman ***p***apilloma***v***irus-16 (HPV-16) E7 oncoprotein (18), (ii) the ***l***atent ***m***embrane ***p***rotein 1 (LMP1)(19) and (iii) 2A (LMP2A)(20) of the ***E***pstein ***B***arr ***v***irus (EBV), involved in viral activation from latency, (iv) the ***c***ell cycle-***d***ependent ***k***inase (CDK) inhibitor p21 (21,22), (v) the extracellular signal-regulated kinase 3, ERK3 (22), the ***i***nhibitors of ***d***ifferentiation (vi) Id2 (23) and (vii) Id1 (24), two proproliferative Helix-Loop-Helix proteins, (viii) hydroxymethyglutaryl-Coenzyme A reductase (HMG-CoA reductase), the first and key regulatory enzyme in the cholesterol biosynthetic pathway (25), (ix) $p19^{ARF}$, the mouse Mdm2 inhibitor and (x) $p14^{ARF}$, its human homologue (26), (xi) the HPV-58 E7 oncoprotein, and (xii) the cell cycle regulator $p16^{INK4a}$ (27). As for HMG-CoA reductase, several specific internal lysine residues have also been shown to be important for its targeting, and therefore the essentiality of the N-terminal residue in the process has to be further substantiated. The case of p21 requires further investigation, as one study reported that its degradation by the proteasome does not require ubiquitination [28], while an independent study has demonstrated a role for Mdm2 in targeting p21, also without a requirement for ubiquitination [29]. As we noted for MyoD, substitution of the internal lysines inhibited slightly (up to twofold) both conjugation and degradation of HPV-16, LMP1, and Id2, suggesting that these residues, probably also by serving as ubiquitin anchors, can modulate the stability of these proteins. It is possible to suppose that N-terminal ubiquitination and modification of internal lysines is catalyzed by different ligases that may be even located in different subcellular compartments (e.g. the nucleus and cytosol). Because of the role that internal

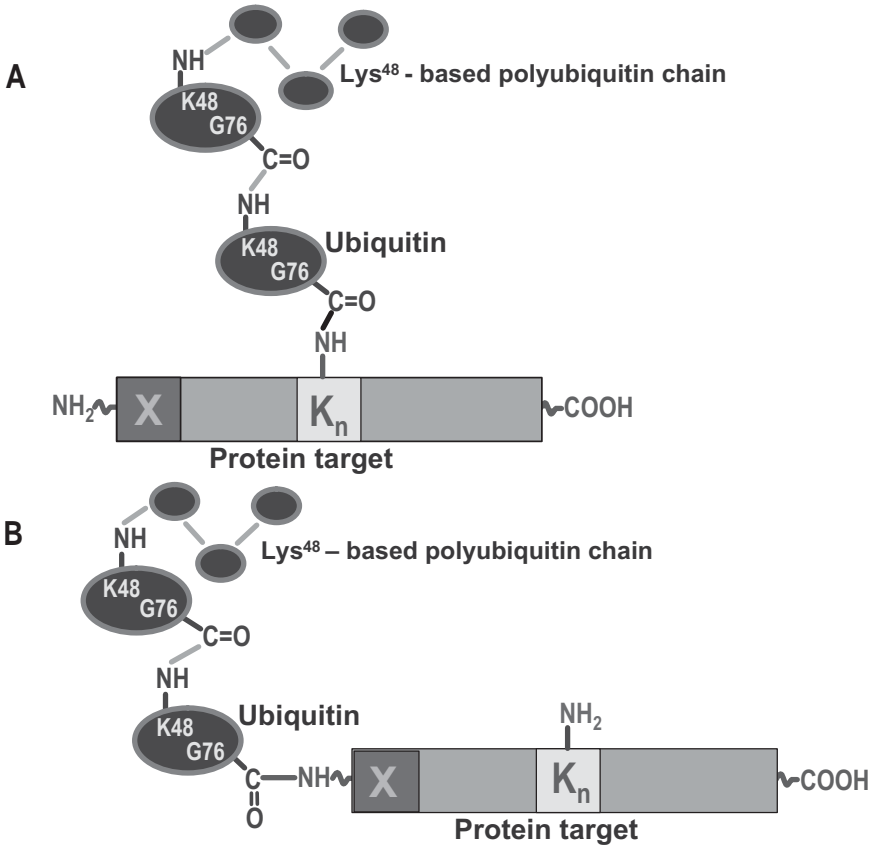

Fig. 2.1. Ubiquitination on an internal lysine and on the N-terminal residue of the target substrate. (A) The first ubiquitin moiety is conjugated, via its C-terminal Gly^{76} residue, to the ε-NH_2 group of an internal lysine residue of the target substrate (K_n). (B) The first ubiquitin moiety is conjugated to a free α-NH_2 group of the N-terminal residue, X. In both cases, successive addition of activated ubiquitin moieties to internal Lys^{48} on the previously conjugated ubiquitin moiety leads to the synthesis of a polyubiquitin chain which serves as the degradation signal for the 26S proteasome.

lysines play in modulating the stability of these proteins, and in order to better understand the physiological significance of this novel mode of modification, it was important to identify proteins whose degradation is completely dependent on N-terminal ubiquitination. An important group of potential substrates for N-terminal ubiquitination is that of ***n***aturally ***o***ccurring ***l***ysine-***l***ess ***p***roteins – NOLLPs. Since these proteins cannot use the "canonical" lysine conjugation pathway, in order to be targeted by the ubiquitin system they must use, an alternative site for their tagging. Searching the database, we were able to identify 177 eukaryotic NOLLPs, 14 of which occur in humans. In addition, we have identified 111 viral NOLLPs. We have shown that two of the proteins mentioned above, the human tumor supressor $p16^{INK4a}$ and the viral oncoprotein HPV-58 E7 are degraded via the N-terminal ubiquitination pathway [27]. Interestingly, we demonstrated that $p16^{INK4a}$ is ubiq-

uitinated and degraded only in sparse cells, and is stable in dense cells. Similar findings were reported for the NOLLPs $p19^{ARF}$ and $p14^{ARF}$ (26).

For E7-16 [18], LMP1 [19], and MyoD (unpublished), it has been shown that truncation of a short N-terminal segment of 10–20 residues stabilized the proteins, suggesting that the entire domain beyond the single N-terminal residue plays a role in governing the stability of these proteins. Such a segment can allow the mobility/flexibility necessary for the N-terminal residue to serve as a ubiquitin acceptor. It can also serve as a recognition domain for the cognate E3. There is no homology between the N-terminal domains of these three proteins, suggesting that if the three N-terminal domains serve as recognition motifs, they recognize different components of the ubiquitin system. Interestingly, the LMP2A E3 was identified as a member of the NEDD4 family of HECT domain ligases, AIP4 and/or WWP2 [20]. A PY motif in LMP2A is recognized by the E3. It resides in the N-terminal domain of the molecule, supporting the hypothesis that in these proteins the E3-binding domain may reside in close proximity to the N-terminal residue ubiquitination site.

Is there any direct evidence for N-terminal ubiquitination? All the different and independent lines of evidence in the various studies strongly suggest that ubiquitination occurs on the N-terminal residue, and any other scenario is highly unlikely. Yet, the only direct evidence must be demonstration of a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of the target substrate. The study on p21 [21] and E7-58 [27] brought us a little closer. Bloom and colleagues [21] transfected cells with N-terminally His-tagged ubiquitin and N-terminally HA-tagged p21 that contained a Factor X proteolytic site immediately after the HA tag and upstream of the p21 reading frame. They then immunoprecipitated and resolved the cell-generated ubiquitin conjugates of p21 and treated the mono-ubiquitin–p21 adduct with Factor-X protease. This treatment released a smaller species of p21 (lacking His-ubiquitin and the HA-tag-Factor X site) and His-ubiquitin-HA-Factor X site, thus demonstrating that the HA-tag-Factor X site, which was previously part of p21, had now become part of the Factor X-cleaved ubiquitin. A similar experimental evidence was brought for the NOLLP HPV E7-58 [27]. Here, Ben-Saadon and colleagues generated two species of the protein containing the eight amino acid sequence of the Tobacco Etch Virus (TEV) protease cleavage site inserted either 21 amino acid residues after the iMet [E7-58-TEV(21)] or immediately after the iMet [E7-58-TEV(1)]. The prediction from this experiment was that if ubiquitin is indeed attached to the N-terminal residue of E7-58, TEV protease-catalyzed cleavage will generate an extended ubiquitin molecule that will also contain the respective N-terminal domain of E7-58 [21 residues or 1 residue, respectively, dependent upon whether the substrate of the reaction is E7-58-TEV(21) or E7-58-TEV(1), and the six amino acids derived from the TEV cleavage site]. Such extended ubiquitin moieties were indeed generated following incubation of the substrate with labeled methylated ubiquitin (which generated mostly the mono-ubiquitin adduct of the E7-58 protein), followed by cleavage of the adduct with TEV. The only conclusion that can be derived from these experiments is that the ubiquitin moiety was fused to any of the amino acid residues of the HA tag-Factor X site at the N-terminal domain of p21, or to any of the first 21 amino acids

of E7 or the TEV site (part of it; the protease cleaves after the sixth amino acids out of eight in the complete site). Such internal modification is unlikely, however, as it must require a novel chemistry since none of the residues in the tags, the protease sites or the E7 N-terminal fragment, is lysine. Yet, formally, it is still possible that such a modification occurs. The HA tag contains, for example, three Tyr residues. Thus, the evidence provided by these two experiments clearly limits an unlikely non-peptide bond ubiquitination, such as esterification, to a much smaller zone in the N-terminal domain of the tagged p21 or the TEV-containing E7-58, but does not demonstrate directly that the modification occurs indeed on the N-terminal residue.

As noted, only identification of a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of the target protein will constitute such an evidence. Ben-Saadon and colleagues have recently isolated the long sought after fusion peptide [27]: mass spectrometric analysis of a tryptic digest of the isolated mono-ubiquitin adduct of HPV-58 E7 revealed a peptide of 11 amino acids, GG-MHGNNPTLR which represents the last two C-terminal amino acids of ubiquitin, GlyGly, and the first nine residues of E7, MetHisGlyAsnAsnProThrLeuArg (MHGNNPTLR). It should be noted that WT E7-58 contains an Arg residue in position 2. It was necessary to substitute this Arg with His, since otherwise the digesting enzyme, trypsin, would have generated a tetrapeptide, GG-MR, that would have been difficult, if not impossible, to identify in the MS analysis. MS/MS analysis of the 11-mer, verified its internal sequence. Coulombe and colleagues were also able to isolate and identify the sequence of a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of HA-tagged p21 that also contained, downstream of the tag, a stretch of residues derived from the N-terminal domain of the native substrate, but without the iMet (which was removed during the construction of the tagged protein) [22].

2.3 Discussion

N-terminal ubiquitination is a novel pathway, clearly distinct from the N-end rule pathway [30]. In the latter, the N-terminal residue serves as a recognition and binding motif to the ubiquitin ligase, E3α; however, ubiquitination occurs on an internal lysine(s). In contrast, in the N-terminal ubiquitination pathway, modification occurs on the N-terminal residue, whereas recognition probably involves a downstream motif. It should be mentioned that in yeast, using the model fusion protein ubiquitin-Pro-X-β-galactosidase (where X is a short sequence derived from the λ repressor), a new proteolytic pathway has been described, designated the UFD (***u***biquitin ***f***usion ***d***egradation) pathway [31]. The stably fused ubiquitin moiety (note that in this exceptional case, with Pro and not any other amino acid residue as the linking residue, the ubiquitin moiety is not cleaved off by ubiquitin C-terminal hydrolases), functions as a degradation signal, where its Lys^{48} serves as an anchor for the synthesized polyubiquitin chain. This pathway involves several enzymes, UFD 1–5, some of which appear to be unique and are not part of the ''canonical''

UPS. It is possible that N-terminal ubiquitination is the most upstream event in the UFD pathway – which was discovered using an artificial chimeric ubiquitin-protein model substrate: the N-terminal ubiquitination pathway can function by providing substrates to the UFD pathway.

The physiological significance of N-terminal ubiquitination is still obscure. Naturally occurring lysine-less proteins, NOLLPs, that are degraded by the ubiquitin system must traverse this pathway. Many such proteins, mostly viral, can be found in the database (see above and in Refs. [26,27]). We believe that many additional lysine-containing proteins, will be discovered to be targeted via this novel mode of modification. Of note is that all the proteins that are N-terminally ubiquitinated must contain a free, unmodified N-terminal residue. Such proteins constitute approximately 25% of all cellular proteins, while the remaining 75% are Nα-acetylated. Whether a protein will be acetylated is dependent on the structure of the N-terminal domain of the mature protein. This is determined by the combined activities of ***m***ethionine ***a***mino***p***eptidases (MAPs) and ***N***-terminal ***a***cetyl***t***ransferases (NATs), which are dependent on the specific sequence of up to the first four N-terminal residues of the target protein substrates (reviewed in Ref. [32]). Thus, it is possible to predict which proteins will be potential substrates of the N-terminal ubiquitination pathway. Internal C-terminal fragments of Nα-acetylated proteins can also be modified by ubiquitin at their ''new'' N-terminal residue following limited processing. Many proteins, such as the NF-κB precursors p105 and p100 or caspase substrates are processed initially in a limited manner, generating a C-terminal fragment with a newly exposed N-terminal residue. For all lysine-containing proteins, the intact free N-termini as well as the products of processing, the assumption is that their internal lysines are not easily accessible, for whatever reason, for ubiquitination, and it is only the N-terminal residue that can be modified. Interestingly, most of the substrates identified thus far have a few lysine residues that might not be accessible to the E3s: For example, MyoD has nine (out of 319), E7 two (out of 97), LMP1 has a single lysine residue (out of 440), LMP2A has three (out of 497), Id2 nine (out of 134) and p21 six (out of 164). From the random discovery of thirteen N-terminally ubiquitinated proteins, it appears that their number could well be larger and that many more will be discovered, which will help in the unraveling of the unique characteristics that distinguish this group of substrates.

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3
Evolutionary Origin of the Activation Step During Ubiquitin-dependent Protein Degradation

Hermann Schindelin

Abbreviations

E1	Activating enzyme of a UbL
E2	Conjugating enzyme of a UbL
E3	Ubiquitin ligase
Moco	Molybdenum cofactor
MPT	Molybdopterin
NEDD8-E1	NEDD8-activating enzyme
Ubiquitin-E1	Ubiquitin-activating enzyme
UbL	Ubiquitin-like protein

Abstract

Ubiquitin and related protein modifiers are activated in an ATP-dependent process, which leads to the initial formation of an acyl adenylate between the C-terminus of the modifier and AMP. The modifier is subsequently transferred onto an active-site cysteine residue in the activating (E1) enzyme via a thioester bond and from there to a conjugating (E2) enzyme. In the case of ubiquitin a large family of ubiqutin ligases (E3 enzymes) primarily ensure specific transfer onto the correct protein substrate. Biosynthesis of the molybdenum cofactor is an evolutionarily conserved pathway present in bacteria, archaea and eukaryotes. The molybdenum cofactor contains a *cis*-dithiolene group and incorporation of the sulfur atoms involves among other proteins, MoaD and MoeB. Structural studies of MoaD revealed that this protein shares the same fold as ubiquitin despite the absence of detectable sequence homology. The crystal structure of the MoaD–MoeB complex in its apo-state defined the structure of MoeB and its corresponding domains in the E1 enzymes. The MoaD–MoeB structures in complex with ATP and after formation of the acyl adenylate identified key residues involved in the catalysis of this enzyme superfamily. The recent crystal structures of the NEDD8 activator confirmed the predictions made on the basis of the MoaD–MoeB complex and describe the more complex architecture of the E1 enzymes. The phylogenetic distribution of the en-

zymes involved in Moco biosynthesis strongly suggest that the two-component systems consisting of a UbL protein and a cognate E1 enzyme, which are present exclusively in eukaryotes, are derived from the simpler and universally distributed MoaD–MoeB pair.

3.1 Introduction

3.1.1 Activation of Ubiquitin and Ubiquitin-like Proteins

The transfer of ubiquitin and related protein modifiers (reviewed in [1]) such as SUMO [2], NEDD8 [3], Apg12 [4], Apg8 [5], ISG15 [6], Urm1 [7] and Hub1p [8] is initiated by an activation step catalyzed by an activating (E1) enzyme, which is specific for the respective modifier [9, 10]. The activation reaction [11, 12] is dependent on ATP, which is hydrolyzed to form an acyl adenylate between the C-terminus of the modifier and AMP. The resulting high-energy mixed anhydride intermediate is nucleophilically attacked by a conserved cysteine residue of the E1 enzyme, leading to the formation of a thioester linkage between this cysteine and the C-terminus of the modifier [11–13]. Subsequently the UbL protein is transferred from the E1 enzyme to a conjugating (E2) enzyme in a *trans*-thioesterification reaction. The thioester linkages preserve the free energy of ATP and facilitate transfer of the modifier onto target proteins in which the modifier is almost always linked with its C-terminus to the side chain of a lysine residue via an isopeptide bond.

In the archetypical transfer of ubiquitin to a target protein via ubiquitin's Lys48 residue and the subsequent elongation of the mono-ubiquitin to an oligo-ubiquitin chain of at least four residues, this modification triggers the proteasome-dependent degradation of the target protein. In addition, ubiquitylation is also involved in DNA repair (summarized in Ref. [14]), receptor endocytosis (reviewed in Ref. [15]), endocytic sorting (reviewed in Ref. [16]), budding of HIV (reviewed in Ref. [17]) and inflammatory responses [18]. While UbLs are generally transferred onto proteins, APG8, which is involved in the process of autophagy, is conjugated to phosphatidylethanolamine [19] through its amino group, thus mimicking the isopeptide linkage typically observed in other UbL–protein complexes. Ubiquitin and its related modifiers are exclusively found in eukaryotes, but are evolutionarily derived from individual steps in ancient metabolic pathways, which lead to the formation of either the molybdenum cofactor, thiamine or certain types of FeS-clusters.

3.1.2 Molybdenum Cofactor Biosynthesis

The molybdenum cofactor (Moco) is the essential component of a group of redox enzymes [20–22], which are diverse not only in terms of their phylogenetic distri-

bution, but also in their architectures, both at the overall structural level and in their active site geometry, and finally in the wide variety of transformations catalyzed by these enzymes. Some of the better-known Moco-containing enzymes include sulfite oxidase and xanthine dehydrogenase in humans, assimilatory nitrate reductases in plants and dissimilatory nitrate reductases as well as formate dehydrogenases in bacteria. Moco consists of a mononuclear molybdenum coordinated by the dithiolene moiety of a family of tricyclic pyranopterin structures, the simplest of which is commonly referred to as molybdopterin (MPT). Moco biosynthesis is an evolutionarily conserved pathway comprising several interesting reactions. Mutations in the human Moco biosynthetic genes lead to Moco deficiency, a severe disease that leads to premature death in early childhood [23]. The affected patients show severe neurological abnormalities such as attenuated growth of the brain, seizures, and, frequently, dislocated ocular lenses.

Genes involved in Moco biosynthesis have been identified in eubacteria, archaea and eukaryotes. Although many details of Moco biosynthesis are still unclear at present, the pathway can be divided into three universally conserved stages (Figure 3.1A). (1) *Conversion of a guanosine derivative into precursor Z*. This aspect is different from other pterin biosynthetic pathways, since C8 of the purine is inserted between the 2′ and 3′ ribose carbon atoms during formation of precursor Z, rather than being eliminated [24, 25]. (2) *Transformation of precursor Z into MPT*. This process generates the dithiolene group responsible for coordination of the molybdenum atom in the cofactor, and is catalyzed by MPT synthase [26–28]. MPT synthase is composed of two subunits encoded in *E. coli* by the *moaD* and *moaE* genes. In its active form, MoaD contains a thiocarboxylate at its C-terminus, which acts as the sulfur donor for the synthesis of the dithiolene group (Figure 3.1B). MoeB activates MPT synthase by transferring a sulfur atom onto the C-terminus of MoaD generating the thiocarboxylate [29]. MoeB exhibits significant sequence similarity (Figure 3.2A) to two segments of the E1 enzyme for ubiquitin (UBA1); one is located close to the N-terminus, while the other is located near the center of the sequence encoding UBA1. A similar relationship also exists with E1 enzymes, which are heterodimeric such as the NEDD8-E1 (Figure 3.2A). The sequence similarities between MoeB and E1 enzymes, in concert with their functional relationship (Figure 3.2B), have fostered speculation regarding an evolutionary link between the two pathways [30]. (3) *Metal incorporation*. The MogA and MoeA proteins together are responsible for metal incorporation [31–33].

Additional steps occur in the case of eubacteria, namely syntheses of various dinucleotide forms of the cofactor, in which the pyranopterin is linked to a second nucleotide via a pyrophosphate linkage [34].

3.2 The Crystal Structure of MoaD Reveals the Ubiquitin Fold

The high-resolution crystal structure of MPT synthase [35, 36] revealed that the enzyme forms an elongated, heterotetrameric molecule (Figure 3.3A) with overall dimensions of $93 \times 28 \times 27$ Å. The small (MoaD) subunits are positioned on oppo-

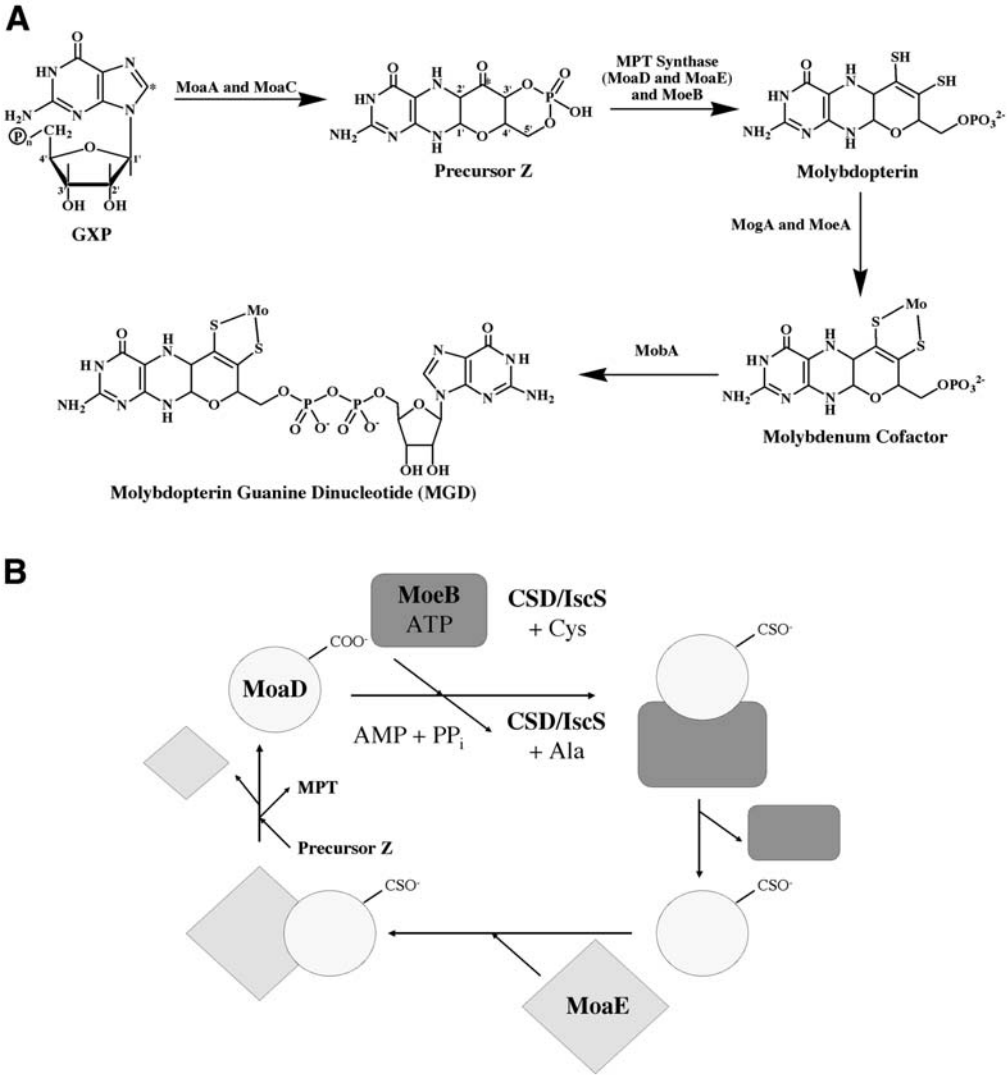


Fig. 3.1. Moco biosynthesis in *E. coli*. (A) The carbon atom at position 8 of the guanosine derivative of unknown composition (GXP), which is the starting structure, is incorporated into precursor Z as indicated by the asterisk. In the mature Moco additional Mo ligands are present besides the dithiolene sulfurs shown here, with the metal being either penta- or hexa-coordinated. (B) Details of step 2, the sulfur incorporation step. MoaD, MoeB and MoaE are represented by a circle, a rectangle and a diamond, respectively.

site ends of the heterotetramer and interact only with the large (MoaE) subunits, which in turn dimerize. The MoaE subunit has an α/β hammerhead fold containing an additional antiparallel 3-stranded β-sheet. The C-terminus of each MoaD subunit is deeply inserted into the active site located in the MoaE monomer. The

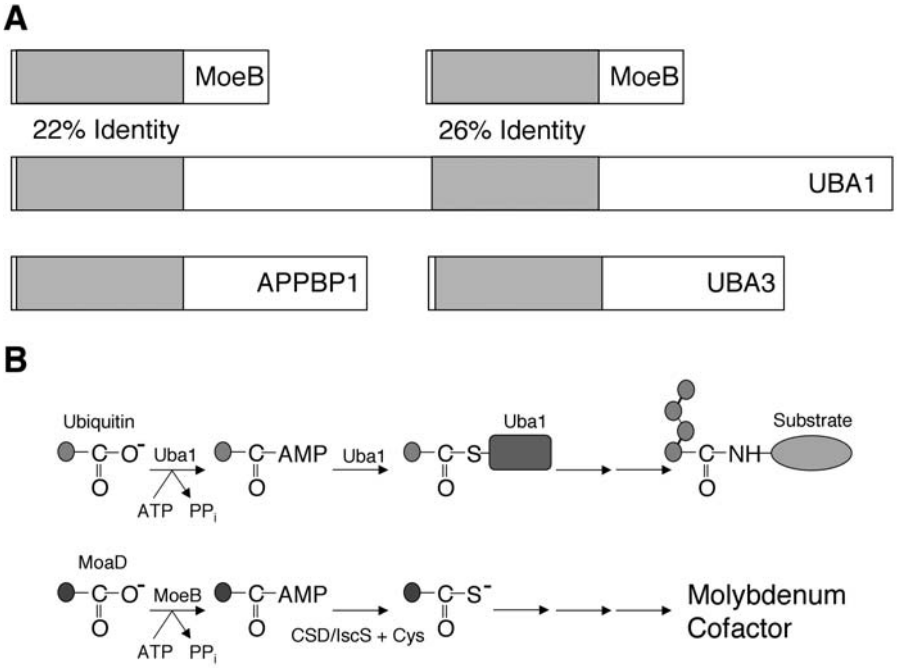


Fig. 3.2. MoeB-E1 sequence relationships. (A) Schematic comparison between MoeB, the ubiquitin-activating enzyme (UBA1) and the heterodimeric (APPBP1–UBA3) NEDD8 activator based on PSI-BLAST [54] sequence analyses. The numbers represent the sequence identities between the first ~170–180 residues of MoeB (shaded) and the corresponding regions in the human ubiquitin-activating enzyme. Residues 3–189 of *E. coli* MoeB can be aligned with residues 47–236 of human ubiquitin E1 (22% identity) and residues 7–175 of *E. coli* MoeB can be aligned with residues 446–620 of human ubiquitin E1 (26% identity). (B) Relationships between the reactions catalyzed by MoeB and the ubiquitin-E1 (UBA1).

interface between the two proteins is quite extensive, burying ~2000 Å^2, and is primarily hydrophobic in character. Although primarily restricted to a single MoaE subunit the active site appears to also involve residues from the distal MoaE subunit. Precursor Z binds to this active site and two sulfur atoms are transferred sequentially from the thiocarboxylated MoaD C-terminus to the substrate to form MPT.

The crystal structure of MPT synthase and the simultaneously determined NMR structure of the MoaD-related ThiS protein involved in thiamine biosynthesis [37] unambiguously demonstrated the evolutionary relationship between a subset of enzymes involved in the biosynthesis of S-containing cofactors (e.g. Moco, thiamine and certain FeS-clusters) and the process of ubiquitin activation. MoaD displays significant structural homology to human ubiquitin (Figure 3.3B and C), resulting in a superposition with a root mean square (rms) deviation of 3.6 Å for 68 equivalent Cα atoms out of 76 residues in ubiquitin. The key secondary structure

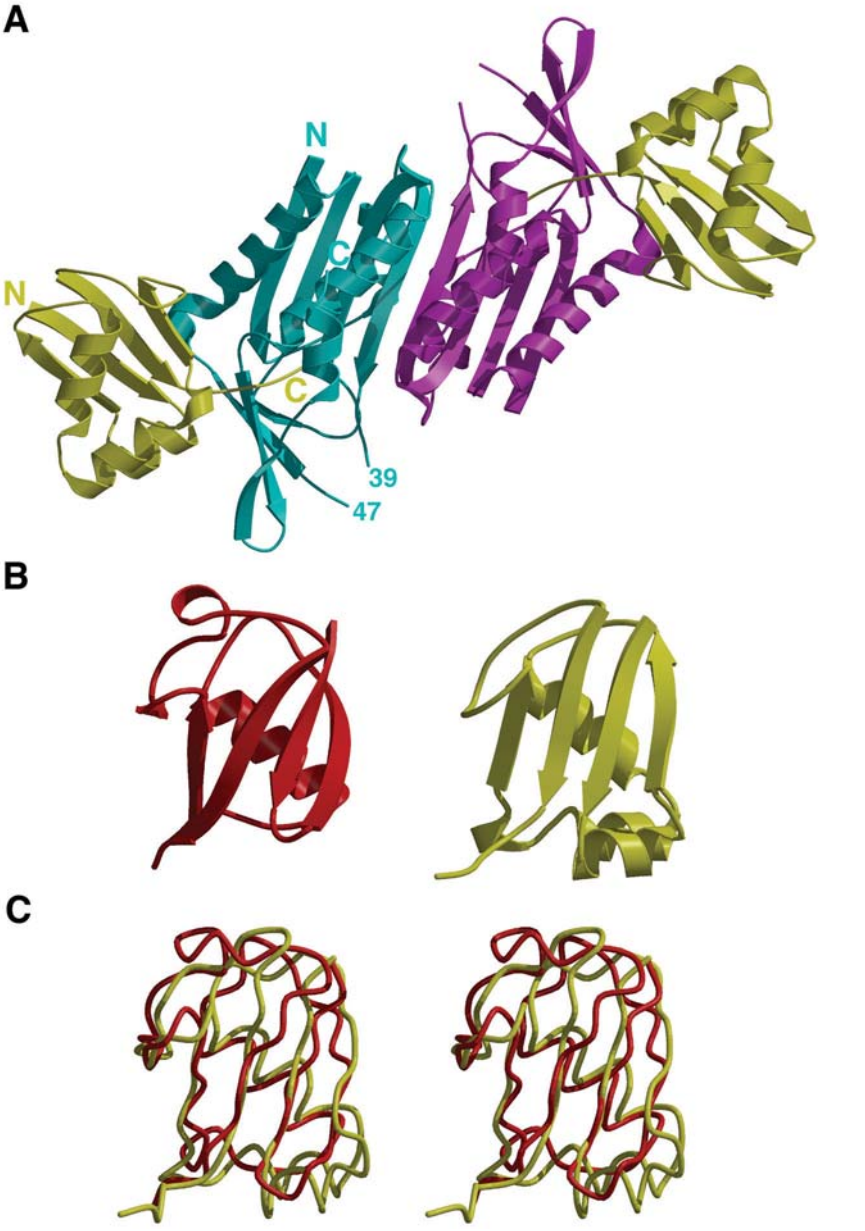


Fig. 3.3. Structure of MPT synthase. (A) Overall structure of the heterotetramer. MoaD subunits are shown in yellow, MoaE subunits in cyan and magenta. The view is along the two-fold axis of symmetry. N- and C-termini and residues adjacent to a disordered loop are labeled in one of the MoaD–MoaE heterodimers. (B) Side-by-side comparison of MoaD (yellow) and ubiquitin (red) in the same orientation. (C) Stereo diagram of a least-squares superposition of MoaD (yellow) and ubiquitin (red).

elements, a five-stranded mixed β-sheet packed against an α-helix, which runs diagonally across one face of the β-sheet, are present in both structures and also in ThiS. However, there is no statistically meaningful level of sequence conservation between MoaD and ubiquitin (7% identity) with the exception of the C-terminal Gly–Gly dipeptide. This dipeptide is a conserved feature present in almost all UbL proteins, but usually requires proteolytic processing to be liberated. Because of the relationship between Moco biosynthesis and ubiquitin-dependent protein degradation described above, this structural homology was not completely unexpected. Together with the known sequence similarities between MoeB/ThiF and the E1 enzymes, this level of structural homology confirmed that the ubiquitin-like protein modifiers and their activating enzymes are the likely evolutionary offspring of the corresponding proteins involved in Moco and thiamine biosynthesis.

3.3
Structural Studies of the MoeB–MoaD Complex

The crystal structure of the MoeB–MoaD complex (Figure 3.4) was determined by multiple isomorphous replacement in its apo-state at 1.7-Å resolution, with bound ATP at 2.9-Å resolution and after formation of the covalent MoaD-adenylate at 2.1-Å resolution [38]. The latter two structures were obtained by soaking either ATP or Mg-ATP into crystals of the apo-complex.

3.3.1
Structure of MoeB

The structure of MoeB consists of eight β-strands that form a continuous β-sheet surrounded by eight α-helices, which are located on opposite sides of the β-sheet (Figure 3.5A). In the N-terminal half of the sheet all β-strands are parallel and reveal a variation of the Rossman fold [39]: the typical $\beta\alpha\beta\alpha\beta$-topology is interrupted between the second β-strand (β_2) and the fourth α-helix (α_4) by the insertion of two 3_{10} helices. The first of these 3_{10} helices contains five residues that are strictly conserved between MoeB and the E1 enzymes. The loop between β1 and α3 contains a highly conserved glycine-rich motif with the sequence Gly–X–Gly–X–X–Gly, which is reminiscent of the P-loop [40] typically found in the superfamily of ATP and GTP hydrolyzing enzymes. The C-terminal half of MoeB contains an antiparallel β-sheet (β5–β8) and is critical for MoeB dimerization and MoaD binding (see below). Two Cys–X–X–Cys motifs are found in this half of the protein and are responsible for coordinating a zinc atom with tetrahedral geometry through their thiolates. This zinc-binding site is quite distant from the active site, thus suggesting a structural rather than a catalytic role for the metal. Moreover, these zinc-binding motifs are only found in 60–70% of the known MoeB sequences and only in a few E1 sequences, indicating that they are non-essential for the catalytic mechanism.

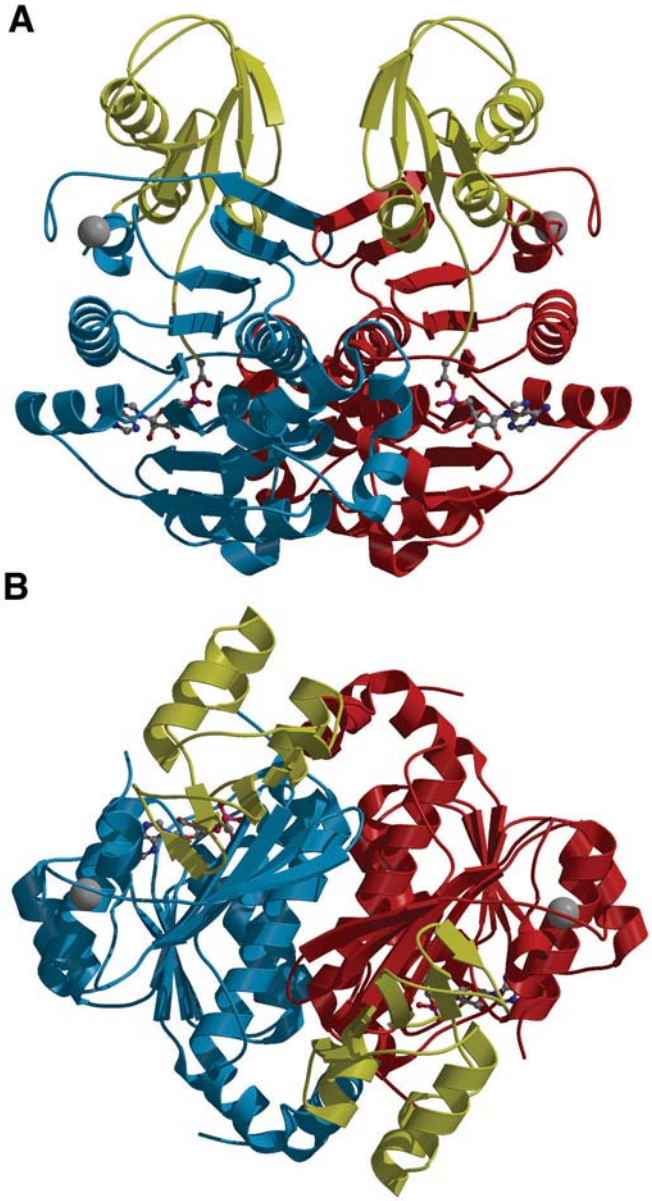


Fig. 3.4. Structure of the MoaD–MoeB complex. (A) Overall structure of the hetero-tetramer. MoaD subunits are shown in yellow, MoeB subunits in red and light blue. The two-fold axis of symmetry is running vertically in the plane of the paper. The AMP is shown in bonds representation together with the covalent link to the C-terminal glycine of MoaD. The Zn ion is shown in van der Waals representation in gray. (B) As in (A) after a 90° rotation around the horizontal axis. The view is now along the two-fold axis of symmetry.

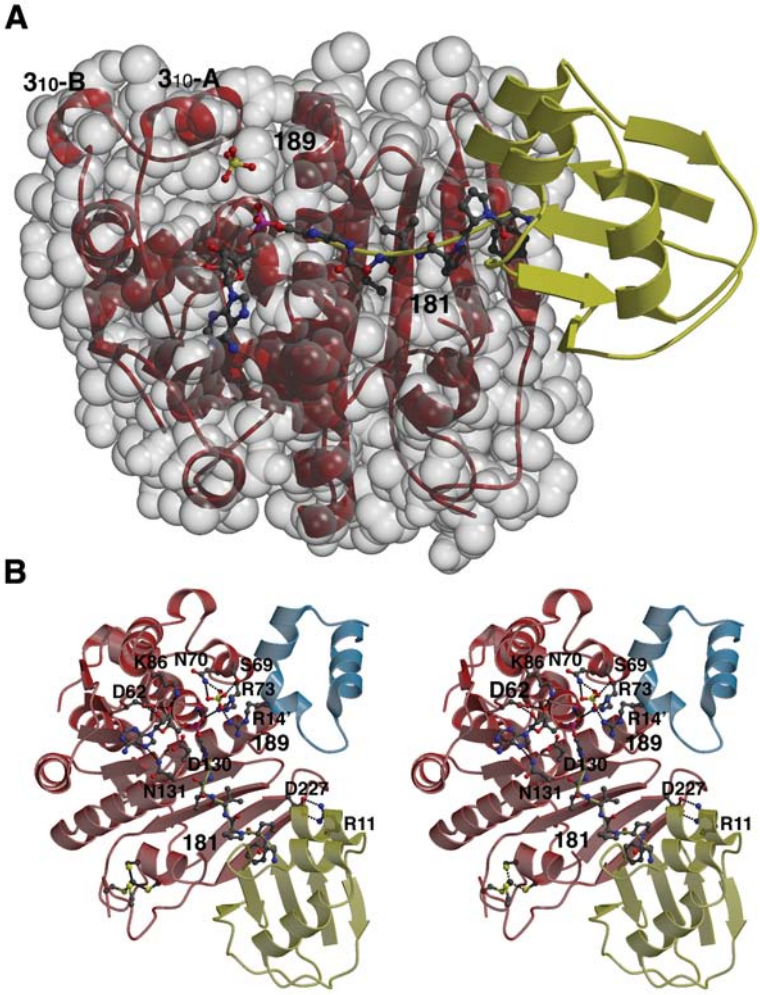


Fig. 3.5. MoeB-catalyzed reaction. (A) Structure of the MoeB–MoaD heterodimer with MoaD in yellow and MoeB in red. Atoms in MoeB are shown as gray spheres representing their van der Waals radii and are rendered transparent. Residues 75 to 81 of MoaD, the AMP and sulfate molecule are shown in ball-and-stick representation. (B) Close-up stereo view of the acyl adenylate intermediate formed during MoaD activation in which AMP is covalently linked at Gly 81 to MoaD (yellow). The principal MoeB subunit with which MoaD interacts is shown in red, while the N-terminus of the second MoeB subunit is shown in blue. Selected residues have been labeled and hydrogen bonds are indicated by dashed lines.

3.3.2 The MoeB–MoaD Interface

As in the interface between MoaD and MoaE, the subunits of MPT synthase, the MoeB–MoaD interface is composed to 65% of hydrophobic interactions. In fact, the hydrophobic core between MoeB and MoaD involves the same region of the

MoaD surface buried in the MPT synthase complex, yet it is slightly more extensive in the MoaD–MoeB complex. Three residues found in MoaD, Phe D7, LeuD59 and Phe D75, are common to both interfaces. Residues in MoaD and MoeB are prefixed with D and B, respectively, to allow differentiation between the two chains. Most of the interactions involving MoaD binding are localized to α7, β7 and β8 of MoeB. In addition to the hydrophobic core, a salt bridge and two hydrogen bonds are formed between Asp B227 Oδ1 and Oδ2 and Arg D11 Nη1 and Nη2 at the periphery of the complex. Additional hydrogen bonds involve the C-terminal tail of MoaD (see below).

Perhaps the most striking feature of the MoeB-MoaD interface is the C-terminal extension of residues 76–81 of MoaD into a cleft on the MoeB surface (Figure 3.5A), which is adjacent to the Gly-rich sequence motif. Proper positioning of the C-terminal Gly–Gly dipeptide appears to be accomplished by hydrogen-bonded interactions. Hydrogen bonds are present between the main chain oxygen of Thr D79 and Nε of Arg B135, which is conserved in MoeB and E1 enzymes, and between the oxygen of Glu D80 and the nitrogen of Ala B154. The C-terminus of MoaD extends over β5 of MoeB, which acts as a structural scaffold. Sequence alignments using MoeB and E1 sequences show a preference for small amino acids (Gly, Ala, Ser) at the center of β5, which appear to allow the insertion of the Gly–Gly motif of MoaD and the UbL into the active site of MoeB and E1. The active-site region in MoeB is delineated by residues found in loops connecting β1 and α3 (containing the modified P-loop motif), β2 and 3_{10}-A, as well as β4 and α6. The strictly conserved residues in helix 3_{10}-A form a region of the active site that binds a sulfate molecule from the mother liquor. In addition, a second sulfate molecule is observed at the active site in close proximity to the incoming Gly–Gly motif of MoaD. Residues 182 to 188 of MoeB are disordered, but owing to the constraints imposed by the adjacent residues (181 and 189) these residues must form a flexible loop crossing over the MoaD C-terminus. One of the residues in this segment, Cys 187, has been proposed to correspond to the active-site Cys in the E1 enzymes and to form a thioester bond with the MoaD C-terminus prior to the formation of the thiocarboxylate [9].

3.3.3 Structure of MoeB–MoaD with Bound ATP

The structure of the MoeB–MoaD–ATP ternary complex reveals that the ATP molecule is bound in a pocket in close proximity to the C-terminus of MoaD. Although the MoaD carboxylate and the α-phosphate are in close spatial proximity, their electrostatic repulsion in the absence of magnesium prevents a nucleophilic attack of MoaD on the α-phosphate. Residues in the modified P-loop motif of MoeB form the floor of the nucleotide-binding pocket. The adenine ring is located in a hydrophobic patch of the pocket, which is created by Phe B63, Leu B109 and Val B134. None of these residues is conserved in MoeB and surprisingly there is only one hydrogen bond to the purine base involving the side chain of Asn 131 and the N7 atom (Figure 3.5B). The ATP appears to be anchored at the active site

through the hydroxyl groups of its ribose ring and the triphosphate moiety. O2′ and O3′ form hydrogen bonds with Oδ1 and Oδ2 of Asp B62, which is strictly conserved in MoeB orthologs and E1 enzymes. Additionally, Nζ of the conserved Lys B86 forms hydrogen bonds with Oδ2 of the ribose. The α-phosphate is buried deeply in the pocket and is involved in main-chain contacts with the nitrogen atom of Gly B41 of the P-loop. Additional MoeB-ATP contacts are seen between the side chains of the strictly conserved Arg B73 and one oxygen each of the α- and β-phosphates and between Lys B86 and the β-phosphate. Interactions with the γ-phosphate involve the side chains of Ser B69 and Asn B70. The overall shape of the binding pocket distorts the ATP molecule and induces a kink between the tightly bound α- and β-phosphates. Arg B14′, a residue from the second MoeB monomer, is inserted at the active site of the first monomer and undergoes a significant conformational change compared to the nucleotide-free structure. In the ATP-bound state, the side-chain atom Nε of Arg B14′ is within hydrogen-bonding distance of two oxygens of the γ-phosphate, whereas some atoms of its side chain are displaced by more than 5 Å in the apo-structure. This residue, though conserved in all MoeB sequences, is not present in the MoeB-like central domain of the ubiquitin-E1 and the UBA3 subunit of the NEDD8-E1.

3.3.4
Structure of the MoaD Adenylate

Soaking MoeB–MoaD cocrystals with ATP and Mg^{2+} led to the visualization of a covalent reaction intermediate (Figure 3.5B). The most striking feature of this structure is the presence of an MoaD adenylate at the active site in which Gly D81 is covalently linked to the α-phosphate through a mixed anhydride. Although the pyrophosphate leaving group is not seen in this structure, a bound sulfate molecule from the mother liquor is presumably mimicking one of the phosphates of pyrophosphate. This sulfate is ligated by Ser B69, Asn B70, and Arg B73 and is observed in a position similar to that occupied by the γ-phosphate in the ATP-bound model. The role of the strictly conserved Asp B130 might be to coordinate the divalent Mg^{2+} ion that appears to be necessary for the turnover of ATP, which in turn would be coordinated by oxygen atoms of the α- and β-phosphate. In contrast to glycyl tRNA synthetase, in which the metal remains bound to the α-phosphate after formation of the glycyl adenylate [41], the structure of the MoaD adenylate provides no evidence of a bound Mg^{2+}.

3.3.5
Fate of the Adenylate

After the formation of an acyl adenylate, the similarities between MoeB and E1 appear to come to an end (Figure 3.2B). In the E1 enzymes an active-site cysteine residue attacks the ubiquitin adenylate forming the E1-ubiquitin thioester. *E. coli* MoeB contains nine cysteine residues, four of which are involved in coordinating the zinc atom. Sequence alignments show that among the remaining cysteines

only Cys B187 is conserved in all MoeB sequences, and might correspond to the active-site cysteine in the E1 sequence family. It has been postulated [9] that the corresponding residue in MoeB also forms a thioester with the MoaD C-terminus, which is then attacked by sulfide to form the thiocarboxylate. Cys B187 in MoeB is part of the consistently disordered loop region, which is located in close proximity to the active site. There appears to be no obvious reason why the side chain of this residue should not be able to attack the acyl adenylate if a thioester is indeed formed during the reaction. While substitution of this cysteine by alanine has been reported not to impair MoeB activity in an *in vitro* system [42], a more recent study [43] does report a 20% reduction in activity. This latter observation is in agreement with mass spectrometric data on ThiF, the MoeB-related protein involved in thiamine biosynthesis, which demonstrated that the corresponding cysteine forms an acyldisulfide linkage with the C-terminus of ThiS [44], suggesting that this residue does indeed have a somewhat similar activity to the active site cysteine present in the E1 enzymes, despite the fact that no thioester intermediate is formed.

In molybdopterin and thiamine biosynthesis the thiocarboxylate sulfur is derived from cysteine by a cysteine desulfurase. Recent findings have implicated IscS in the biosynthesis of thiamine [45]. IscS mobilizes sulfur from cysteine forming an IscS persulfide that is subsequently shuttled to ThiI, a rhodanese-like enzyme, to form a putative ThiI persulfide. This moiety is responsible for attacking the ThiS adenylate, which is bound at the ThiF active site, leading to the formation of the thiocarboxylate product. IscS has also been implicated in Moco biosynthesis together with the related CSD protein [29], although ThiI is not involved. It is interesting to note that some MoeB orthologs, including human MoeB, contain an additional C-terminal domain, which shares distant sequence relationships with rhodaneses. In fact, a C-terminal truncation of the *A. nidulans* MoeB homolog, CnxF, has been shown to abrogate enzyme function as evidenced by the lack of MPT production [46]. Recently, the rhodanese-like domain of MOCS3, the human MoeB ortholog, was shown to be able to transfer the sulfur required for thiocarboxylate formation of MOCS2A, the small subunit of human MPT synthase [43]. Furthermore, site-directed mutagenesis revealed that the conserved cysteine residue in the rhodanese domain is essential for activity. The fact that residues 182 to 188 of MoeB are disordered could indicate that they are involved in protein–protein interactions with either the rhodanese domain or the cysteine desulfurase. Cys187 of MoeB could either cleave the acyldisulfide between the cysteine of the rhodanese domain and the MoaD C-terminus as suggested by Matthies et al. [43], or could itself form an acyldisulfide bond with the MoaD C-terminus prior to formation of the MoaD thiocarboxylate.

3.4 Structure of the NEDD8 Activator

NEDD8 (Rub1 in yeast) is a UbL, which is attached to cullins following activation by a specific E1 and transfer by an E2 enzyme [47]. Cullins are subunits of the SCF

(***S***kp1-***C***ullin-***F***-box protein) family of ubiquitin ligases (E3) and this modification results in an increase in the ubiquitin ligase activity of these enzymes [48, 49]. The E1 enzyme responsible for activation of NEDD8 is a heterodimer composed of the APPBP1 (534 residues in humans) and UBA3 (442 residues in humans) subunits, which share homology (Figure 3.2A) with the N- and C-terminal regions of ubiquitin's E1 enzyme (UBA1). The reactions catalyzed by the E1 for ubiquitin, SUMO and NEDD8 are more complex than in the case of MoeB as established for ubiquitin's E1 [11–13]. While the initial formation of the acyl adenylate between ubiquitin and AMP is a conserved feature, the ensuing covalent attachment of the UbL to an active-site cysteine is specific to the E1 enzymes. This covalent E1–ubl complex subsequently binds another UbL and ATP leading to the formation of a second acyl adenylate. After transfer of the first UbL onto an E2 enzyme, the second adenylated UbL is transferred to the active-site cysteine thereby re-forming the thioester linkage. The crystal structures of the heterodimeric APPB1–UBA3 NEEDD8 activator in its apo-state [50] and in complex with NEDD8 and ATP [51] were reported in 2003.

3.4.1 Overall Structure of the NEDD8-E1

The crystal structure of the heterodimeric NEDD8-specific E1 in the absence of NEDD8 has been determined at 2.6-Å resolution [50]. The complex consists of three structural entities (Figure 3.6A): (1) An adenylation domain formed by the MoeB-like repeats in both APPBP1 and UBA3. As predicted on the basis of the MoaD–MoeB crystal structure the two MoeB-like repeats are arranged in exactly the same way as they are in the MoeB dimer. However, as will become evident shortly, only one active site is present in this heterodimer. In addition, APPBP1 contains a four-helix bundle domain (residues 407 to 485, shown in dark blue in Figure 3.6A), which replaces the disordered loop of MoeB and occupies the region in which the MoaD subunit is located in the MoaD–MoeB complex. This feature contributes to the fact that the APPBP1 subunit is catalytically incompetent. (2) A catalytic domain responsible for thioester formation, which contains the active-site cysteine (Cys 216). This domain is formed by ~80 residues from UBA3 (residues 209–287, shown in light green in Figure 3.6A) including the essential cysteine, and an additional ~225 residues from APPBP1 (residues 169–393, shown in dark green in Figure 3.6A). Both of these domains are α-helical; the segment belonging to UBA3 folds into four α-helices and is inserted at the position of the disordered loop in MoeB (residues 182 to 188), while the additional domain in APPBP1 contains eleven α-helices and is inserted between the β-strand and α-helix corresponding to β6 and α7 of MoeB. (3) Finally, a small, UbL domain (starting at residue 348, shown in orange in Figure 3.6A) is present at the C-terminus of UBA3. These three domains are arranged such that a large groove is formed with the adenylation and catalytic domains on opposite sides and the C-terminal UbL domain on the UBA3-side of the groove. With the exception of a crossover loop, which connects the adenylation domain and the catalytic domain of UBA3, the groove is fully ac-

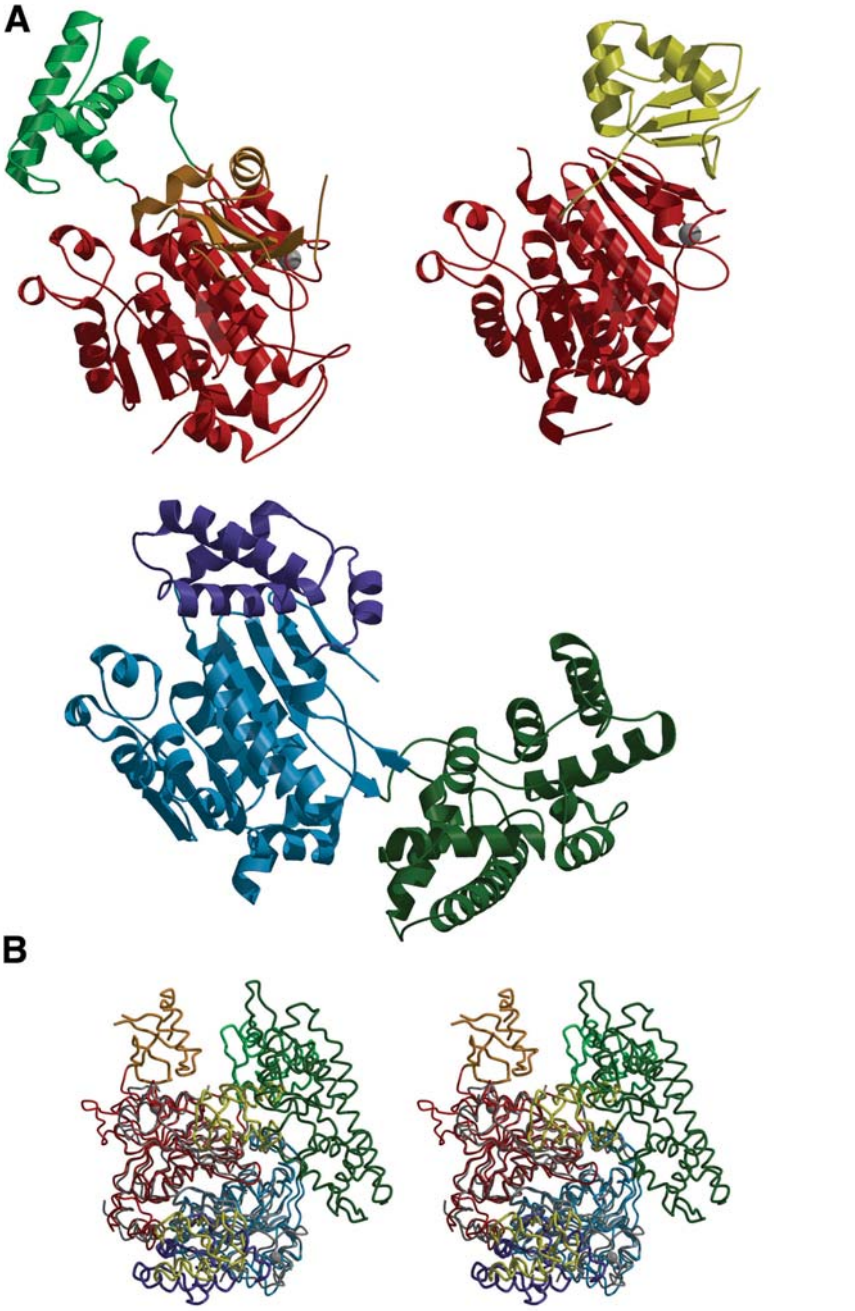


Fig. 3.6. Structure of the NEDD8 activator. (A) Subunit structure of the NEDD8 activator. The UBA3 subunit (upper left) is shown with the MoeB-related adenylation domain in red, the helical insertion containing the active-site cysteine in light green and the C-terminal UbL domain in orange. The APPBP1 subunit (bottom) is shown with the MoeB-related

cessible along its entire length. The groove can be subdivided into two clefts, each of which is of sufficient size to allow binding of either two NEDD8 molecules or one E2 enzyme. Cleft 1 contains the glycine-rich loop involved in ATP binding, while cleft 2 points toward the catalytic cysteine of UBA3. This residue is located at the center of the catalytic domain, in an interface between the segments originating from the UBA3 and APPBP1 subunits, although it is at a substantial distance from the cleft.

Subsequently, the crystal structure of the complex between APPBP1/UBA3 and NEDD8 was determined at 3-Å resolution [51]. NEDD8 is bound in the middle of the groove formed by APPBP1/UBA3 and interacts with the adenylation domain of UBA3 and the large extra-domain of APPBP1 (Figure 3.6A). NEDD8's extended C-terminus is threaded underneath UBA3's crossover loop and extends towards the Gly-rich loop of UBA3. A total of $\sim$3400 Å^2 or 34% of NEDD8's surface area is buried in the complex, and residues involved in these interactions can be mapped to two different areas on the surface: (1) A hydrophobic area including Leu 9, Ile 44, His 68, Val 70, Leu 71 and Leu 73 on NEDD8 interacts with the adenylation domain of UBA3 in a manner analogous to that observed in the MoaD–MoeB interface. This region includes the C-terminus of NEDD8, which contacts the crossover loop and extends into the active site of UBA3. (2) A charged surface patch containing residues Arg 25, Glu 28, Arg 29, Glu 31 and Glu 32, located in the lone α-helix of NEDD8 on the opposite side, contacts the large helical domain of APPBP1. This interaction motif appears to be specific for E1 proteins and is not found in the MoaD–MoeB complex, which explains why significantly less surface area is buried in the MoaD–MoeB complex.

NEDD8 binding is accompanied by conformational changes in NEDD8 and the UBA3 subunit of the activator. The most notable change in NEDD8 involves the C-terminal tail, which rotates around Leu 69 by about 30°. Binding reduces the inherent flexibility, leading to a visualization of the last three residues that contrasts with the structure of free NEDD8 [52]. Conformational changes in the activator are evident in the domain containing Cys 216, which moves away from the adenylation domain, thereby facilitating NEDD8 binding in the widened groove. At the same time the crossover loop moves closer to the floor of the groove (by 2.5 Å) and clamps down on the NEDD8 C-terminus.

The structure also provides insights into how different E1 enzymes discriminate between different UbLs. Ala 72 of NEDD8 appears to be a key determinant of spe-

domain in light blue, the helical extension, which forms part of the catalytic sub-domain in dark green and the additional helical sub-domain in dark blue. The MoaD–MoeB complex (upper right) is shown in the same orientation as the UBA3 and APPBP1 subunits with MoeB in red and MoaD in yellow. The Zn atoms in the UBA3 and MoeB subunits are shown as gray spheres. (B) Stereo diagram of a superposition of the APPBP1–UBA3–NEDD8 (color-coded as defined in (A)) and hetero-tetrameric MoaD–MoeB complexes (MoaD subunits are shown in yellow and MoeB subunits in gray). While the MoaD subunit bound to the MoeB subunit corresponding to UBA3 (red) fits reasonably well, the second MoaD subunit overlaps dramatically with the four-helical insertion (dark blue) in APPBP1.

cificity. This residue participates in van der Waals interactions with Leu 206 and Tyr 207 of UBA3's crossover loop. Ala 72 is replaced by arginine in ubiquitin and when modeled in the APPBP1–UBA3–NEDD8 complex this residue would be forced into close contact with Arg 190 of UBA3. The residue corresponding to Arg190 in the E1 for ubiquitin, however, is a glutamine, which on the basis of the APPBP1–UBA3–NEDD8 complex is predicted to interact favorably with Arg 72 of ubiquitin, a prediction that was confirmed by biochemical studies [51].

In addition to the ternary APPBP1–UBA3–NEDD8 complex the quarternary complex with bound ATP has been described at 3.6-Å resolution [51]. Because Cys 216 was changed to alanine and Mg^{2+} was not present in the crystals, ATP hydrolysis could not take place, thus preventing formation of a NEDD8 adenylate and subsequent thioester formation. ATP is bound in the nucleotide-binding pocket of UBA3 in close proximity to the NEDD8 C-terminus. The adenine base interacts with several hydrophobic residues (Met 80, Ile 127, Leu 145 and Ala 150), which are at least type-conserved in other E1 enzymes. In contrast to ATP binding by MoeB an adenine-specific hydrogen bond is formed between the exocyclic amino group and a glutamine residue (Gln 128 in UBA3); however, this residue is not conserved in other UbL activators. The remaining interactions involve residues that are highly conserved between E1 enzymes and MoeB. Of particular interest are Asp 146 (Asp 130 in MoeB), which is proposed to ligate the Mg^{2+} ion and two Arg residues: Arg 90 (Arg 73 in MoeB) and Arg 15 from APPBP1 (Arg 14 in MoeB). The latter residue is the only one in the ATP-binding site of the NEDD8 E1 that originates from APPBP1. In MoeB this residue is contributed from the other monomer of the MoeB dimer where it contacts the ATP in a similar fashion. As already envisioned based on the NEDD8-E1 structure alone, the active site cysteine is at the significant distance of $\sim$32 Å from the α-phosphate.

3.4.2 Comparison with the MoaD–MoeB Complex

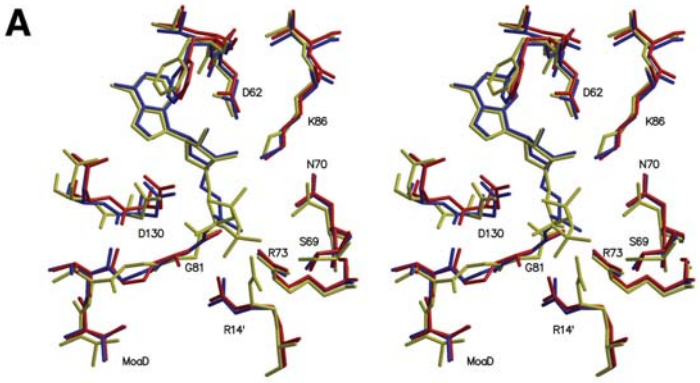
The adenylation domains of APPBP1 and UBA3 are in fact remarkably conserved with MoeB (Figure 3.6A and B). In the case of APPBP1 the regions corresponding to MoeB involve residues 6–168 at the N-terminus (corresponding to residues 5–167 of MoeB), residues 394–404 (169–179 of MoeB) following the larger first insertion and, at the C-terminus, residues 486–534 (189–238 in MoeB) following the smaller second insertion. Overall, the Cα atoms of 220 out of 240 structurally observed residues of MoeB can be superimposed with an rms deviation of 1.6 Å resulting in 19% overall sequence identity. A similar picture is evident when MoeB and UBA3 are compared: at the N-terminus UBA3 residues 12–208 correspond to MoeB's residues 4–181 (an insertion of 12 residues in UBA3 is primarily responsible for the offset in the numbers at the C-terminal ends of the corresponding stretches), while the structural similarities at the C-terminus, following the insertion of the domain containing the catalytic cysteine, involve residues 288–347 of UBA3, which align with residues 189–248 of MoeB. In this case the rms deviation after superposition of the Cα atoms for 230 out of 240 structurally observed resi-

dues are 1.9 Å with 23% overall sequence identity. Another feature that is conserved between MoeB and the UBA3 subunit of the NEDD8 activator is the presence of a bound Zn ion, which is ligated in an analogous manner by the thiolates of four Cys residues.

3.4.3 Conformational Changes during the Formation of the Acyl Adenylate

The available structures of the NEDD8 activator and the MoaD–MoeB complex provide valuable insights into the universally conserved adenylation step catalyzed by members of the E1 enzyme superfamily. The following discussion focuses on the available MoaD–MoeB structures, but also applies to the E1 enzymes. A view into the active site of the apo, ATP-bound, and acyl adenylate forms of the MoeB–MoaD complex reveals subtle conformational changes in the protein (Figure 3.7A). Interestingly, the active sites of the apo and acyl adenylate models have remarkably similar structures. Both contain a sulfate molecule from the mother liquor interacting with Arg B14′, Ser B69, Asn B70 and Arg B73. The positions of the sulfates in each model are nearly identical and correspond to the γ-phosphate in the ATP-bound complex. The only noticeable difference in the two models is seen in the conformation of the MoaD C-terminus where Gly D80 and Gly D81 clearly adopt different conformations as a result of the covalent linkage between the C-terminal glycine and the α-phosphate. The active site of the ATP-bound model shows the most pronounced structural changes. The side chain of Arg B14′, which complements the active site across the MoeB dimer interface, exhibits the largest conformational change. Additionally, the side chains of Ser B69 and Lys B86 adopt different conformations compared to the apo and acyl adenylate models. Finally, the C-terminal Gly–Gly motif of MoaD adopts a different conformation owing to a flip of the peptide bond between Thr D79 and Gly D80.

Multiple sequence alignments using various MoeB and E1 sequences reveal a remarkable degree of conservation for the residues surrounding the active site. In light of the structural data on the MoaD–MoeB and APPBP1–UBA3–NEDD8 complexes, it is possible to assign functional roles to most of the residues in these conserved regions. The loop regions between β_1 and α_3 (secondary structure elements and residue numbers refer to MoeB, but the corresponding regions/residues are also present in the APPBP1–UBA3–NEDD8 complex) consist of a glycine-rich nucleotide-binding motif that facilitates entry of ATP into the respective active sites. The loop region between β2 and helix 3_{10}-A is critical for binding the ribose of ATP. The highly conserved residues forming helix 3_{10}-A are essential for binding the β- and γ-phosphates of ATP and, more importantly, stabilize the pyrophosphate leaving group upon attack by the MoaD or UbL carboxylates. Residues in the loop between β4 and α6 are responsible for the proper positioning of Asp B130 adjacent to the α-phosphate of ATP, and this residue is predicted to be involved in Mg^{2+}-ligation. Arg B135 found inside helix α6 properly orients both the incoming C-terminal extension of MoaD and strand β5 of MoeB, which serves to support the C-terminal Gly–Gly dipeptide.


A
D62
K86
N70
D130
S69
R73
G81
R14'
ModD
D62
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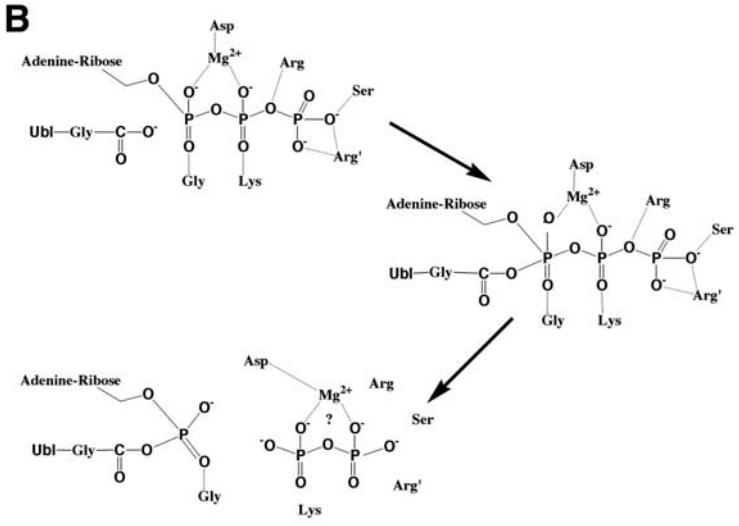
B
Asp
Mg2+
Adenine-Ribose
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Ubl—Gly—C—O-
Gly
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Arg'
Asp
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Arg
Ser
Ubl—Gly—C—O
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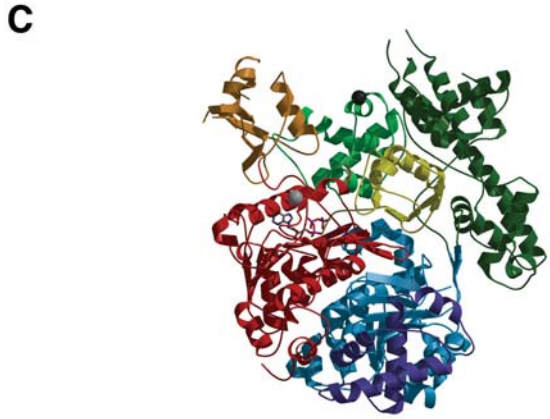
C

A reaction mechanism for UbL activation can be formulated based on the MoaD–MoeB and the APPBP1–UBA3–NEDD8 structures (Figure 3.7B). After Mg^{2+}-ATP and UbL binding at the active site, the carboxylate oxygen of the terminal glycine of the UbL attacks the α-phosphate of ATP creating a transient pentacovalent intermediate. The divalent metal appears to be required to overcome the electrostatic repulsion between the UbL C-terminus and the α-phosphate. Subsequently, the bond between the α- and β-phosphates is cleaved, which could be facilitated by the strained conformation of the triphosphate observed in the ATP-bound structures of the MoaD–MoeB and APPBP1–UBA3–NEDD8 complexes. Furthermore, the developing negative charge on the β-phosphate appears to be stabilized by two arginine residues, which in MoeB correspond to Arg B73 and Arg B14′ of the second MoeB monomer. In the E1 enzymes the second arginine, corresponding to Arg B14′ of MoeB, is contributed from the second MoeB-like repeat in the case of the single subunit E1s such as the ubiquitin activating enzyme, or the second subunit as is the case for the APPBP1 subunit of the hetereodimeric NEDD8-E1. The importance of Arg14, Arg73 and Asp130 of MoeB has been demonstrated by site-directed mutagenesis and a nitrate-reductase overlay assay [38].

Following the formation of the acyl adenylate the reactions will proceed along different paths. In the MoaD–MoeB complex a thiocarboxylate will be formed and the current knowledge regarding this step has been summarized (Section 3.3.5.). In contrast, the E1 enzymes will form the covalent thioester linkage between their active site cysteine and the UbL C-terminus. A remarkable observation of the NEDD8–APPBP1–UBA3–ATP complex in this context has been the large distance (~32 Å) between this cysteine and the α-phosphate of ATP, which apparently requires substantial conformational changes for the reaction to proceed. As mentioned earlier a second UbL protein will bind to the E1 enzymes and this process could trigger the necessary conformational changes leading to the formation of the thiocarboxylate. Another issue that is poorly understood is binding of the cognate E2 enzyme and the resulting transfer of the UbL to E2. Clearly more structural and mechanistic studies are required to understand the complex mechanism of the E1 enzymes in detail.

Summary

Recent structural studies of the bacterial MoaD–MoeB system have demonstrated that the E1-catalyzed activation of UbL proteins is derived from a more ancient and

Fig. 3.7. Adenylation reaction. (A) Stereo representation of a superposition of the MoaD–MoeB complex in its apo-state (red), in complex with ATP (yellow) and after formation of the acyl adenylate (blue). (B) Proposed reaction scheme for the formation of the acyl adenylate. Arg′ refers to the second arginine originating either from the second subunit in case of MoeB and hetereodimeric E1 enzymes, or the N-terminal MoeB-repeat in single subunit E1 enzymes. (C) The structure of the NEDD8 activator in complex with NEDD8 (yellow) and ATP (all-bonds representation) is shown with the same subunit and domain color code defined in Figure 3.6A. The active-site cysteine and the bound Zn are indicated as black and gray spheres, respectively.

widespread step during Moco biosynthesis, namely the temporary incorporation of sulfur as a thiocarboxylate. The MoaD protein is thus the evolutionary ancestor of ubiquitin and other UbLs and due to its near-universal presence in all phylogenetic kingdoms occupies a position ubiquitin was envisioned to assume when it originally received its name and was thought to be present in all kingdoms of like [53]. On the other hand, the MoeB protein, as already deduced by sequence comparisons, is the ancestor of the E1 enzymes. All E1-catalyzed reactions including MoeB involve the formation of an acyl adenylate with the C-terminus of a small protein, but subsequently diverge with the formation of either a thiocarboxylate or a thioester. The factors dictating whether a thiocarboxylate or a thioester is formed are not fully understood at present. After the activation step was adopted to fulfill secondary functions in eukaryotes an additional transfer step, catalyzed by the E2 enzymes, was presumably added and, in the case of ubiquitin, also the complex diversity of the E3 enzymes evolved, which ensures that only selected proteins are degraded at the appropriate time by the proteasome.

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4
RING Fingers and Relatives: Determinators of Protein Fate

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4.1
Introduction and Overview

As recently as 1998, the RING finger was a structure without known function, and was often confused with the zinc finger. Three years later this compact structure was rapidly becoming one of the most widely studied protein modules because its presence in proteins has strong predictive value for ubiquitin ligase activity. An exhaustive treatment of RING finger ubiquitin ligases would warrant an entire volume by itself. Therefore, this chapter will provide general information on the RING finger and its structural relatives. This is followed by brief synopses of several well-studied families of RING finger proteins implicated in cell regulation and signaling. These examples are Siahs, IAPs, TRAFs and Cbls. These are intended to illustrate the biological importance and complexities of RING finger proteins. We will then provide more detailed discussions on two RING finger proteins: Parkin, associated with autosomal recessive juvenile Parkinson's disease and Mdm2, the most well-known and extensively studied cellular ubiquitin protein ligase for the tumor suppressor p53.

4.1.1
Historical Perspective

Ubiquitylation of proteins, i.e. their conjugation with ubiquitin, has dramatic effects on their fate and function. Its most well-described role involves proteasomal degradation as a consequence of modification with K48-linked chains of ubiquitin, but it has other roles not linked to proteasomal degradation. These include enhancement of endocytosis and targeting to lysosomes (vacuoles in yeast), DNA repair, transcriptional regulation and kinase activation. Ubiquitylation is a hierarchical, multi-step process generally involving, at a minimum, enzymes known as ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s) and ubiquitin protein ligases (E3s). E1 forms a thiolester linkage with the C-terminus of ubiquitin via an ATP-dependent reaction. Ubiquitin is then transferred to one of

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over 30 (in mammals) E2s where a second thiolester linkage is formed. E3s interact with E2 and substrate, which then mediate the transfer of ubiquitin to substrate [1–5].

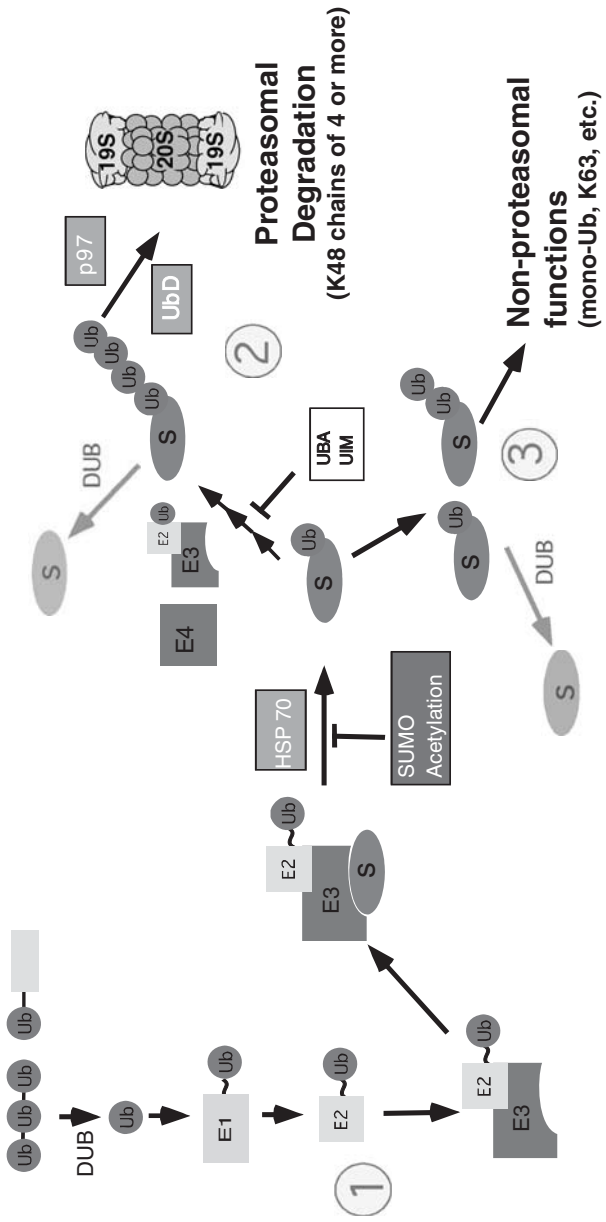
Because of the large number of known ubiquitylation substrates and since E3s recognize specific proteins or modified forms of proteins, the number of E3 specificities is necessarily enormous. However, until recently few E3 had been molecularly characterized. By 1992, for example, 13 E2s had been described in yeast [6]. Of these, 11 function with ubiquitin and one with each of two different ubiquitin-like (UbL) proteins, Sumo and Nedd8/Rub1 [2]. However, while several yeast and metazoan E3s had been identified biochemically by that time, the primary amino acid sequence was known only for a single E3 [7]. This was the *Saccharomyces cerevisiae* Ubr1p, which had been studied extensively by Varshavsky and co-workers in delineation of the N-end rule [8, 9]. Its mammalian ortholog, E3α, was the prototypical E3 used in the description of the basics of the ubiquitin pathway by Hershko and Ciechanover [1, 10].

A major advance in the identification of E3s came with the discovery by Howley and co-workers of E6-AP (***E6*** protein-***a***ssociated ***p***rotein). This cellular protein functions as an E3 for p53 in human papilloma virus (HPV)-infected cells. Specifically, oncogenic strains of HPV (HPV-16 or -18) express a protein, E6, that usurps the E3 function of E6-AP [11, 12]. E6-AP was found to resemble other deduced protein sequences in cDNA databases. This led to the identification in 1995 of a family of proteins characterized by a C-terminal 350 amino acid HECT (***h***omologous to ***E***6-AP ***c***arboxyl ***t***erminus) domain [13]. Many of these have now been shown to be E3s. HECT E3s generally recognize substrates through their N-terminal halves. HECT domains interact with E2s and form transient thiolester linkages with ubiquitin before transferring ubiquitin to target substrates (see Chapter 5). Based on searches using the BLAST program [14, 15], there are 27 HECT proteins encoded within the human genome, not considering splice variants.

As HECT domain function was being elucidated, studies in the mid-1990s on cell-cycle regulation led to the initial description of SCF E3s. These were first characterized as containing ***S***kp1, ***C***ullin-1, and an ***F***-box-containing protein [16, 17]. Also identified during the mid-1990s was the APC (***a***naphase ***p***romoting ***c***omplex – also known as the cyclosome), another multi-subunit cullin-containing E3 that mediates ubiquitylation of mitotic cyclins [18, 19]. Mdm2, initially thought to be a HECT domain variant, was shown in 1997 to have E3 activity towards p53 *in vitro* [20]. For the non-HECT E3s no common structural feature had been detected. Thus, by 1997 the only defined molecular signature for E3 activity was the HECT domain.

In 1999, the world of E3s began to change dramatically. Using a yeast two-hybrid approach, a protein of unknown function, AO7, was identified in the Weissman laboratory as a binding partner for a human E2, UbcH5B, and was found to mediate UbcH5B-dependent self-ubiquitylation [21]. These properties depended upon a motif known as the RING finger, which has the general sequence $CX_2CX_{(9-39)}CXHX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$ (Figure 4.2).

The term RING finger was first coined by Freemont and colleagues referring to a gene product in the MHC class I locus [22]. Their apparent exuberance led to the


Ub
DUB
E1
E2
E3
1
HSP 70
SUMO
Acetylation
S
E4
UBA
UIM
2
Proteasomal
Degradation
(K48 chains of 4 or more)
19S
20S
19S
p97
UbD
DUB
3
Non-proteasomal
functions
(mono-Ub, K63, etc.)

descriptor ***R***eally ***I***nteresting ***N***ew ***G***ene 1 (RING1). Little did they know how interesting it would be! Determination of the structure of several RING fingers [23–29] revealed that the RING finger consists of two Zn binding sites with a total of eight cysteine and histidine ligands. They may be ordered either as C3H2C3 (RING-H2) or C3HC4 (RING-HC) with the resultant fold assuming a cross-braced type arrangement (Figures 4.2 and Figure 4.3). AO7 was determined, specifically, to be a RING-H2 protein. Strikingly, each of four other RING-H2 proteins, for which there was no prior evidence for roles in ubiquitylation, were also demonstrated to have RING finger-dependent E3 activity. The RING-HC proteins Brca1 (***Br***east ***c***ancer ***a***ssociated protein 1) and Siah1 (***s***even ***i***n ***a***bsentia ***h***omolog 1) were found to behave in a similar manner [21].

Coincidentally, while initial E3 studies were ongoing, the Ashwell lab (next door to the Weissman lab), had evidence for a potential role for proteasome activity in IAP (***i***nhibitor of ***ap***optosis) function. A connection became clear with the determination that most IAPs include RING-HC fingers and have E3 activity [30]. It shortly became apparent through studies by a number of groups that many proteins whose functions had been associated with ubiquitylation, such as Cbls, Mdm2, Ubr1p/E3α and Hrd1p/Der3p are RING finger E3s [31–34]. Similarly, the presence of a small RING finger protein, Apc11, within the multi-subunit APC took on new significance [35].

Remarkably, at the same time, several groups independently determined that there was a previously undiscovered component to the SCF complex – a small non-canonical RING finger protein known variably as Rbx1, Roc1 or Hrt1 [36–

Fig. 4.1. Fundamentals of the ubiquitin system. Adapted from Ref. [5]. Figure 4.1 shows the fundamentals of the ubiquitin system. (1) Ubiquitin is synthesized in linear chains or as the N-terminal fusion with small ribosomal subunits that are cleaved by deubiquitylating enzymes to form the active protein. Ubiquitin is then activated in an ATP-dependent manner by E1 where a thiolester linkage is formed. It is then transthiolated to the active-site cysteine of an E2. E2s interact with E3s and with substrates and mediate either the indirect (in the case of HECT E3s) or direct transfer of ubiquitin to substrate. A number of factors can affect this process. We know that interactions with Hsp70 can facilitate ubiquitylation in specific instances and competition for lysines on substrates with the processes of acetylation and sumoylation may be inhibitory in certain instances. (2) For efficient proteasomal targeting to occur chains of ubiquitin linked internally through K48 must be formed. This appears to involve multiple cycles of E3-mediated transfer of ubiquitin or in some cases other factors known as E4s may play a role in facilitating the processivity of polyubiquitin-chain formation. Interactions with proteins containing UbDs (***ub***iquitin ***d***omains), including some E3s, may facilitate targeting to the proteasome. For a number of subsrates an ATPase known as p97 (also known as VCP or in yeast as Cdc48p) facilitates transport to proteasomes. (3) The factors that influence the balance between K48 chains and mono-ubiquitin or other linkages, such as K63, are poorly understood. However, ubiquitin-binding domains such as the UBA or UIM could influence this balance in cells by blocking K48 on ubiquitin and thus favoring chain termination or other linkages [82] – this is a point that is far from being established with certainty. Anywhere along the pathway deubiquitylating enzymes may reverse the process, including at the level of the proteasome itself.

(A)
RING, PHD, FYVE fingers
Cross-brace Zn-binding structures
U box
cross-brace
Hydrogen bonds/Salt bridges
Zinc Finger
(B)
BRCA1 RING
c-cbl RING
APC11 RING
Praja1 RING
gp78 RING
RING Motif
KAP1 PHD
MEKK1 PHD
MF-X1 PHD
AIRE PHD
PHD Motif
HRS FYVE
EEA1 FYVE
AMKHZM FYVE
KUFT1 FYVE
FYVE Motif
Ufd2 U box
CHIP U box
PRP19 U box
KIAA0684 U box
Consensus

39]. It was also found that Rbx1 was central to the E3 activity of the newly described CBC^{VHL} complex [40, 41]. During this time, others demonstrated binding of E2s to RING finger proteins – although in these cases the connection with ubiquitylation was not made [42]. So, by mid-2000 there was a new family of over 300 additional potential human E3s.

Things did not stop with the identification of the RING finger. The PHD (***p***lant ***h***omeo***d***omain) or LAP (***l***eukemia-***a***ssociated ***p***rotein) domain represents a variation on the RING finger [43, 44]. A second more distant RING finger relative has been identified as the U-box (see below) [45]. Members of both of these have now been shown to have E3 activity [46–51]. Thus, from 1998 to 2002 we had gone from a family of perhaps 27 potential human E3s, which could be discerned based on their primary amino acid sequence, to over 400. Additionally, substantial heterogeneity in substrate recognition is provided by the cullin-containing E3s.

4.2
RING Fingers as E3s

4.2.1
General Considerations

It is reasonable to divide RING finger E3s into two major types, cullin and non-cullin E3s, the latter being the primary focus of this chapter. However, to put the full range of RING finger E3 specificities into perspective some mention of the cullin E3s is required. cullin-containing E3s are remarkable in having the capacity to bind to and ubiquitylate multiple different targets through use of different substrate-recognition subunits. These E3s have a small RING finger protein (in most cases Rbx1/Roc1/Hrt1) associated with a specific member of the cullin family.

Fig. 4.2. Comparison of RING finger and RING finger-related motifs. (A) Schematic representation of RING, PHD and FYVE fingers (left), U-box (center), and tandem classical zinc fingers for comparison (right). (B) Multiple alignments of representative RING, PHD, and FYVE, fingers. Adapted from Ref. [46]. In Figure 4.2A, numbered residues in RING fingers represent metal coordinating residues. A canonical RING finger has histidine in position 4, cysteine in positions 1–3, 6–8. RING fingers are classified as RING-H2 or RING-HC depending on whether position 5 is occupied by histidine (-H2) or cysteine (-HC). Canonical PHD finger consensus has histidine in position 5. FYVE finger includes (R/K)(R/K)(HHCR) insertion indicated in purple. PHD finger includes invariant tryptophan two amino acids before the seventh coordinating residue indicated in blue. In the U-box the predicted conformation is conferred by hydrogen bonding and salt bridges, indicated schematically by dotted lines. In Figure 4.2B, predicted metal-coordinating residues are indicated in blue boxes. Consensus RING, PHD and FYVE finger motifs are indicated below each grouping. The position of tryptophan conserved in PHD fingers is indicated in blue. The FYVE finger insert is in purple. The canonical RING, PHD and FYVE finger motif is indicated under each set of alignments. For the U-box, conserved residues are indicated in red.

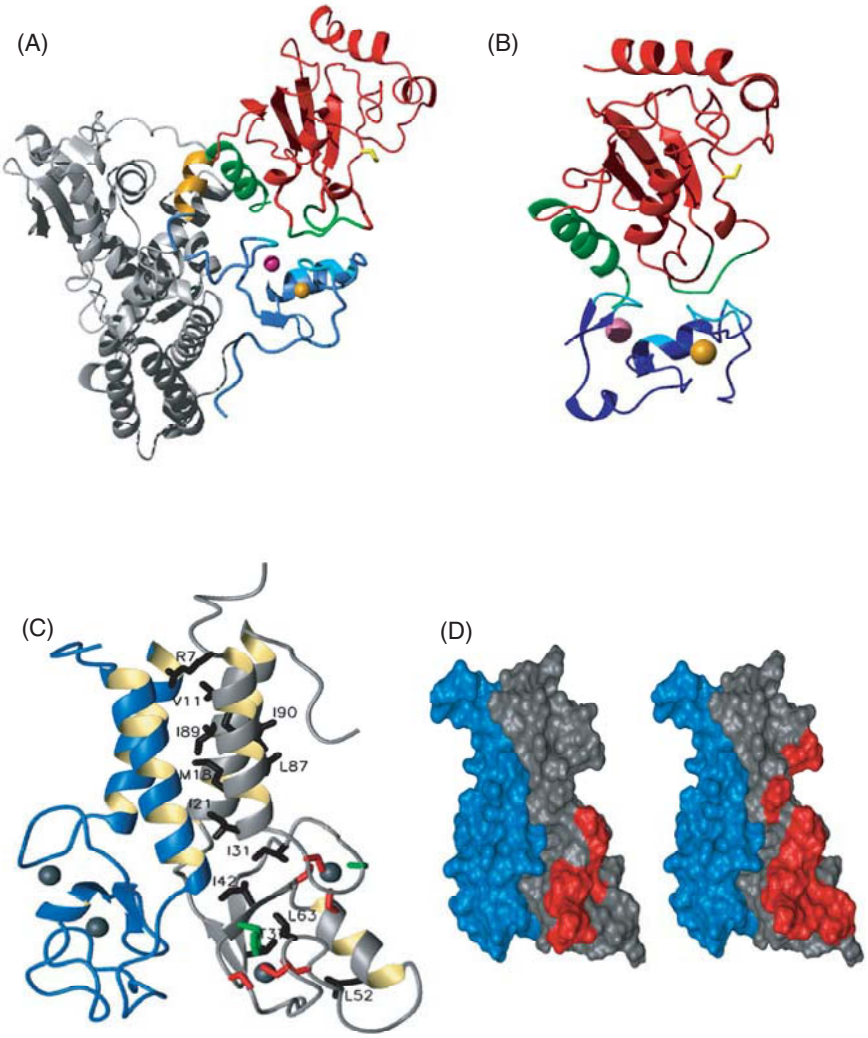


Fig. 4.3. Structures of RING-E2 complexes and RING dimers. (A) c-Cbl and UbcH7 based on crystal structures [23]. (B) Cnot4 and UbcH5B based on NMR solution structures and molecular modeling [28, 70]. (C) Solution structure of Brca1–Bard1 dimer [25, 77]. (D) Space-filling model of the Brca1–Bard1 dimer showing (right) sites of interaction with UbcH5C with which it functionally interacts, and (left) less extensive sites of interaction with UbcH7, for which there is no evidence of functional interaction. (C and D adapted from ref. 25 and ref. 77 with permission). In Figure 4.3A, Cbl is shown in silver, its RING finger region is shown in blue, and E2 in red. Points of interaction of UbcH7 on c-Cbl (RING finger and linker α-helix) are indicated in cyan and Cbl-interacting sites on E2 are shown in green. Note that most interactions involve a linker α-helix of Cbl (shown in orange), loops including the cysteine pairs that coordinate the first (shown in pink) and last (shown in orange) Zn molecule and the central α-helix of the RING finger. In Figure 4.3B, E2, RING, active sites, contacts and Zn residues are colored as in Figure 4.3A. Interactions involve the same general regions of the E2 and RING finger with no evidence of involvement of regions outside

cullin-containing E3s include the SCF E3s, where F-box proteins recognize substrates and Skp1 connects the Cul1 core to F-box proteins [17, 24, 52–55]. For the CBC (elongin ***C***, elongin ***B***, ***C***ul2 or Cul5) E3s the dimer of elongin C and elongin B play an analogous role to Skp1. VHL (***V***on-***H***ippel-***L***indau), the first defined substrate-recognition element for the CBC complex, or other members of the SOCS (***s***uppressor ***o***f ***c***ytokine ***s***ignaling) box proteins, serve to recognize the substrate [40, 56, 57]. More recently the Cul-3 BTB/POZ (***B***ric-a-Brac ***T***ramtrack and ***B***road Complex/***Po***x virus and ***Zn*** finger) family of E3s has been identified. In these complexes BTB proteins both recognize substrate and link to Cul3, combining the roles played by the Skp1 and F-box proteins of the SCF [58–60]. The most complex cullin-containing E3 is the anaphase promoting complex, APC. Apc2 is a cullin, and Apc11 is a small RING finger protein. The APC complex also includes at least nine other essential subunits, a large number of which include TPR (***t***etratrico***p***eptide ***r***epeat) protein–protein interaction domains. Tandem TPR repeats generate a right-handed helical structure with an amphipathic channel that is thought to accommodate an α-helix of a target protein [61]. These subunits may therefore serve to hold the APC complex together or to bind substrates. Other known APC substrate-recognition elements include Cdc20 and Cdh1/Hct1 [62–64]. In general, the cullin-containing E3 is written with the family name followed by the substrate-recognition element in superscript. Thus, the SCF E3 for IκB may be written as $SCF^{\beta TRCP}$, while the E3 that recognizes hypoxia inducible factors, HIF-1α and HIF-1β, is CBC^{VHL}. As shorthand, cullin-containing E3s are often referred to as "multi-subunit E3s" to underscore the need for multiple subunits for discernable activity. This is clearly an over-simplification, as single subunit, non-cullin, E3s can also function in the context of more complex assemblies. For more detail on cullin-containing E3s see Chapters 6 and 7.

An obvious question is whether all consensus RING fingers mediate ubiquitylation. Our view, based on the literature, our experience and discussions with colleagues, is that the large majority do. A few RING proteins persist in showing no E3 activity, as defined by the minimal criteria of E2-dependent self-ubiquitylation *in vitro*, despite experimental evaluation by multiple laboratories. However, almost all of these persistent negatives influence the ubiquitin pathway. The best example of this is Bard1 (***B***rca1-***a***ssociated ***R***ING ***d***omain 1). Inactive as an E3 by itself, Bard1 dimerizes with Brca1 and greatly enhances the latter's E3 activity [21, 65, 66] (Figure 4.3C and D). Furthermore, failure to express Bard1 markedly destabilizes Brca1 [67]. Until recently, MdmX had not been reported to be an E3. However, there is now evidence that MdmX has at least a small amount of ligase activity

the RING finger. Figure 4.3C shows Brca1 in silver, Bard1 in blue. Zn-binding residues are indicated in green. Known mis-sense mutations of Brca1 are labeled. Mutations of residues marked in red have been linked to familial cancers; other mutations (in black) have been observed in patients with sporadic breast or ovarian cancer. Extensive E2-interacting regions on Brca1 extend beyond the RING finger to include regions in the α-helices N- and C-terminal to the RING that form the four-helix dimerization interface.

[68]. Perhaps, analogous to Bard1, MdmX may modulate the E3 activity of Mdm2 towards p53 through dimerization (see below). Ironically, PML (***p***ro***m***yleocytic ***l***eukemia), one of the first RING finger proteins to be characterized has, to date, not shown E3 activity (see below).

4.2.2 Structural Analysis and Structure–Function Relationships

How do RING fingers function to mediate ubiquitylation? Unlike HECT domains, which function as catalytic intermediates in the transfer of ubiquitin from E2 to substrate, there is no evidence that RING fingers play such a role. A minimalist view is that RING fingers act as E2 docking sites. As such, they would position the complex of E2 and ubiquitin allowing for nucleophilic attack from a substrate lysine onto the C-terminus of ubiquitin [69]. In this model, the RING finger is not expected to affect the catalytic reaction required for ubiquitin transfer. Consistent with a docking role, the crystal structure of the c-Cbl RING finger with an E2, UbcH7, provides no evidence for a catalytic role in RING finger function (Figure 4.3A). This is similarly the case for the solution structure of the Cnot4 RING finger with UbcH5B. The Cnot4 RING finger is atypical in having only cysteine residues coordinating Zn, but otherwise has a consensus RING finger sequence [70] (Figure 4.3B). For both of these the active site cysteine of the E2 projects away from, and is not in close proximity to, the RING finger. Thus, based on these structures, there is little to suggest direct interactions between the RING finger and the site of ubiquitin attachment on E2.

Despite the structural data, this docking-site model may be an over-simplification of how RING fingers function. There is evidence that E2 binding without an intact RING finger is not sufficient to target substrates for degradation. This is based on the analysis of other E2 binding domains, including those found within the yeast protein Cue1p [34, 71] and in gp78 [72]. The latter, also known as AMFR (the ***a***utocrine ***m***otility ***f***actor ***r***eceptor), is a mammalian E3 involved in ERAD (***e***ndoplasmic ***r***eticulum ***a***ssociated ***d***egradation). It includes both a RING finger and a distinct E2 binding site [72], both of which are required for proper E3 activity in the presence of its cognate E2. Also contrary to the minimalist view are instances where E2–RING finger interactions occur but lack functional activity. This is the case for UbcH7 and the Brca1/Bard1 heterodimer (see below) [23, 25]. It is, therefore, reasonable that the RING finger might act as an allosteric co-factor whose binding to E2-Ub alters the E2 so as to facilitate transfer of ubiquitin to substrate.

4.2.2.1 RING finger–E2 Interactions

For both Cnot4 and c-Cbl there are several points of contact between the E2 (shown in red) and the RING finger (shown in blue in Figure 4.3A and B). These include residues in the first α-helix of the E2 and two loops that extend down from the core

of the E2 into the RING finger (shown in green). One precedes and one immediately follows the active-site cysteine (yellow side chain). The first E2 α-helix contacts residues close to the first cysteine pair of the RING finger (shown in cyan around the first Zn molecule, which is depicted in pink). The first loop of the E2 interacts with the central α-helix of the RING finger at one or more points (contacts in cyan), while the second loop of the E2 contacts residues near the final cysteine pair (contacts in cyan). However, there is variation between the two E2–RING finger pairs as to the specific residues involved. In addition, c-Cbl also contacts the first α-helix of the E2 through a linker α-helix immediately N-terminal to the RING finger (indicated in orange). On the other hand, for Cnot4 all of the E2 contacts are confined to the RING finger. While Cnot4 exhibits robust activity with UbcH5B *in vitro*, it is notable that there is little evidence for direct functional interactions between c-Cbl and UbcH7.

A third RING finger protein for which E2 interaction data is available is Brca1. Notably, mutations of Zn-coordinating and other residues in the Brca1 RING finger are associated with familial breast and ovarian cancers [73–76]. By itself, Brca1 has weak E3 activity, which is dramatically enhanced by dimerization with Bard1, which also contains a RING finger. While the two RING fingers are juxtaposed in the dimeric structure (Figure 4.3C), the major determinants of dimerization are α-helices N- and C-terminal to each RING finger. These form a four α-helix bundle [25]. NMR studies have examined the binding of the Brca1-Bard1 heterodimer to both UbcH5C, with which this E3 functions, and UbcH7, which has no activity with the heterodimer as assessed by self-ubiquitylation [77].

Although the Brca1-Bard1 heterodimer contains two RING finger subunits, UbcH5C and UbcH7 interact only with the Brca1 subunit. One reason for this may be the lack of a central α-helix in the Bard1 RING finger. These central α-helices may be one essential element required for E2 contact. UbcH5C contacts the Brca1 RING finger through an extended 17 amino acid interface on the RING finger (Figure 4.3C and 3D). Points of contact on the Brca1 RING finger are more extensive than those seen for c-Cbl and Cnot4, although similar regions of the RING finger are involved. Interactions with UbcH5C extend beyond the Brca1 RING finger forming additional interfaces on the two adjacent Brca1 α-helices. In contrast to UbcH5C, UbcH7 forms a single, less extensive interface involving 10 Brca1 RING finger residues. Despite the differences in interactions, both E2s show only weak affinity for the Brca1 RING finger, and NMR titration experiments found no large difference in affinity between the two E2s. These observations may be explained if UbcH5C and UbcH7 bind to Brca1 in similar, but not identical, orientations.

Interestingly, Ile26, which immediately precedes the second cysteine of the Brca1 RING finger, is a contact point for both UbcH5C and UbcH7. While not required for the coordination of Zn, this residue is also essential for maintaining E3 activity. Conversely, mutations in other E2-contact residues in the RING finger, particularly those in the central α-helix of Brca1 can be altered with little functional consequence [77]. The residues corresponding to Ile26 in both Cnot4 (Leu16) and c-Cbl (Ile83) are also included in E2 interaction interfaces. Again, however, there is

substantial variation in E2 contact points on the central α-helices of these RING fingers. Consistent with the structural observations, inspection of a number of RING proteins (Figure 4.2) reveals a high degree of conservation of the residue immediately preceding the second Zn-coordinating cysteine. Similar conservation is found surrounding other Zn-coordinating residues. With exceptions [23, 31], this is not the case for amino acids corresponding to the central α-helix of RING fingers.

While the major site of interaction for each E2–E3 pair is within the RING finger, there are additional binding surfaces for E2 on c-Cbl and Brca1 outside of this region. These contacts vary between RING finger proteins. For example, those c-Cbl residues implicated in E2 binding that are N-terminal to its RING finger, do not correspond to those non-RING finger residues implicated in Brca1–UbcH5C interactions. The structural data summarized in Figure 4.3 also provides mechanistic insights into the sequential actions of E1, E2 and E3 enzymes. In addition to interacting with RING fingers (and HECT domains), the first α-helix of E2 is also a major site of interaction with E1 [78]. This supports a mechanism where E2 must receive ubiquitin from E1 prior to association with E3. In considering the structures depicted in Figure 4.3, one should note that all were determined using E2 without bound ubiquitin. The possibility that E2 loaded with ubiquitin will provide different results cannot be overlooked.

As noted above for Brca1, E2–RING finger interactions do not need to be of high affinity to be productive. While UbcH5B binding is easily detectable for the RING finger protein AO7, stable binding of E2s to RING fingers is not generally the norm. In considering this generality one needs to keep in mind that generation of poly-ubiquitin chains on proteins requires repetitive transfers of ubiquitin from E2 to substrate. As such, E2s exist in both ubiquitin-bound and -unbound states. It follows that an ideal RING finger, whether as an E2 binding platform or as a means to decrease the activation energy for ubiquitin transfer, must not bind the E2 so tightly as to limit its dissociation after transfer of ubiquitin to the target protein. Consistent with this notion are observations that mutations outside the RING of AO7 that reduce E2 binding correlate with increased self-ubiquitylation (Lister, K. M., Lorick, K. L., Jensen, J. P. and Weissman, A. M., unpublished observations) and that release of E2-ubiquitin by the SCF complex E3 appears to be an important first step in substrate ubiquitylation [79].

One point that arises from study of these E2–E3 structures, is that there is a degree of specificity in their physical interactions. The biochemical data extends the idea of physical specificity in E2–E3 interactions to functional specificity. An example of this is the Brca1-Bard1 heterodimer, which interacts physically with UbcH7 and UbcH5C, but is only active with UbcH5C. There appear to be two consequences of E2 specificity: variability in the strength of a particular E3 response and variability in the type of ubiquitin modification.

The Snurf (***s***mall ***nu***clear ***R***ING ***f***inger) protein has E3 activity using at least six different E2s [80], all of which appear to have different patterns of ubiquitin modification. On the other hand AO7 and Brca1 only appear active with members of the

UbcH5 family [21, 80, 81]. Presumably, the ability to interact productively with a smaller number of E2s will restrict E3 activity to situations where the RING finger protein and the E2 are co-expressed and co-localized.

Because ubiquitylation is not limited to degradation of substrate proteins – a process primarily utilizing K48-linked poly-ubiquitin chains [82] – the use of different E2s may control the type of ubiquitin modification or ubiquitin chain formed. Brca1-Bard1 has been shown to form K6-linked and K29-linked ubiquitin chains when employing UbcH5C as the E2 [83]. When employing the closely related but distinct UbcH5B, the same E3 can form K63-linked chains [66]. The RING finger protein, Rad5 binds to Mms2-Ubc13 to form K63-linked ubiquitin chains in the DNA repair process [84, 85]. This linkage requires the complex of another RING finger protein, Rad18, and an additional E2, Rad6 [85]. While there is no data to suggest the E3 proteins involved in this process form other ubiquitin linkages, Rad18 has been implicated in proteasomal degradation of Ho endonuclease in yeast [86], implying that it may form K48-linked chains.

4.2.3
Other Protein–Protein Interaction Motifs in RING finger Proteins

A remarkable feature of RING fingers is their small (up to 70 amino acids), compact nature. This is in contrast to the considerably larger elongated HECT domain (~350 amino acids). Perhaps one consequence of this small size is its inclusion into a large number of proteins having a number of different protein-interaction modules. The RING finger thereby provides ubiquitin ligase activity to a wide range of otherwise functionally divergent proteins. Accordingly, the means by which RING finger E3s interact with substrates are highly variable and run the gamut of protein–protein interactions (http://home.cancer.gov/lpds/weissman). As discussed above, small RING finger proteins can interact with a large number of substrates in the context of a cullin-containing complex. For others, the size and complexity of the ligase presents the possibility for many different interactions. Well-characterized examples of this include Cbl proteins [87] (see below) and the Brca1-Bard1 heterodimer [88]. Protein interaction domains found in this dimeric E3 include: the Brca1 RING finger, which, in addition to interacting with E2, has been reported to bind the de-ubiquitylating enzyme Bap1 [89]; BRCT (***Br***ca1 ***c***arboxyl-***t***erminal) domains, found in both Brca1 and Bard1, which bind to basal transcription machinery [90]; the large non-conserved central region of Brca1, whcih binds DNA repair enzymes and transcription factors [91–93]; and the Bard1 ankyrin repeats, which may interact with a number of different proteins and may induce apoptosis [94].

RING finger proteins may have other domains associated with signal transduction, such as SH3 and STAT domains and domains that bind and effect hydrolysis of nucleotides in signal transduction or for other purposes. These include ATPases, ATP synthases, serine/threonine kinases, GTP-binding domains, ADP-ribosylation domains and AAA-superfamily ATPases (http://home.cancer.gov/lpds/weissman).

One example that encompasses two of these in one protein is Ard1 (***ADP-r***ibosylation factor ***d***omain 1), a 64-kDa protein with an ADP-ribosylation factor domain linked to an N-terminal GTPase domain [95, 96]. The Ard1 GTPase domain physically binds its ADP-ribosylation factor domain, stimulating hydrolysis of bound GTP. It has been suggested that this protein plays a role in vesicular trafficking, and it is also a member of the TRIM family of RING finger proteins (see below). The RING finger is found in other proteins associated with organelle transport, including those with kinesin motor domains, peroxisome domains (Pex3, Pex10 and Pex12), and clathrin heavy chain repeats (Vps11) (http://home.cancer.gov/lpds/weissman).

The RING finger motif is also found in numerous proteins having domains associated with nuclear functions (e.g. helicases, DNA repair ezymes). It is also found in proteins having domains associated with the establishment and maintenance of intracellular and extracellular matrices (e.g. scaffold/matrix specific factors, Band4.1, ezrin/radixin homologs). A number of other protein-interaction domains in RING finger proteins, such as WD40 repeats, PDZ domains, sterile α motifs or TPR domains, are not easily pigeonholed as being either organelle- or function-specific. Curiously, RING fingers are frequently found in proteins with a variety of other Zn-binding structures (Table 4.1). In many cases these cysteine-rich domains have no effect on E3 activity, but may mediate interactions with proteins and nucleic acids.

There are a number of RING finger E3s that contain transmembrane domains, including some without characterized substrates, such as Kf-1 and Trc8 [21]. One example for which a direct substrate interaction has been determined is Rnf5, which interacts with and mediates ubiquitylation of paxillin [97]. However, for other transmembrane RING finger E3s, the means of interaction with a substrate does not involve easily traceable direct or even indirect protein–protein interactions. The best example of this is in ERAD, where a single E3 has the potential to target multiple structurally unrelated substrates. For one such RING finger E3, the yeast Hrd1p/Der3p, there is evidence for specific indirect interactions through Hrd3p in targeting yeast HMGCoA reductase [98]. For other Der3p substrates, including the test substrate CPY*, there is little evidence for substrate–ligase interactions [99]. This is also the case for the mammalian transmembrane ERAD E3, gp78 and its substrates such as the T cell antigen receptor CD3-δ subunit and Apolipoprotein B [72, 100]. Whether ERAD substrates are recognized by co-localization in membrane sub-domains, through adaptors or chaperones, or a combination of these remains to be determined.

Some RING finger and related E3s possess regions related to ubiquitin, known as ubiquitin domains (UbD). Examples of these are Parkin and Hoil-1, both of which also contain two RING fingers (see below). There is increasing evidence that at least one function of UbDs is to mediate interactions with proteasomes [101–103]. There is also now a growing list of protein domains that bind ubiquitin. Of these the structurally related UBA (***ub***iquitin-***a***ssociated) domain and Cue domains are found in two of the Cbl family members (see below) and in gp78, respectively.

Tab. 4.1. Alternative zinc-binding domains in RING proteins.

Description (CD ID); Consensus	*Function*	*Examples in RING proteins (Genebank Accession)*	*Zn-binding domain References*
ZnF-RanBP (smart00547) **C**PA**C**TFLNFASRSK**C**F A**C**GAP	Zn-coordinating RNA-binding domain, in RAN-binding protein binds RAN-GDP.	MdmX (NP_002384), Mdm2 (NP_002383), UbcM4-IP 3 (NP112506)	357, 358
Rad18-like CCHC Zn finger (smart00734) LVQ**C**PV**C**FREVPENLI NS**H**LDS**C**L	Yeast Rad18p functions with the RING finger protein Rad5p in error-free post-replicative DNA repair.	Yeast Rad18 (NP_009992); human Xpcc (NP_004619)-Xeroderma pigmentosa complementation group C protein	359, 360
B-Box-type Zn finger (smart00336) QAAPK**C**DS**H**GDEPAE FF**C**EE**C**GALL**C**RD**C**D EAE**H**RG**H**TVVLL	B-boxes have seven potential Zn-coordinating residues but only four bind zinc. Characteristic of TRIM proteins.	Human Murf2 (NP_908975); (Ifp1) Inerferon-responsive finger (NP_569074)	361, 362
TRAF-type Zn finger (pfam02176) HEKT**C**PFVPVP**C**PNK **C**GKKILREDLPD**H**LSA D**C**PKRPVP**C**PFKVYG **C**KVDMVRENLQ	Found in Trafs. Protein–protein interactions	Human Traf4 (NP_004286); Traf5 (NP_004610); Traf6 (NP_004611)	363, 364
ZZ Zn finger (smart00291) V**HH**SVS**C**DT**C**GKPIV GVRY**HC**LV**C**PDYDL **C**ES**C**FAKGG**HH** GE**H**SM	Zn-binding domain, present in CBP/p300 and Dystrophin. In Dystrophin, domain is implicated in Calmodulin binding. Missense mutation of conserved cysteine correlates with Duchenne muscular dystrophy.	Mindbomb (NP_065825)-involved in neural development; SWIM domain containing protein 2 (NP_872327)	365, 366
C1 domain (smart00109); **HHH**VFRTFTGKPTY**C** **C**V**C**RKSIWGSFKGGL R**C**SW**C**KVK**CH**KK**C**A PKVPKP**C**	Protein kinase C conserved region 1 (C1) domains (Cysteine-rich domains); Some bind phorbol esters and diacylglycerol. Some bind RasGTP.	Mll3 (NM_170606)-mixed lineage leukemia protein	367, 368
ZnF_NFX (smart00438); **C**GI**HTC**EKL**CH**EGD**C** GPVS**C**R**C**	Found in the transcriptional repressor NF-X1, a PHD finger/RING finger protein.	NF-X1 (NP_002495)-MHC class 1 X-box binding factor	369

Tab. 4.1. *(continued)*

Description (CD ID); Consensus	*Function*	*Examples in RING proteins (Genebank Accession)*	*Zn-binding domain References*
ZnF_UBR1 (smart00396) **C**TYKFTGGEVIYR**C**K T**C**GLDPT**C**VL**C**SD**C**F RSN**CH**KG**H**DYSLKTS RGSGI**C**D**C**GDKEAWN EDLK**C**KA**H**	Domain is involved in recognition of N-end rule substrates.	Yeast Ubr1(NP_011700); Human E3-α I (NP_777576) and II (NP_056070)	370
ZnF_UBP (smart00290); R**C**SV**C**GTIENLWL**C**LI **C**GQVG**C**GRYQLS**H**A LE**H**FEETG**H**PLVVKL GTQRV	Found in ubiquitin hydrolases and other proteins. In BRAP2, this domain binds ras-GTP.	Brap2/Imp (NP_006759)-Brca1-associated protein, impedes Raf signaling.	365, 366
FYVE finger (smart00064) **C**MG**C**GKEFNLTKRR **HHC**RN**C**GRIF**C**SK**C**S SKKAPLPKLGNEKPV RV**C**DD**C**YENLNG	Implicated in endosomal targeting. Recent data indicates that these domains bind PtdIns(3)P	Sakura/Fring (NP_476519); Riff/Momo (NP_919247) a suspected inibitor of apoptosis.	371–374
In-between-Ring fingers (IBR) domain (smart00647); KW**C**PAPD**C**SAAIIVTE EEG**C**NRVT**C**PK**C**GFS F**C**FR**C**KVEW**H**SPVS**C**	The IBR (C6HC or DRIL) domain is found to occur between pairs of RING fingers (pfam00097). The function of this domain is unknown.	Ariadne1 (NP_005735), parkin	375
PHD Zn-finger (smart00249) Y**C**SV**C**GKPDDGGELL Q**C**DG**C**DRWY**H**QT**C**L GPPLLlEEPDGKWY**C**P K**C**K	Found in nuclear proteins and implicated in chromatin-mediated transcriptional regulation.	Tif1-α (NP_003843)	43
Zn finger C3H1 (smart00356); KYKTEL**C**KFFKRGN**C** PYGDR**C**KFA**H**PL	Implicated in DNA binding. Found in proteins controlling cell cycle or growth. Shown to interact with the 3′ UTR of mRNA. Often found in tandem.	Zfp 183 (NP_849192), makorin1 (NP_038474)	376, 377
C2HC Zn finger (smart00343) K**C**YN**C**GKPG**H**IARD**C** PS	Found in the Nucleocapsid protein of retrovirus. Also found in eukaryotic RNA- or ssDNA-binding proteins	Human Rb-BP 6 (NP_008841)-retinoblatoma-binding protein 6	378, 379
ZnF_C2H2 (smart00355) YR**C**PE**C**GKVFKSKSA LQE**H**MRT**H**	First identified in the *Xenopus* transcription factor TFIIIA. Found in numerous nucleic acid-binding proteins.	Rag1 (NP_000439), Strin/Rfp138 (NP_057355)	380

In addition to containing protein–protein interaction motifs, E3–substrate specificity may be affected by post-translational modifications. In particular, phosphorylation can alter E3–substrate interactions. One example is p53 where certain phosphorylations inhibit its direct binding to Mdm2, while others indirectly enhance their association by promoting nuclear localization of p53 [104–106]. Phosphorylation also directly enhances substrate interactions, as exemplified by the Cbls, which include phospho-tyrosine binding domains (see below) [107].

4.2.4
Variations on the RING Finger

In addition to RING-HC and RING-H2, there is one example of a RING-CC, Cnot4. Cnot4, part of a transcriptional repressor complex, has potent E3 activity that is highly specific for UbcH5B (Figure 4.3B). In addition, there are other variations on the RING finger that also demonstrate ubiquitin ligase activity. These include the PHD finger and the U-box.

The PHD finger closely resembles the RING finger in having eight cysteines and histidines that bind Zn in a cross-brace pattern (Figure 4.2). Differences with the RING finger include a variation in spacing between the coordinating residues; a cysteine in the fourth and a histidine in the fifth (C4HC3) coordinating residues; and an invariant tryptophan two amino acids before the seventh Zn-coordinating residue. NF-X1 is an example of a protein with co-linear consensus sequences for PHD and RING fingers. We have shown that the PHD arrangement – not the RING finger – is essential for *in vitro* E3 activity [46]. Another PHD finger E3 is Mekk1 (***m***itogen-activated protein kinase/***E***RK ***k***inase ***k***inase 1) [48]. Mekk1 functions not only as an activating kinase for Erk (***e***xtracellular signal ***r***egulated ***k***inase) and JNK (***J***u***n*** ***N***-terminal ***k***inase) through its kinase domain, but also as a negative regulator by targeting Erk1/2 for degradation. Similarly, the AIRE (***a***uto***i***mmune ***re***gulator) protein, which contains two PHD fingers, has ubiquitin ligase activity. This E3 activity requires only the first PHD finger sequence, which, of the two, more closely resembles the PHD consensus [49]. The murine γ-herpesvirus-68 K3 was originally described as a PHD/LAP finger protein [51]. It was later suggested that this is a RING finger variant distinct from the PHD [108]. K3 localizes to the endoplasmic reticulum membrane and binds the cytoplasmic tail of nascent MHC class I H-2D(b), targeting it for ubiquitylation and degradation [50, 109, 110]. Similar PHD finger variant proteins from Kaposi Sarcoma Herpes virus, MR1 and MR2, lead to internalization and lysosomal degradation of cell-surface molecules including Class I MHC, B7.2 and ICAM-1 [51].

The relation of the U-box motif to the RING finger is far less obvious than that of the PHD finger. The first U-box protein implicated in ubiquitylation was CHIP (***c***arboxy-terminus ***H***sc70-***i***nteracting ***p***rotein) [111–113]. Subsequently, a yeast protein, Ufd2p, was shown to enhance the processivity of the ubiquitin chain formed by the HECT E3 Ufd4p. This led Jentsch and co-workers to coin the term E4 in reference to this function of Ufd2p [114]. Aravind and Koonin then identified a motif common to CHIP, Ufd2p and more than 10 other proteins. They predicted that the

U-box would conform to a cross-brace structure similar to the RING finger, although it lacks the canonical cysteine and histidine residues for Zn-binding. This motif is predicted to fold using salt bridges and other hydrophilic interactions in order to achieve the structure provided to the RING finger by its Zn coordinating residues [45]. The E3 activity of this family was subsequently verified in the same way that the activity of RING fingers was established, although there is yet to be direct structural verification of its predicted RING finger-like topology [47, 115].

It may be that not all domains that resemble RING fingers are E3 modules. The FYVE finger binds phosphoinositides to effect protein transport. It bears similarity to the RING finger in the use of eight Zn-coordinating residues and in its cross-brace structure [116–118]. The FYVE finger is, however, quite distinct. It employs only cysteines to coordinate Zn, has a different spacing for its Zn-binding residues and contains a short basic amino acid residue sequence preceding the third Zn-coordinating cysteine (Figure 4.2) [119]. FYVE fingers have not, so far, been shown to have E3 activity.

4.2.5
High-order Structure of RINGs – TRIMs

RING fingers exist in structural contexts crucial to their function. One example is the relatively small but medically relevant family of TRIAD proteins, which will be discussed in a separate section in the context of Parkin. The largest family of higher order RING finger-containing proteins are the tripartite motif (TRIM) or RING/B-box/Coiled Coil (RBCC) proteins. An example, Midline1 (Mid1) is shown in Figure 4.4A. TRIM proteins constitute up to one-fifth of the nearly 300 known RING finger proteins in the human genome. As expected, RING finger domains of TRIM proteins can serve as ubiquitin ligase modules.

The two B-box Zn-finger domains (Table 4.1) bind phosphoproteins and, similar to TFIIA Zn fingers, they may also bind nucleic acids. The binding of TRIM proteins to DNA through B-boxes may aid in the modification of transcription factors and histones by ubiquitin. Consistent with this, several TRIMs are known transcriptional regulators. For example, Trim24/Tif1-α is believed to bind to the AF2 (***a***ctivation ***f***unction 2) region of the estrogen, retinoic acid and vitamin D receptors [120]. The ***e***strogen-responsive ***f***inger ***p***rotein (Trim25/Efp) is a transcription factor that is postulated to mediate estrogen action in breast cancer [121, 122]. Another example of a DNA-interacting TRIM protein is Trim32/Hta (***H***IV ***T***AT-***a***ssociated), which translocates from cytoplasm to nucleus in HIV-infected cells. In the nucleus, Hta appears to aid in regulating TAT-mediated transcription [123]. On the other hand, Trim22/Staf50 (***S***timulated ***t***rans-***a***ctivation ***f***actor 50 kD) down-regulates transcription from the HIV-1 LTR promoter region in response to interferon. Staf50 might mediate interferon's antiviral effects [124].

The coiled-coil domain frequently mediates hetero- or homodimerization. About two-thirds of a large number of TRIM proteins tested dimerize [125]. As a number of other RING finger proteins either homo- or hererodimerize, dimerization may facilitate optimal E3 activity of TRIMs. While most proteins that carry the TRIM

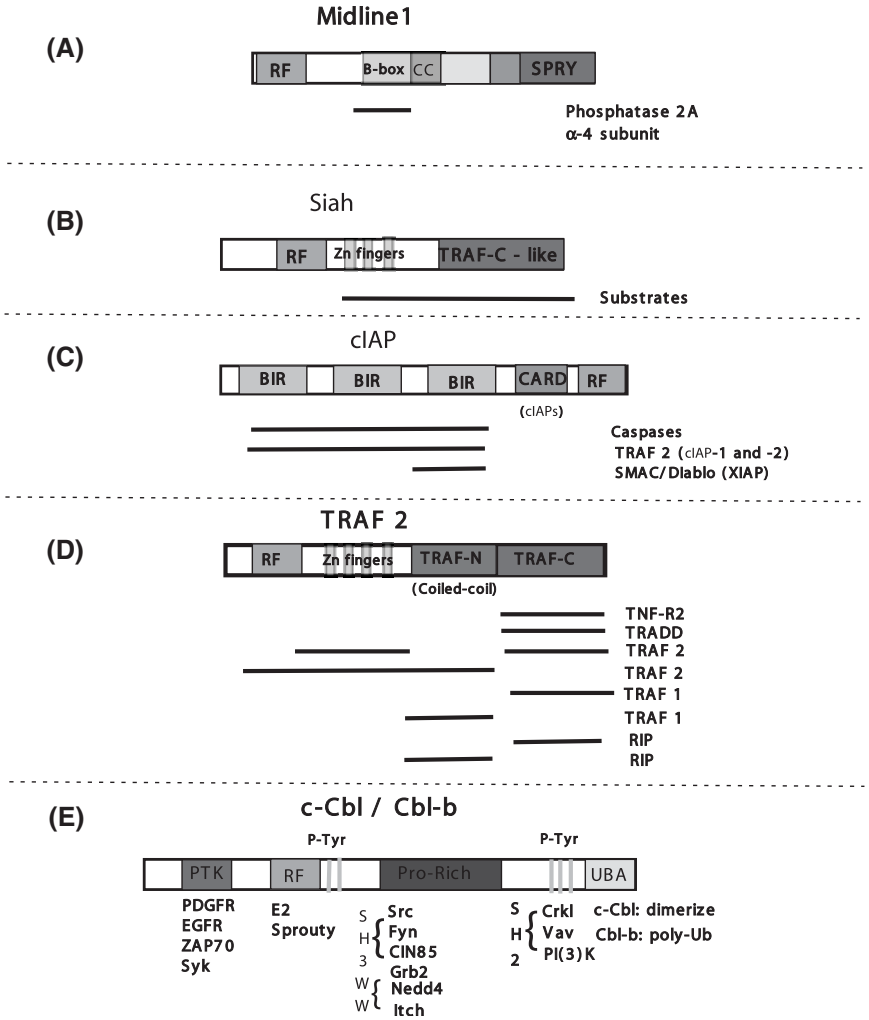


Fig. 4.4. Representative domains and interactions of members of RING finger families. In (A–D) underlines indicate areas of interactions with other proteins. In (E) representative interacting proteins are listed directly below the various domains. RING finger indicated by RF. CARD domain not found in XIAP.

designation have RING fingers, there are variants that retain all of the TRIM domains except the RING finger. In some cases these form heterodimers with RING finger-containing TRIMs via their coiled-coil domains. TRIMs without RING fingers may help modulate substrate interactions, or serve as substrates themselves. An excellent example for study is the RING fingerless TRIM29 or ATCD (***a***taxia ***t***elangiectsia ***c***omplementation group ***D*** protein). ATCD forms heterodimers with RING-containing TRIMs including Trim1/Mid2, Trim10/hematopoietic RING finger, Trim22/Staf50 and the ret finger protein [125]. The significance of these in-

teractions is not yet known. However, ATCD mutations found in ataxia telangiectsia patients makes alteration of E3 activity an attractive mechanism for disease pathogenesis.

A number of other TRIM-containing proteins are associated with genetic disorders. For example, mutations of TRIM37 (Mul1) correlate with mulibrey (***mu***scle-***li***ver-***br***ain-***ey***e) nanism, an autosomal recessive disorder involving tissues of mesodermal origin [126, 127]. Although Mul1 E3 activity is yet to be established, it is known to affect induction of NF-κB by TRAF2 and TRAF6 [128]. Mid1 (Figure 4.4A) mutations are associated with the X-linked Opitz syndrome, which is characterized by severe midline abnormalities. Mid1 forms both homo- and heterodimers with Mid2 (TRIM1), which also contains a RING finger [129]. Mid1/Mid2 dimers are involved in the formation of microtubule anchors. The ***p***rotein ***p***hosphatase 2A (Pp2A) regulatory subunit, α4, is attached to microtubules by the B-box regions of Mid1/Mid2. Pp2A is also a substrate for this E3 [130]. While not yet implicated in human disease, Murf-1 (***Mu***scle-specific ***R***ING ***f***inger 1) is a TRIM E3 whose overexpression is associated with muscle atrophy in rodents. MURF1$^{-/-}$ mice exhibit protection from muscle atrophy [131].

PML is a TRIM protein that is an exception to the rule – one of the few RING finger proteins for which no E3 activity has yet been detected. There are fourteen TRIM-containing PML splice variants. Phosphorylated PML localizes to PML nuclear bodies, where numerous roles have been ascribed to it. These roles include transcription factor [132], tumor suppressor [133] and regulator of p53 response to oncogenic signals [134]. Though PML is not known to be a sumo ligase, it is sumoylated at multiple sites, including on its RING finger. This modification affects its ability to bind Mdm2 and presumably regulates p53 response [135].

4.3 RING Fingers in Cell Signaling

Families of RING finger proteins play important roles in cell regulation, signal transduction and apoptosis. Some of the more prominent families are the Siahs, IAPs, TRAFs, and Cbls (Figure 4.4). Short summaries of these are presented below.

4.3.1 Siahs

The Siah mammalian RING finger E3s are homologs of *Drosophila* seven-in-absentia (Sina) and are represented in plants by the Sinat family. Before RING fingers were known to be E3s, Sina was implicated in Tramtrack degradation in *Drosophila* [136]. Further, Siahs were found to be involved in the proteasomal degradation of the DCC (***d***eleted in ***c***olorectal ***c***arcinoma) gene product, the Netrin receptor [137]. In humans, there are two family members, Siah1 and Siah2. In mice, in addition to Siah2, there are two highly homologous forms of Siah1 (a and b). In plants, there are five Siah relatives, Sinat1–5. Siah proteins are generally ~280 amino acids and characterized by variable N-terminal extensions followed by a

RING finger (Figure 4.4B). The RING finger is followed by a cysteine-rich Zn-finger-containing region. Siahs are known to dimerize, and there is biochemical and structural evidence that this primarily involves the Zn-finger region and part of the more C-terminal coiled-coil domain [138–140]. A role for dimerization in Sinat5 E3 activity has been established [141]. Consistent with biochemical studies, the crystal structure of the C-terminal region, lacking the RING finger, has revealed that it exists as a dimer [142]. Interestingly, this domain bears substantial similarity to the C-terminal regions of TRAF proteins.

The C-terminal domain of Siah proteins is also referred to as the substrate-binding domain (SBD). Siahs target a wide array of divergent substrates for degradation. Directly recognized substrates include the netrin receptor, c-Myb, Bob/Obf1, Peg3/Pw1, Synphillin-1 and TRAF2 [137, 143–147]. However, Siahs can also exist in the context of an SCF-like complex that includes Skp1, Ebi, Sip (***S***iah-***i***nteracting ***p***rotein) and the adenomatous polyposis coli protein. This complex serves as an alternative to $SCF^{\beta TRCP}$ in targeting β-catenin for ubiquitylation [148]. In addition, a consensus Siah-binding sequence, RPVAxVxPxxR, has been identified with the core sequence PxAxVxP. This sequence is found in Sip as well as a number of Siah substrates, including netrin receptor. Sequences slightly degenerate from this are found in other Siah substrates including ***n***uclear receptor ***co-r***epressor (Nco-R), Kid motor protein and Numb. However, other Siah substrates, such as adenomatous polyposis coli protein, Synaptophysin, and group 1 metabotropic glutamate receptors contain no similar sequence, revealing the complexity of Siah interactions [145].

The biological consequences of Siah E3 activity, apparent from the number of its substrates, are significant. Siahs are implicated in mitosis and meiosis [149, 150]. In plants, Sinat5 targets the transcriptional activator Nac1, thereby attenuating auxin-mediated signaling and modulating lateral root development [141]. In mouse, deletion of both Siah1 α and Siah2 results in embryonic lethality [147]. Siahs also modulate tumor necrosis factor receptor (TNFR) function by promoting degradation of TRAF2 [151].

Most recently Siah2 has been shown to target prolyl-hydroxylase family members for degradation. Furthermore, Siah2 is transcriptionally induced in response to hypoxia. Proline hydroxylation has been observed on Hif-1α and Hif-2α (hypoxia inducible factor-1α and -2α) during normoxia. Thus, hypoxia may result in increased Siah2 and decreased prolyl-hydroxylases [152]. This would lead to decreased targeting of Hif-1α and -2α by CBC^{VHL} and, consequently, an increase in levels of VEGF (***V***ascular ***e***ndothelial ***g***rowth ***f***actor) and other Hif-1α and -2α targets.

4.3.2 IAPs

The *sine qua non* of the IAPs (***i***nhibitors ***o***f ***a***poptosis) is one or more copies of a Zn-finger-containing domain referred to as a BIR (***B***aculovirus ***I***AP ***R***epeat; Figure 4.4C). This name was derived from their initial identification in baculovirus, where they prevent host-cell apoptosis, allowing viral replication. At least 10 mammalian IAPs have been described. Many have C-terminal RING fingers [153]. A major

function of IAPs, including XIAP, cIAP-1 and cIAP-2, all of which are active E3s [30], is to bind to and inhibit the tonic activation of caspases. The most extensively studied and potent mammalian IAP is XIAP, which binds and inhibits processed Caspase 9 as well as activated Caspase 7 and Caspase 3 [154] – the latter being an important effector caspase common to both the intrinsic (mitochondrial) and extrinsic (death receptor initiated) apoptotic pathways. Our understanding of how inhibition of caspases by XIAP is regulated has been greatly assisted by the identification of two XIAP-interacting proteins Smac/Diablo and HrtA2/Omi. These are released from mitochondria in response to permeability changes induced by pro-apoptotic stimuli. Also released on disruption of mitochondrial integrity is cytochrome c, which together with Apaf-1 and Caspase 9 leads to the activation of the effector caspase, Caspase 3. Smac/Diablo and HrtA2/Omi bind XIAP, and possibly other IAPs, resulting in the release of caspases from the IAP [155–157]. This may explain the paradox that, in certain XIAP-expressing cells, death-receptor-mediated apoptosis (i.e. the extrinsic pathway) depends on pro-apoptotic Bcl-2 family members that increase mitochondrial outer membrane permeability. In *Drosophila*, in addition to Smac/Diablo and HrtA2/Omi, induction of apoptosis requires three other proteins: Reaper, Hid and Grim. Upon over-expression, these result in excess cell death, which is suppressed by co-expression of the *Drosophila* IAP, DIAP1 [158–160]. Reaper, Hid and Grim bind DIAP1 and promote its ubiquitylation and degradation in a RING finger-dependent manner. This enables them to promote self-ubiquitylation and degradation of the IAPs. These findings are consistent with the initial observation of IAP E3 activity, where activation of apoptosis via the intrinsic pathway resulted in degradation of IAPs and activation of caspase activity [30]. Both of these functions are RING finger dependent, as expressing RING fingerless IAPs delays apoptosis. Thus, a scheme emerges where IAPs continually inhibit caspases until their dissociation is promoted by proteins such as Smac/Diablo. In this context, self-ubiquitylation is possibly potentiated by Reaper, Hid and Grim and their mammalian orthologs. Ubiquitylation of Caspase 9 by XIAP and of Smac/Diablo by cIAP1 and cIAP2 has also been reported. While such findings are consistent with anti-apoptotic roles of the IAPs, their overall significance is unclear [30, 161–163].

It should be stressed that IAPs are not just caspase inhibitors. cIAP-1 and cIAP-2 are recruited to TNFRs in response to activation by ***t***umor ***n***ecrosis ***f***actor (Tnf) [164]. As discussed below, cIAPs apparently play roles in attenuating TNF signaling by contributing to TRAF2 ubiquitylation. At face value this might be construed as a pro-apoptotic role in that it can contribute to down-regulation of NF-κB activation. However, we are just beginning to scratch the surface as to the roles of the IAPs so judgments should probably be kept in reserve.

4.3.3 TRAFs

TRAFs (***T***NF ***r***eceptor ***a***ssociated ***f***actors) are a family of signaling molecules characterized by a conserved C-terminal TRAF domain (Figure 4.4D) [165], which is

divided into TRAF-N and TRAF-C. TRAF-N includes a coiled-coil domain. TRAF-C is structurally similar to the C-terminal region of Siahs. TRAF domains mediate many TRAF interactions, including their association with receptors, oligomerization (e.g. trimerization of TRAF2), and interaction with IAPs. The name TRAF was coined because the two prototypic members of the family, TRAF1 and TRAF2, were first found to associate directly with type II TNFR (TNFR-2) [164]. Interactions with TNFR-1 occur through the adaptor molecule Tradd. Six mammalian TRAFs (TRAF1–6) have been identified. They function as critical signal transducers for the TNFR family and the IL-1/Toll-like receptor family and, therefore, affect a wide range of biological processes, such as embryonic development, innate immunity, inflammation and bone homeostasis [166]. TRAFs2–6 all contain RING fingers at their N-termini, which under many circumstances are required for receptor-mediated signaling.

TRAF6 promotes its own ubiquitylation *in vitro* [167]. This ubiquitylation has been shown to utilize an E2 consisting of Ubc13 (also know as Bendless) dimerized with Mms2/Uev1a (***U***biquitin ***E***2 ***v***ariant 1A). Mms2, like Tsg101 (below), includes a core 14-kDa UBC domain common to E2s but lacks the canonical active-site cysteine. Association of TRAF6 with this dimer results in K63-linked polyubiquitin chains. It has more recently been shown that ubiquitylation of TRAF6 with K63-linked polyubiquitin chains is required for activation of the kinase Tak1. Tak1, in turn, activates IκB kinase as well as the kinase(s) that activates the JNK and p38 kinase systems [168–170].

The large amount of data available for TRAF2 in comparison to TRAF6 makes its story more complex, but perhaps also more informative. CD40 engagement results in redistribution of TRAF2 to lipid rafts. This correlates with its ubiquitylation and subsequent degradation. Presumably, TRAF2 degradation prevents prolonged JNK activation. Expression of EBV-Lmp1 (***E***pstein–***B***arr ***v***irus ***l***atent ***m***embrane ***p***rotein 1) also results in redistribution of TRAF2. However, Lmp1-associated redistribution does not lead to TRAF2 degradation. This may help explain the prolonged activation of downstream signaling pathways by this viral protein [171, 172]. It has not been determined directly whether ubiquitylation of TRAF2 is a function of its intrinsic activity or due to ubiquitylation by other E3s. In fact, cIAP-2 can ubiquitylate TRAF2 following TNFR stimulation and Siah2 can bind to and promote TRAF2 ubiquitylation under stress conditions [151, 173]. Furthermore, there is little evidence for *in vitro* activity of TRAF2 in a purified ubiquitylation system.

Interestingly, recent reports indicate that ubiquitylation of TRAF2 in the presence of Ubc13 and Mms2 is also required for activation of the downstream kinases Gckr (***g***erminal ***c***enter ***k***inase-***r***elated) and JNK [174, 175]. This suggests that K63-linked polyubiquitylation of TRAFs may be a common mechanism for members of this family to activate kinases. This story becomes more intriguing since the presence of the TRAF2 RING finger domain is necessary for JNK, p38 and NF-κB activation. However, of the three, only JNK activation requires intact E3 activity of TRAF2 and expression of Ubc13. This TRAF2- and Ubc13-dependent activation of JNK correlates with redistribution of ubiquitylated TRAF2 into an insoluble com-

partment, reminiscent of CD40-mediated signaling [175]. Thus, it seems that JNK activation may involve K63-linked polyubiquitylation and redistribution of TRAF2 to selective membrane microdomains or insoluble compartments. On the other hand, activation of the p38 and NF-κB pathways requires neither K63-linked chains nor TRAF2 E3 activity. However, it does require the physical presence of the RING finger region of TRAF2 [175]. It may be that TRAF2 and TRAF6 mediate assembly of K63-linked polyubiquitin chains that are either directly or indirectly required for activation of some of their targets. However, interactions with IAPs and Siahs may be required to synthesize K48-linked polyubiquitin chains necessary for TRAF2 and TRAF6 proteasomal degradation. Between these two ubiquitin-generating events there may also be a requisite disassembly of the K63-linked chains by deubiquitylating enzymes. While we await elucidation of the details, what is emerging is a complex set of interrelationships between the Siahs, IAPs and TRAFs in mediating signaling though TNFRs and related receptors.

4.3.4 Cbls

The Cbls play crucial roles in signaling by determining the fate of tyrosine kinase receptors as well as non-receptor tyrosine kinases. The importance of Cbls first became apparent with the discovery that the *v-Cbl* oncogene (***C***asitas ***B-l***ineage Lymphoma) is transduced by the Cas NS-1 retrovirus, resulting in lymphomas of B cell lineage and fibroblast transformation. v-Cbl corresponds to the N-terminal region of c-Cbl. v-Cbl includes the phospho-tyrosine binding domain but lacks other domains including the RING finger [176]. The Cbls were first implicated in cell signaling with the finding that the *Caenorhabditis elegans* Cbl protein, Sli1, rescued a loss of function phenotype of the EGFR (***E***pidermal ***G***rowth ***F***actor ***R***eceptor) ortholog Let-23 [177]. In mammals there are three Cbls. c-Cbl is the cellular ortholog of v-Cbl [178], Cbl-b is highly homologous to c-Cbl [179] and the most recently characterized family member is a shorter cousin known as Cbl-3 or Cbl-c [180, 181].

Cbls share a common architecture [107]. All members of the family have an N-terminal ***p***hospho-***t***yrosine ***b***inding (PTB) site that includes a four α-helix bundle, an EF hand and an atypical SH2 domain. This is followed by a RING finger. C-terminal to the RING finger are proline-rich regions – more extensive in c-Cbl and Cbl-b than in the shorter Cbl-3. The proline-rich region provides interaction sites for SH3 proteins including constitutive interactions with Grb2 and others (Figure 4.4E). Moving further towards the C-terminus, the two longer members of the family include a number of sites for tyrosine phosphorylation that bind heterologous SH2 domains. c-Cbl and Cbl-b include C-terminal UBA domains. As with a subset of other UBA domains including Rad23, the c-Cbl UBA domain mediates homodimerization, apparently functioning akin to a leucine zipper [182]. Recently, the UBA of Cbl-b has been shown to bind polyubiquitin without evidence for a role in dimerization [183].

Because of their many interactions, Cbls were logically thought of as scaffolds for signaling and endocytosis. It is now apparent that Cbls target many tyrosine

kinases for ubiquitylation. Extensively characterized substrates include receptor tyrosine kinases such as EGFR and PDGFR (*p*latelet-*d*erived *g*rowth *f*actor *r*eceptor) [31, 184–186], and non-receptor tyrosine kinases, such as Src and Lck [186–189]. Generally, recognition of receptor tyrosine kinases occurs through the PTK-binding domain. Interactions with Src-family tyrosine kinases may occur between the proline-rich regions of Cbls and SH3 domain of the kinases [107, 190]. Cbl may also play ubiquitin-independent roles related to endocytosis. This has been postulated for the SH3-dependent interactions of c-Cbl with Cin85, which may provide a bridge from receptors to endophillins [191, 192].

The first clue to a relationship between Cbl and ubiquitylation came from Stanley and co-workers who demonstrated that c-Cbl was mono-ubiquitylated in response to CSF-1 (*c*olony *s*timulating *f*actor-1) [193]. Yarden and colleagues demonstrated an association between Cbl recruitment to receptors and their ubiquitylation and down-regulation. C-Cbl E3 ligase activity was subsequently demonstrated by several groups [31, 184, 186].

Cbl-mediated ubiquitylation illustrates several important points related to the varied effects of ubiquitylation. The first is that transmembrane receptors targeted for degradation by Cbl proteins are largely targeted to lysosomes [194–196]. Second, as with ubiquitylation of yeast cell surface transporters and receptors by the HECT E3 Rsp5, this targeting does not require formation of K48-linked polyubiquitin chains [197]. Additionally, in response to receptor–ligand binding, multiple components of the receptor signaling complex are targeted for degradation by Cbls. Included among these are the Cbls themselves and the associated proteins, Grb2 and Shc [198]. In contrast to transmembrane receptors, ubiquitylation of non-receptor tyrosine kinases appears to be associated with proteasomal degradation [186, 199, 200; A. Magnifico, S. Lipkowitz, A. M. Weissman, unpublished observations]. Further, there is evidence to suggest that Cbl-mediated ubiquitylation of PI3 kinase does not target it to either lysosomes or proteasomes but leads to its redistribution to CD28 or to T-cell antigen receptors and results in the attenuation of its activity [201]. Thus, Cbls provide an example of the range of different effects that can be mediated by a single ubiquitin ligase. Additionally, all three members of the Cbl family bind members of the WW domain class of HECT E3s including Nedd4 and Itch and are subject to ubiquitylation by these HECT E3s, providing yet another level of regulation [202].

There are still a number of questions regarding Cbl proteins. Where in the process leading from movement off the cell surface to lysosomes does Cbl-mediated ubiquitylation have its effects? Some studies suggest a role distal to internalization [189, 203] while others suggest a role at the cell surface [204]. Another question pertains to why receptor degradation in response to Cbl-mediated ubiquitylation is abrogated by inhibitors of both proteasomes and lysosomes. Is there a short-lived proteasomal protein essential to the process or are some components of the activated receptor complex targeted to proteasomes while the receptor itself is degraded in lysosomes? Is there an interdependent process of degradation of the signaling complex with some substrates "peeled off" and sent to proteasomes while others go on to lysosomes and multi-vesicular bodies? Finally, why is there a re-

quirement for three distinct family members? A partial explanation may be their differential transcription in various tissues. Both c-Cbl and Cbl-b are widely expressed and, from analysis of mouse models, appear to have at least partially redundant developmental functions [205]. However, it is also clear that there are tissue-specific differences. c-Cbl is found to a greater extent in immature thymocytes and Cbl-b is found in more mature T cells [205–207]. In contrast, Cbl-3 exhibits a much narrower range of distribution [180]. There is much more to be learned about this family of complex regulatory proteins.

4.4 Multi RING finger Proteins

4.4.1 Mindbomb and TRIADs

Oligomerization is a common feature of RING finger proteins; examples include Brca1-Bard1 [208], Siahs [142], Mdm2-MdmX [209] and c-Cbl [182]. Additionally, some evidence suggests a propensity for RING fingers to form higher order aggregates *in vitro* [210]. A number of proteins contain multiple RING fingers in the same polypeptide. The most striking example of this is Mindbomb (Mib), one of several E3s implicated in Notch signaling. In *Drosophila* this protein plays a role in ubiquitylation and internalization of Delta, a Notch co-receptor. Mib includes three RING finger motifs, only the most C-terminal of which has been shown to have E3 activity. Mutations in the second RING finger have adverse developmental effects, but the relationship of this to ubiquitylation is as yet unclear [211]. Searches of Genbank additionally reveal at least three families of proteins, including homologs of the Icbp90 transcription factor (Np95), where a RING is found in the same polypeptide chain as a PHD domain. Similarly, the protein KIAA0860 contains both a RING finger and a U-box.

The most well characterized and apparently largest group of multi-RING proteins is the family of 13 human and at least 16 *Arabidopsis thaliana* E3s that generally include two RING fingers or RING finger-like consensus sequences with an intervening cysteine-rich sequence. This intervening sequence is referred to as the IBR (***i***n ***b***etween ***R***INGs). These proteins are variously referred to as TRIAD (***T***wo ***R***ING fingers and ***I***ntervening-***A***ssociated ***D***omain), DRIL (***d***ouble ***RI***NG-finger ***l***inked), or RBR (***R***ING-***b***etween ***R***INGs-RING). The TRIAD motif has a general, although far from absolute, pattern of conserved residues, C3HC4-C6HC-C3HC4.

The most extensively studied TRIAD is Parkin, shown schematically in Figure 4.5A. The N-terminus contains a region homologous to ubiquitin called the ***ub***iquitin ***d***omain (UbD), which interacts directly with proteasomes. The C-terminus contains two RING fingers (R1, R2) separated by a cysteine-rich ***i***n-***b***etween ***R***ING (IBR) region. This TRIAD motif mediates E3 activity and interacts with molecular chaperones. The last three amino acids of Parkin interact with a PDZ domain and possibly function to anchor Parkin to lipid microdomains.

Other multi-RING proteins with demonstrated E3 activity include HHARI, the

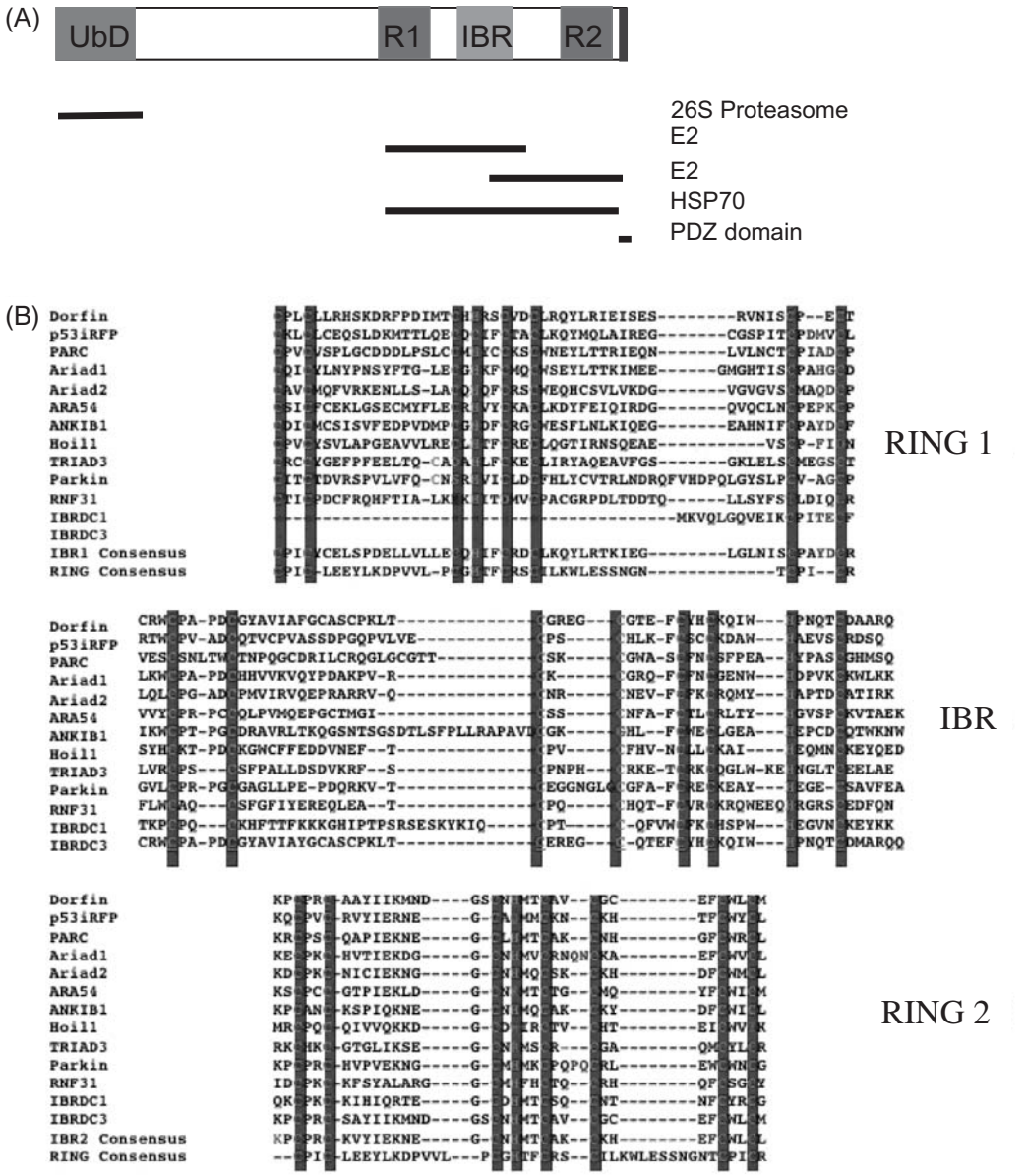


Fig. 4.5. Parkin and RBR proteins (A) Schematic of Parkin. (B) Alignment of RING-IBR-RING domains of RBR proteins.

human homolog of the Drosophila retinoic acid-inducible protein, Ariadne1. HHARI promotes ubiquitylation and degradation of a protein homologous to translation initiation factor 4E [212]. Parc is a TRIAD protein that binds p53 in the cytoplasm and shows E3 activity *in vitro*, although its *in vivo* targets are un-

known [213]. Dorfin promotes the ubiquitylation of Synphilin-1, which is also a Parkin substrate [214]. Dorfin also promotes the ubiquitylation and degradation of mutant copper/zinc ***s***uper***o***xide ***d***ismutase (Sod-1) [215]. In familial ALS and transgenic models, mutant Sod-1 is misfolded and aggregates in inclusions that also contain ubiquitin, proteasome and Hsc70 [216–219]. Hoil-1 is a ubiquitin ligase for ***i***ron ***r***egulatory binding ***p***rotein 2 (Irp2), which binds to iron responsive elements in RNA to alter the stability of RNAs encoding ferritin and transferrin receptor. Hoil-1 recognizes and ubiquitylates Irp2 under conditions of high cellular iron [220–221]. Some other members of the TRIAD family are ***a***ndrogen ***r***eceptor-***a***ssociated protein 54 (Ara54), which interacts with and functions as a co-activator for androgen receptor and Ariadne2, one of the first family members described.

Analysis of the primary amino acid sequence of the thirteen TRIAD proteins reveals striking differences compared to consensus RINGs in both the first and second RING fingers (Figure 4.5B). Two (Parkin, Triad3) lack the predicted Zn-coordinating cysteine at the third position of the first RING, although other potential coordinating residues can be identified for both. Three lack identifiable first RING fingers (Ibrdc1, Ibrdc3, Rnf31). Seven of the remaining eight (Ara54, Ariadne1, Ariadne2, Parc, p53iRfp, Ankib1, Hoil-1) also have atypical first RINGs in having one or two extra amino acids inserted between the seventh and eighth predicted Zn-coordinating cysteines. In addition, p53iRFP has a cysteine rather than the canonical histidine at position four. Of the thirteen, only Dorfin has a first RING finger motif that fits the general consensus.

The second RING finger is also divergent from RING finger consensus sequences. Ara54, Triad3, Hoil-1 and p53iRfp all lack a histidine in the fourth coordination site with only a lysine, arginine, tryptophan and glutamine respectively available to substitute – none would be predicted to be a good Zn ligand. Ariadne1 and Parkin have two extra amino acids inserted between the fifth and sixth predicted coordinating residues. While the remaining seven proteins may be described as having a consensus RING, the distance between C6 and C7 is abbreviated – only four amino acids as compared to a consensus of ten randomly selected, non-IBR RING finger proteins, where the shortest stretch is eight amino acids. Based on structures of RING fingers this might be expected to impact on coordination of the second Zn. Accordingly, a solution structure of the second RING region of Ariadne1 lacks classic RING finger topology and there is no evidence of a second coordinated Zn. Strikingly it can still mediate ubiquitylation [222]. These findings underscore that when one is dealing with RING finger-like structures, ultimately it is function rather than variation from canonical structures or consensus sequences that counts.

The differences between TRIAD proteins and simpler RING fingers, and among the TRIAD proteins themselves, suggest distinct functions for the two RING finger domains in the context of the complete RBR, and perhaps different functions from non-IBR RINGs. Consistent with this, the proximal HHARI1 RING cannot be substituted for by the c-Cbl RING finger. However, the proximal RING finger of Parkin does not restore function either [223]. Although exceptions exist, RING finger

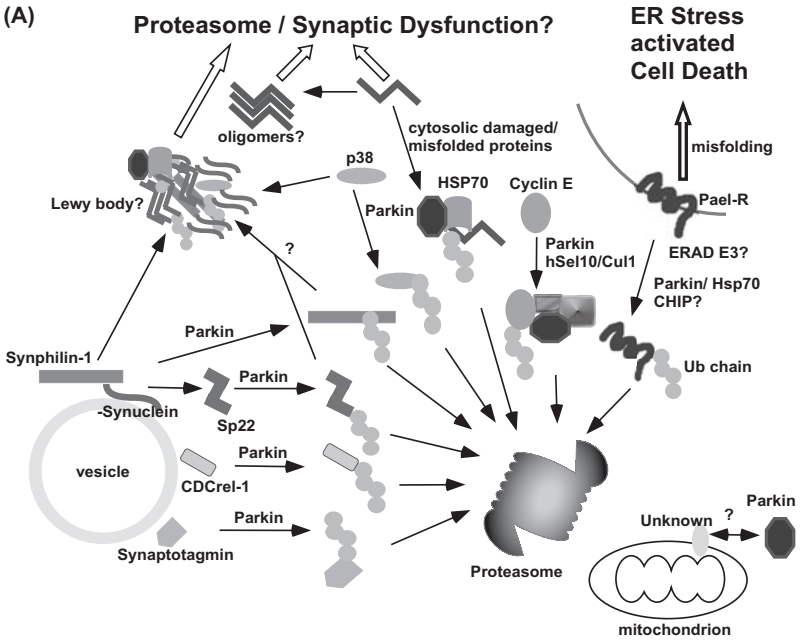
domains isolated from these proteins are generally not sufficient for E2 interactions: the Parkin-UbcH7 interaction requires the entire RBR domain [224], while the interaction of HHARI with UbcH7 or UbcH8 requires the proximal RING finger and part of the IBR [41]. Similarly, the association of the distal RING finger of Parkin with UbcH8 [225] is enhanced by the IBR. E2s other than UbcH7 and UbcH8 also interact with RBRs. Ara54 binds UbcH6, Ube2E2 and Ube2E3 but not UbcH7 [226]. Functional interactions with Parkin have been detected with other E2s, including the mammalian orthologs of the yeast ERAD E2s, Ubc6p and Ubc7p (MmUbc6/Ube2J2 and MmUbc7/Ube2G2) and with UbcH5B [224]. How the RBR functions as a unit for each of the family members remains to be determined. Whether the two RING fingers bind two E2s simultaneously or sequentially to facilitate chain formation, or whether they function more like Brca1-Bard1, where one RING finger enhances the E3 activity of the other without directly binding an E2, also remains an open question.

4.4.2
Parkin and Parkinson's Disease

Parkin has been extensively studied because of its linkage to autosomal recessive juvenile-onset Parkinsonism (ARJP). It may be the most commonly mutated gene in familial Parkinson's disease (PD) [229]. Most disease-associated mutations result in an inactive E3. In addition to being a TRIAD protein, Parkin also includes an ubiquitin domain (UbD), which binds proteasomes [230]. A UbD mutation that may affect proteasome interactions is linked to early-onset PD [231], suggesting that this is crucial to normal function. Notably Hoil-1 also has a UbD. Interestingly, Bag-1 also binds the proteasome via its UbD. Bag-1 recruits a complex consisting of CHIP (a U-box E3) and the chaperone Hsp70. This complex has been proposed to mediate efficient degradation of misfolded proteins [232, 233]. This may be enhanced through non-canonical K33-linked polyubiquitin chains assembled on Bag-1 by CHIP [234].

Loss-of-function mutations in the *Parkin* gene correlate with ARJP with an average age of onset of 26 years. ARJP shares clinical features with autosomal dominant and sporadic PD, which generally present much later in life. Additionally, both diseases respond to L-Dopa treatment. However, while there is loss of dopaminergic neurons in the *substantia nigra* of ARJP patients, there is a general absence of Lewy bodies, the inclusion bodies characteristic of adult PD [235–238]. As a result, Parkin-linked PD is often assumed to lack inclusion bodies. However, there is little autopsy material available from the brains of patients with ARJP and there is at least one case where Lewy bodies have been observed [239]. Thus, it may be too early to draw conclusions.

It has also been proposed that *Parkin* is a tumor suppressor gene [240]. It is included in the fragile site FRA6E [241] and down-regulation of Parkin protein due to exon duplication or deletions has been observed in several cancer cell lines and tumors [241].


(A)
Proteasome / Synaptic Dysfunction?
ER Stress activated Cell Death
oligomers?
cytosolic damaged/ misfolded proteins
misfolding
p38
HSP70
Cyclin E
Pael-R
Lewy body?
Parkin
Parkin hSel10/Cul1
ERAD E3?
?
Parkin/ Hsp70 CHIP?
Parkin
Synphilin-1
Ub chain
Parkin
-Synuclein
Sp22
vesicle
Parkin
CDCrel-1
Parkin
Unknown
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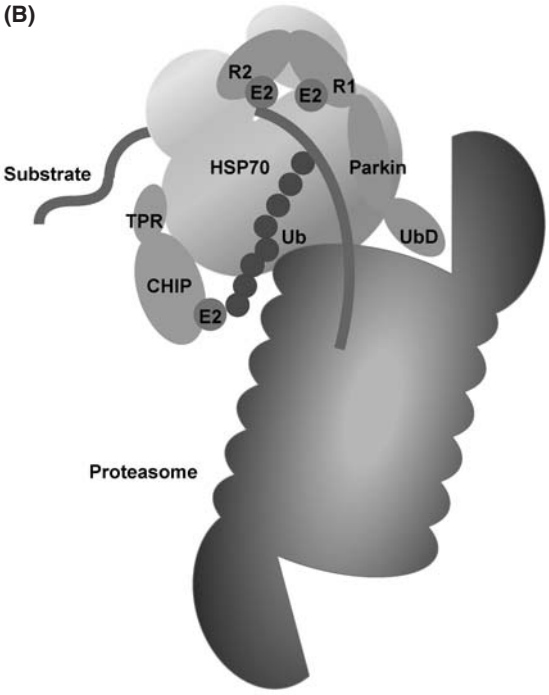
Parkin
Synaptotagmin
Proteasome
mitochondrion


(B)
R2
E2
E2
R1
Substrate
HSP70
Parkin
TPR
Ub
UbD
CHIP
E2
Proteasome

4.4.2.1 Parkin Substrates

There is great interest in identifying Parkin substrates (Figure 4.6A). The first of these, CDCrel-1, was identified as a yeast two-hybrid binding partner [225]. CDCrel-1 is a septin involved in vesicle cycling. CDCrel-1 over-expression inhibits dopamine release in PC12 cells and causes dopamine-dependent degeneration in rats [242]. However, CDCrel-1 null mice show no defect in development or dopamine release [243]. A second vesicle-related substrate is Synaptotagmin (Syt) XI [244], which interacts with Parkin via domains common to many Syt family members, suggesting that Parkin may target multiple Syts [244]. Although Parkin is largely cytosolic, there is evidence for association with synaptic vesicles mediated through Parkin's C-terminal PDZ-domain-binding motif. The PDZ-binding domain of Parkin has also been shown to interact with Cask, a lipid raft-associated protein [245].

α-Synuclein is a cytoplasmic vesicle-associated protein first found in autosomal-dominant PD. It is a prominent component of Lewy bodies and mutations are associated with genetic PD. A rare 22-kDa form of *O*-glycosylated α-synuclein (αSp22) is a Parkin substrate [246] and has also been shown to accumulate in ARJP. However, this finding remains to be generally established. The modified form represents a relatively small sub-population of α-synuclein and there is little evidence that the more common non-glycosylated form is a substrate. Interestingly, Parkin over-expression protects dopaminergic neurons in *Drosophila* over-expressing α-synuclein [247] and also has protective effects in cell-culture models of α-synuclein over-expression [248]. In these models no αSp22 has been observed. Parkin also promotes ubiquitylation and proteasomal degradation of Synphilin-1 [146], an α-synuclein interacting protein that associates with Parkin through its ankyrin repeats [249]. When over-expressed with α-synuclein, Synphilin-1 promotes the formation of inclusion bodies. Notably, over-expression of Synphilin-1 alone results in aggresome formation in HEK293 cells, suggesting Synphilin-1 itself is an aggregation-prone protein [250]. The significance of Synphilin-1 ubiquitylation in PD remains to be determined. Interestingly, at least two other RING E3s, Siah1 and Dorfin, can mediate Synphilin-1 ubiquitylation.

Vesicular trafficking and inclusion body formation are both dependent on the integrity of microtubules and other cytoskeletal components. Parkin has been shown to target misfolded tubulin for degradation [251] (Figure 4.6B) and to interact with centrosomes upon proteasomal inhibition [252]. Whether this reflects association with specific substrates or co-localization with proteasomes in centrosomes re-

Fig. 4.6. Models of Parkin Function. (A) Parkin substrates. (B) A model for Parkin-dependent degradation of misfolded proteins. Parkin recruits a complex containing molecular chaperones and the unfolded substrates to the proteasome. Degradation may be facilitated by direct coupling of ubiquitylation and degradation of the substrate with chaperone assistance. The complex may also recruit CHIP, a U-box protein, to enhance substrate degradation.

mains an open question, as does the significance of its association with actin [253]. The range of membrane-associated Parkin substrates is not limited to vesicles. Pael-R (***P***arkin-***a***ssociated ***E***ndothelin-***l***ike ***R***eceptor) [228] is a putative G-protein-coupled receptor that is enriched in dopaminergic neurons. When overexpressed, Pael-R misfolds and forms insoluble aggregates in the endoplasmic reticulum causing endoplasmic reticulum stress-activated apoptosis. Parkin promotes Pael-R ubiquitylation and degradation from the endoplasmic reticulum. Pael-R also accumulates in some ARJP brains. In *Drosophila*, over-expression of human Parkin protects dopaminergic neurons from toxicity induced by Pael-R [247].

Other cytoplasmic Parkin substrates have been identified. Parkin promotes ubiquitylation of the p38 subunit of aminoacyl-tRNA synthetase [254], which can be found in Lewy bodies. When over-expressed in COS-7 cells, p38 results in aggresome-like inclusions that include Hsp70 and Parkin. Parkin also promotes the ubiquitylation of polyglutamine (polyQ)-expanded Ataxin-3 *in vitro* and facilitates degradation of an Ataxin-3-derived fragment containing an expanded polyQ tract [230]. Over-expression of Parkin suppresses cell death and ameliorates proteasome inhibition and Caspase12 activation induced by expression of the polyQ-expanded proteins. Another putatative Parkin substrate is an, as yet, unidentified, protein on mitochondrial membranes that may modulate mitochondrial permeability. Parkin has also been demonstrated to facilitate the targeting of Cyclin-E for ubiquitylation. In this case Parkin exists as a part of a novel SCF-like E3 complex along with the F-box/WD40 protein hSel-10 and Cul-1 [255]. This raises the provocative possibility that Parkin may be associated with other, as yet to be defined, SCF-like E3 complexes.

4.4.2.2 **Parkin Animal Models**

At present, there are two reports of Parkin$^{-/-}$ mice. One study reports that Parkin$^{-/-}$ mice have motor impairments but no dopaminergic degeneration [256]. No accumulation of CDCrel-1, Synphilin-1 or α-synuclein [256] is observed. Intriguingly, however, they exhibit increased striatal extracellular dopamine and reduced striatal neuronal excitability. This is consistent with a model in which loss of Parkin results in altered dopamine re-uptake or clearance and post-synaptic down-regulation. How this might happen is unclear. However, since Parkin is involved in the degradation of misfolded proteins [230, 257], oxidatively damaged synaptic proteins may accumulate in Parkin null animals. Alternatively, there may be an indirect effect of substrate accumulation with failure of proteasomal function due to aggregation/accumulation of misfolded proteins. In a different study, Parkin mutants show reduced body weight and learning deficits [258]. Diminished motor activation with amphetamine suggests a reduced cytoplasmic pool of dopamine. Increased oxidation of dopamine is also observed, enhancing the potential for free-radical-mediated damage. Interestingly, there is increased glutathione in the striatum, which may partially compensate for increased oxidative stress. No degeneration of dopaminergic neurons is found. However, there are reduced levels of dopamine and vesicular monoamine transporters. These could either be contribu-

tory to the abnormalities observed or be an early indicator of degeneration of nigrostriatal terminals. Recently, the quaking (viable) mice, which show demyelination in the central nervous system, have been shown to have spontaneous deletions of *Parkin* and *Parkin co-regulated gene* (*PACRG*) in addition to the *Quaking* gene (qKI) [259, 260]. Again, the quaking (viable) mutants show no dopaminergic degeneration or α-synuclein accumulation.

Loss of a *Parkin* gene ortholog in *Drosophila* results in flies with a reduced life span and male sterility due to a defect in late spermatogenesis [261]. However, there is no overall neuronal degeneration or dopaminergic cell loss, even though dopaminergic neurons in the dorsomedial cluster show cell-body shrinkage and reduced tyrosine hydroxylase staining in proximal dendrites of aged flies [261]. Interestingly, this is the area most affected by α-synuclein transgenics [262]. Parkin null alleles also confer locomotor defects in climbing and flight due to loss of muscle integrity. This is associated with swollen mitochondria with disrupted cristae [261]. In PC12 cells, Parkin over-expression is associated with delayed mitochondrial swelling and cytochrome c release upon exposure to C2-ceramide [263] and there is evidence for Parkin association with the mitochondrial outer membrane where it may protect cells from ceramide toxicity [263]. This protective effect is dependent on Parkin's E3 activity and intact proteasome function. One may expect Parkin null animals to be more susceptible to oxidative stress, which in *Drosophila* may manifest as muscle defects.

Although these animal models show abnormalities, they fail to reproduce the PD phenotype. Still, they suggest a role for Parkin in synaptic function and maintenance of mitochondrial integrity.

4.4.2.3 **Possible Pathogenic Mechanisms in ARJP**

While possibly providing clues regarding Parkin's role in ARJP, animal models have led to few clear insights. The brain differs from most other organs in its lack of regenerative capacity, so that the damage accumulated from low-level chronic insults may be increased. All forms of PD are primarily manifested in a cell type prone to oxidative insults from decades of dopamine production. It therefore becomes evident why short-lived animals, such as mouse and fly, are of limited utility in testing the role of specific proteins as etiologic factors of this disease. These limits apply even for ARJP, which does not manifest clinically, on average, until the third decade. Additionally, the range of Parkin substrates identified to date, which include proteins associated with PD and other neurodegenerative disorders, are not necessarily derived from unbiased approaches.

The association of Parkin both with dopamine-containing vesicles and with mitochondria is consistent with a role in quality control within axons and dendrites – disposing of proteins that have been subject to oxidative damage. How does Parkin recognize a set of structurally diverse proteins? One mechanism might be through direct recognition of oxidatively-induced modifications or exposed epitopes indicative of misfolded or damaged proteins. Alternatively, Parkin may not directly recognize substrates but rather function together with chaperones, particularly Hsp70,

that directly associate with altered proteins (Figure 4.6A and B). Support for indirect recognition includes the finding that although the proximal RING finger of Parkin is implicated in some substrate interactions, there is little to suggest that these are direct [249]. However, this RING finger does directly bind Hsp70 [230, 264]. Additionally, for some substrates, such as polyQ-expanded Ataxin 3, Hsp70 is crucial for ubiquitylation [230]. Other Parkin substrates including Pael-R, Synphilin-1 and p38 are also Hsp70-associated [254, 264; Y. C. Tsai, unpublished result]. There are striking parallels between Parkin and the U-box E3 CHIP. CHIP interacts with the UbD protein Bag-1 to target multiple Hsp70-bound proteins for proteasomal degradation [112, 113, 233]. Also, there is evidence that Parkin functionally interacts with CHIP to enhance Parkin-dependent degradation of Pael-R [264], with CHIP playing an E4-like role. Thus, Parkin may be a fusion of chaperone-binding, ubiquitin-ligase and proteasome-targeting motifs.

It is possible that a subset of TRIAD proteins may play overlapping roles in intracellular protein quality control. In this regard, it is provocative that *Drosophila* Ariadne-1 mutants show motor impairments and defects in muscle and neuronal development [265]. Also in neuronal cultures, over-expression of Hsp70 suppresses aggregation of the Dorfin substrate, mutant Sod-1 [266]. Furthermore Dorfin, like Parkin, has the capacity to target Synphilin-1 for proteasomal degradation. Thus, multiple members of the TRIAD family might function as crucial intermediaries between chaperones, the ubiquitylation machinery and proteasomal degradation in protein quality control. While *Parkin* mutations are associated with ARJP, it remains to be determined whether damaged Parkin contributes to disease progression in other forms of PD. Notably, Parkin and the other TRIAD proteins are cysteine-rich and may be particularly susceptible to oxidation or nitrosylation on these residues. This could result in dysfunction of protein quality control. The atypical nature of TRIAD RING fingers, with evidence for diminished stable Zn coordination, may further increase the propensity of TRIADs to be damaged. One can therefore envision an amplifying effect where failure to maintain vesicular or mitochondrial integrity by mutant or damaged Parkin leads to increased potential for oxidative damage of other cellular proteins.

4.5
Regulation of p53 by Mdm2 and other RING finger Proteins

4.5.1
Mdm2

p53 is maintained at low levels in normal proliferating cells. Increased p53 levels and altered transcription from p53-responsive genes are associated with response to cellular stress and DNA damage [267, 268]. The role of p53 in regulating genes that effect cell-cycle arrest, allowing for DNA repair if possible, and in regulating genes that induce apoptosis in tumor cells, has earned it the apt title of "guardian of the genome" [269]. The significance of losing p53 activity in cancer is under-

scored by the finding that up to 50% of cancers have inactivating mutations in the *p53* gene [270]. Many other cancers have wild-type p53 yet there is still a failure to activate a p53 response. We now know there are several mechanisms responsible for this. The one that has attracted the most attention is increased levels of the cognate p53 ubiquitin ligase Mdm2 [106]. This E3 binds to the trans-activation domain of p53 through its N-terminus and mediates p53 ubiquitylation through its C-terminus [271].

Mdm2, which was one of the first characterized p53-responsive proteins, was identified in a spontaneously transformed cell line, 3T3DM, as the product of a gene amplified on the mouse double-minute (MDM) chromosome [272]. Its oncogenic property was demonstrated by the finding that over-expression immortalizes rodent primary fibroblasts [271, 273]. The discovery that this 90-kDa protein binds to p53 and inhibits p53 transactivation [274] demonstrated a feedback loop between Mdm2 and p53 [275, 276]. The importance of regulating p53 by Mdm2 is underscored by the observation that early embryonic lethality of Mdm2 deficient mice is rescued by simultaneous deletion of p53 [277, 278]. Moreover, the *Mdm2* gene is amplified in approximately one-third of human sarcomas, and is over-expressed, with or without gene amplification, in a wide range of human tumors [279, 280]. A majority of these cancers retain wild-type p53, which is inactivated by Mdm2, thus allowing for survival of transformed cells.

Since Mdm2 binds the p53 transactivation domain, it directly interferes with the interaction between p53 and basal transcription factors. This leads to a block of transcription. However, additional mechanisms may contribute to inhibition of p53-dependent trans-activation. The N-terminal region of Mdm2 has intrinsic transcriptional repression activity [281] and more recent studies found that Mdm2 recruits a transcriptional co-repressor, CtBP2 (***C-t***erminal ***b***inding ***p***rotein 2) [282]. Interestingly, this association is abolished by NADH, which changes the conformation of CtBP2 [282]. Since NADH is increased in hypoxia, it is conceivable that dissociation of CtBP2 from Mdm2 may contribute to hypoxia-induced p53 activation.

Besides its role in transcription, Mdm2 also promotes the ubiquitylation and degradation of p53. This function is central to the role of Mdm2 in maintaining the low levels of p53 that allow cell proliferation [283, 284]. The initial study demonstrating Mdm2 ubiquitin-ligase activity *in vitro* provided evidence that it could be a HECT domain variant [20]. However, we now know that its E3 activity towards p53 is dependent on its atypical RING finger [32, 285] (Figure 4.7). Moreover, the intrinsic ligase activity of Mdm2 induces its own ubiquitylation and degradation [32]. This self-ubiquitylation of Mdm2 may allow for proper regulation of the p53 response.

Mdm2 is expressed at low levels throughout embryonic development and in most adult tissues [286]. It is transcribed from two promoters. The more distal, located between the non-coding exon I and exon II, is p53 responsive [275, 276]. Over 40 alternatively and aberrantly spliced variants of *Mdm2* mRNAs have been detected [287]. Many encode proteins that are deficient in binding to and therefore regulating p53. Others may function as dominant negative regulators, preventing the inhibitory action of Mdm2 toward p53 [288–290]. Still others promote cell

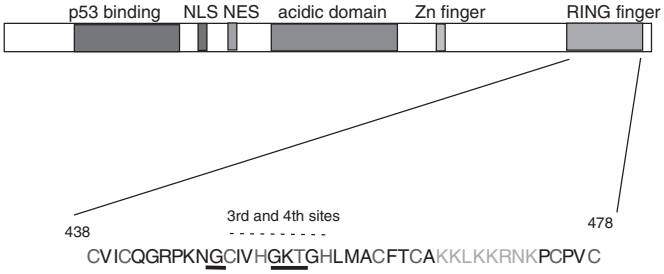


Fig. 4.7. Schematic representation of Mdm2 domains and RING finger. Mdm2 binds p53 as well as p63 and p73 through an N-terminal region of the protein. An acidic central domain, not found in MdmX, is essential for function as is its C-terminal RING finger. Nuclear import and export signals are indicated in Figure 4.7. The RING finger of Mdm2 includes 10 residues that could potentially represent sites of Zn coordination for this atypical RING finger (red), including four that have been variously proposed to represent the third and fourth Zn coordinating residues. The nucleolar localization sequence that is revealed upon binding of Arf or upon adenine nucleotide binding is shown in green and the Walker A site of nucleotide binding is underlined.

growth and tumor formation in a p53-independent manner [291]. The contribution of these to tumor development and prognosis is unknown.

The Mdm2 RING finger is unusual for the spacing of its putative Zn coordinating residues. Four amino acids have been implicated, in one way or another, as being the third and fourth coordinating residues [32, 292, 293]. These include a threonine, in addition to cysteine and histidine residues (Figure 4.7). This raises the possibility that the Mdm2 RING may exist in multiple conformations. Elucidation of the structure of its RING finger will hopefully clarify this. The RING finger of Mdm2 also includes a nucleolar localization signal in the region between the sixth and seventh coordinating residues. This region is apparently revealed when Mdm2 binds to the Arf (***a***lternative ***r***eading ***f***rame) protein [294]. Interestingly, the Arf–Mdm2 interaction does not directly involve the RING finger (see below) (Figure 4.7). Further, it has recently been determined that this nucleolar localization signal can also be exposed in an Arf-independent manner by binding of adenine nucleotides through a Walker A motif within the RING finger (Figure 4.7) [295]. These findings reinforce the idea that the RING finger may exist in multiple states. The novel nature of the Mdm2 RING finger is underscored by the finding that substitution of a heterologous RING finger results in a chimera that promotes self-ubiquitylation but not ubiquitylation of p53 [32]. The specificity for the Mdm2 RING in p53 ubiquitylation suggests that the RING finger may have to be oriented in a specific manner to present E2-Ub to the substrate. Consistent with this, the ubiquitylation sites on p53 appear limited to a cluster of six lysine residues located near its C-terminus [296]. These also represent potential acetylation sites, which would preclude ubiquitylation, further adding to the myriad ways by which p53 is regulated [297]. The specificity in ubiquitylation sites may provide an explanation

for the finding that Mdm2 also binds to p53 family members p63 and p73, but does not promote their ubiquitylation and degradation [296]. These two family members lack the lysine-rich region near the C-terminus [298, 299].

Much attention has been focused on whether Mdm2 can mediate formation of polyubiquitin chains on p53. Studies using purified p53, Mdm2 and ubiquitin with K48 or K63 mutations found that Mdm2 only catalyzes the addition of single ubiquitin to multiple lysine residues of p53 [300]. Another *in vitro* study, however, suggested that Mdm2 induces both mono- and polyubiquitylation of p53, depending on the level of Mdm2 [301]. It is unclear to what extent these *in vitro* observations reflect the *in vivo* setting. Regardless, there is now evidence to suggest that additional factors may work together with Mdm2 to promote polyubiquitylation and proteasomal degradation of p53. p300 has now been reported to have ubiquitin-ligase activity for p53 or to facilitate polyubiquitylation of p53 together with Mdm2 [302]. This suggests that p300 could be an E4 for p53. Since p300 is a transcription co-activator, a possibility is that Mdm2-p300 promotes the selective polyubiquitylation and degradation of p53 that is actively engaged in transcription.

4.5.2 Pirh2

Pirh2 is a RING finger protein that binds p53 and has been shown to mediate its ubiquitylation, independent of Mdm2 [303]. Down-regulation of endogenous Pirh2 increases p53, whereas expression of Pirh2 leads to a decrease in p53. Again, this leads to repression of p53-induced trans-activation and reversal of growth inhibition. Interestingly, the Pirh2 binding site on p53 (residues 82–292) does not overlap the Mdm2 binding site (residues 1–51). Thus, both Mdm2 and Pirh2 might bind a single p53 molecule and cooperate to mediate its ubiquitylation. The existence of an additional E3 for p53 may explain the observation that JNK activation can lead to p53 ubiquitylation and degradation in an Mdm2-independent manner [304]. Like Mdm2, Pirh2 is also transcriptionally up-regulated by p53 [303]. Another RING finger protein that interacts with p53 is a TRIAD protein, Parc, which binds p53 in the cytosol [213]. Whether Parc contributes to p53 ubiquitylation remains to be determined.

4.5.3 MdmX

MdmX (also known as Mdm4) is a close relative of Mdm2. Analogous to Mdm2, MdmX binds p53 through its N-terminus and includes a RING finger at its C-terminus [305]. In addition to direct binding of p53, MdmX heterodimerizes with Mdm2. This interaction appears to be mediated through their RING fingers. Although MdmX does not effectively promote p53 ubiquitylation [209], $MdmX^{(-/-)}$ mice, like those negative for Mdm2, undergo embryonic death that is rescued by

loss of p53 [277, 278, 306–308]. Thus, MdmX is also an essential negative regulator of p53 activity. Accordingly, the *MdmX* gene is amplified and over-expressed in certain human malignant gliomas that express wild-type p53 [309]. Therefore, it is possible that MdmX functions as a critical regulator of Mdm2 to regulate the level and activity of p53. There are several lines of evidence supporting this. Down-regulation of MdmX causes a decrease in Mdm2 and an increase in p53. This leads to a subsequent increase in the sensitivity of cells to UV-induced apoptosis [307, 310]. In $p53^{-/-}/MdmX^{-/-}$ MEFs, re-expression of MdmX increases the half-life of co-transfected Mdm2 and enhances the degradation of re-expressed p53 [310]. These results suggest that the formation of Mdm2–MdmX heterodimers selectively inhibits Mdm2 self-ubiquitylation and stabilizes Mdm2, leading to increased ubiquitylation of p53. Over-expression of MdmX appears to reverse nuclear export of p53 promoted by Mdm2 [209]. Intriguingly, Mdm2 also promotes the ubiquitylation and degradation of MdmX [311, 312]. It is conceivable that this represents another mechanism for cells to down-regulate Mdm2 activity, ensuring the increase of p53 in response to stress stimuli.

The structural similarities and functional distinctions between Mdm2 and MdmX lend themselves to "mix and match" studies. MdmX alone has very low E3 activity towards either itself or p53. Interestingly, when the RING finger of MdmX is replaced with that of Mdm2, the chimeric molecule can not ubiquitylate p53 despite binding to p53 and mediating its own self-ubiquitylation [313]. However, if the central acidic domain of Mdm2 is also fused to the N-terminal portion of MdmX along with the Mdm2 RING, the resultant chimeric protein now becomes competent for p53 ubiquitylation. The importance of this acidic domain is further indicated by the finding that Mdm2 lacking this domain does not ubiquitylate p53. However, co-expression of the acidic domain *in trans* restores the ability of mutant Mdm2 to ubiquitylate p53 [313]. The underlying mechanism for this complementation is not yet clear. If nothing else it underscores the complexities and nuances of substrate ubiquitylation *in vivo*.

4.5.4
Arf and Other Modulators of Mdm2 Activity

Another member of the Mdm2–MdmX–p53 cast already mentioned above is Arf. This is a small basic protein (pI > 12) encoded by the *Ink4a* locus, which also encodes the cyclin-dependent kinase inhibitor p16Ink4a [314]. Shortly after their identification, both human and mouse Arf (p14Arf and p19Arf respectively) were found to interact with Mdm2 in a region N-terminal to the RING finger, between amino acids 235 and 289 in the central acid domain [315]. This interaction blocked Mdm2-mediated p53 degradation [316–319]. Inhibition of p53 degradation by Arf was the result of its direct inhibition of Mdm2 ubiquitin-ligase activity [320, 321]. However, Arf may also inhibit Mdm2 by promoting Mdm2-mediated ubiquitylation of MdmX [322], thereby lessening the activity of Mdm2 towards p53. Additionally, over-expressed Arf resides in the nucleolus and appears to reveal a nucleolar

localization signal contained within the Mdm2 RING finger, although its binding site is N-terminal to the RING finger [294]. This change in Mdm2 localization correlates with inhibition of p53 degradation, presumably by separating Mdm2 from p53 or possibly by preventing nuclear export of the Mdm2–p53 complex [323–325]. However, relocation of Mdm2 to the nucleolus is apparently not essential for the inhibition of Mdm2 by Arf, although it may contribute to the suppression of Mdm2 under certain circumstances [326–328]. Further adding to the complexity of its function is the observation that Arf interacts with the Sumo E2 UbcH9, and mediates sumoylation of Mdm2 [329]. How sumoylation, which is frequently correlated with nuclear transport, might contribute to the observations obtained with Arf is another question to be answered.

Interacting with many other proteins, such as ribosomal components, steroid receptors and tumor suppressor gene products, provides additional ways to modulate the activity of Mdm2. Ribosomal proteins L5, L11 and L23 have all been found to bind Mdm2 and the Mdm2–p53 complex [330–332]. Enforced expression of L11 inhibits Mdm2-induced p53 ubiquitylation and degradation, leading to accumulation and activation of p53. L11 also stabilizes expressed Mdm2, suggesting that it may act by inhibiting the E3 activity of Mdm2 [333]. This prediction remains to be directly demonstrated through *in vitro* experiments. Low concentrations of actinomycin D disrupt ribosomal function and increase the levels of L11 bound to Mdm2. It is therefore conceivable that L11 plays an important role in stabilizing p53 in response to perturbations of ribosome integrity and activity. Tsg101 (***t****umor* ***s****usceptibility* ***g****ene 101* product) is an E2-like (Uev) protein, which includes a UBC core structure that, like Mms2, lacks an active site cysteine. Tsg101 also binds to and regulates the function of Mdm2 [334]. Its over-expression results in an increase in Mdm2 and a reciprocal decrease of p53 in cells. Tsg101$^{-/-}$ mice accumulate p53 and exhibit early embryonic death [335]. Notably, Tsg101 plays an important role in endosomal trafficking and down-regulation of membrane receptors [336]. The significance of this in relation to Mdm2 regulation is unclear. Whether regulation of Mdm2 contributes to the proposed tumor-suppressor function of Tsg101.

The level and activity of Mdm2 are regulated by a variety of signals. The p53 inducer nitric oxide down-regulates Mdm2 at a post-transcriptional level [337]. Phosphorylaton plays major roles in Mdm2 regulation. In response to DNA damage, Atm (***a****taxia-****t****elangiectasia* ***m****utated*) phosphorylates Mdm2 on Ser395, impeding Mdm2-mediated nuclear export and degradation of p53 [338]. There is also evidence indicating that Atm activates c-Abl, which phosphorylates Mdm2 at Tyr394 and prevents its interaction with p53 [339]. However, phosphorylation may also enhance the activity of Mdm2. For example, the growth-factor-activated kinase Akt (***AK****R mouse strain* ***t****hymoma*) phosphorylates Mdm2 at Ser166 and Ser186, which promotes its nuclear translocation, leading to increased p53 ubiquitylation and degradation [340, 341]. This may contribute to the anti-apoptotic action of growth factors such as IGF1 and EGF. Consistent with this, the tumor suppressor Pten (***p****hosphatase and* ***ten****sin homolog*), a phosphatase that dephosphorylates the Akt

activator PIP3, protects p53 from Mdm2 and enhances p53-mediated transcription [342, 343]. Recently, Merlin, the product of *Neurofibromatosis 2* tumor-suppressor gene, was also found to down-regulate Mdm2. This led to inhibition of Mdm2-mediated p53 degradation and an increase of p53 transcriptional activity [344]. Therefore, blocking Mdm2 E3 activity toward p53 appears to be a common mechanism utilized by a number of tumor suppressors.

4.5.5
Other Potential Mdm2 Substrates

In addition to p53 and MdmX, a number of other proteins have been identified as potential Mdm2 substrates. Included among these are *β*-arrestin, *β*2-adrenergic receptor, androgen receptor, glucocorticoid receptor, histone acetyl transferase Tip60 and PCAF [322, 345–349]. While the physiological or pathological significance of the ubiquitylation of these proteins by Mdm2 remains to be further explored, these findings are consistent with the notion that Mdm2 has p53-independent functions in cells.

4.5.6
Mdm2 and Therapeutic Intervention in Cancer

Given its importance to cancer, a thorough understanding of how p53 levels and transcriptional activity are regulated is of practical significance. Accordingly, interventions that disrupt the capacity of Mdm2 to modulate both activity and levels of p53 become clinically important. Reagents have now been identified that block the physical interactions of these two proteins [350] and we and others have taken an interest in identifying small molecule inhibitors that might block Mdm2's E3 activity [106, 351, 352]. Whether reagents that inhibit Mdm2's ubiquitin-ligase activity will have therapeutic utility, especially if the self-ubiquitylation activity of Mdm2 is similarly inhibited, remains to be determined. The reason this becomes an issue is that such inhibition may result in the accumulation of p53 bound to Mdm2, which would be incapable of its crucial trans-activation functions. Thus, combinations of blockers, such as the recently identified Nutlins [353], which bind to p53 and block interactions with Mdm2 and *bona fide* specific Mdm2 E3 inhibitors are an attractive combination.

However, the complexity of p53 regulation increases the likelihood that therapies aimed at particular targets in this pathway could give unexpected results. p53 exists as a tetramer, each tetramer can potentially directly bind a combination of four Mdm2 and MdmX molecules and each of these has the potential to bind another Mdm2 or MdmX through RING finger-mediated dimerization. The stoichiometry of these p53–Mdm2–MdmX arrangements could potentially alter the balance between p53 stabilization and degradation. Adding to this complexity are the roles played by p300 and Pirh2 as well as other regulators such as Arf and ribosomal

proteins. As if this were not enough, the issue of which E3 adds ubiquitin to p53 and how many ubiquitins each E3 adds awaits elucidation. Also, whether ubiquitin modification of p53 occurs in the nucleus, cytosol or both is unknown.

Underscoring the nuances involved in p53 regulation are studies on the deubiquitylating enzyme HAUSP (***h***erpesvirus-***a***ssociated ***u***biquitin-***s***pecific ***p***rotease) – newly published as this chapter was being completed. This deubiquitylating enzyme was first found to stabilize p53 presumably by reversing the effects of Mdm2 [354]. HAUSP has effects on p53 when its expression is ablated, either through RNA interference or in HAUSP null mice. However, the predominant effect is the opposite of what was expected. The primary target of HAUSP appears to be Mdm2 and not p53. Thus, loss of HAUSP stabilizes p53 and enhances the presumed self-ubiquitylation and consequent proteasomal degradation of Mdm2 [355, 356]. As with most things related to p53, each additional piece of information pertaining to control of its degradation alerts us to the complexities of regulating this "guardian of the genome".

4.6 Conclusion – Perspective

At the beginning of the 1990s there was only a rudimentary appreciation of the importance of protein degradation as a means to control protein levels in a temporally and spatially defined manner. We now understand that degradation of regulatory proteins plays important roles in almost all cellular processes. Similarly, degradation of misfolded proteins, unassembled proteins or proteins without useful functions is crucial to normal cellular processes. Further, an increasing number of disease states are found to be associated with dysfunction of these degradative processes. While the common final pathway by which most non-cell-surface proteins are degraded is via ubiquitin modification in the 26S proteasome, it is clear that ubiquitin ligases are indispensable regulatory arbiters for both proteasomal and lysosomal targeting. It is through their recognition and targeting of substrates that the destiny of proteins, and by extension the fate of the cell, is decided. Ubiquitn ligases, however, are proving to be even more important than simple arbiters of protein destruction. Non-degradative ubiquitin modification appears to play additional roles in kinase activation, DNA repair and other cellular pathways.

The largest class of E3s, by far, is represented by the RING finger and its structural relatives the PHD finger and the U-box. Among these, further division can be made between the multi-subunit cullin-containing complexes and those non-cullin E3s in which protein–protein interaction domains and the RING, PHD or U-box co-exist in the same polypeptide. Non-cullin E3s, which constitute the large majority of RING finger proteins, have the flexibility to recognize substrates either directly or in the context of protein complexes. In some cases, these RING finger proteins can target specific individual substrates. In other cases, such as CHIP and possibly Parkin, these E3s target multiple proteins through cellular quality

control systems. Further, there is now evidence to suggest that E3s not usually thought of as being components of SCF complexes, such as Siah and Parkin, may under certain circumstances function in this manner. The interaction of ubiquitin ligases with specific subsets of E2s may contribute to the processivity and type of ubiquitin modification that the E3 generates. It is also apparent that, in many cases, common features can be found in E3s that contribute to their activities or specificities, such as heterologous Zn-binding domains, coiled-coil domains, UbDs and ubiquitin-binding domains. How the RING finger and each of these contributes to substrate selection, the type and length of ubiquitin chain formed, E2 interactions, and approximating the substrate–E3 complex with the proteasome or other cellular structures are all questions that require analysis. Further insights into the remarkable regulatory pathways mediated by ubiquitylation will emerge as we begin to develop an increased understanding of individual substrate–E3 pairs.

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5
Ubiquitin-conjugating Enzymes

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5.1
Introduction

In this chapter we review the biochemical, structural, and biological properties of ubiquitin-conjugating enzymes (also called E2 enzymes). Because length restrictions preclude a comprehensive treatment, we focus on key findings that have revealed important general insights and principles. Throughout the piece we try to point out important unanswered questions concerning the E2 enzyme family.

A few words about nomenclature are necessary. The yeast E2 genes were numbered in the order of their discovery, but the situation is more complicated in mammals. There are currently three naming systems in use for human E2s: one based on protein molecular mass (e.g. $E2_{25K}$ is a 25-kD E2), one based on temporal order of gene cloning (e.g. UbcH10 is specified by the tenth E2 gene cloned in humans), and one based on relationship to yeast E2s (e.g. HR6A is one of two human homologs of yeast Rad6/Ubc2). The second system is the least ambiguous, but also the least informative. In this chapter, we generally name mammalian E2s according to their relationship to yeast E2s. When this is not possible, we use one of the published names.

5.2
Historical Background

Ubiquitin's best-understood function is that of a protein cofactor in an intracellular protein-degradation pathway that terminates with the destruction of ubiquitin-tagged substrates by 26S proteasomes [1]. The discovery in 1980 that this 76-residue protein is conjugated to proteolytic substrates through the formation of a peptide-like bond, and in an ATP-dependent manner, suggested that ubiquitin activation would be part of the conjugation process [2, 3]. A ubiquitin activating enzyme (E1) was soon identified and shown to employ an aminoacyl-tRNA-synthetase-like mechanism [4]. E1 first catalyzes the addition of an adenylate moiety to the carboxyl group of ubiquitin's C-terminal residue, G76. The AMP-bound

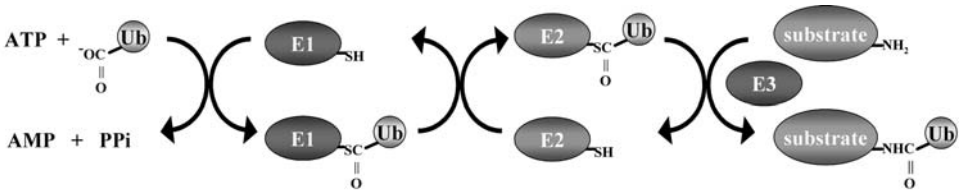

Fig. 5.1. The ubiquitin-conjugation pathway. Steps in ubiquitin activation and substrate modification. E1, ubiquitin activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase. Atoms involved in the thiol ester and amide bonds are shown.

ubiquitin is then transferred to a cysteine residue in the E1 active site, concomitant with the formation of a new molecule of ubiquitin adenylate. The thiol-linked ubiquitin is the proximal source of activated ubiquitin for downstream steps.

From a chemical point of view, the E1/ubiquitin thiol ester should be competent to donate ubiquitin to a substrate amino group. In fact, aminoacyl-enzyme thiol esters are used in exactly this way in non-ribosomal polypeptide synthesis, a process that was discovered around the same time as ubiquitin–protein conjugation [5]. In spite of the attractive simplicity of this model, however, biochemical reconstitution studies showed that besides E1 two additional fractions were required to conjugate ubiquitin to a model substrate. They were called ubiquitin carrier protein (E2) and ubiquitin-protein ligase (E3), respectively, since the respective factors seemed to act sequentially [6]. Interestingly, the E2 factor apparently formed a thiol ester with ubiquitin. Based on these results, Hershko and co-workers proposed the ''ubiquitin conjugation cascade'' (Figure 5.1).

Multiple thiol ester-forming proteins were present in the E2 fraction [6], but only the smallest of them reconstituted substrate ubiquitination catalyzed by the then-known E3 [7]. This result suggested that there could be multiple E2s with distinct functional properties. Confirmation of this hypothesis came with the cloning of the first two E2 genes, *RAD6/UBC2* and *CDC34/UBC3*, which indeed encoded homologous yeast proteins with a signature cysteine-containing active-site motif [8, 9]. The two E2s functioned in distinct biological processes – DNA damage tolerance [8, 10] and cell-cycle control [9] – providing the first hint that ubiquitination might regulate a broad range of biological processes. A family of E2 enzymes naturally suggested that there would also be a family of E3 enzymes. This prediction has since been strikingly confirmed. We now know that specific E2/E3 complexes function to modify specific substrates with ubiquitin.

5.3 What is an E2?

A protein can be identified as an E2 enzyme according to several different criteria. Functionally, the E2 occupies an intermediate position in the conjugation cascade –

that is, it acts between the E1 and the E3 (Figure 5.1). This property accounts for the original name of ubiquitin carrier protein, which drew an analogy to the acyl carrier proteins used in fatty acid biosynthesis and non-ribosomal peptide synthesis [6]. Subsequently, with the recognition that E2 enzymes often play an active role in conjugation, the conjugating enzyme name gained favor.

Mechanistically, the E2 first participates in a thiol ester transfer reaction, in which the activated ubiquitin is moved from the active-site cysteine of E1 to that of the E2 (Figure 5.1). The E2/ubiquitin thiol ester intermediate is strictly required for downstream steps, as shown by ablation of substrate ubiquitination following mutation of the active-site cysteine residues of different E2s (see, for example, Refs. [11, 12]). The ubiquitin is then transferred from the E2 active site to the ε-amino group of the substrate's lysine residue, forming an isopeptide bond. The conjugation site can also be a specific lysine on a previously conjugated ubiquitin, which leads to polyubiquitin chain elongation; chains linked through K48 are the principal signal for targeting substrates to proteasomes [1]. Transfer of ubiquitin to the substrate requires the assistance of the E3 [1, 6]. If this enzyme belongs to the HECT domain family (***H***omologous to ***E***6AP ***C***-***T***erminus), the ubiquitin is first transferred to an active-site cysteine residue of the E3; if the E3 belongs to the RING domain family (***R***eally ***I***nteresting ***N***ew ***G***ene), ubiquitin is transferred directly to the substrate's amino group (Section 5.6.3). Collectively, these properties constitute the biochemical definition of an E2 enzyme: it is a protein that accepts ubiquitin in thiol ester linkage from E1, and cooperates with an E3 enzyme to deliver this ubiquitin to the substrate.

The functional specialization of individual E2s (Section 5.4) reflects the specificity of interaction of each E2 with its cognate E3(s), in conjunction with the E3's substrate specificity. Therefore an E2 enzyme can also be defined according to the cognate E3(s) with which it interacts. The E3 partners of many of the eleven ubiquitin-dedicated E2s in *Saccharomyces cerevisiae* are conserved in higher organisms (Section 5.4). However, both the E2 and E3 families are much larger in higher organisms than in yeast. Present accounting suggests that there are 50–70 E2s, and hundreds of E3s, in mammals [13, 14].

The amino acids surrounding the thiol ester-forming cysteine residue are particularly highly conserved, but there is sequence similarity throughout the ~150-residue E2 core domain (Figure 5.2). This bioinformatic definition makes it easy to identify E2 genes in sequenced genomes [13, 14]. In fact, many E2s consist of just this core domain (Figure 5.2). The fact that such E2s are often functionally distinct from one another indicates that modest sequence variation within the core domain can be highly significant. Structural biology has begun to shed light on this structure/function correlation (Section 5.6). Other E2s display N- and C-terminal extensions to the core domain (Figure 5.2), which may play a role in E3 and/or substrate specificity (see Refs. [15–19]).

Structural biology provides a final way to define an E2 enzyme. As expected from the strong sequence conservation, the E2 core domain adopts a conserved fold. At the time this article was being prepared, twelve different E2 structures had been deposited in the Protein Data Bank. The average root-mean-square deviation of

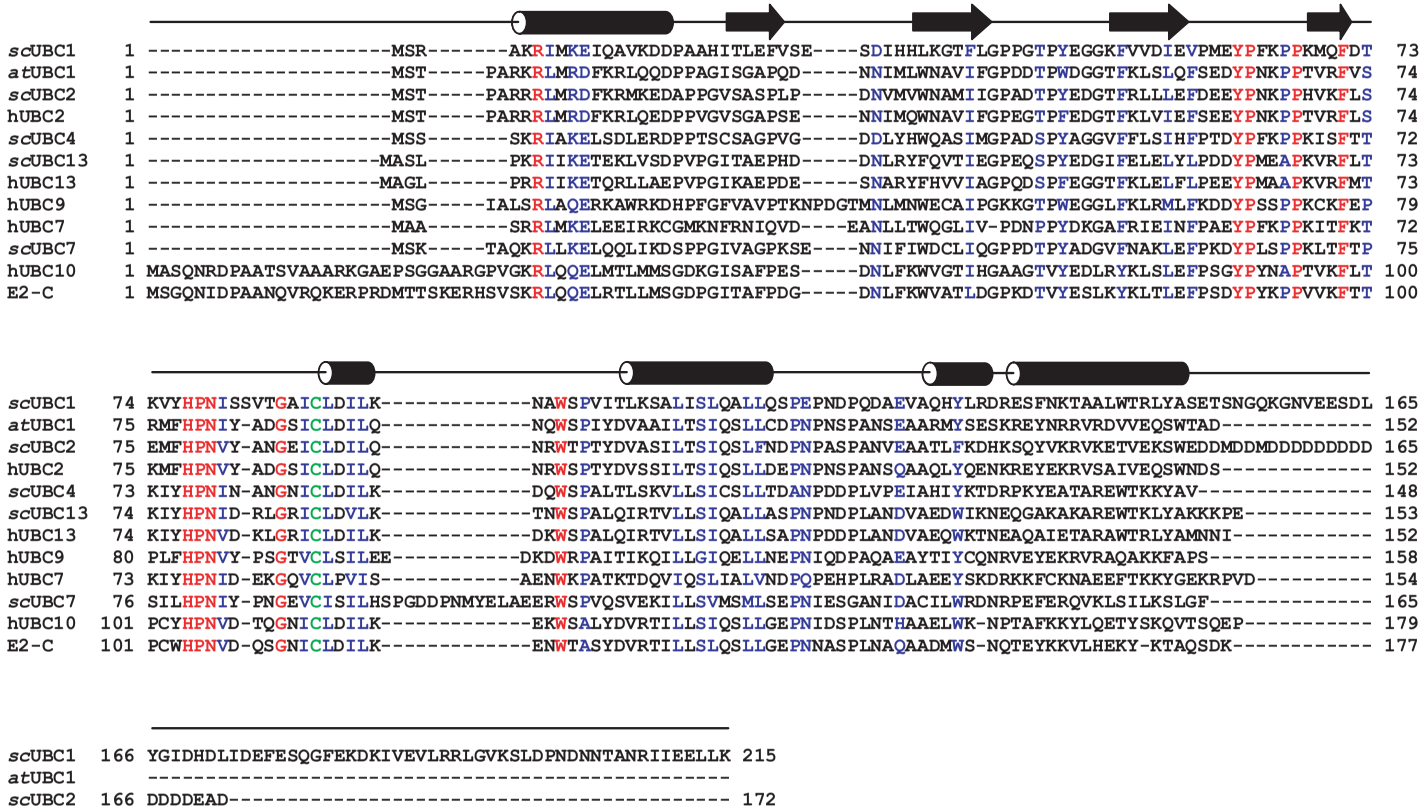

Fig. 5.2. E2 sequence alignments. Sequences of the twelve E2s found in the PDB. The active-site cysteine is colored green, identical residues colored red, and conserved residues colored blue.

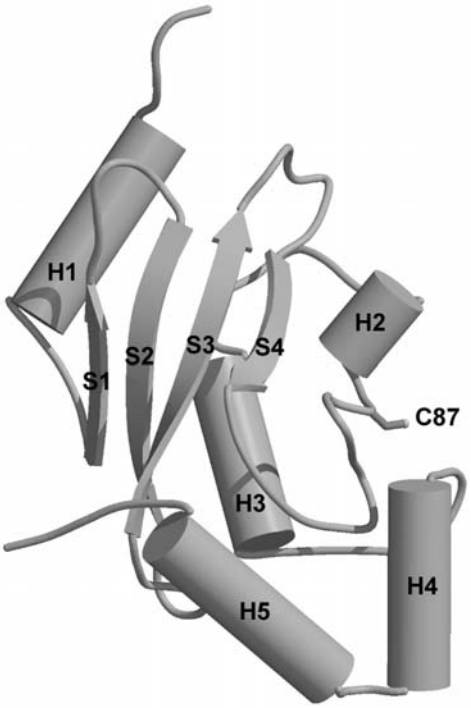

Fig. 5.3. Ubc13 (1JBB). Canonical α/β E2 fold with the active-site cysteine shown in ball-and-stick.

the 150 Cα positions of these structures is less than 2 Å. E2s are α/β proteins containing a central anti-parallel four-stranded β-sheet (S1–S4), four α-helices (H1, H3, H4, H5), and a small 3_{10} helix (H2) (Figure 5.3) [20, 21]. The cysteine is located on an extended loop after β-strand 4 and immediately before the short 3_{10} helix H2. The active-site cysteine sits in a shallow groove composed of residues from the H3–H4 and S4–H2 loops. The canonical α/β E2 fold is highly versatile, allowing E2 enzymes to associate with several different proteins in the ubiquitin conjugation cascade without any perturbation of the E2's tertiary structure (Section 5.6). Residues occupying the face opposite the active site are less conserved than those surrounding the cysteine [21]. Sequence variation in this region contributes to the functional diversity of the E2 family by permitting specific interactions of individual E2s with cognate E3s and (perhaps) substrates.

5.4
Functional Diversity of Ubiquitin-conjugating Enzymes

The functional range of the ubiquitin-conjugating enzyme family is easily appreciated by considering the family members in a single organism. Table 5.1 summa-

Tab. 5.1. E2 enzymes of the yeast *Saccharomyces cerevisiae*.

Gene	*Amino acids*	*Cognate E3*	*Functions and substrates*
UBC1	215 [28]		Short C-terminal tail harbors ubiquitin-associated (UBA) domain [155]
		Unknown	Essential in *ubc4Δubc5Δ* genetic background, suggesting a redundant role with Ubc4/5 in proteasomal turnover of short-lived and abnormal proteins [28]
		Hrd1 [39]	Role in ERAD that is not fulfilled by Ubc4/5 [39, 40]
UBC2 (*RAD6*)	172 [8]	Ubr1 [156]	Proteasomal degradation of N-end rule [158] substrates, including cohesin fragment [51]
		Ubr1 [52]	Proteasomal degradation of Cup9 transcriptional repressor regulates peptide import
		Rad18 [73, 157]	DNA-damage tolerance [8] via mono-ubiquitination of PCNA [65] (non-proteolytic function)
		Bre1 [67, 69]	Ubiquitination of histone H2B [68] regulates gene transcription and silencing [71] (non-proteolytic function)
UBC3 (*CDC34*)	295 [9]	SCF E3s	Essential gene; long C-terminal tail; targets diverse substrates for proteasomal degradation [30, 34, 36]; regulation of cell-cycle progression
UBC4	148 [24]	Unknown	Proteasomal degradation of diverse short-lived proteins [24]
		Doa10	Proteasomal degradation of MATα2 transcriptional repressor [49]
		Rsp5	Endocytosis of membrane proteins [60, 61]; protein trafficking (see Ref. [63])
UBC5	148 [24]	See *UBC4*	92% identical to Ubc4; functionally redundant [24]
UBC6	250 [46]		C-terminal tail provides anchoring to ER membrane [46]
		Unknown	Together with Ubc7, proteasomal degradation of some ERAD substrates [47, 48, 159]
		Doa10 [42]	Proteasomal turnover of Ubc6 is Ubc6-, Ubc7-, and Doa10-dependent [42, 50]
		Doa10 [42]	In conjunction with Ubc7, proteasomal turnover of MATα2
UBC7	165 [25]		Localized to ER membrane via Cue1 [37]
		Hrd1 [39]	Role in proteasomal degradation via ERAD [38, 48] confers resistance to cadmium and other ER stresses [25]
		Doa10 [42]	In conjunction with Ubc7, proteasomal turnover of MATα2
UBC8	206 [160]	Unknown	Glucose-induced proteasome degradation of fructose-1,6-bisphosphatase [57]
UBC10	165 [75]	Unknown	Also called Pas2/Pex10. Peroxisome biogenesis [75]; Pex10 is a candidate E3 [78]

Tab. 5.1. *(continued)*

Gene	*Amino acids*	*Cognate E3*	*Functions and substrates*
UBC11	156 [84]	Unknown	Unknown; similar to clam E2-C (E2-C functions in mitotic cyclin turnover [79], but Ubc11 is dispensable for this process in yeast [84])
UBC13	153		Heterodimerizes with Mms2 (UEV) [72]
		Rad5 [73]	DNA-damage tolerance [72] via polyubiquitination of PCNA [65] (non-proteolytic function)
UBC9	157 [161]		Essential gene; E2 dedicated to Smt3 (SUMO) [163]
		Siz1/2 [162]	Septin modification
UBC12	188 [164]		E2 dedicated to Rub1 (Nedd8)
		SCF E3s	Modification of specific cullin lysine residue activates cullin-based E3s [165]

rizes key properties of the complete set of E2s in the yeast *S. cerevisiae*, including notable structural features, known cognate E3(s) and their key substrates, and biological functions (see also [22, 23]). We cannot give a comprehensive review of E2 functions in higher organisms, but we do comment on some notable instances of functional conservation, expansion, and divergence (see also [23]).

5.4.1 Functions Related to Proteasome Proteolysis

In many cases, the specific function(s) of a given E2 enzyme reflect its role in targeting one or more substrates for degradation by 26S proteasomes. The scope of this function varies considerably between E2 family members, however. At one extreme, the functionally redundant enzymes Ubc4 and Ubc5 are necessary for the turnover of many substrates, as shown by a marked reduction in the rate of turnover of endogenous short-lived and abnormal proteins in a *ubc4Δubc5Δ* yeast strain [24]. The slow growth and stress sensitivity of this strain [24, 25] can also be ascribed to inhibition of proteasomal proteolysis since these phenotypes are characteristic of proteasome mutants [22, 26]. Despite the important role of Ubc4/5 in proteasome degradation, few E3 partners relevant to this function have been identified. One is Ufd4, a HECT-domain E3 that mediates the degradation of linear ubiquitin fusion proteins [27]. A *ufd4Δ* strain grows normally, however, indicating that Ubc4 has other cognate E3s. Rsp5, an essential HECT-domain E3, is one likely candidate since this E3 partners with Ubc4 in other pathways (see below). *UBC1* is essential for viability in the *ubc4Δubc5Δ* strain, suggesting that Ubc1 shares substantial functional overlap with Ubc4/5 in directing substrates to proteasomes for degradation [28].

The Ubc4/5 sub-family of E2s is much larger in mammals, where it includes

both constitutively and selectively expressed enzymes. Notable human E2s in this group are UbcH5a/b/c, UbcH7, and UbcH8 (see Ref. [23]). The expansion is likely to reflect the much larger size of the E3 family in higher organisms. However, while the results of *in vitro* conjugation assays and protein–protein interaction studies suggest that certain E3s partner specifically with individual Ubc4/5 subfamily members, the degree of E3 (hence, functional) selectivity in the cellular setting remains quite uncertain (discussed in Refs. [23, 29]). RNA interference studies and mouse knockout models may be helpful in addressing this question in the future.

Ubc3/Cdc34 supports the proteasome-mediated proteolysis of numerous substrates through its role as the specific E2 partner of a large family of multi-subunit RING E3s called SCF E3s (***S***kp-***C***ullin-***F***-box, Section 5.6.3). This role is preserved in higher organisms [30]. Yeast *ubc3* mutants arrest in G1 phase of the cell cycle because they fail to degrade Sic1 [31], an inhibitor of the G1/S transition that is recognized and polyubiquitinated by a specific SCF E3 [32, 33]. Although this is the only essential function of yeast Ubc3 [31], this E2 partners with many other SCF E3s to target diverse substrates for degradation by proteasomes (see Refs. [30, 34–36]). Although studies in yeast suggest that Cdc34 is the main E2 partner of SCF E3s, some SCF E3s seem to partner with Ubc4/5-type E3s (see Refs. [23, 36]). Ubc3 has a long C-terminal tail (Table 5.1), making it the most distinctive yeast E2 in terms of primary structure. A chimeric E2 in which the Ubc3 tail is appended to the core domain of Ubc2 fulfills the essential function of Ubc3 in yeast, suggesting that the tail of Ubc3 is necessary for key interactions with the E3 or Sic1 [15, 16].

Ubc7 is localized to the endoplasmic reticulum (ER) through an interaction with a partner protein, Cue1 [37], and plays a major role in proteasome degradation. Ubc7 acts on misfolded proteins of the ER, which are ejected from this compartment as a prelude to ubiquitination at the cytosolic face of the ER membrane and degradation by cytosolic proteasomes [38]. Ubc7's role in ERAD (***ER A***ssociated ***D***egradation) explains why a *ubc7Δ* strain is conditionally sensitive to agents that induce protein misfolding in the ER [25, 39–41]. Ubc7 frequently partners with Hrd1, an ER-localized RING E3 [39], but some ERAD substrates of Ubc7 seem to be recognized in cooperation with a different ER-localized RING E3, Doa10 [42]. Consistent with Ubc7's prominent role in ERAD, the yeast *UBC7* and *CUE1* genes are induced as part of the ***U***nfolded ***P***rotein ***R***esponse (UPR) and there is a synthetic lethal relationship between certain ERAD and UPR genes [40, 41]. Yeast Ubc1 also plays a significant, but poorly-defined, role in ERAD [39, 40]. Mammalian Ubc7 also functions in ERAD [43–45].

Ubc6 localizes to the ER through its own C-terminal membrane anchor [46]. Although Ubc6 plays a role in ERAD, its function in this process is less conspicuous than that of Ubc7 [43, 47, 48]. Interestingly, Ubc6 and Ubc7 both contribute to the Doa10-dependent degradation of a soluble nuclear protein [42, 49], and Ubc6 is itself rapidly degraded by proteasomes in a manner that depends on its own active-site cysteine, its C-terminal membrane anchor, functional Ubc7, and Doa10 [42, 50]. The purpose of this instability remains mysterious.

Ubc2 functions rather selectively in proteasome proteolysis. In yeast, two specific E3 partners are known, leading to proteasome degradation events that regulate chromosome stability [51], peptide import [52], and homing endonuclease stability [53]. Mammals have two closely-related Ubc2 isoforms, each of which complements most of the functions of the yeast *ubc2Δ* strain [54]. But the mammalian Ubc2 isoforms also have specialized functions – one of them is required for spermatogenesis in the mouse [55] and at least one of them can be inferred to be necessary for cardiovascular development [56].

So far, Ubc8 has been implicated in the regulated turnover of just one substrate, and its E3 partner(s) remain unknown [57]. Interestingly, the closest mammalian relative of yeast Ubc8 is expressed with a restricted tissue specificity and (in some tissues) in a regulated manner [58, 59].

5.4.2 Endocytosis and Trafficking

Just because an E2 functions in proteasome proteolysis does not mean that its functions are limited to this pathway. This is because the E2's functional range is largely determined by the substrate specificity of its E3 partner(s). For example, yeast Ubc4 and Ubc5 play a prominent role in proteasome degradation, but they also cooperate with a HECT E3, Rsp5, to mono-ubiquitinate certain plasma membrane receptors [60–62]. This modification signals receptor endocytosis, leading to degradation in the vacuole (equivalent to the mammalian lysosome). Ubc1 is partially redundant with Ubc4/5 in endocytosis [61, 62], as seen in ubiquitination reactions leading to proteasome degradation (above). Thus, Ubc1, 4, and 5 may be able to substitute for one another in complexes involving many different E3s, probably reflecting the strong conservation of the core domain between Ubc1 and Ubc4/5. Ubc4 and Ubc5 may also act with Rsp5 to regulate protein trafficking downstream of endocytosis (reviewed in Ref. [63]).

5.4.3 Non-proteolytic Functions

As mentioned in Section 5.2, Ubc2 is the defining player in a conserved DNA damage-tolerance pathway [8, 10, 64]. Here Ubc2 partners with a RING E3, Rad18, to modify a DNA polymerase processivity factor with a single ubiquitin [65]. This modification signals error-prone bypass of DNA lesions [66]. Ubc2 partners with a different RING E3 (Table 5.1) to mono-ubiquitinate histone H2B [67–69]. This modification promotes histone methylation, which in turn regulates transcription and silencing [70, 71].

Ubc13 participates in the same DNA damage-tolerance pathway as Ubc2 [72]. Ubc13 collaborates with two enzyme partners (Table 5.1) to modify the DNA polymerase cofactor (above) with a K63-linked polyubiquitin chain, which signals error-free lesion bypass [65, 72, 73]. In higher organisms, Ubc13 also helps to synthesize K63-linked polyubiquitin chains in a second non-proteolytic signaling pathway (see

Ref. [74]). The function and mechanism of Ubc13 are discussed in more detail below (Section 5.7).

5.4.4 E2s of Uncertain Function

Ubc10 is required for the biogenesis of the peroxisome, an oxidative organelle [75]. This E2 plays a role in peroxisomal protein import [76] and is recruited to the peroxisomal membrane through an interaction with a partner protein [77]. Membrane-localized Ubc10 also seems to be spatially proximal to Pex10, which has a RING-like domain [78]. Whether Pex10 is a cognate E3 of Ubc10 remains to be determined, as does the mechanistic role of ubiquitin conjugation in peroxisome biogenesis.

The function of the remaining yeast E2, Ubc11, remains uncertain. Ubc11 is very similar to E2-C, a clam E2 that acts with an essential multi-subunit RING E3, the ***a***naphase ***p***romoting ***c***omplex (APC) or cyclosome, to ubiquitinate mitotic cyclins [79]. This reaction leads to cyclin degradation by proteasomes, which drives exit from mitosis [35, 80]. Mitotic cyclin ubiquitination can be reconstituted *in vitro* with apparent amphibian and fission yeast orthologs of either Ubc11 or Ubc4 [81, 82] and other data implicate both E2s in this process in higher cells [82, 83]. However, mitotic cyclins are efficiently degraded in budding yeast *ubc4Δ* and *ubc11Δ* strains, indicating that other E2 enzymes can support this essential function in *S. cerevisiae* [84].

5.4.5 E2 Enzymes and Disease

There are now several striking examples of disease-related defects in ubiquitin conjugation, but most of them involve E3s rather than E2s. This is not surprising given the paramount role of E3s in substrate selection and the corresponding intensity of research effort that has been focused on E3s. Still, there are several hints that defects at the E2 level of the conjugation cascade can also contribute to disease.

Many viruses subvert the ubiquitin system to evade the host cell's defenses or modulate the cellular environment so as to promote viral replication (see Refs. [85, 86] and Section 5.7). The genome of African swine fever virus encodes an E2 enzyme that is somewhat similar to yeast Ubc3 [87, 88]. This enzyme might alter the activity or specificity of the host cell's conjugation cascade so as to benefit the virus, or it could act on specific viral proteins. ***H***erpes ***S***implex ***V***irus-1 (HSV-1) encodes an E3 enzyme that specifically binds the host cell's Ubc3/Cdc34 enzyme and targets this E2 for ubiquitination and (presumably) degradation – events that may help to stabilize specific cyclins and promote viral replication [89].

A different kind of relationship between an E2 enzyme and disease is exemplified by the finding that the Alzheimer's amyloid-β peptide induces the expression of $E2_{25K}$, a mammalian relative of yeast Ubc1 [90]. $E2_{25K}$ was found to play a major role in amyloid-β-dependent neuronal cell killing. This effect may be related

to the $E2_{25K}$-dependent production of aberrant polyubiquitin chains, leading to the inhibition of proteasomes [90, 91]. Other studies showed that the human homolog of yeast Ubc11 is over-expressed in numerous cancer cell lines and primary tumors and that forced over-expression of this E2 in cultured cells can drive proliferation and transformation [92]. Similarly, transformation and chromosomal abnormalities were observed following over-expression of human Ubc2b [93]. Such disease-related over-expression effects could arise in two different ways. The higher E2 concentration could lead to a relaxation of specificity – that is, pairings with non-cognate E3s – leading to inappropriate ubiquitination events. Alternatively, specificity could be maintained, but an inappropriately high flux through the normal E2/E3 pathway could lead to the excessive ubiquitination of cognate substrates.

5.5 E2 Enzymes Dedicated to Ubiquitin-like Proteins (UbLs)

Ubiquitin is just one member of a family of protein modifiers that share a common fold and a common mechanism of isopeptide tagging [70, 94, 95]. Like ubiquitin, individual UbLs are activated at a C-terminal glycine residue by a specific E1 enzyme. Often, the next step is transfer to a specific E2 enzyme. Certain UbL-specific E2 enzymes are so similar to ubiquitin-conjugating enzymes that they were initially thought to be members of the ubiquitin-conjugating enzyme family. This was true of yeast Ubc9 and Ubc12, which are dedicated to Smt3/SUMO and Rub1/Nedd8, respectively (Table 5.1). SUMO modifies numerous cellular proteins and has a broad functional range [94], but the only known target of Nedd8 is a specific lysine residue in one subunit (the cullin) of SCF E3s. Nedd8 modification activates these E3s (see Ref. [70]).

The reader should consult earlier reviews [70, 94, 95] and other chapters in this volume for a detailed discussion of UbL biology and biochemistry. There are two important points for the current discussion. First, the conjugation cascades of UbLs differ from that of ubiquitin chiefly in terms of complexity – there is one conjugating enzyme per UbL, and many fewer E3s. Second, because modifier proteins (including ubiquitin) do not interact strongly with their dedicated E2s (Section 5.6.1), it is believed that E1 enzymes play the major role in matching E2s with the correct modifier protein (see Ref. [96]).

5.6 The Biochemistry of E2 Enzymes

5.6.1 E1 Interaction

An E2 needs to associate with several different proteins in the course of the ubiquitin conjugation cascade, with the first being the E1. Mutational studies con-

ducted with the SUMO-specific E2 Ubc9 suggest that free Ubc9 associates with its free cognate E1 through a surface of Ubc9 that includes the C-terminal residues of α-helix H1 and residues in a loop between β-strands S1 and S2 (Figure 5.3) [97]. This surface of Ubc9 is also important for thiol ester bond formation [97]. Several residues in α-helix H1, particularly the C-terminal residues, are poorly conserved among E2s, and Ubc9 contains a five-residue insertion in the loop between β-strands S1 and S2 (Figure 5.2). Thus, this region of E2s may contribute to specificity for their cognate E1s. Consistent with this idea, the N-terminal helix (H1) of a ubiquitin E2 was found to be important for E2/ubiquitin thiol ester formation [98]. One cautionary note is that Ubc9 displays a substantial affinity for its free E1 [97], whereas ubiquitin E2s bind tightly to their E1 only after it has been loaded with ubiquitin [6, 99, 100]. The structural basis of this effect remains to be determined.

5.6.2 Interactions with Thiol-linked Ubiquitin

As a consequence of interacting with ubiquitin-loaded E1, the E2 accepts the activated ubiquitin at its active-site cysteine residue. This thiol ester complex, although biochemically detectable [6], has not been crystallized because it is labile in comparison to the requirements of structural biology. However, NMR chemical shift perturbations have been used to map the binding surface of ubiquitin onto human Ubc2b [101], yeast Ubc1 [102], and human Ubc13 [103]. All three models map the ubiquitin-binding surface of the E2 to a common area that includes parts of α-helix H3, the loop between α-helices H3 and H4, and residues around the active-site cysteine in the extended S4-H2 loop, including part of the 3_{10} helix H2 (Figures 5.2 and 5.4). The C-terminus of ubiquitin extends around part of the E2 and is constrained in a cleft [102, 103]. Although this contact surface is detectable in the thiol ester, free E2s display a negligible affinity for free ubiquitin [6]. Thus, the covalent E2/ubiquitin bond enables the formation of these non-covalent contacts.

5.6.3 E3 Interactions

After E2/ubiquitin thiol ester formation, the ubiquitin must be transferred to the substrate, which is sometimes another ubiquitin. An E3 is usually required for this reaction *in vitro*, and is always required *in vivo*. There are three known types of E3s: the RING domain, HECT domain, and U-box (UFD2 homology) families. RING and U-box E3s act as bridging factors for E2s and substrates, but HECT E3s use a different mechanism, adding an extra step to the pathway (Section 5.6.3.3).

5.6.3.1 RING E3/E2 Interactions

The small RING domain coordinates two zinc ions in a cross-brace arrangement [104]. The domain is defined by the presence of eight zinc-binding groups (cysteines and histidines) with a conserved spacing, such that the distance between

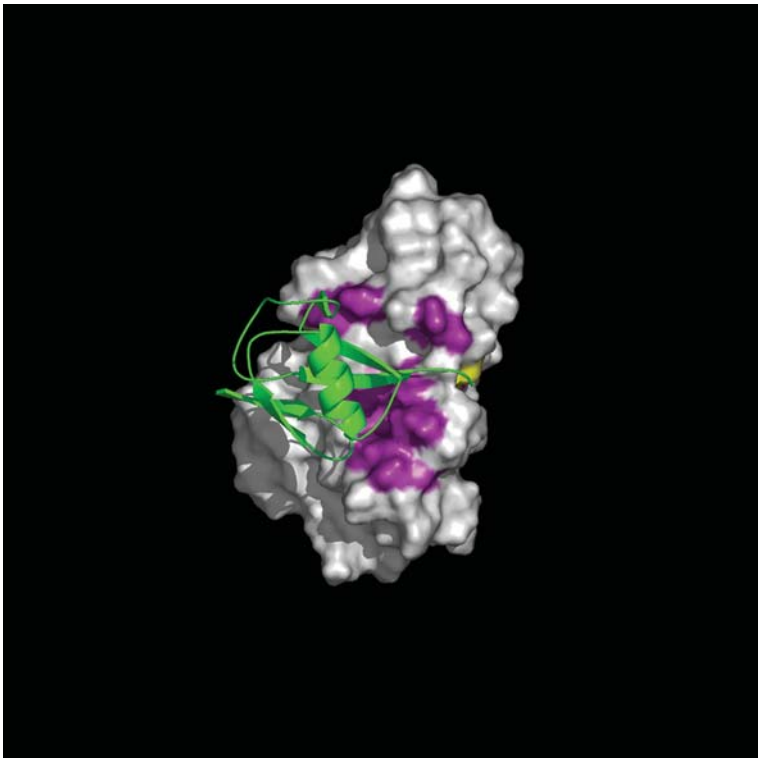

Fig. 5.4. Ubc1/ubiquitin thiol ester complex model (1FXT). The surface of Ubc1 is shown with residues implicated in ubiquitin binding colored purple and the active-site cysteine colored yellow. Ubiquitin is colored green.

the two zincs is conserved at 14 Å [104]. Sequence conservation between RING domains is otherwise minimal. The RING-domain fold consists of a central α-helix and several small β-strands separated by loops with variable lengths [105].

RING E3s can be either single-subunit or multi-subunit enzymes. The crystal structure of UbcH7 complexed to a single-subunit RING E3, c-Cbl, shows that the RING domain is the main site of contact, although there are a few intermolecular hydrogen bonds to a non-RING helix of the E3 [106] (Figure 5.5). The structure of the E2 in the c-Cbl/UbcH7 complex is unchanged relative to free E2 structures. The main basis for the interaction is the packing of several hydrophobic residues of UbcH7, notably F63, into a shallow groove on the RING domain surface. These residues come from the S3–S4 and H2–H3 loops (Figure 5.3). The α-helix H1 of UbcH7 makes the hydrogen-bond contacts to the non-RING α-helix. Interestingly, even though the c-Cbl/UbcH7 structure undoubtedly shows conserved RING/E2 contacts, this complex is catalytically inactive (cited in Ref. [107]). Therefore additional E2/RING contacts may be needed for catalytic competence.

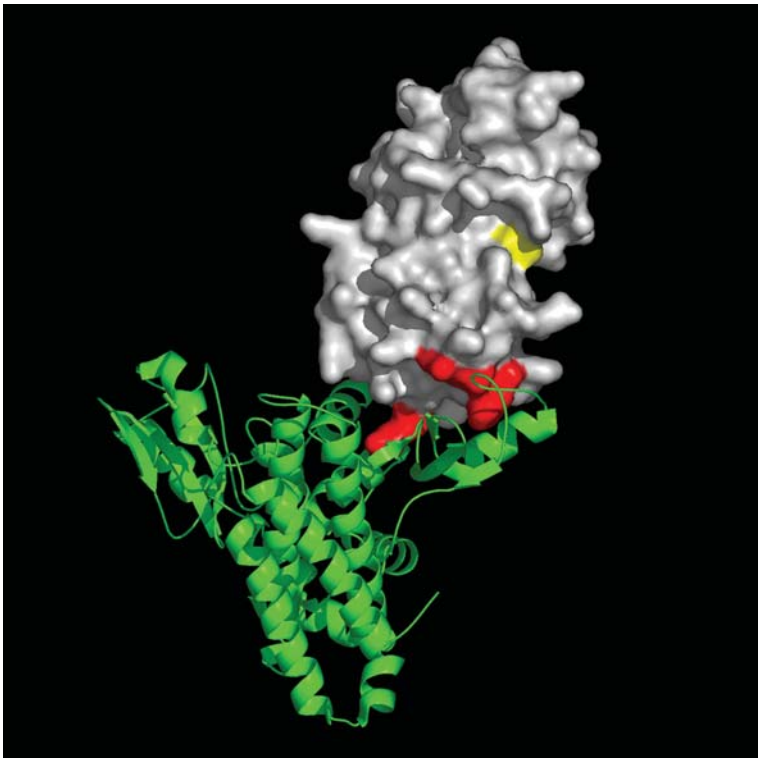

Fig. 5.5. UbcH7/c-Cbl complex (1FBV). The surface of UbcH7 is shown with residues interacting with the c-Cbl RING domain shown in red and the active-site cysteine shown in yellow. c-Cbl is colored green.

The surface of UbcH7 that contacts c-Cbl does not overlap with the E2 surface that contacts ubiquitin (Figures. 5.4 and 5.5; see also Ref. [108]), confirming that the E2/ubiquitin thiol ester can associate with a RING E3. The E2 surface that contacts c-Cbl does, however, overlap the E2 surface implicated in E1/E2 interactions (Section 5.6.1). Thus, the E1 may have to depart from the E2/ubiquitin complex before E2/E3 interactions can take place.

The closest approach of a RING-domain residue to the active-site cysteine of UbcH7 is about 15 Å, arguing against a role for RING E3s in chemical catalysis [106]. Instead, RING E3s have been proposed to facilitate ubiquitination by inducing physical proximity of the E2/ubiquitin thiol ester and the substrate [23, 30, 106, 109]. Catalysis would result from the increased local concentrations of the two reactants (discussed further below).

RING/E2 interactions have also been studied with BRCA1. This E3 is unique in that it must heterodimerize with a second RING-domain protein, BARD1, in order to display maximal E3 activity [110]. Even though the heterodimer interface leaves

both RING domains available for interaction [110], UbcH5c binds exclusively to the BRCA1 RING [107]. The interacting surface of UbcH5c is homologous to the surface of UbcH7 that contacts the c-Cbl RING domain in that several residues of UbcH5c pack into a cleft on the BRCA1 RING domain. But UbcH5c also makes several contacts with the C-terminus of the BRCA1 RING domain and with a non-RING region of the heterodimer [107]. These extra contacts are not observed when UbcH7 binds to the BRCA1/BARD1 complex [107]. Because the BRCA1/BARD1/UbcH7 complex is inactive, the extra contacts observed with UbcH5c may help to create a competent E2/E3 complex.

Despite the greater complexity of multi-subunit RING E3s, a common theme is evident – all SCF E3s, as well as several other types of cullin-based E3s, utilize a common RING-domain subunit, the small protein Rbx1 (reviewed in Refs. [30, 111]). Four subunits compose the minimal SCF E3 ligase complex: a cullin scaffold, Rbx1, an adaptor protein (Skp1), and a substrate-binding subunit that connects to the adaptor through a conserved domain called the F-box (see Ref. [30]). The cullin acts as a scaffold, with Rbx1 binding to one end to form a cullin/Rbx1 subcomplex that recruits the E2 and, in many cases, displays a substrate-independent ubiquitin-ligase activity (see Refs. [23, 30]).

The crystal structure of the mammalian SCF^{Skp2} ($Skp1/Cul1/F\text{-}box^{Skp2}/Rbx1$) E3 ligase shows a remarkably rigid, elongated complex [112]. The Cul1 scaffold contains three cullin-repeat motifs that span ~110 Å, with Rbx1 binding to a discrete C-terminal α/β domain. The $Skp1/F\text{-}box^{Skp2}$ complex binds to the opposite (N-terminal end) of the cullin. Rbx1 displays a hydrophobic groove, as seen previously in the c-Cbl RING domain [106, 112]. In c-Cbl, this groove provides an interaction surface for UbcH7 and it is reasonable to assume a similar mode of interaction in the case of Rbx1. Interestingly, the site where Nedd8 modifies the cullin is close to where the E2 binds, consistent with data which suggest that neddylation modulates E2 binding or activity [113, 114].

A model of the full SCF/E2 complex [112] shows that the end of Skp2 which binds the substrate is pointed toward the Rbx1-bound E2, with a 50-Å gap between the two. Models based on two other SCF structures show similar distances between the F-box protein and the E2 [109, 115]. Whether this gap can be bridged by the bound substrate is currently unclear. It has been suggested that the E2 may bind to Rbx1 somewhat differently than UbcH7 is observed to bind in the c-Cbl RING/UbcH7 complex, but it is not obvious that this can lead to a 20 Å movement of the E2 toward the bound substrate as suggested [109].

One could also imagine that the bound substrate and E2 ''meet'' through conformational changes of the SCF complex. However, the rigid separation produced by the Cul1 scaffold seems to be important for activity – introducing a flexible linker into the center of Cul1 produced a protein that could still bind an E2, but did not catalyze substrate ubiquitination [112]. An interesting study established a positive correlation between the rate of dissociation of the Ubc3/ubiquitin intermediate from the RING domain and the rate of Sic1 ubiquitination catalyzed by SCF^{Cdc4} [116]. The authors proposed that the role of the RING domain is to bring the

charged E2 into the vicinity of the SCF-bound substrate, but that release of the charged E2 is important to bridge the gap and enable multiple substrate lysines to be targeted. However, although these mechanisms may place the substrate's lysine residue in the general vicinity of the E2's active site, it is unclear that they can establish an effective orientation of the lysine residue and the thiol ester bond. Since there is no known consensus site for ubiquitination [23, 117, 118], it is unlikely that specific molecular contacts in the vicinity of the substrate's lysine residue are used to position this attacking group. Overall, it remains unclear how the substrate's lysine residue approachs the E2 active site.

5.6.3.2 U-box E3/E2 Interactions

The U-box family of E3s bind E2s through the small U-box domain [119]. Some U-box E3s do not seem to have their own cognate substrates, but instead promote polyubiquitination of the substrates of other E3s [120]. Other U-box E3s have defined cognate substrates and behave in a canonical manner [121, 122].

An NMR structure [123] confirmed an earlier prediction [124] that the U-box domain has a RING-domain-like fold. Remarkably, the U-box domain uses hydrogen-bonding networks in place of zinc coordination to support the characteristic cross-brace arrangement. These interactions stabilize a globular fold consisting of a central α-helix surrounded by several β-strands, which are separated by loops of variable length [123]. There is a shallow groove in the surface located in a position homologous to the E2-interacting surface of RING domains. Mutational studies have linked E3 ligase activity to some of the residues in the surface groove [123, 125]. Since these mutations do not disrupt the U-box fold, they are likely to abrogate E2 binding. Although it is likely that E2s bind similarly to the U-box and RING domains, no E2/U-box structure has been reported so far.

5.6.3.3 HECT E3/E2 Interactions

HECT-domain E3s are defined by the presence of a domain of ~350 residues that is homologous to the C-terminus of the founding family member, E6AP (E6 ***A***ssociated ***P***rotein [126]). E6AP is known for its role in binding the E6 protein of oncogenic human papilloma viruses and targeting the p53 tumor suppressor for ubiquitination and degradation [127]. HECT-domain E3s possess an active-site cysteine residue positioned ~35 residues upstream of the C-terminus; a thiol ester with ubiquitin is formed at this site and is required for substrate ubiquitination [128].

The crystal structure of an E6AP/UbcH7 complex showed that the HECT domain is L-shaped, with a large, mostly α-helical, N-terminal lobe and a small C-terminal lobe with an α/β structure [108] (Figure 5.6). UbcH7 binds to the end of the N-terminal lobe and somewhat parallel to the C-terminal lobe, forming an overall U-shaped complex. UbcH7 binds in a large hydrophobic groove in the N-terminal lobe [108]. As seen in other E2/E3 structures, neither the E2 enzyme nor the HECT domain changes its overall fold upon binding. UbcH7 contacts its binding groove with residues from the S3–S4 loop and the H2–H3 loop (Figure 5.3). A few contacts are also made with the C-terminal portion of α-helix H1.

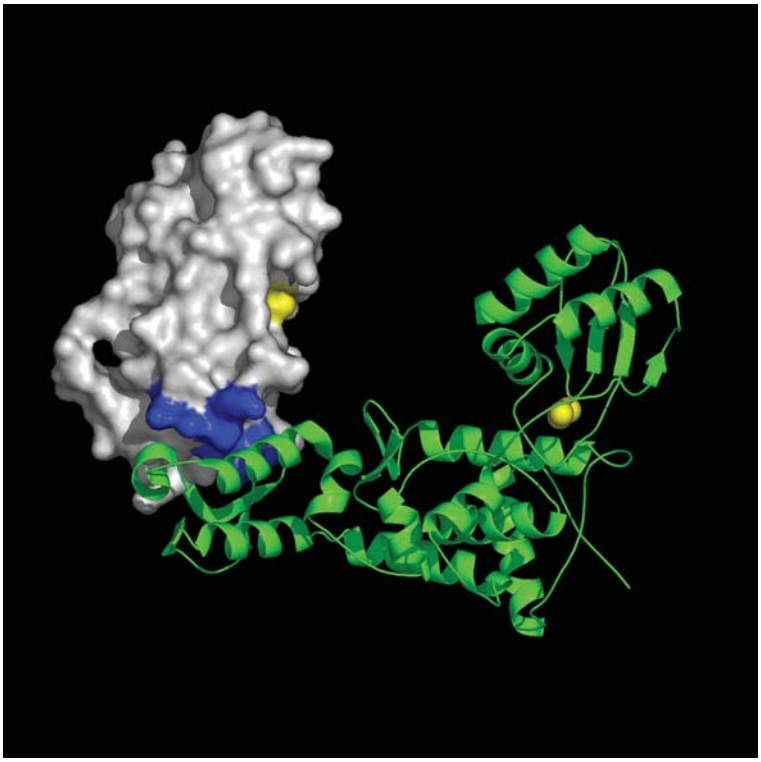

Fig. 5.6. UbcH7/E6AP (1C4Z). The surface of UbcH7 is shown with residues interacting with the E6AP HECT domain shown in blue and the active-site cysteine shown in yellow. E6AP is colored green with the active-site cysteine between the N- and C-lobe shown in yellow.

Remarkably, these are the same two loops and helix that bind the RING domain in the c-Cbl/UbcH7 structure [106]. That two different E3s contact a largely similar surface on UbcH7 (Figures 5.5 and 5.6) can be explained through the nature of key side-chain contacts. The S3–S4 loop seems to provide most of the specificity, as it contains the F63 residue that is present in all E2s that are known to bind both HECT E3s and c-Cbl [106, 108]. In c-Cbl, the main contacts for F63 are isoleucine and tryptophan residues located in the RING groove [106]. Both F63 and its contact site in c-Cbl are seen to vary in other E2/RING E3 pairs, suggesting that interactions between these three specific residues are needed, but that the nature of the contact can vary [106]. In other words, a different E2 could bind to a different E3 with a similar geometry, but through different types of side-chain contacts. UbcH7-F63 makes specific contacts with six E6AP residues, so this interaction is likely to be important for all HECT/E2 pairs [108]. The H2–H3 loop that makes the other major contacts with the HECT domain is part of the more variable E2 surface (Sec-

tion 5.3). The specific contacts made between the H2–H3 loop of UbcH7 and the E6AP HECT domain could be used to correctly predict the E2 preferences of E6AP and Rsp5 [108]. Thus, residues in the S3–S4 and H2–H3 loops play an important role in determining the specificity of E2/E3 interactions.

Positioned near the bend between the two lobes of the HECT domain is its active-site cysteine [108]. This residue is 41 Å away from the active-site cysteine of UbcH7 (Figure 5.6), suggesting that a large conformational change is needed to bring about transfer of ubiquitin from E2 to E3. Such a mechanism was confirmed in the crystal structure of another HECT domain [129]. The WWP1-HECT domain resembles the E6AP HECT domain in having two lobes, but their relative positions differ. In the WWP1-HECT domain the two lobes form an inverted T-shape as opposed to the L-shape seen in the E6AP HECT structure [108, 129]. This conformational change can be brought about by a rotation around three residues in a hinge loop connecting the two lobes. Modeling in the E2 in a position homologous to that seen in the E6AP/UbcH7 structure, the distance between the two active-site cysteines decreases to 16 Å [129]. With additional rotation around the hinge loop the WWP1 active-site cysteine can be brought within 5 Å of the E2's active-site cysteine. Mutational studies suggested that the flexibility of this hinge loop is indeed important for ligase activity [129].

This flexibility requirement points to possible models for ubiquitin transfer and polyubiquitin chain elongation. One possibility is that the HECT domain adds ubiquitin to target substrates one at a time. This would imply that the E3 changes specificity – from recognizing the substrate to recognizing the ubiquitin – following transfer of the first ubiquitin to the substrate. A different possibility is that the chain is built up by the HECT E3 first, and then transferred as a unit to the substrate. This would require two active sites, one to hold the growing polyubiquitin chain, and the other to hold the next ubiquitin to be added. HECT E3/E2 complexes would satisfy this condition. RING E3/E2 complexes cannot, and thus would have to utilize another mechanism, presumably building on their rigid architecture. In an attractive model [129], the HECT cysteine could hold the first ubiquitin (and later the growing chain), while the C-lobe could rotate to position the first ubiquitin's target lysine near the thiol ester bond of the bound E2/ubiquitin intermediate. Subsequent rounds of ubiquitin addition to the chain terminus would require the C-lobe to keep rotating, ultimately ending in steric problems for the chain which could favor its transfer to the substrate.

A third general model for polyubiquitin chain extension is that the initiation and elongation phases of the reaction involve different E2 enzymes, different E3 enzymes, or different E2/E3 complexes. The modification of a substrate with a non-canonical polyubiquitin chain follows the third model. The Rad6/Rad18 complex ligates the first ubiquitin, while the Mms2/Ubc13/Rad5 complex extends the chain [65, 73]. The extension of K48-linked chains from ubiquitin fusion proteins seems to involve the sequential action of two different E3s with the same E2 [120]. In another possible example, two E2s (orthologs of Ubc11 and Ubc4) have been suggested to act sequentially with the APC in the polyubiquitination of mitotic cyclins in fission yeast [82].

5.6.4 E2/Substrate Interactions

With HECT domain E3s, all of the chemistry of isopeptide bond formation occurs at the E3 active site. With RING and U-box E3s, however, the E2 participates directly in this chemical reaction, so the substrate's lysine must closely approach the E2's active site. A crystal structure of the SUMO E2 Ubc9, complexed with a large fragment of RanGAP1 (an efficient sumoylation substrate), reveals the specificity of this interaction [130]. Unlike ubiquitination, sumoylation is site-specific. The target lysine for sumoylation lies within a tetrapeptide sequence motif Ψ-K-X-D/E, where Ψ is a hydrophobic residue, K is the target lysine, and X is any residue. Ubc9 makes specific interactions with each of these consensus-motif residues in a manner that places the lysine ε-amino group within 3.5 Å of the Ubc9 active-site cysteine [130]. The lysine approaches the cysteine from what is expected to be its unencumbered (by SUMO) side [102]. The interacting surface on Ubc9 involves α-helix H4, the loop preceding it, and the extended S4–H2 loop, including the active-site cysteine [130] (Figure 5.3). This surface does not overlap with the presumptive binding surface for SUMO. This mode of interaction is unlikely to hold with ubiquitin E2s, since no general consensus site for ubiquitination is known.

A model for ubiquitin E2/substrate interactions has also been proposed for the special case in which the substrate is ubiquitin [131]. The crystal structure of the Mms2/Ubc13 complex led to the modeling of an E2/UEV/ubiquitin (donor)/ubiquitin (acceptor) model. As discussed in Section 5.7, UEV (***U***biquitin ***E***2 ***V***ariant) proteins such as Mms2 are homologous to E2s, but lack the active-site cysteine residue. Known UEV/Ubc13 complexes act as E2 enzymes specialized for the synthesis of K63-linked polyubiquitin chains [72, 132]. In the model [131], Ubc13 is bound to the donor ubiquitin through a thiol ester bond in a manner that agrees well with inferences from NMR analysis of the Ubc1/ubiquitin thiol ester [102] (Figure 5.7). The position of the non-covalently bound acceptor ubiquitin is determined by the orientation of Mms2 on Ubc13 (Figure 5.7). The acceptor ubiquitin has its K63 side chain placed to enter the active site of Ubc13 to form a diubiquitin conjugate. The model suggests that K63 of ubiquitin is selected as the conjugation site through steric exclusion of other lysines, as determined by an interaction between Mms2 and a region of ubiquitin that is distant from K63 [131]. Recent NMR studies have confirmed and refined this model [103]. Thus, the substrate lysine is presented to the active-site cysteine through an indirect mechanism, in contrast to the Ubc9/RanGAP1 example in which the E2 interacts directly with the lysine residue itself [130]. Unlike most ubiquitination reactions, the modification of ubiquitin itself is often site-specific. The Mms2/Ubc13/ubiquitin model can help to explain this phenomenon.

5.6.5 E2 Catalysis Mechanism

Chemical catalytic mechanisms in the ubiquitin conjugation cascade have proved difficult to decipher. The reactions leading to E2/ubiquitin thiol ester and isopep-

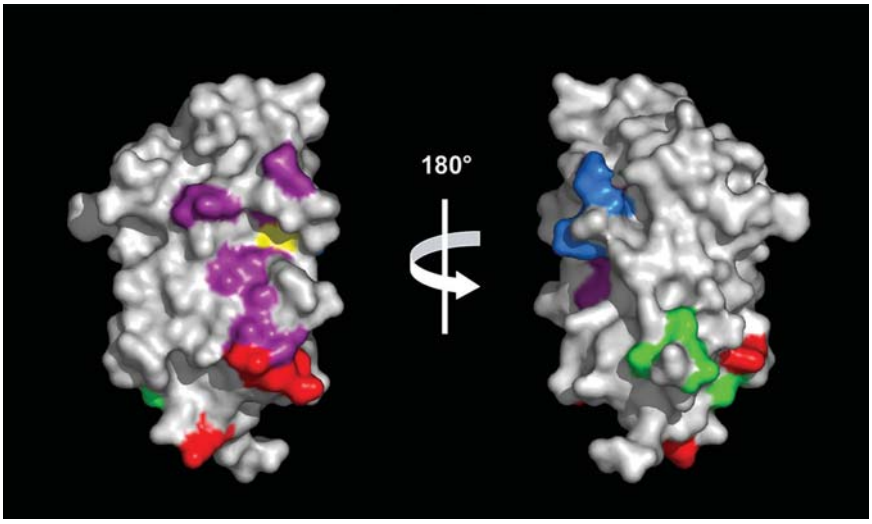

Fig. 5.7. Ubc13 interaction surface. The interacting surfaces have been mapped onto Ubc13. The active-site cysteine is shown in yellow. Colored surfaces contact: covalently bound ubiquitin (purple); RING domains (red); E1 (presumptive, green); acceptor ubiquitin involved in K63-linked polyubiqutin-chain synthesis (blue).

tide bond formation would be facilitated by electrostatic stabilization of the oxyanion and deprotonation of the attacking amino group (isopeptide bond formation) by a general base [133, 134]. However, while the sequence conservation around the E2 active site is very high (Figure 5.2), all E2 structures show a lack of candidate catalytic residues close to the cysteine (see Refs. [23, 135]). Although catalytic residues could be contributed by other enzymes in the cascade or by the E2 backbone, structural data argue strongly against a chemical catalytic contribution where it may be needed most – in reactions involving RING and U-box domain E3s.

Recent studies [136] addressed the role of a strictly conserved asparagine positioned just upstream of the active-site cysteine (N79 in Ubc1 numbering, Figure 5.2). In existing E2 structures the asparagine is hydrogen-bonded to the backbone or a side chain. It is distant from the E3 contact surface and, as expected, it is dispensable for E2 binding to RING domain E3s. However, the asparagine is critical for E2-catalyzed and RING E3/E2-catalyzed ubiquitin conjugation reactions. The similar effect of asparagine mutation on the two types of conjugation reactions is reasonable given that E2s do not experience structural perturbations upon binding to E3s (above). The data suggest that an intrinsic catalytic role of the asparagine side chain is brought into play through RING-mediated recruitment of the catalytically competent E2/ubiquitin thiol ester. The asparagine is dispensable for upstream and downstream thiol transfer reactions, suggesting that catalytic residues for these reactions may be located in the E1 and HECT E3 active sites.

A specific proposal for the role of the asparagine was developed in a model which breaks the hydrogen bonds to the backbone and rotates the asparagine to-

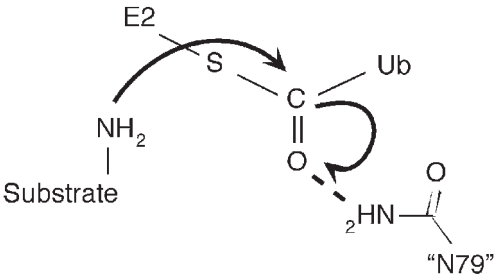

Fig. 5.8. Model for catalytic role of E2 active-site asparagine. The side chain of the asparagine in the conserved ''HPN'' motif (Figure 5.2) stabilizes the oxyanion that forms when the substrate's lysine attacks the E2/ubiquitin thiol ester bond. N79 is numbering for Ubc1 (Figure 5.2).

ward the active-site cysteine [136]. Molecular modeling suggested that the asparagine can be positioned to donate a hydrogen bond to the oxyanion (Figure 5.8) [136]. Many cysteine proteases, including deubiquitinating enzymes, use an amide side chain in this manner [134, 137–139]. Structural studies of a deubiquitinating enzyme have shown that the entry of ubiquitin into the active site causes a histidine and an asparagine to shift their positions so that the histidine becomes the general base and the asparagine provides the oxyanion hole [137]. Similarly, ubiquitin binding in the E2 active site could be a trigger that repositions the asparagine. So far, no general base is evident in E2s, but this group may not be needed due to the lability of the thiol ester bond.

5.7 Functional Diversification of the E2 Fold

Increasing evidence suggests that evolution has used (and is using) the E2 fold for new purposes. In one apparent example of functional expansion, E2 core domains have been observed to be embedded within much larger polypeptide chains [140, 141]. The functional properties of these massive E2s remain poorly characterized, and it is likely that more of them will be discovered. But the clearest case of functional diversification is provided by the UEV proteins. UEVs are related to E2s in their primary, secondary, and tertiary structures, but they lack an active-site cysteine residue and therefore cannot function as canonical E2s [142]. Nonetheless they play several different roles in ubiquitin-dependent pathways.

Mms2 and its close (mammalian) relative Uev1a form heterodimers with Ubc13 [72, 132]. Each complex plays a key role in the synthesis of K63-linked chains, which act as non-proteolytic signals in different cellular pathways. The Mms2/Ubc13 complex participates in the *UBC2/RAD6*-dependent DNA damage tolerance pathway by polyubiquitinating the DNA polymerase processivity factor called PCNA

(***P***roliferating ***C***ell ***N***uclear ***A***ntigen) [65, 72]. To be activated for this pathway, PCNA is first mono-ubiquitinated by the Rad6/Rad18 complex, and then modified with a K63-linked polyubiquitin chain by the Mms2/Ubc13/Rad5 complex (Rad18 and Rad5 are RING E3s) [65, 73]. The Mms2/Ubc13 complex has a core ubiquitin polymerization activity [72]. Rad5 might stimulate this activity [132] or target the Uev/E2 complex to PCNA [73], or both.

The related human Uev1a/Ubc13 complex is involved in NFκB signal transduction [132]. It plays an intermediate role in the signaling cascade that starts with a proinflammatory cytokine signal and culminates in the nuclear translocation of the active NFκB transcription factor. In this pathway the Ubc13/Uev1a complex modifies a RING E3, Traf6, with K63-linked polyubiquitin chains [132]. This modification is linked to Traf6 oligomerization. It instigates a cascade of kinase reactions ultimately cause the ubiquitination and degradation of NFκB's inhibitory partner, IκBα [74, 143].

The crystal structure of Mms2 has been solved alone and in complex with Ubc13 [131, 144]. The overall fold is similar to that in E2s, containing a central four-stranded anti-parallel β-sheet surrounded by α-helices. Differences include the absence of the C-terminal α-helix H5 in the shorter Mms2 protein. The helical N-terminus of Mms2 is also extended compared to Ubc13, and this region plays the major role in heterodimer formation. Two ubiquitins can bind to the heterodimer (Section 5.6.4) and the surfaces they contact do not overlap with the surface contacted by Rad5 [131, 145].

Ubiquitin plays a crucial role in a protein-trafficking pathway that delivers specific cargo proteins to regions of the late endosome membrane that invaginate into the lumen, thereby targeting these proteins to the vacuole/lysosome (reviewed in Ref. [146]). A different UEV protein, called Tsg101 in humans (***T***umor ***S***usceptibility ***G***ene) and Vps23 in yeast (***V***acuolar ***P***rotein ***S***orting), is part of a large complex that plays a critical role in the sorting step. Cargo proteins are selected based on their conjugation to mono-ubiquitin; the specific role of the UEV protein is to bind the cargo-linked mono-ubiquitin moiety [147]. HIV-1 and certain other viruses subvert this function of Tsg101 in order to bud from the plasma membrane [148–150]. Mechanistically, Tsg101 is recruited to the virus budding sites by binding to a tetrapeptide "PTAP" motif in the late domain of viral proteins such as HIV-1-GAG. Tsg101 is essential for virus budding from the plasma membrane [148], so it is possible that the endocytic budding machinery is hijacked to the plasma membrane via the Tsg101/GAG interaction [85].

The solution structure of the Tsg101 UEV domain has been solved alone and in complex with a PTAP-containing peptide [151, 152]. Human Tsg101 contains 390 amino acids, with the UEV domain located at the N-terminus [152]. The UEV domain is the minimal region needed to bind HIV-1 GAG, and is also the domain involved in mono-ubiquitin recognition and binding [153]. The overall fold of the UEV domain is similar to that of E2s. One notable difference is the presence of an extra N-terminal α-helix on Tsg101 [152]. The other major difference is the absence in Tsg101 UEV of the two C-terminal α-helices of the E2s – a truncation that was

also seen in the Mms2 structures. This truncation appears to be a special trait of UEV proteins [154]. In Tsg101 UEV, the absence of the C-terminal helices helps to create the binding site for the PTAP peptide [151].

When aligning the structures of a canonical E2, Mms2, and the Tsg101 UEV domain, the hydrophobic core and the region surrounding the vestigial active site are quite similar, but the Tsg101 UEV domain differs from Mms2 and canonical E2s in the positions of the first two β-strands [152]. In Tsg101 they are elongated and shifted toward the N-terminus, forming a β-hairpin that extends 11 residues outside the main body of the domain [152]. This loop is important for ubiquitin binding by Tsg101 [152]. As determined by chemical shift mapping and mutagenesis studies, the Tsg101/ubiquitin binding interface involves the bottom half of the four-stranded β-sheet, including the β-hairpin (loop S1–S2). The binding interface for ubiquitin on Tsg101 is distinct from the surface that Mms2 uses to position the acceptor ubiquitin within the Ubc13/Mms2 complex [131, 144]. Thus, two different UEVs bind ubiquitin in two different ways. The structural biology and biochemistry of UEVs illustrates how modest changes to an E2-like module can create new, functionally important interaction sites. The UEV domain is just one of a growing set of small domains that can endow other protein domains with ubiquitin-binding capability (reviewed in Ref. [63]). Such binding elements are likely to play important roles in transducing ubiquitin signals in diverse cellular pathways.

5.8 Concluding Remarks

We have emphasized the biochemical properties of E2s, particularly interactions with other factors in the conjugation cascade, because these properties are central to the biological actions of E2s. We have tried to give a flavor of the ''creativity and economy'' [103] with which E2s have evolved to maximize the interaction potential of a relatively small and conserved surface (Figure 5.7). Owing to the large scope of the relevant literature and the limited length of this chapter, we have not done full justice to the biological breadth of the E2 enzyme family. For example, we have focused on yeast and mammalian enzymes, but ubiquitin conjugation is increasingly being studied in other model organisms, including flies, worms, and plants. These systems offer powerful tools to address outstanding questions about ubiquitin-dependent pathways in general and E2 enzymes in particular. What are some of those questions? Significant uncertainties remain concerning E2 catalysis and mechanism, as discussed in Section 5.6. Another important question has been largely ignored in this review – exactly why are there so many E2s? One appealing model is that the identity of the E2 can modulate the substrate specificity of the E3, but experimental evidence for this model remains sparse. Another possibility is that the E2 has little or no influence on substrate choice, but rather helps to control the flux of activated ubiquitin to its cognate E3. In view of the remarkable developments in ubiquitin biology over the last decade, we should be prepared for both interesting and unexpected answers to these (and other) questions.

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6
The SCF Ubiquitin E3 Ligase

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6.1
Introduction

One of the most effective ways to activate or inactivate a biological process rapidly is to specifically eliminate through proteolysis the critical proteins that regulate or participate in the process. Eukaryotic cells utilize ubiquitin-dependent proteolysis to regulate responses to diverse signals during development and metabolism [1, 2]. With more than 30 000 genes encoded in the human genome, selective degradation of a particular protein in response to a regulatory signal poses a great challenge to the cell. The ubiquitin-dependent proteolysis pathway ensures that each protein is degraded in a temporal and spatially regulated fashion in response to such diverse signals or environmental cues [2]. In this system, the doomed protein is specifically modified by ubiquitin, a small peptide consisting of 76 amino acid residues [1]. The enzymatic cascade is set in motion when ubiquitin is first activated by an activating enzyme, E1, at the expense of ATP. The activated ubiquitin, which is covalently linked to the E1 enzyme by a thioester bond, is transferred to a member of a family of ubiquitin E2-conjugating enzymes. Last but not least, the doomed protein substrate is recognized by an ubiquitin E3 ligase, which often aids in ubiquitin transfer from E2 to substrate. Polyubiquitinated proteins are then degraded by the 26S proteasome. Since E3 ligases define the substrate specificity, studies suggest that intricate and fascinating mechanisms specify a large number of ubiquitin E3 ligases for the selective and timely elimination of a particular substrate through ubiquitin-dependent proteolysis [1–3]. In this chapter, we will focus on the function and regulation of the SCF (***S***kp1, ***C***ul1/*C*dc53, ***F***-box proteins) family of ubiquitin E3 ligases. Unlike the HECT-domain E3 ligases, which consist of a single polypeptide, the SCF E3 ligase is composed of multiple protein subunits. This multiprotein complex regulates many important biological processes such as the cell cycle, transcription, and inflammation response. In addition, SCF is subject to regulation at various levels by complex signaling processes, and some of the regulatory mechanisms are exclusive to this class of E3 ligases. Accordingly, alteration of the function and regulation of the SCF ubiquitin E3 ligase has been associated with human diseases such as cancer.

6.2 Discovery of the SCF Complex

One of the largest ubiquitin E3 ligase families, SCF ubiquitin E3 ligases are assembled from Skp1, Cul1/*C*dc53 an F-box protein, and Roc1 (also called Rbx1 or Hrt1) [3]. Skp1 and the F-box protein Skp2 (***S***-phase ***k***inase associated ***p***rotein 1 and 2), were initially identified during analysis of the cyclin A/CDK2 complex [4]. Skp2 expression was found to be highly elevated in many cancer cells and is required for G1 cells to enter S phase. However, Skp1 and Skp2 can form a complex independent of cyclin A/CDK2, suggesting that this binary complex may have a cell-cycle function independent of the cyclin A/CDK2 kinase activity.

Yeast Skp1 was isolated as a high copy suppressor of yeast cdc4 temperature-sensitive mutant at restrictive temperature, and as a protein that interacts with human cyclin F, a protein that can also suppress the cell-cycle defects of cdc4 mutant when it is expressed in high copy in yeast [5]. Skp1 also directly interacts with the yeast *C*dc4 protein, which encodes eight WD40 repeats (WD repeats), and cyclin F. Since Skp1 also directly binds to Skp2 which contains seven ***l***eucine-***r***ich ***r***epeats (LRR), these observations suggest that Skp2, *C*dc4, and cyclin F may share a common mechanism for Skp1 binding. Indeed, sequence alignment of all three proteins indicates that they possess a relatively conserved 40–45 amino acid motif which mediates the binding of Skp1 [5] (Figure 6.1). This motif had been previously identified in some WD repeat-containing proteins but its significance was unknown [6]. This motif is therefore called the F-box, after the cognate region in cyclin F, and is present in a wide variety of otherwise unrelated proteins [5, 7–9]. Accordingly, the proteins that contain this motif are called F-box proteins [5]. The function of Skp1 was further revealed by earlier observations that yeast *C*dc4, *C*dc53, and *C*dc34 temperature-sensitive mutants all fail to perform yeast Start-related events (G1 progression into S phase, nuclear DNA replication, and spindle formation) and accumulate yeast CDK inhibitor $p40^{Sic1}$ at the restrictive temperature [10–12]. Since *C*dc34 encodes an ubiquitin E2-conjugating enzyme [10], *C*dc34, *C*dc4, and *C*dc53 are likely to act in concert to regulate the G1/S transition by controlling the ubiquitin-dependent proteolysis of $p40^{Sic1}$. Certain Skp1 mutants also accumulate $p40^{Sic1}$ and expression of Skp1 in cdc4 mutants is sufficient to suppress the accumulation of $p40^{Sic1}$ in cdc4 mutants at restrictive temperature [5]. These observations suggested that Skp1 is involved in the *C*dc4-, *C*dc53-, and *C*dc34-mediated ubiquitin-dependent proteolysis of $p40^{Sic1}$.

Cullin-1 (Cul1) was originally isolated from *Caenorhabditis elegans* as a negative regulator of cell proliferation during development [13]. Loss of Cul1 (or *lin-19*) in

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Skp2:  D S L P D E L L L G I F S C L C L P E L L K V S G V C K R W Y R L A S D - E S L W (a.a.98-137)
Cdc4:  T S L P F E I S L K I F N Y L Q F E D I I N S L G V S Q N W N K I I R K S T S L W (a.a.276-316)
CycF:  L S L P E D V L F H I L K W L S V E D I L A V R A V H S Q L K D L V D N H A S V W (a.a.33-73)
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Fig. 6.1. The F-box motif in human Skp2, budding yeast Cdc4, and human cyclin F (CycF). The conserved amino acids are highlighted.

C. elegans causes hyperplasia in all tissues. In the proliferating cells, the progression from G1 to S phase is accelerated. The normal developmentally programmed mitotic arrests are overridden, with additional cell divisions that produce abnormally small cells. It was found that Cul1 belongs to a conserved family of cullins that share extensive homology [13]. The cloning of yeast *C*dc53 revealed that it is an ortholog of Cul1. Biochemical analyses suggested that *C*dc53, *C*dc4, and *C*dc34 form a protein complex [11]. These studies laid the foundation for the more detailed studies of SCF ubiquitin E3 ligase and related cullin-containing ubiquitin E3 ligases.

6.3 The Components of the SCF Complex

The essential components of the SCF ubiquitin E3 ligase include Skp1, Cul-1/*C*dc53, one of many F-box proteins, and the RING-H2-finger protein Roc1 (Rbx1 or Hrt1) (Figure 6.2). Although initial studies did not reveal the presence of a fourth component of the SCF complex [14, 15], later work showed that a RING-H2-finger protein, Roc1, is an essential subunit of the SCF complex [3]. The SCF complex thus contains three invariable components (Roc1, Cul1, and Skp1) which provide a core structure to which one of the many substrate-specific subunits (F-box proteins) binds. The Roc1–Cul1–Skp1 core also independently interacts with the ubiquitin E2-conjugating enzyme to couple ubiquitin transfer to the substrates [3]. One of the F-box proteins binds directly to a specific substrate and such interaction facilitates the polyubiquitination of the substrate by ubiquitin

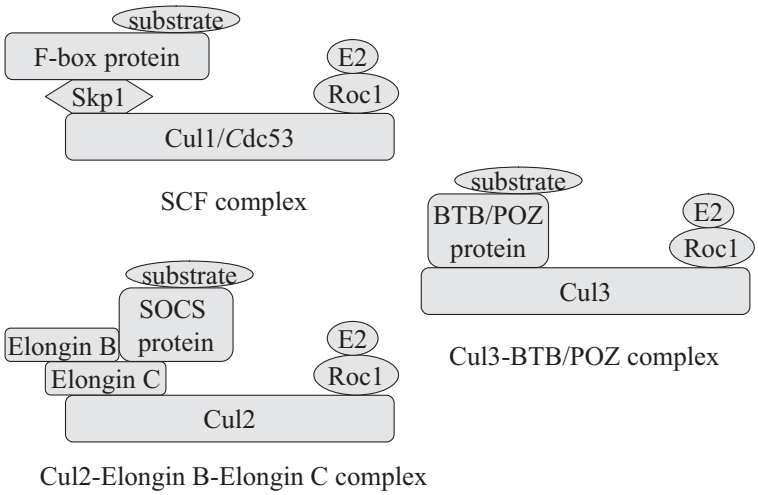

Fig. 6.2. The structures of SCF, Cul2–Elongin B-C, and Cul3–BTB/POZ ubiquitin E3 ligase complexes with the bound substrates and E2 enzymes.

transfer from the ubiquitin-charged E2. Since there are multiple F-box proteins [3], this mechanism illustrates how the same core complex can control the abundance of a diverse spectrum of substrates. Related E3 ligases built with a conserved cullin as a core protein employ similar strategies to extend the substrate specificity.

6.3.1 Roc1/Rbx1/Hrt1

The RING-H2 protein, Roc1 (also called Rbx1 and Hrt1, but Roc1 hereafter) was identified through its interaction with either mammalian Cul1, Cul2, or yeast *C*dc53 [3]. It was found that addition of Roc1 stimulates the polyubiquitination activity of SCF complexes. Roc1 and its homologs are highly conserved in evolution. Roc1 contains a cysteine-containing and zinc-binding RING-finger domain in its C-terminal half that is distinct from but related to other RING-finger E3 ligases such as c-Cbl. A close homolog, Roc2/Rbx2/Hrt2 (also called Sag), also exists [3]. Both Roc1 and Roc2 can bind to Cul1 and related cullins (Cul2–5, Cul7, and Cul8) through a highly conserved C-terminal region, the cullin homology domain [3, 16, 17]. A more distant Roc1 homolog, Apc11, is a component of the ***A***naphase ***p***romoting ***c***omplex/***C***yclosome (Apc/C) [3], an ubiquitin E3 ligase complex that regulates mitosis. Apc11 binds to a distant cullin-related protein, Apc2, in the Apc/C complex [18]. Genetic studies confirmed the essential role of Roc1 in the SCF E3 ligase complex. Roc1 also binds to ubiquitin E2 ligases such as *C*dc34, and thus serves as the link between the E2 (*C*dc34) conjugating enzyme and Cul1/*C*dc53. The RING-H2 domain of Roc1 has been shown to be required for the E2-binding and ubiquitin-ligation reaction. However, although biochemical studies suggest that Roc1 and Roc2 share the same biochemical properties of cullin binding and act as a link between the cullin E3 ligases and the E2 enzymes, the physiological roles of Roc1a (a Roc1 ortholog) and Roc1b (a Roc2 ortholog) appear to differ in *Drosophila melanogaster* [19]. Drosophila Roc1a is required for cell proliferation, and cells lacking Roc1a fail to proliferate during development. However, expression of Roc1b under the control of Roc1a promoter does not rescue the Roc1a-deficient phenotype. In addition, Roc1a deficiency causes the differential accumulation of SCF substrates in *Drosophila*. While F-box protein Slimb/β-Trcp is required for both the proteolytic degradation of Armadillo/β-Trcp (Arm) and the proteolytic processing of the Cubitus interruptus (Ci) [20], Roc1a null mutants only accumulate Ci but not Arm. These studies suggest that an additional mechanism may exist to distinguish between Roc1a and Roc1b and various SCF substrates. *Drosophila* encode a third Roc1-like protein, Roc2, but its function in the cullin ubiquitin E3 ligase is not clear.

6.3.2 Cullin-1 (Cul1)

In the SCF complex, Cul1 forms the core scaffold that associates with Roc1 at the extreme C-terminal region [3]. At its the amino terminal region, Cul1 interacts

with Skp1. The Roc1-binding domain exhibits the highest conservation among cullins and was initially identified as the cullin homology domain [3, 13]. The conservation of this domain is consistent with the fact that Roc1 can bind to almost all cullins (Cul1–5, Cul7, and Cul8) and this binding couples cullin E3 ligases to the ubiquitin E2-conjugating enzymes.

The N-terminal region for Skp1 binding is conserved between orthologs of Cul1 from different species, and also displays significant homology in the equivalent regions among other cullins such as Cul2, Cul3, Cul4, and Cul5 [21]. In Cul2 and Cul5 this region has been shown to interact with Elongin C [22, 23], a protein that shares considerable homology with Skp1. In another parallel, the N-terminal region of Cul3 has been shown to interact with BTB/POZ proteins that display a similar three-dimensional crystal structure to that of Skp1 [24].

The crystal structure of Cul1 has been resolved [21] and found to resemble a long stalk connecting two protein-interaction domains at either end of the stalk. The globular domain of 360 amino acids at the C-terminal region of Cul1 forms a complex with Roc1. In this region, a four-helix bundle and an α/β domain form two winged-helix domains. This creates a V-shaped groove in which the C-terminal half of Roc1, containing the RING-finger domain, is situated. This Cul1 region spans the cullin homology domain which is highly conserved between all cullins, being present even in the more distantly related cullin homolog Apc2, a subunit of the Apc/C ubiquitin E3 ligase. This sequence conservation suggests that other cullins also use the same strategy to interact with RING-H2 proteins such as Roc1 [21]. Overall, the globular domain of Cul1/Roc1 generates a surface area for interaction with the E2 enzyme [21]. Although deletion mutant analysis of Cul1 suggests that the first 219 amino acids are required to bind Skp1 [3], the crystal structure of the N-terminal region of the Cul1 protein reveals that Cul1 forms three cullin repeats (about 120 amino acids each) in an arch-like shape [21]. The first N-terminal repeat forms the domain for Skp1 interaction while the other two repeats form a long stalk between the Skp1/F-box protein-binding domain and the Roc1 interaction domain. In the crystal structure of Cul1, it does not seem that the F-box proteins such as Skp2 bind or interact with Cul1 [21]. This is consistent with the biochemical analysis that F-box proteins require Skp1 for binding to Cul1 [5].

6.3.3 Skp1

Skp1 serves as an adaptor protein that provides a molecular link between Cul1/Roc1 and the F-box proteins [4, 5]. The Skp1 protein contains two separate protein-interaction domains that are conserved among its family members between species [21]. The N-terminal region of Skp1 (~1–70 a.a.) interacts with Cul1 while the C-terminal half (100–163 a.a.) binds the F-box proteins [21]. The use of Skp1 as an adaptor to link the core ubiquitin E3 ligase components of Cul1/Roc1 with numerous and diverse substrate-targeting subunits, the F-box proteins, represents a strategy to specifically target many proteins for ubiquitination

[3]. The role of Skp1 is to bring the substrate-targeting subunit, the F-box protein, into proximity with the Cul1/Roc1/E2 complex to promote ubiquitin transfer from the E2-ubiquitin to the F-box protein-bound substrates.

The crystal structure of Skp1 reveals that it contains a BTB/POZ-like domain at the N-terminal region [25]. It has been found that BTB/POZ proteins bind to Cul3 and act as the substrate-targeting subunits for Cul3 E3 ligase-mediated ubiquitin-dependent proteolysis [24]. Thus the similarity between Skp1 and BTB/POZ proteins is significant. The structure of Skp1 also confirmed its similarity with Elongin C, a Skp1-like protein that interacts with Cul2. Both Skp1 and Elongin C also share structural resemblance to the potassium channel tetramerization domain, which also belongs to the BTB/POZ superfamily [25, 26]. Thus the BTB/POZ-like structures determine the interaction between cullins and their adaptor proteins such as Skp1, Elongin C, and other BTB/POZ-containing proteins.

Interestingly, although in mammals and single-cell organisms such as yeast there is only one conserved Skp1 homolog, other multi-cellular organisms encode multiple Skp1-like proteins. In *Arabidopsis thaliana*, at least 19 Asks (***A***rabidopsis ***Sk***p1-like) are predicted and genetic studies suggest that Asks1 is part of SCF^{Tir} and SCF^{Coi1} complexes that regulate the responses to the plant hormones auxin and jasmonate, respectively [27, 28]. It also regulates vegetative and flower development and male meiosis. Other Asks exhibit different abilities to interact with F-box proteins. In addition, seven Skp1 homologs have been identified in *Drosophila melanogaster* and at least 21 ***Sk***p1-***r***elated proteins (Skrs) have been identified in *C. elegans* [29–31]. In *C. elegans*, while loss of Skr7, 8, 9, and 10 results in slow growth and morphological abnormalities, Skr1 and Skr2 are essential for embryonic development. The presence of such large families of Skp1-related proteins in these organisms suggest that selective expression of these Skp1-related proteins during development or in a particular tissue may represent an additional level of regulation for their protein substrates.

In addition to targeting substrate proteins for degradation, Skp1 has been associated with certain activities that remain to be further characterized. For example, Emi1 (also called FBX5), an F-box protein that binds to Skp1 [32], contains a zinc-binding region near its carboxy terminus that is separate from its F-box region. This zinc-finger domain is required for binding to *C*dc20 or Cdh1, substrate-targeting subunits of Apc/C ubiquitin E3 ligase. The binding of Emi1 to *C*dc20 or Cdh1 inhibits Apc activity and thus regulates mitosis [32]. Furthermore, yeast Skp1 also binds to the kinetochore Cbf3 complex and is essential for the yeast kinetochore/centromere function in G2 [3]. However, the precise roles of Skp1 in these biological processes still remain unclear.

6.3.4
F-box Proteins

F-box proteins serve as the substrate-targeting subunit of the SCF ubiquitin E3 ligase [5]. They are structurally diverse but they all contain a relatively conserved signature motif of about 45–50 amino acids [5]. This motif, the F-box, was initially

identified among human Skp2, yeast *C*dc4, and human cyclin F, which all bind to Skp1 [5]. F-box proteins also contain a separate protein–protein interaction domain that mediates the binding to various substrates [5, 14, 15]. The binding of F-box proteins to their selected substrates usually targets the respective substrate for polyubiquitination and subsequent proteolysis through the 26S proteasome. However, F-box proteins can also mediate the processing of certain protein precursors to their cleavage products through limited ubiquitin-dependent proteolysis [20, 33].

The existence of a large repertoire of F-box proteins means that SCF E3 ligase is one of the largest E3 ligase families (other large E3 families such as the Cul2- and Cul3-containing ubiquitin E3 ligases are also related to the SCF E3 ligase) [2, 24]. In the yeast *Saccharomyces cerevisiae*, at least 11 F-box proteins that contain conserved F-box domains have been identified [7]. In *Drosophila* at least 22 F-box proteins exist, while more than 326 F-box proteins are predicted in the genome of *C. elegans* [34]. In human and mouse, the presence of at least 38 conserved F-box proteins has been reported [4, 5, 8, 9]. However, many F-box proteins may contain a less-canonical F-box motif [35]. In such cases, identification of the candidate F-box proteins in the protein databases using the standard sequence search algorithms is quite difficult. The classification of such a protein as a member of the F-box family relies on confirming its association with the other components of the SCF complex and its activity towards a particular protein substrate [35]. The prototypical F-box proteins such as Skp2, *C*dc4, or *β*-Trcp have been relatively well studied. These studies clearly indicate that F-box proteins act as the substrate-targeting subunit of the SCF ubiquitin E3 ligases [3].

In addition to the F-box motif, many conserved F-box proteins contain either ***l***eucine-***r***ich ***r***epeats (LRR) such as Skp2 or yeast Grr1, or WD40 repeats, which are present in *C*dc4 or *β*-Trcp [5]. In human and mouse, seven F-box proteins contain WD40 repeats (Fbws) while 10 F-box proteins have LRR repeats (Fbls) at their C-terminal domain [8, 9]. However, a large number of F-box proteins contain other protein–protein interaction modules or unknown domains (Fbxs). The LRR or WD-40 repeats of F-box proteins have been shown to mediate the interaction between F-box proteins and their respective substrates through phosphorylated serines or threonines [34]. The differential binding specificities of protein–protein interaction domains found in F-box proteins confers the substrate specificity of the SCF ubiquitin E3 ligase.

6.4
E2-conjugating Enzymes for the SCF E3 Ligases

The function of the SCF E3 ligase complex is to facilitate ubiquitin transfer from the E2-conjugating enzymes to the protein substrates. Although more than two dozen E2s exist, genetic studies suggest the *C*dc34 E2 conjugation enzyme is especially involved in regulating SCF substrates [12]. These observations are further strengthened by the association between *C*dc34 and components of SCF complexes. From yeast to human, this highly conserved E2 is also required for the *in*

vitro polyubiquitination reactions for the substrates of SCF E3 ligases [14, 15, 36, 37]. However, other E2 enzymes, such as human UbcH5, can also function *in vitro* for polyubiquitination of certain substrates of the SCF complexes with similar [38], if not greater, efficiency than *C*dc34.

6.5 Substrates and Substrate Recognition

Both genetic and biochemical analyses suggest that the SCF E3 ligase targets a wide spectrum of important proteins for ubiquitin-dependent proteolysis (see Table 6.1 for examples). A common feature of the physiological substrates of various SCF

Tab. 6.1. F-box proteins.

Protein	*Species*	*Substrates*	*Function of substrates*
Skp2	*H. sapiens*	p27	CDK inhibitor
		p21	CDK inhibitor
		Rb2/p130	Rb-related protein, CDK inhibitor
		Orc1	Component of origin recognition complex
Beta-Trcp	*H. sapiens*	β-catenin	Transcription factor
		IκB	Inhibitor of transcription
		CD4	HIV Vpu target, surface receptor
		Emi1	Inhibitor of anaphase-promoting complex/cyclosome
		CDC25A	Phosphatase, positive regulator of Cdks
hCdc4/Fbw7	*H. sapiens*	Cyclin E	G1 cyclin
		Notch	Receptor
		Presenilin 1	Familial Alzheimer's disease gene
Tome-1	*X. laevis*	Wee1	CDK inhibitory kinase
Slimb (beta-Trcp homologue)	*D. melanogaster*	Armadillo	β-catenin homolog
		Cubitus interruptus	Transcription factor
Archipelago (Ago) (hCdc4/Fbw homologue)	*D. melanogaster*	Cyclin E	G1 cyclin
Cdc4	*S. cerevisiae*	Cdc6	Replication initiation protein
		Sic1	Cdk inhibitor
		Far1	Cdk inhibitor
		Gcn4	Transcription repressor
Grr1	*S. cerevisiae*	Cln1	G1 cyclins
		Cln2	G1 cyclin
Met30	*S. cerevisiae*	Met4	Transcription factor
		Swe1	Wee1-like kinase
Pop1/Pop2	*S. pombe*	Rum	Cdk inhibitor
Tir1	*A. thaliana*	AXR2/IAA7	Auxin response
		AXR3/IAA17	Auxin reponse
Ebf1/Ebf2	*A. thiana*	EIN3	Transcription factor in ethylene response

ligases such as CDK inhibitors $p40^{Sic1}$ or $p27^{Kip1}$, β-catenin, or IκB shows a requirement for phosphorylation of substrates on either serine or threonine for SCF-mediated ubiquitin-dependent proteolysis [2, 3]. The WD40 and LRR repeats in the F-box proteins bind phosphorylated substrates independent of the F-box, which interacts with Skp1. The binding of various F-box proteins to phosphorylated serines or threonines within a particular substrate appears to be quite specific [36–39], suggesting that the phosphorylation-mediated binding of F-box proteins is dependent on the peptide sequences surrounding the phosphorylation site of protein substrates. The binding of F-box proteins to the phosphorylated substrates promotes the interaction of Skp1 and its associated Cul1/Roc1 with the substrates, facilitating ubiquitin transfer from the E2-conjugating enzymes to the substrates [3]. Subsequent polyubiquitination of the substrates is coupled to their proteolysis by the 26S proteasome.

However, studies also suggest that many F-box proteins can bind and target a number of proteins for polyubiquitination in a phosphorylation- and sequence-dependent manner [34, 36, 37, 40–43]. Furthermore, evidence also suggests that some F-box proteins can selectively bind to several sites containing phosphorylated serines/threonines within a single protein substrate [44, 45]. These observations raise the question of how substrate specificity is defined. In several cases, additional mechanisms appear to be involved in specifying substrate selection by the F-box proteins. Several well characterized examples of SCF substrates will be presented to highlight our current understanding of the substrate-specificity of the SCF ubiquitin E3 ligase.

6.5.1 Skp2 and Its Substrates

One of the best characterized SCF complexes is SCF^{SKP2}. Skp2 was originally isolated by its highly elevated expression in many cancer cell lines and by its association with Skp1 and cyclin A/Cdk2/Cks1 [4]. Skp2 was also found to be critical in regulating the progression of mammalian G1 cells into S phase. One of the critical G1 cell-cycle regulators is $p27^{Kip1}$. In the cell cycle, p27 protein levels are regulated by ubiquitin-dependent proteolysis, being high in G0 and early G1 and low in late G1 and throughout S phase [46]. The high G1 level of p27 is required for preventing the premature activation of cyclin E/CDK2 or other G1- or S-specific cyclin/Cdks. In late G1, p27 is rapidly proteolyzed through ubiquitin-dependent degradation [46], promoting the release of active cyclin E/Cdk2 kinase and consequently the S phase entry. p27 degradation requires a single phosphorylation site at threonine 187 located at the C-terminal end of the protein [47]. Cyclin E-associated kinases can phosphorylate p27 at this critical site *in vitro* and in transfection systems. Conversion of threonine 187 to non-phosphorylatable alanine in p27 greatly stabilizes this protein. The F-box protein Skp2 specifically binds to the phosphorylated threonine 187 in p27 and targets p27 for polyubiquitination and subsequent proteolysis while other F-box proteins such as β-Trcp do not [36, 37]. Deletion of the F-box region in Skp2 promotes the interaction between Skp2 and the phosphorylated p27 but causes stabilization of p27 *in vivo* [37]. A particularly unique re-

quirement for substrate binding and recognition by Skp2 involves an accessory protein, $p9^{Cks1}$ [48, 49]. Cks1 was previously identified as a Cdk-binding and Suc1-like protein, and initially isolated as a suppressor of certain *C*dc28/*C*dc2 mutants in yeast [50]. SCF^{Skp2}-mediated p27 polyubiquitination requires Cks1, and the activity associated with p27 polyubiquitination is independent of Cdk binding but depends on its direct and specific interaction with Skp2. A close Cks1 homolog, Cks2, cannot substitute for Cks1 in this reaction. The polyubiquitination of p27 has been reconstituted with purified Skp2, Skp1, Cul1, Roc1, Cks1, cyclin E/CDK2, *C*dc34, and E1 in the presence of ATP and ubiquitin [36, 49]. Overexpression of Skp2 is sufficient to induce p27 down-regulation and in some cases, induces S phase [51]. Strikingly, genetically engineered Skp2 knockout mice and Cks1 deficient mice share similar phenotypes [49, 52]. Mouse embryonic fibroblasts derived from $Skp2^{-/-}$ and $Cks1^{-/-}$ mice both accumulate p27 and its binding partner cyclin E [49, 52]. Thus it appears that Cks1 is specifically evolved in mammals to facilitate Skp2-mediated substrate polyubiquitination. Interestingly, Skp2 was isolated as a protein complex that contains cyclin A/Cdk2/Cks1 [4]. One possible role for cyclin A/CDK2 is recruitment of Cks1 into the proximity of Skp2 to facilitate Skp2 binding to phosphorylated p27. Alternatively, cyclin A/CDK2 can also bind and phosphorylate p27 at threonine 187 to promote Skp2 binding.

Although p27 remains the critical target of Skp2 in late G1, additional polyubiquitinated substrates of Skp2 have been identified. These include the retinoblastoma-related protein Rb2/p130 [41], Cdk inhibitor p21 [40], and other proteins. Characterization of Skp2 binding sites in these proteins reveals that while phosphorylation of serine 130 in p21 is required for Skp2 binding and polyubiquitination, phosphorylation of serine 672 in p130 is essential for the interaction with Skp2 and p130 polyubiquitination. One common feature among these characterized Skp2 substrates is the presence of minimum serine/threonine followed immediately by a proline residue (S or T/P) in the Skp2-binding motifs. However, it is still not clear how Skp2 selects its binding site after the phosphorylation of serines or threonines in these and other substrates.

6.5.2 *β*-Trcp and Its Substrates

The substrate consensus sequences are best illustrated in the case of *β*-Trcp (also called Fbw1, FWD1, and Hos in vertebrates, and Slimb in Drosophila), an F-box protein that contains seven WD40 repeats at its C-terminal region [20]. Initial genetic evidence in *Drosophila* suggests that the Drosophila *β*-Trcp homolog Slimb regulates proliferation and axis formation during development through the Wingless/Wnt and Hedgehog signaling pathways [20]. Genetic mosaic analysis of Slimb *Drosophila* mutants indicates that the slimb defect causes the abnormal accumulation of Armadillo, the Drosophila homolog of *β*-catenin, a transcription factor involved in the Wingless/Wnt pathway. In human, *β*-catenin is the target of the human tumor suppressor protein ***a***denomatous ***p***olyposis ***c***oli (APC) which is often mutated in familial colorectal cancers [53]. In the absence of wingless signaling, the cytoplasmic *β*-catenin is unstable and is degraded by ubiquitin-dependent pro-

teolysis. However, an active Wingless signaling pathway stabilizes β-catenin, which is subsequently transported from the cytoplasm into the nucleus to activate Wingless transcription programs. β-catenin is destabilized by phosphorylation on two conserved serines (serines 33 and 37, underlined in the sequence of DSGIHS) catalyzed by the ***g***lycogen ***s***ynthase ***k***inase-3b (GSK-3b) and ***c***asein ***k***inase ***I***ε (CKIε), through binding of the scaffold protein Axin and APC [53]. Phosphorylation of these two conserved serines in this N-terminal region of β-catenin triggers its ubiquitin-dependent proteolysis mediated by $SCF^{\beta\text{-Trcp}}$ [39]. In addition to β-catenin, β-Trcp also binds to two phosphorylated serines (serines 32 and 36, DSGLDS) of IκB [38, 39], an inhibitor of NFκB, and regulates the NFκB-mediated inflammatory and other responses. Initial studies on the binding sites of β-catenin, IκB, and HIV-1 protein Vpu (another β-Trcp-binding protein when it is phosphorylated at two serines on DSGNES) [54], suggest that dual phosphorylation of serines within a consensus sequence of DSGXXS is sufficient for β-Trcp binding. This binding triggers the polyubiquitination of β-catenin and IκB. In addition to Armadillo, slimb mutation in *Drosophila* also causes the abnormal accumulation of Cubitus interruptus (Ci), producing phenotypes that resemble the ectopic activation of the Hedgehog signaling pathway [20]. In the absence of Hedgehog signal, full length Ci (p155) is processed by the proteasome in a β-Trcp-dependent manner to generate a smaller p55 form of Ci, which acts as a repressor for Hedgehog-regulated transcription. Mammalian β-Trcp exhibits similar processing activity towards NFκB1 [33]. However, studies on the phosphorylation-dependent processing of NFκB1 demonstrate that it occurs when the serines in the DSGXXXS motif of these proteins are phosphorylated [33]. The extra amino acid between the two phosphorylated serines suggests a certain tolerance by β-Trcp. More strikingly, characterization of the β-Trcp-mediated polyubiquitination of *C*dc25A in response to DNA damage indicates that higher tolerance of the spacer between the dual phosphorylated serines exists [42, 43]. In this case, β-Trcp binds and targets *C*dc25A for polyubiquitination once the two serines in DSGXXXXS are phosphorylated. The tolerance of two additional amino acid residues in the spacer region between the two phosphorylated serines suggests that β-Trcp is substantially flexible in its binding to substrates within the consensus sequence. Intriguingly, *C*dc25A degradation is triggered after its phosphorylation at serine 76 by the DNA damage checkpoint kinase CHK1 in response to DNA damage. This phosphorylation, which precedes the serine 79 and serine 82 utilized for phosphorylation-dependent binding of β-Trcp in the DSGXXXXS motif, is essential for the DNA-damage-induced *C*dc25A proteolysis by the $SCF^{\beta\text{-Trcp}}$ E3 ligase. The mechanism by which the phosphorylated serine 76 triggers the β-Trcp-mediated *C*dc25A polyubiquitination is not clear. These findings suggest that a more complicated regulation exists for the polyubiquitination of *C*dc25 by the $SCF^{\beta\text{-Trcp}}$ E3 ligase.

6.5.3
Yeast *C*dc4 and Its Substrates

The substrate recognition mechanisms discussed above suggest that phosphorylation at a particular site (or sites) is sufficient to bind the F-box proteins Skp2 or β-

Trcp to their respective substrates. These studies also suggest certain flexibilities in the binding of these F-box proteins to their substrate consensus sites. The yeast F-box *C*dc4, which contains eight WD40 repeats, has been implicated in mediating the ubiquitin-dependent degradation of Cdk inhibitor $p40^{Sic1}$ and Far1, replication initiation protein *C*dc6, and transcription repressor GCN4. Characterization of the yeast SCF^{Cdc4}-mediated polyubiquitination of yeast CDK inhibitor $p40^{Sic1}$ provides additional insights into the mechanism of the interaction between the phosphorylated substrates and F-box proteins.

It has been established that phosphorylation of Sic1 is absolutely required for SCF^{Cdc4}-mediated polyubiquitination [3, 45]. Sic1 is phosphorylated by the Cln/*C*dc28 kinase at the minimum consensus sequences of serine/threonine followed immediately by a proline (S/TP) [3]. In addition, the Cln/*C*dc28 kinase preferentially phosphorylates the S/TP site containing basic amino acid residues (arginines or lysines) [3]. Initial characterization of Sic1 phosphorylation sites reveals that at least nine such sites exist for Cln/*C*dc28 phosphorylation and subsequent *C*dc4 binding [3, 45]. Since Skp2 binds to p27 only when threonine 187 of p27 is phosphorylated [36, 37], a systematic characterization of Sic1 phosphorylation sites was conducted to determine which one of these nine sites is critical for the binding of yeast *C*dc4 F-box protein [45]. Initially, all of the potential serine/threonine phosphorylation sites were removed by site-directed mutagenesis of the Sic1 protein. This mutant is extremely stable and cannot be degraded by SCF^{Cdc4} *in vivo*. Systematic re-addition of serine/threonine phosphorylation sites to the Sic1 mutant protein suggests that while addition of any single serine or threonine is not sufficient to trigger its degradation, re-addition of at least six phosphorylation sites of the potential nine serines/threonines restores the Sic1 sensitivity to SCF^{Cdc4}. This differs from the polyubiquitination of the substrates of SCF^{Skp2}, in which a single phosphorylation constitutes the binding site for F-box protein [45]. However, the requirement of multiple phosphorylation sites is not unique to yeast Sic1 and its F-box protein *C*dc4. The ubiquitin-dependent proteolysis of yeast Cln2, which is mediated through the F-box protein Grr1 encoding seven LRRs also depends on the phosphorylation of a cluster of at least four serines/threonines in the Cln2 protein [44]. The requirement of at least six phosphorylation sites in Sic1 suggests that these sites may cooperate to allow the multiply phosphorylated Sic1 to bind to *C*dc4 and raises the question of how *C*dc4 can count the number of phosphorylations to properly target Sic1 for polyubiquitination.

It turns out that not all the phosphorylated sites are created equal. The mechanism for binding cooperativity by multiple phosphorylation sites in Sic1 is demonstrated in part by the observation that a high affinity phosphorylated *C*dc4-binding site on human cyclin E (threonine 380) is sufficient to destabilize Sic1 that lacks all original nine phosphorylation sites through *C*dc4 [44]. Thus it is unlikely that the eight WD40 repeats of *C*dc4 contain six or more phosphorylation binding modules for the binding of multiply phosphorylated Sic1 to *C*dc4. Rather, it appears that a single phosphorylation site is necessary for Sic1 binding to *C*dc4.

The co-crystal structure of yeast *C*dc4 and its phosphorylated substrates, as well as that of *β*-Trcp and its phosphopeptide substrate derived from *β*-catenin, have

been resolved [55, 56]. The β-Trcp and *C*dc4 proteins contain either seven or eight WD40 repeats, respectively, which correspond to the formation of seven- or eight-blade β-propeller structures. The phosphopeptide substrates lie across the top surface, in alignment with the active E2-binding site in the Roc1-binding domain located at the C-terminal end of Cul1. All seven or eight β-propeller blades of β-Trcp or *C*dc4 interact with the phosphorylated peptide substrates. One significant feature of the co-crystal structure of *C*dc4 with its respective substrate phosphopeptides is that there is only one phosphorylation-binding site on the surface of the WD40 propeller repeats in *C*dc4 [55]. This phosphorylation site is reminiscent of the β-Trcp site which binds the phosphorylated serine 37 in β-catenin [56]. Consistent with the genetic and biochemical characterization, the structure of *C*dc4 does not suggest that it can contain six phospho-binding modules for Sic1 [55].

How then can we explain the observed cooperativity of Sic1 phosphorylation in binding *C*dc4? A model was proposed to explain this cooperativity [45, 55]. It hypothesizes that while each single phosphorylation site in Sic1 may constitute a suboptimal binding site for *C*dc4, the binding and subsequent release of each phosphorylated site will somehow increase the local concentration of Sic1 near *C*dc4 [45, 55].

The presence of the multiply phosphorylated suboptimal sites in Sic1 should accelerate binding and dissociation cycles of Sic1 within the WD40-repeat domain. In proximity to *C*dc4, this should elevate the effective concentration of this form of Sic1 above its simple diffusional rate. In terms of *C*dc4 binding, this process should favor multiply phosphorylated Sic1 over those containing fewer phosphates. Biologically, Sic1 prevents premature entry into S phase in yeast by inhibiting the S phase cyclin/Cdk kinase, Clb5/6/*C*dc28. Thus, multiple phosphorylations of Sic1 may require a higher level of G1 Cln/*C*dc28 and promote a shaper transition of G1 to S phase. Consistent with this possibility, conversion of the positively charged arginine or lysine residues downstream of the serine/threonine-proline (S/TP) sites to neutral amino acids (such as alanine) in Sic1 reduces the number of phosphorylated sites in Sic1 required for the binding of *C*dc4. Such changes may convert the suboptimal *C*dc4-binding sites to high affinity ones for the binding and ubiquitination of Sic1 by SCF^{Cdc4}. While this possibility may explain the Sic1 and *C*dc4 interaction, it also provides an interesting model for substrate recognition and selection by other F-box proteins in which a degenerate phosphorylation consensus site is present in the substrates.

6.6 Structure of the SCF E3 Ligase Complex

Elucidation of the structure of the SCF ubiquitin E3 ligase complex and their substrates should help resolve certain issues regarding the mechanism by which SCF E3 ligase promotes ubiquitin transfer from the E2 enzyme to the protein substrate. Recently, the structures of Cul1/Roc1 in complex with Skp1 and Skp2, as well as that of Skp1/β-Trcp and *C*dc4, have been reported [21, 25, 55, 56]. These studies

suggest a rigid structure for the SCF complexes and thus provide an insight into the mechanism by which the SCF E3 ligase modulates the polyubiquitination of the protein substrates.

The overall shape of SCF^{Skp2} consists of an elongated structure with Cul1 as the scaffold protein [21]. The structure of Cul1 displays a long stalk connecting protein interaction domains at either end of the stalk. While the C-terminal region of Cul1 forms a complex with the RING-H2 protein Roc1, the extreme and opposite N-terminal region of the Cul1 protein forms the domain for Skp1 interaction. The other two cullin repeats connecting these two functional domains of Cul1 adopt an arch-like shape. One surprise is that there is a substantial space of about 50 Å between the Skp1/F-box protein-binding domain and the Roc1 interaction domain, which recruits the active E2. In addition, a prominent feature of the SCF structure is the rigidity of the Cul1 stalk. This rigidity appears to be required to separate the substrate-binding domain of Skp1 from the E2-binding domain of Roc1. Attempts to alter the distance or the rigidity of Cul1 by incorporating a more flexible swivel in the connecting Cul1 stalk results in loss of SCF ubiquitin E3 ligase activity towards its physiological substrates. This rigid structure has also been observed in c-Cbl RING-finger protein which represents an independent E3 ligase family [57].

The structures of Skp2/Skp1, and Skp1/*β*-Trcp containing the phospho-*β*-catenin substrate peptide, and Skp1/yeast *C*dc4/phospho-substrate peptides have been reported [21, 25, 55, 56]. While the conserved F-box regions of Skp2, *β*-Trcp, and *C*dc4 interact with Skp1, additional linker repeats between the F-box and the LRR or WD40 repeats also support the interaction with Skp1. In addition, the C-terminal tail of Skp2 also folds back into the linker repeats between the F-box and the linker region to provide additional interaction with the C-terminal region of Skp1. These conformations suggest that the Skp1 and Skp2 interaction, as well as Skp1 binding to *β*-Trcp and *C*dc4, is relatively rigid. This rigid structure may position the LRR or WD40 domains of these F-box proteins to orient the substrates in a particular direction towards the E2 site by Cu11/Roc1 interface. Thus the structural rigidity of the SCF E3 ligases and the existence of a substantial distant gap between the substrate-binding domain and the E2/RING-H2 domain may be a common feature required for the polyubiquitination of the substrates. However, the distance between the F-box-bound substrates and the Cul1/RING-H2 protein-bound E2 poses a structural limit for their direct interaction and thus the ubiquitin-transfer reaction from E2 to substrates.

The SCF E3 ligase, unlike other E3 ligases such as the HECT E3 ligase, does not appear to form a covalent thioester bond between ubiquitin and the E3 ligase [2]. This may suggest that the SCF E3 ligase could use a different mechanism to drive the ubiquitin-transfer reaction. Interestingly, it has been found that although the *C*dc34 E2-conjugating enzyme binds to Roc1, which in turn binds to Cul1, the covalent linkage between ubiquitin and *C*dc34 leads to an increased dissociation of the ubiquitin-charged *C*dc34 from the Roc1/Cul1 interaction [58]. These observations suggest that *C*dc34 may be constantly dissociated from the Roc1/Cul1-binding domain in a cyclic fashion during the ubiquitin-transfer reaction to extend the elongating ubiquitin chain on the substrate [58]. Thus the func-

tion of Roc1/Cul1 is probably to bring *C*dc34 into the vicinity of the SCF-bound substrates, which lie on the top surface of the β-propeller repeats of WD40 or LRR in line with the E2 active site [21, 55, 56]. The formation of a thioester bond between ubiquitin and *C*dc34 facilitates *C*dc4 release from the Roc1/Cul1 interaction. Once the ubiquitin-charged *C*dc34 is released into the region surrounding the substrate, the resulting increase in the effective concentration of ubiquitin-*C*dc34 near the substrates promotes ubiquitin transfer from *C*dc34 to the substrates. This model appears to provide a mechanistic explanation for the distance between the F-box protein-bound substrates and the Roc1/Cul1-bound E2, and the requirement for the rigid structure of SCF complexes. In addition, it also helps to address how the E2 protein can cope with the increasing distance between the elongating polyubiquitin chain and the fixed positions of the Roc1/Cul1-bound E2 and the F-box-bound substrates in bringing about the ubiquitin-transfer reaction. This cyclic association and dissociation of the E2 enzyme may also help interpret some observations for the effects of ***C***op9-***s***ig***n***alosome complex (Csn) [59]. Although biochemically Csn plays an inhibitory role towards SCF through deneddylation of Cul1 and deubiquitination of the ubiquitinated substrates [59], the loss of Csn often produces accumulation of the SCF substrates. Since one function of Cul1 neddylation is thought to increase the binding of E2 to Roc1, loss of Csn *in vivo* may affect the cyclic binding of E2 to SCF complex during the polyubiquitination reaction of SCF substrates [59].

The rigid structure of the SCF complexes may also underlie the observed selectivity of the lysine residues in the substrates [21, 56]. It was found that only a subset of lysines in the SCF substrates such as p27 or Sic1 can be efficiently polyubiquitinated by SCF-E2 enzymes [60, 61]. Conversion of these lysines to arginines stabilizes these proteins even though they still contain other lysines. A single polyubiquitination chain on one of these critical lysines appears to be sufficient for substrate degradation by the 26S proteasome [60]. It thus appears that only those lysines in the SCF substrates that are in sight of or in the vicinity of the E2 enzyme may be used as ubiquitination receptors during the SCF-mediated ubiquitin-transfer reaction. The rigidity of the SCF complex is likely to contribute to such a restriction on the use of lysines in the substrates.

However, it is still possible that other mechanisms may exist to bridge the gap between substrate and E2 in the SCF-mediated ubiquitin-transfer reaction. For example, reports suggest that SCF may form higher order structures to facilitate the degradation of protein substrates. The *S. pombe* F-box proteins Pop1 and Pop2 have been shown to form heterodimers, and evidence suggests that these interactions may be important for the degradation of their *in vivo* substrates [62].

6.7 Regulation of SCF Activity

Several mechanisms have been shown to regulate the activity of the SCF complex. The expression of F-box proteins such as Skp2 is regulated by cell-cycle-dependent transcription [4]. The expression of Skp2 is high in late G1, S, and G2/M phase but

low in the early G1 phase. In addition, Skp2 expression appears to be regulated by cell attachment and by the Pten/PI-3 kinase signaling pathway in certain cells [63, 64]. Tome-1, an F-box protein that triggers mitosis by targeting the mitotic inhibitory kinase Wee1 for proteolysis, is regulated by ubiquitin-dependent degradation through the Apc/C-Cdh1 E3 ligase in the G1 cells [35].

The expression of Cul1 has also been reported to increase in cycling cells after growth factor stimulation as compared with that of G0 cells [65]. In *C. elegans* and other organisms where multiple Skp1-related genes exist, the expression of individual Skp1-related genes is also regulated in a development- or cell-specific manner [27]. The regulated expression of these genes may play an important role in controlling the temporal functions of these SCF complexes in cell-cycle, development, and tissue specificity.

Another level of control is mediated through the control of F-box protein stabilities by the SCF complex using an auto-ubiquitination mechanism. Deletion of the F-box motif of various F-box proteins such as yeast *C*dc4 or *β*-Trcp abolishes the interaction between Skp1/Cul1 and the F-box proteins [66]. Consequently the F-box proteins become more stable. This regulation may provide a means to recycle the components of SCF complexes between different F-box proteins. In addition, the levels of a particular F-box protein may be in part regulated by the balance between autoubiquitination and substrate-specific ubiquitination and thus could be sensitive to the presence of the substrates.

Cul1, together with other cullin family members, is uniquely regulated by a covalent modification with an ubiquitin-like protein, Nedd8 (Rub1 in yeast), at the conserved lysine 720 or equivalent sites in other cullins [59]. Neddylation appears to be important for the activity of SCF complexes and is essential for many organisms. The neddylation of Cul1 depends on the neddylation-activating E1 (APPBP1 and UBA3) and its specific conjugating E2 enzyme (UBC12). It was found that Csn, which shares substantial homology with the lid subcomplex of the 26S proteasome, exhibits an intrinsic peptidase activity towards neddylated Cul1 and other cullins [67]. Csn binds to cullins and this binding promotes the deneddylation of Cul1 and other cullins [67]. Csn may also play a role in recruiting the deubiquitination enzymes to reverse the ubiquitination of the SCF substrates [68]. One function of neddylation may be associated with the recruitment of E2 to the Cul1/Roc1 complex [68]. This possibility is also consistent with the structural determination that the neddylation receptor lysine 720 of Cul1 lies within the same surface as the Roc1-binding site in the C-terminal domain of Cul1 [68]. Binding of Roc1 to Cul1 or Cul2 can promote the neddylation of these cullins in plants and animals [68]. The binding of Roc1 to Cul1 may provide an open configuration for the neddylation of lysine 720 in Cul1, which lies close to the Roc1-binding site.

In addition, studies suggest that the association between Skp1/F-box proteins and Cul1/Roc1 is highly regulated. In particular, reports suggest that the binding of a Cul1-binding protein, Cand1/Tip120, to Cul1 or Cul1/Roc1 complexes causes the dissociation of Skp1/F-box proteins from Cul1. Cand1 (***c***ullin-***a***ssociated ***n***edd8-***d***issociated protein 1) or Tip120 was isolated as a protein that binds to the Cul1/Roc1 complex only when Cul1 is not neddylated [69, 70]. Both *in vitro* and *in vivo*,

there is a dynamic equilibrium between Cand1, Cul1 neddylation/deneddylation, and Skp1/F box protein binding. Assembly of the SCF complex is in part regulated by Cand1 and Cul1 neddylation. In the absence of Cul1 neddylation, Cand1 binds to the Cul1/Roc1 complex and such an interaction dissociates Skp1/F-box proteins from the Cul1/Roc1 complex. Neddylation of Cul1 promotes Cand1 dissociation, and facilitates the incorporation of Skp1/F-box proteins and SCF complex assembly. However, it remains unclear whether Cand1 is required for each cycle of ubiquitin transfer by the SCF and E2 enzymes to the substrate. Although Csn acts as an inhibitor of Cul1 and related cullin E3 complexes, loss of Csn activity often results in the accumulation, rather than enhanced degradation, of the SCF substrates [59]. The accumulation of SCF substrates in CSN mutants suggests that neddylation of Cul1 may be required for the repeated cycles of ubiquitin transfer *in vivo*.

6.8 The SCF Complex and the Related Cullin-containing Ubiquitin E3 Ligase

The SCF ubiquitin E3 ligase serves as the prototype of many related ubiquitin E3 ligases containing one of the cullin family members (Figure 6.2). So far, eight highly conserved cullins (Cul1–8) are found from yeast to human [3, 16, 17, 71]. All cullin-containing E3 ligases appear to bind one of the RING-H2 proteins, Roc1 or Roc2, and are subject to modification and regulation by the Nedd8 pathway. These multiprotein complexes probably represent the largest branch of the ubiquitin E3 ligases owing to their utilization of distinct substrate-targeting subunits.

Among these E3 ligases, Cul2 and Cul5 bind to Elongin C, an Skp1-like protein that is highly conserved between yeast and human [22, 23]. Elongin C also binds to Elongin B, an ubiquitin-like protein that is absent in SCF complexes. The Cul2/ElonginB/ElonginC complex interacts with the ***v***on ***H***ippel–***L***indau tumor suppressor (VHL), a substrate-targeting subunit that regulates the stability of the hypoxia-inducible transcription factor HIFα in response to oxygen levels [23, 72]. Mutation of VHL is associated with many renal cell carcinomas and these mutations affect the VHL activity as the substrate-targeting unit of the Cul2/ElonginB/C complex. VHL also belongs to the large family of SOCS box proteins which are candidate substrate-targeting subunits of the Cul2/ElonginB/C complex that regulates signal transduction and many other biological processes [2]. For example, studies have shown that Cul2/Elongin C maintains germ cell fate in *C. elegans* by selectively targeting the germ cell-specific zinc-finger proteins for ubiquitin-dependent proteolysis in the soma cell but not in the germ cell [73].

Studies indicate that Cul3 binds to and employs BTB/POZ proteins as substrate-targeting subunits [24]. Cul3 has been shown to regulate mammalian embryonic cyclin E levels and also meiosis in *C. elegans*. A large number of BTB/POZ proteins exist. In human, more than 200 BTB/POZ proteins have been identified while more than 100 BTB/POZ proteins exist in *Drosophila* and *C. elegans*. The association of the BTB/POZ protein with Cul3 also has structural relevance to the SCF and

Cul2/Elongin B/C E3 complexes, since both Skp1 and Elongin C show the BTB/POZ-like protein fold. Thus the presence of the large family of cullin-containing ubiquitin E3 ligases suggests their profound regulatory roles in various important biological processes.

6.9 Perspectives

The SCF complex represents one of the largest ubiquitin E3 ligase families. The diversity of the F-box proteins allows the involvement of SCF in regulating various biological processes. So far, only a small number of F-box proteins have been characterized; a large body of work remains to further identify the substrates and regulation of other F-box proteins at cellular and organismal levels. In addition, the SCF E3 ligase represents the prototype of an extended family of E3 ligases that contain cullins. These cullin-containing E3 ligases, with at least hundreds of subunit-targeting subunits, are involved in a spectrum of biological events encompassing cell cycle, cell fate, and various signaling pathways. Alterations of many F-box proteins and SCF-regulated pathways are also associated with human diseases [74]. Understanding the function and regulation of this ubiquitin-dependent proteolysis mechanism should provide new insight into the treatment of human diseases such as cancer.

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7
The Structural Biology of Ubiquitin–Protein Ligases

Ning Zheng and Nikola P. Pavletich

7.1
Introduction

Ubiquitination, the conjugation of ubiquitin to proteins, is a major post-translational modification mechanism that regulates a broad spectrum of biological functions [1]. One of the major functional roles of ubiquitination is to control the turnover rate of the substrate proteins, whereby the substrates are targeted to the 26S proteasome and proteolytically degraded. By adjusting the abundance of key proteins in cellular pathways, ubiquitination can switch many regulatory circuits to different states. Ubiquitination has also been found to regulate proteins through processes other than targeting proteins to the proteasome. These include endocytotic pathways, where ubiquitination serves as a sorting signal, and the DNA-damage response, where ubiquitination has a poorly understood but essential role [2].

The fundamental step of protein ubiquitination involves the formation of an amide bond between the ubiquitin C-terminus and the ε-amino group of a substrate lysine residue. Variations in the way additional ubiquitin molecules are conjugated to the substrate-linked ubiquitin confer different cellular functionalities on the modifier. To target proteins for proteasome-dependent degradation, a poly-ubiquitin chain is assembled on the substrates through the successive conjugation of ubiquitin to the K48 residue of the previous ubiquitin. A similar ubiquitin chain assembled through conjugation to the K63 residue, or the absence of any ubiquitin chain extension, on the other hand, lead to non-proteolytic signaling events.

The conjugation of ubiquitin to proteins is mediated by ubiquitin–protein ligases, which function at the last step of a three-enzyme reaction (Figure 7.1) [1, 3]. In the first reaction, the ubiquitin-activating enzyme, E1, activates free ubiquitin by utilizing ATP to form a high-energy thioester bond between the ubiquitin C-terminus and an E1 cysteine residue. The activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme, E2, which forms a similar thioester linkage between its own active-site cysteine and ubiquitin. The final step of protein ubiquitination is catalyzed by the ubiquitin–protein ligases, E3, which bind both an E2 and a protein substrate and promote the ubiquitin transfer from the E2 to the sub-

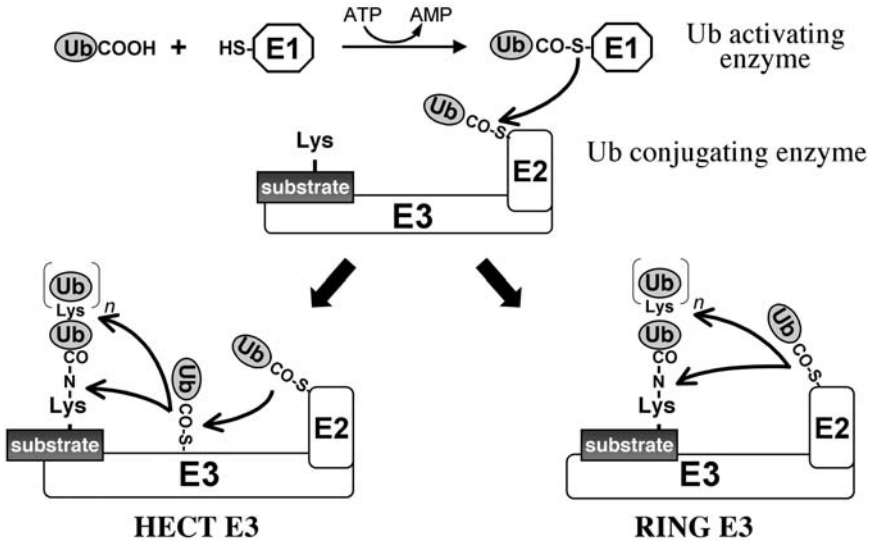

Fig. 7.1. The E1–E2–E3 enzyme cascade of ubiquitin conjugation.

strate. The importance of the ubiquitin–protein ligases in ubiquitination is underscored by their roles in both determining the specificity of the modification and catalyzing the ubiquitin-transfer reaction. Coupled to various cellular signaling events, ubiquitin ligases ensure that the ubiquitination process is temporally controlled and tightly regulated with a high degree of substrate specificity. Not surprisingly, ubiquitin–protein ligases serve as the key regulators in many cellular pathways. Abnormal ubiquitin–protein ligase activity has been implicated in numerous human diseases such as cancer and neurological disorders [4].

Although the first E3 ligase activity was described in the early 1980s [5], it is only more recently that the E3s have emerged as a large superfamily of proteins and protein complexes. Central to this was the realization that the RING domain is a common motif in many E3s (reviewed in Ref. [6]). The rapidly growing number of ubiquitin ligases and the increasing recognition of their biological importance in recent years have been followed by several structural studies of E3s and of E3 complexes. In this chapter, we will review the recent advances in the structural biology of ubiquitin–protein ligases, focusing on their general architecture, their substrate recognition and E2-binding activities, and, importantly, the mechanistic insights into E3-catalyzed protein ubiquitination provided by these structures.

7.2
The Two Major Classes of Ubiquitin–Protein Ligases

The E3 ubiquitin–protein ligases represent a large and diverse family of proteins and protein complexes [3]. The human genome alone is estimated to code for hun-

dreds of E3s, whereas the number of E2s is estimated at around thirty, and there is only one identified E1. The diversity of the E3 ubiquitin–protein ligases is reflected in their early loose definition as proteins or protein complexes that are required, in addition to the E1 and E2 activities, for the ubiquitination of a substrate. Today, we know that most E3s carry out three functions. They bind the substrate, thus conferring substrate specificity to the ubiquitination pathway, they bind a cognate E2, and they promote the ubiquitination of the substrate [1]. Identification and the realization of a common structural motif, namely the RING domain, in many otherwise divergent ubiquitin ligases have greatly facilitated the classification of known E3s [3, 6]. To date, all characterized ubiquitin ligases can be grouped into two major classes: the HECT (***H***omologous to ***E***6AP **C**-***T***erminus) class and the RING/RING-like class. These two classes of E3s contain different signature domains and mediate substrate ubiquitination in functionally distinct ways.

HECT E3s share a conserved ~40-kDa C-terminal catalytic HECT domain, preceded by divergent N-terminal domains that bind different protein substrates [3]. To mediate ubiquitination, HECT E3s first form a thioester intermediate between their active-site cysteine and the ubiquitin C-terminus and then transfer ubiquitin to the substrate (Figure 7.1) [7]. The RING class E3s do not form such a thioester intermediate with ubiquitin. Instead, they promote the direct transfer of ubiquitin from the E2 to the substrate. RING E3s are structurally diverse, containing from one to over ten subunits, yet they all have a RING domain in common [6, 8]. The RING domain is a ~60-amino acid structural domain stabilized by two to three zinc atoms. In most cases that have been studied, the RING domain has the main E2-binding activity. The U-box, which is structurally related to the RING domain but lacks the zinc ligands, has recently also been shown to assemble into RING-like E3s [9].

Despite the difference in sequence conservation, structure, and the way they mediate ubiquitination, all ubiquitin ligases functionally share two common activities. They bind the substrate, conferring specificity to the reaction, and they also bind a cognate E2 (Figure 7.1). In regulating protein stability, the ability of E3s to interact with the substrate protein is often governed by the phosphorylation or other post-translational modification of the substrate. E3s studied to date are highly specific for either the Ubc2 or Ubc4 class of E2s, but generally appear to be less specific for individual E2s within each class, at least *in vitro* [10].

7.3 Mechanistic Questions About E3 Function

One of the central mechanistic questions regarding ubiquitination has been whether the reaction utilizes general acid/base catalysis, possibly in a manner analogous to the catalysis of peptide-bond cleavage. For example, an acidic catalytic residue could deprotonate the substrate lysine and make it a better nucleophile for attacking the ubiquitin thioester bond. In addition, a basic catalytic residue could polarize the thioester bond making the carbonyl carbon a better electrophile, and

it could also stabilize the likely tetrahedral intermediate resulting from the nucleophilic attack. This is still an unanswered question for the HECT E3s.

In the case of RING E3s, three distinct mechanisms can be envisioned. One mechanism is recruitment and positioning. In principle, the RING E3 could promote ubiquitination by simply increasing the effective concentration of the entire substrate protein around the E2. Such an effect could be stereochemically more precise. For example, the E3 could increase the effective concentration of a portion of the substrate that includes the ubiquitination-site lysine at the E2 active site, or position and orient the lysine ε-amino group next to the ubiquitin-E2 thioester bond optimally for nucleophilic attack. The second possible mechanism is that the RING E3 could provide amino acids that act as acid/base catalysts at the active site of the E2, perhaps in a manner analogous to the ***G***TPase ***a***ctivating ***p***roteins (GAPs) [11]. The third possibility is that E3-binding could cause significant conformational changes of the E2 to activate the enzyme for ubiquitin transfer. This was initially proposed based on the observation that RING domains of ubiquitin ligases can catalyze the polymerization of ubiquitin in a substrate-independent manner [12, 13]. As will be discussed later, the results of structural studies favor the model that the E3 raises the effective concentration of the lysine-containing substrate portion as the likely mechanism of catalysis. This, however, does not exclude the possibility that ubiquitination requires acid/base catalysis. Catalytic groups can, in principle, be provided by the E2. Although the vicinity of the E2 active-site cysteine is devoid of solvent-exposed polar or charged residues that are also conserved [10], a catalytic role could be played by E2 backbone groups, or by conserved residues elsewhere on the E2 that are brought into the active site through a hypothetical conformational change [14]. An alternative possibility is that the reaction is substrate catalyzed, with ubiquitin providing catalytic residues.

Another question regarding the mechanism of ubiquitin transfer is how the specificity for the ubiquitinated lysine is achieved. Until now, no sequence of motifs has been found that dictates which lysine on a protein is ubiquitinated. Therefore, it is possible that any lysine residue on a substrate could be modified. This is supported by studies showing that mutation of a large fraction of all lysines on a protein is necessary to reduce ubiquitination of certain proteins to a detectable extent (see, for example, Refs. [15, 16]). In other cases, however, ubiquitination of substrate proteins showed clear lysine specificity. Early work by Alexander Varshavsky and colleagues indicated that the polyubiquitin chain was conjugated to two specific lysine residues in the 1045 amino acid model substrate, β-galactosidase fusion protein [17]. More recently, lysine specificity for ubiquitination has been demonstrated in several physiological ubiquitination substrates. One of the best-characterized cases is IκBα, which gets ubiquitinated only at two adjacent lysines out of a total of 51 lysines. Mutation of both IκBα lysines completely abolishes its ubiquitination and degradation [18, 19]. The lysine specificity of ubiquitination in some proteins could be functionally required for several reasons. First, like protein phosphorylation, the ubiquitination of certain proteins is reversible with deubiquitination being mediated by protein-specific ubiquitin hydrolases [20]. Modification of a specific lysine residue might be favored by the system to gain precise

control. Second, ubiquitination is one of many post-translational modifications of proteins that occur at lysine side chains. These include conjugation of other ubiquitin-like proteins, acetylation, and methylation [21]. Competition among these modifications at a specific lysine residue might be utilized as a mechanism to integrate signals from different pathways. For example, the same two lysines of IκBα that get ubiquitinated can also be modified by the ubiquitin-like protein SUMO (***s***mall ***u***biquitin-related ***mo***difier), and this has been shown to block the ubiquitination and destruction of IκBα [22]. Site-specific acetylation of the p53 tumor suppressor and the E2F-1 transcription factor has also been shown to block their ubiquitination [23, 24].

Ubiquitin–protein ligases promote not only the attachment of ubiquitin to the protein substrates but also the extension of the ubiquitin chain. What determines the choice between mono- *vs.* polyubiquitination is not well understood. It is possible that certain E3s catalyze only mono-ubiquitination. Alternatively, factors other than E3s might be responsible for the attachment of a single ubiquitin. For example, ubiquitin-binding accessory proteins have been suggested to block extension of the ubiquitin chain [25], whereas E3-associated ubiquitin hydrolase could trim down the polyubiquitin chain. The identification and characterization of ***u***biquitin ***E***2 ***v***ariant proteins (UEVs) have provided an explanation for the assembly of K63-linked polyubiquitin chains [26, 27]. As discussed later, UEVs can be considered as special E3s, with the ubiquitin chain as their substrates.

Unlike many other enzymatic reactions, ubiquitination involves multiple enzymes and proteinaceous reactants. Although the structures of some individual components of the enzymatic system, such as the E2 and the RING fold, had been long known [28, 29], basic questions such as how the ubiquitin ligases recognize their substrates and recruit their cognate E2s, and how the E3 ligases couple ligand binding to ubiquitin transfer, could be best answered when the structures of several E3-substrate and E3–E2 complexes became available. These structures also set the framework for addressing questions about the mechanism of ubiquitin transfer.

7.4
The E6AP HECT Domain in Complex With UbcH7

First identified as a protein associated with the ***h***uman ***p***apilloma ***v***irus (HPV) E6 [30], E6AP has been shown to be the cellular E3 that cooperates with the viral E6 protein to ubiquitinate p53 in HPV-infected cells [31]. It has since become the prototypical HECT E3, and in fact the E3–ubiquitin thioester intermediate was first discovered using E6AP [7]. E6AP was independently identified as the gene mutated in ***A***ngelman ***S***yndrome (AS), an inherited developmental syndrome characterized by severe motor dysfunction and mental retardation [32]. The endogenous substrates of E6AP involved in AS remain unknown.

Similar to other HECT E3s, E6AP consists of a ~40-kDa C-terminal HECT domain and an N-terminal region containing sequences involved in binding E6-p53,

and presumably the endogenous substrate(s). Distinct from many other HECT E3s, which have protein–protein interaction motifs such as WW domains, E6AP has no recognizable motifs in the N-terminal region [7]. E6AP also lacks the C2 domain, which is found in some HECT E3s that act on membrane-bound substrates. Nevertheless, biochemical studies have demonstrated that the E6AP HECT domain has the following activities that are likely to be common to all HECT E3s. The E6AP HECT binds a cognate E2 and accepts ubiquitin from the E2, forming a ubiquitin–thioester intermediate with its active-site cysteine. It then transfers ubiquitin to the ε-amino groups of lysine side chains on the substrate by catalyzing the amide bond formation, and subsequently transfers additional ubiquitin molecules to the growing end of the polyubiquitin chain (Figure 7.1) [33, 34].

The Ubc4 but not the Ubc2 family of E2s has been shown to function with E6AP and several other HECT E3s. E6AP appears to have preference for specific E2s within the Ubc4 family, and this has been shown to be due to specificity in the HECT domain–E2 interactions [33, 35–37]. In a yeast-two-hybrid assay, the closely related UbcH7 and UbcH8 E2s, but not the UbcH5, bind to E6AP. When tested *in vitro*, UbcH7 and UbcH8 showed the highest rates of ubiquitin–thioester intermediate formation with E6AP, whereas UbcH5 supports E6AP–ubiquitin–thioester formation at lower rates [37].

The crystal structure of the E6AP HECT domain bound to UbcH7 was the first solved structure of an E3 catalytic domain and of an E3–E2 complex. While the structure revealed the fold of the HECT domain, the details of the HECT catalytic site, the E3–E2 interactions, and the basis of specificity of E6AP for its cognate E2, it also raised many new questions. The structure showed that the HECT domain consists of two loosely packed lobes (Figure 7.2) [38], with an elongated N-terminal one (N-lobe) interacting with the E2, and a globular C-terminal one (C-lobe) bearing the active-site cysteine (Cys820). The C-lobe packs at one end of the N-lobe, forming an overall L-shape with the N-lobe being the base. The active-site cysteine of the HECT E3 is found near the junction of the two lobes, where a shallow and broad cleft is formed at the interface. The E2 active-site cysteine has an open line-of-sight to the E3 cysteine, the two being separated by $\sim$40 Å (Figure 7.2).

The large distance between the E2 and E3 active sites in the complex was unexpected, as trans-thioesterification is thought to proceed through an associative mechanism. For the HECT domain to take the ubiquitin from the E2, the E3 active cysteine has to be in close vicinity to the E2's active site. It has therefore been hypothesized that the HECT domain must undergo a significant conformational change to accept ubiquitin from the E2 [38]. Support for this model came from a subsequent report of the structure of the HECT domain from WWP1, determined by Joseph Noel and coworkers [39]. Although the structures of the individual N and C lobes of the two HECT domains are very similar, the relative orientation and position of the two lobes is very different in the two structures. Instead of packing at one end of the N-lobe, the C-lobe of the WWP1 HECT is interacting with the middle part of the N-lobe, at a position spatially related to that of the E6AP HECT C-lobe by a $\sim$100° rotation around the hinge loop connecting the lobes. Under this structural arrangement, the two active-site cysteines are brought

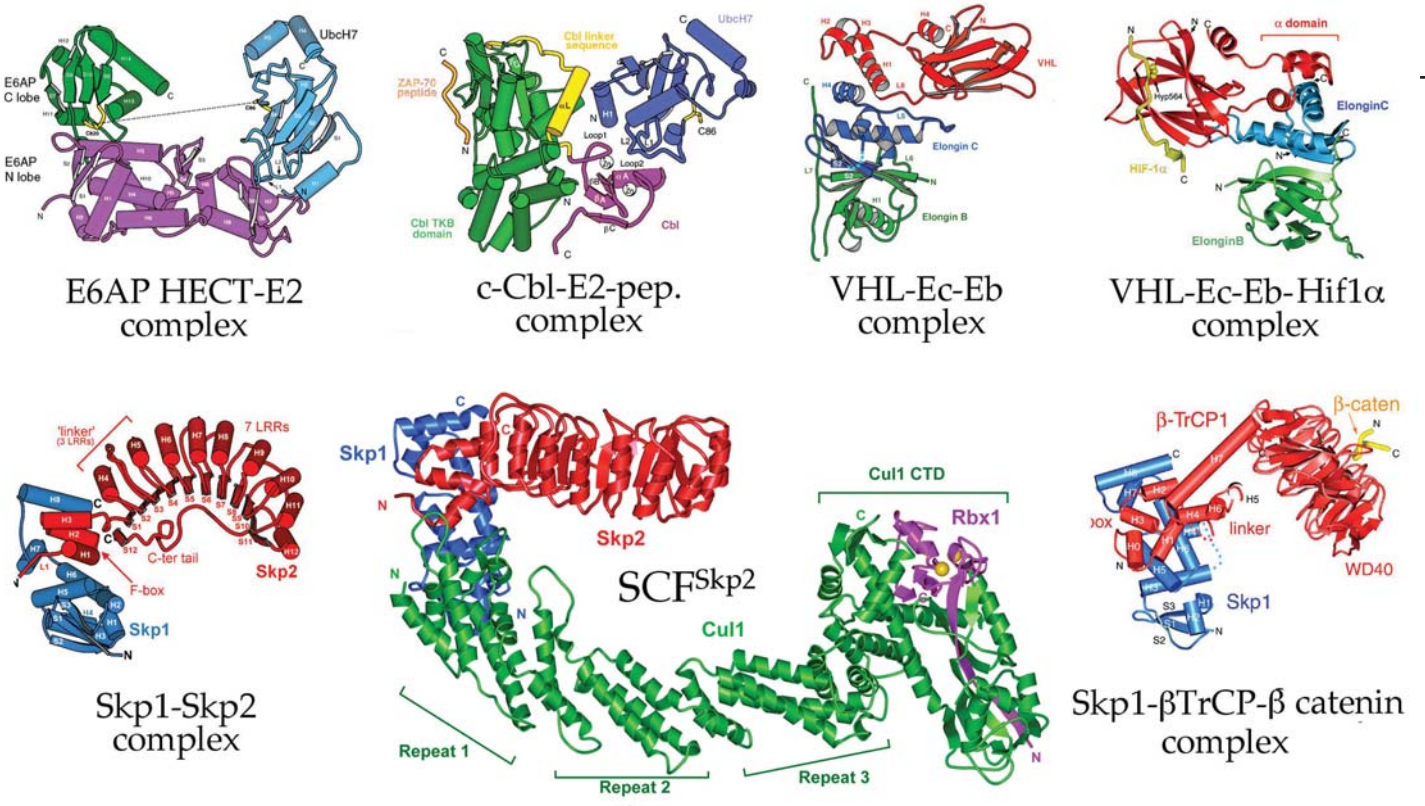

Fig. 7.2. Crystal structures of ubiquitin ligases discussed in this chapter. The structures are colored as following: The HECT N lobe is purple, C lobe green, and the E2 blue, the c-Cbl TKB domain is green, the linker yellow, RING domain purple, the phosphopeptide orange, and the E2 blue. In the VHL complexes, VHL is red, ElonginC blue, ElonginB green and Hif1α orange. In the Skp1–Skp2, SCF^{Skp2} and Skp1–βTrCP-β catenin complexes, Skp1 is blue, the F box protein red, Cul1 green, Rbx1 purple, and β-catenin orange.

much closer to each other, although the two are still 16 Å apart (Figure 7.3). Thus, the HECT must undergo an additional conformational change during catalysis [39]. In principle, the conformations observed in the two crystal structures could be induced by crystal packing and are not biologically relevant. However, the junction of the two lobes in both structures is lined with highly conserved residues from both sides in spite of the loosely packing interface (Figure 7.3), suggesting that this part of the structure, its potential conformational changes, and the specific structural configuration observed in the crystal are functionally important [38]. Moreover, conserved residues from both the N- and C-lobes, including a subset of those found in E6AP, are also juxtaposed at the active site cleft in the WWP1 HECT domain, again implying that the conformation seen in the WWP1 HECT crystal is functionally relevant. It is thus more likely that the conformations of the HECT domain seen in these two structures represent different steps of the ubiquitin-transfer reaction. Although the number of other steps involved remains to be determined, the large movement of the C-lobe relative to the N-lobe around the hinge loop is very likely required during the transfer of ubiquitin from the E2 to the HECT E3. Indeed, mutations that restrain the flexibility of the hinge loop between the two lobes caused significant decrease of the ubiquitin-transfer activity of the WWP1 HECT [39]. So far, neither the substrate-binding domain nor the substrate of any HECT E3s has been successfully co-crystallized with the HECT domain. It remains unclear how the HECT and its active site are orientated relatively to the substrate. It is conceivable that the movement of the HECT C-lobe might also be involved in transferring the ubiquitin it has taken from the E2 to the substrate and/or the growing polyubiquitin chain.

In many enzymes, conserved residues near the active site often participate in the catalytic reaction. To investigate the possible involvement of acid/base catalysis, mutagenesis studies of the E6AP active site have been carried out. Although mutations of several polar or charged residues reduced the efficiency of ubiquitin transfer, a residue that would be consistent with a role in deprotonating the substrate lysine has not been found [38]. Intriguingly, unlike the RING domain, the HECT domain of all known HECT E3s is always found at the C-terminus of the polypeptide. The extreme C-terminus of HECT is located near the active-site cysteine. A frameshift mutation of E6AP resulting in the extension of the C-terminus by 16 amino acids has been found in AS cases. Together, these lines of evidence raise the possibility that the C-terminal carboxylic acid group might participate in the reaction, especially in deprotonating the substrate lysine residue attacking the ubiquitin–thioester bond.

In the crystal of the E6AP-UbcH7 complex, the E3-bound E2 has an α/β structure very similar to the structures of other E2s crystallized by themselves, arguing against the model that E3s activate the E2 by causing conformational changes. The E6AP E3 interacts with one end of the overall elongated UbcH7 through a shallow hydrophobic groove on the N-lobe of its HECT domain (Figure 7.3). The major E2–E3 contacts are made by side chains from two E2 loops, termed L1 and L2 loops, while a portion of the N-terminal α-helix of the E2 is involved in minor intermolecular contacts (Figure 7.3). The structure indicates that the primary determinants of

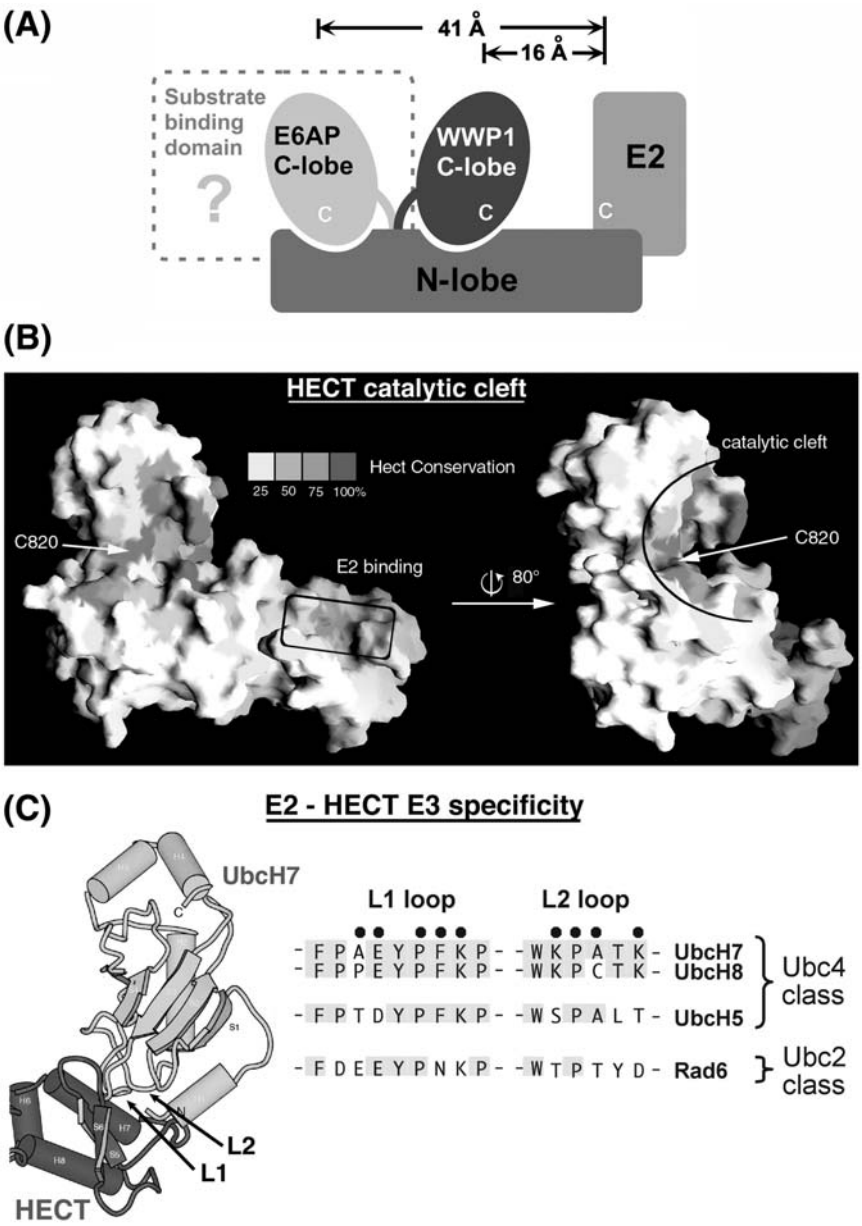

Fig. 7.3. Conformational changes and E2-binding specificity of the HECT domain. (A) Schematic diagram of the structures of E6AP and WWP1 HECT domains with their N-lobes superimposed indicating the potential large domain movement of the HECT E3. (B) Surface representation of the E6AP HECT domain, showing that conserved HECT domain residues map to the catalytic cleft defined by the active-site cysteine (Cys820). (C) Interactions of UbcH7 with a hydrophobic groove of the E6AP HECT domain through the E2's L1 and L2 loops. Alignment of L1 and L2 loop sequences from representative E2s shows that E6AP contacting residues (indicated by dots) are mostly conserved in the E2s that function with E6AP.

E6AP's specificity for UbcH7 are in the L1 and L2 loops of the E2. The hallmark of the interface is an E2 L1 loop phenylalanine (Phe63), which inserts its side chain into the center of the hydrophobic HECT groove. This phenylalanine residue is highly conserved in the Ubc4 but not the Ubc2 E2 subfamily, explaining the specificity of E6AP for the Ubc4 class of E2s. The preference of E6AP for UbcH7/UbcH8 over UbcH5 within the Ubc4 class can be explained by the additional contacts made by the L1 and L2 loops. For example, two L2-loop lysines that contact E6AP are conserved between UbcH7 and UbcH8 but not in UbcH5 (Figure 7.3).

7.5
The c-Cbl–UbcH7 Complex

The product of the proto-oncogene *c-Cbl* negatively regulates activated ***r***eceptor ***t***yrosine ***k***inases (RTKs) such as PDGFR, EGFR, CSF-1R, and Met, by promoting their ubiquitination and subsequent degradation [40]. Although it was initially thought that c-Cbl mediates the polyubiquitination of the RTKs, recent studies have suggested that mono-ubiquitination of RTKs is sufficient for their internalization and degradation [41, 42]. Therefore, one of the functional roles of c-Cbl in RTK down-regulation is likely to mediate their mono-ubiquitination, possibly at multiple sites, providing a signal for their endocytosis and degradation in the lysosome. Consistent with this hypothesis, it has been shown that in addition to ubiquitinating the activated RTKs, c-Cbl also recruits and mono-ubiquitinates adapter proteins involved in endocytosis [43–45].

c-Cbl is a member of the Cbl protein family that is found in most multicellular organisms. In mammals, the Cbl family consists of two additional members (Cbl-b and Cbl-3), whereas in invertebrates such as *Drosophila melangogaster* and *Caenorhabditis elegans*, only one member (D-Cbl and SLI-1, respectively) has been identified [46]. All these Cbl proteins share three highly conserved structural domains, including an N-terminal SH2-containing ***t***yrosine ***k***inase-***b***inding (TKB) domain, a RING domain, and a ~40-residue short linker region connecting the two. Two recognizable sequence motifs, including a proline-rich region and a ubiquitin-associated (UBA) domain, are also found in some but not all Cbl family members. c-Cbl recognizes activated RTKs by binding a phosphotyrosine sequence motif through its TKB domain, whereas it binds an E2 through its conserved RING domain [12, 47]. The structure of the TKB domain bound to a phosphopeptide derived from the non-receptor tyrosine kinase ZAP-70 has been reported by Michael Eck's group [48]. In that structure, the TKB domain is formed by three interacting sub-domains comprising a four-helix (4H) bundle, a calcium-binding EF hand, and a variant SH2 domain. Building on this work, we subsequently determined the structure of a nearly intact c-Cbl including both the TKB and RING domains bound to the same ZAP-70 phosphopeptide as well as the UbcH7 E2 [49].

The c-Cbl–E2–ZAP70 peptide complex adapts a compact structure with multiple inter- and intra-molecular interfaces (Figure 7.2) [49]. The RING domain is anchored on the TKB domain through extensive interactions with the 4H bundle, while the linker forms an ordered loop and an α-helix, which packs closely with

the TKB domain next to the RING. As expected, the E2 predominantly interacts with the c-Cbl RING domain, but it is also in contact with the c-Cbl linker helix (Figure 7.2). The E2 active-site cysteine is located on the side of the complex opposite where the phosphorylated substrate peptide binds.

The structure of the complex provides several insights into the mechanism by which the RING E3 mediates ubiquitination. First, the structure helped to rule out the model of acid/base catalysis, as there were no c-Cbl residues in the vicinity of the E2 active-site cysteine (the closest c-Cbl residue is 15 Å away). Second, as there was no significant conformational change within the UbcH7 E2 upon binding to the Cbl protein, the structure helped to rule out the possibility that the RING E3 activates the E2 by causing conformational changes. Therefore, the mechanism underlying the catalysis could only be plausibly explained by the general recruitment model [49]. Intriguingly, it has been reported that mutations in the linker region inactivate c-Cbl and render it tumorigenic in mice, presumably through a dominant negative effect. In particular, one of the mutations mapping to a tyrosine (Y371) at the linker–TKB interface had been shown to abolish c-Cbl function without qualitatively affecting binding to either RTKs or E2 [12]. This suggests that the Cbl E3 may do more than just recruit the protein substrate to the E2 [49]. In addition to bringing together the substrate and the E2, the precise relative position and orientation of the substrate-binding and E2-binding domains might be important to the functions of the RING E3. In fact, this is consistent with the rigid appearance of the c-Cbl structure, where all functional domains are packed closely to each other. Since the binding site of the substrate phosphopeptide is located far from the E2, the precise positioning of the rest of the substrate would require additional contacting surface from the E3. Strikingly, the molecular surface of c-Cbl revealed a surface channel lined with conserved residues (Figure 7.4). The channel runs from the substrate peptide-binding site to the general vicinity of the E2 active site, suggesting that it might be involved in directing the substrate polypeptide chain towards the E2. Taken together, these structural features of the E3–E2 complex suggest that RING E3s may serve to position and orient the substrate optimally for ubiquitin transfer [49]. Furthermore, the c-Cbl E3 may provide steric and distance restraints that determine which substrate lysine(s), relative to the c-Cbl binding phosphotyrosine epitope, will be ubiquitinated at the highest rates [49].

The structure of the c-Cbl–E2 complex revealed that the c-Cbl RING domain binds to essentially the same structural elements of the E2 as the E6AP HECT domain does (Figure 7.4). These include both the L1 and L2 specificity loops and the N-terminal E2 helix. Similar to the HECT domain, the RING domain forms a shallow hydrophobic groove on its surface, accommodating the L1 and L2 loops. The same E2 L1 loop phenylalanine (Phe63) inserts into the center of the groove. Although with a different side, the N-terminal E2 helix, which packs against the HECT domain, also interacts with the linker helix of the RING (Figure 7.4). The similarities of E2–E3 interactions between the two cases are even more striking considering that the E2-binding grooves of the RING and HECT domains are structurally unrelated. Therefore, it is likely that most E2–E3 interactions will occur the

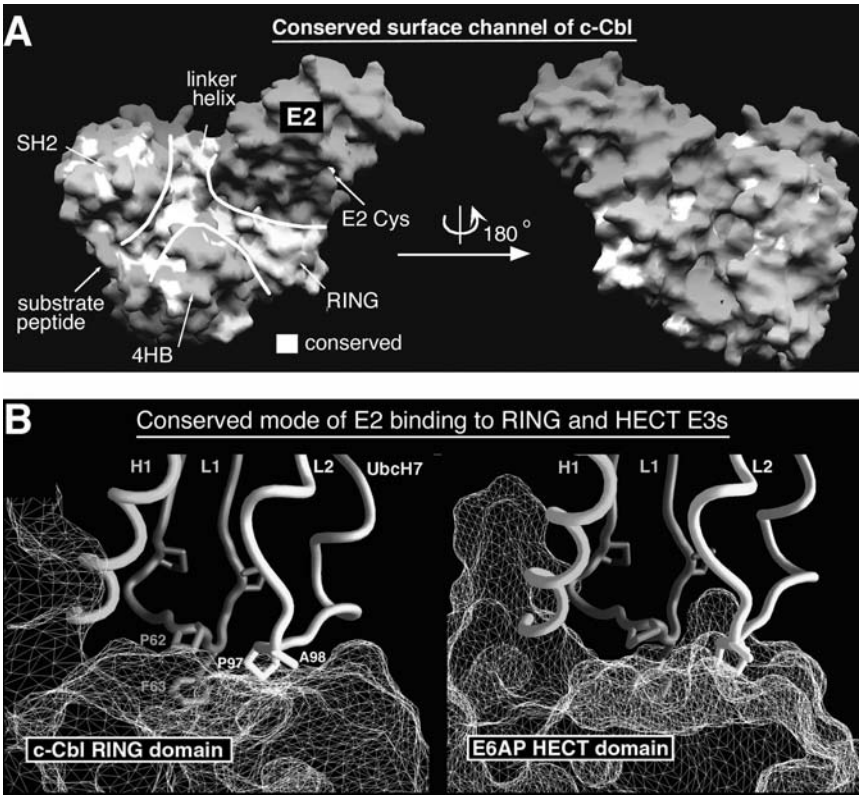

Fig. 7.4. Structural features of the c-Cbl RING E3 for substrate and E2 binding. (A) A conserved surface channel is found at one side of c-Cbl running from the peptide-binding site to the vicinity of the E2 active site. (B) The c-Cbl RING domain recognizes the same structural elements of the E2 as the E6AP HECT domain does despite the completely different folds of the two E3s (surfaces represented as a white net).

same way and that the L1 and L2 loops of the E2 are the principal determinants of E2–E3 specificity [49].

7.6 The SCF E3 Superfamily

The SCF and SCF-like complexes are multi-subunit RING-type E3s that represent the largest E3 family known to date. This superfamily of E3s are involved in regulating cell-cycle progression, signal transduction pathways, transcriptional control, and multiple aspects of cell growth and development (reviewed in Ref. [50]). All members of this E3 superfamily contain two basic components, a member of the cullin protein family and a RING-domain protein. The cullin subunit serves as the

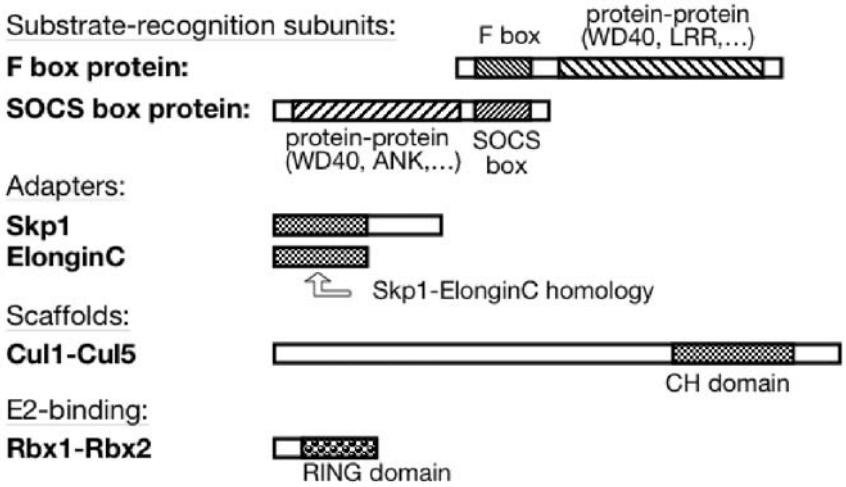

Fig. 7.5. Subunit families of the SCF and SCF-like E3s.

scaffold of the E3 complex, whereas the RING-domain protein recruits the E2. In the human genome, five cullins (Cul1–Cul5) and two cullin-interacting RING proteins (Rbx1 and Rbx2) have been identified (Figure 7.5). In addition, three more genes have been found to share sequence homology to a part of the cullins, termed the ***C***ullin-***H***omology (CH) domain (APC2, KIAA0708, and KIAA0076). Among these three, APC2 has been shown to interact with an Rbx-homologous protein, APC11, together forming the core of the ***a***naphase-***p***romoting ***c***omplex (APC), an important E3 complex regulating the cell cycle [51–53]. KIAA0076 has been shown to interact with Rbx1 (also called Roc1 and Hrt1) and renamed as Cul7 [54].

The prototype of this E3 superfamily is the SCF complex, whose scaffold and RING subunits are Cul1 and Rbx1, respectively. An SCF complex also contains two more components, an adapter protein Skp1, which interacts with the scaffold subunit, and an interchangeable substrate-binding subunit, termed the F-box protein (Figure 7.5). A similar SCF-like complex is formed based on Cul2, in which Elongin C serves as the adapter protein, whereas the substrate-binding subunit is one of the SOCS-box proteins. Recent studies have revealed a new family of SCF-like complexes built on Cul3 [55, 56]. In these complexes, the adapter and substrate-binding functions are combined in a single polypeptide, which is a member of the emerging BTB protein family.

The F-box protein family is the largest substrate-recognition subunit family. It enables the eukaryotic cells to use the SCF E3 machinery to ubiquitinate a large number of diverse protein substrates. So far, over 70 F-box proteins have been identified in the human genome [57, 58]. F-box proteins all share an ~40-amino acid F-box motif, which is usually followed by a C-terminal protein–protein interaction domain such as the WD40 repeats β-propeller (Fbw subfamily) and ***l***eucine-***r***ich ***r***epeats (LRRs; Fbl subfamily; Figure 7.5) [59, 60]. F-box proteins interact with

the Skp1 adapter protein through their F-box motif to assemble with the rest of the SCF complex. F-box proteins play a central role in the phosphorylation-controlled destruction of regulatory proteins. For example, SCF^{Skp2} (superscript denotes the F-box protein) recognizes $p27^{Kip1}$ only after the latter has been phosphorylated during the G1–S transition [61, 62]; SCF^{Fbw7} binds only phosphorylated CyclinE [63–65]; and $SCF^{\beta TrCP}$ recognizes a doubly-phosphorylated destruction motif sequence in the β-catenin and IκBα proteins [66].

The SOCS-box protein family plays a similar role in the Cul2-based E3 complexes to the F-box proteins in the SCF complex [60, 67]. The adapter protein ElonginC shares limited sequence homology over ~115 amino acids with Skp1 and also requires an obligate partner ElonginB for its function (Figure 7.5) [67]. SOCS-box proteins bind ElonginC through their common SOCS-box motif and bind substrates through their N-terminal protein–protein interaction domains such as ankyrin repeats (ASB subfamily) and WD40 repeats (WSB subfamily) (Figure 7.5). Initially identified as suppressors of cytokine signaling, the SOCS-box protein family has expanded to over 40 members in the human genome [68]. Whether all SOCS-box proteins can assemble into E3 complexes remains to be tested, but a large body of studies has demonstrated that the SOCS box protein VHL mediates the ubiquitination of Hif1α in oxygen-response pathways [69]. Furthermore, SOCS-1 and SOCS-3 have been shown to ubiquitinate the ***i***nsulin ***r***eceptor ***s***ubstrate 1 and 2 (IRS1 and IRS2) in response to insulin stimulation [70].

Crystal structures of a number of sub-complexes of the SCF and SCF-like E3s have been reported. These include the VHL–ElonginC–ElonginB complex bound to a Hifα peptide, Skp1–Skp2, Skp1–β-TrCP bound to a β-catenin peptide, Skp1–Cdc4 bound to a consensus peptide, and Cul1–Rbx1–$Skp1–F\text{-}box^{Skp2}$. Together, these structures have not only revealed the general architecture of the SCF complex and delineated the structural and functional roles of each subunit, but also shed light on how these multisubunit RING E3 complexes mediate substrate ubiquitination.

7.6.1 The VHL–ElonginC–ElonginB–Hif1 α Complex

The VHL gene was first identified by positional cloning as a tumor suppressor gene whose germline mutation is associated with the rare inherited von Hippel–Lindau cancer predisposition syndrome [71]. The disorder is characterized by tumors of the central nervous system, kidney, retina, pancreas, and adrenal gland. Soon after, VHL was found to bind the ElonginC and ElonginB proteins [72], which were known previously as factors that stimulated the transcriptional elongation factor ElonginA *in vitro*. Although the association of VHL with ElonginC and ElonginB suggested that VHL may function in transcription elongation, the subsequent detection of Cul2 in the same complex, together with the sequence homology between Skp1 and ElonginC, indicated that these four proteins form an SCF-like ubiquitin ligase complex [73].

To date, the best-characterized substrate of the ***V***HL–Elongin***B***–Elongin***C***–

Cul2–***R***bx1 (VBC-CR) E3 complex is the α-subunit of the heterodimeric ***h***ypoxia-***i***nducible ***f***actors (HIFs). HIFs are transcription factors that play a central role in the cellular response to low oxygen levels (hypoxia) by activating the expression of genes involved in angiogenesis, erythropoiesis, and energy metabolism [75]. VHL recognizes the ***o***xygen-dependent ***d***egradation ***d***omain (ODD) of Hif1α only when a proline residue (Pro564) of the ODD is hydroxylated [75, 76]. This proline hydroxylation modification is carried out by a family of recently identified ***H***IF ***p***rolyl ***h***ydroxylases (HPHs) only in the presence of oxygen [77, 78]. Under normal conditions (normaxia), where there is enough oxygen delivered, Hif1α is constantly synthesized, hydroxylated, ubiquitinated, and degraded. Under hypoxic conditions, however, HPHs fail to hydroxylate Pro564 of Hif1α owing to the lack of oxygen, which allows Hif1α to escape from VBC-CR-mediated ubiquitination and be stabilized. This explains why VHL-associated tumors often have constitutively high levels of Hif1α and is associated with the development of highly vascularized tumors.

The crystal structure of the VHL–ElonginC–ElonginB complex reveals that VHL has two structural domains, an N-terminal β-domain rich in β-sheet and a C-terminal α-domain adopting a three-helix cluster structure [67]. The α-domain, where the SOCS box motif is found, interacts with ElonginC, whereas the β-domain is not involved in any intermolecular contacts (Figure 7.2). The α- and β-domains are connected by two linkers and an extensive interface with a network of hydrogen bonds, indicating that the two are rigidly connected. Several residues mutated in VHL tumors are found at the interface, including the most frequently mutated one (Arg167). This suggests that the relative arrangement of the substrate- and ElonginC-binding domains is important for VHL function, a structural feature also observed in the c-Cbl structure [67].

Mapping of the tumor-derived VHL mutations on the VHL structure revealed two patches of solvent-exposed residues (Figure 7.6). One patch is located on the portion of the α-domain involved in ElonginC binding, confirming the role ElonginC binding plays in the tumor suppressor function of VHL. The second patch,

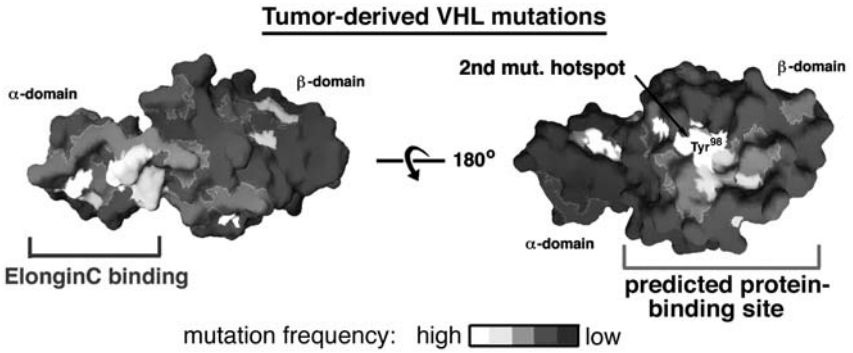

Fig. 7.6. Mis-sense mutations derived from VHL tumors map either to hydrophobic core-residues of the α- and β-domains, or to two clusters of surface residues. One of these clusters corresponds to the ElonginC-binding site, while the other maps to the β-domain, suggesting the presence of a protein-binding site.

which includes the second most frequently mutated VHL residue (Tyr98), is mapped onto the β-domain, strongly suggesting that this domain has a protein-binding site [67]. This prediction was confirmed by the crystal structure of the VBC complex bound to a hydroxyproline-containing Hif1α peptide reported later (Figure 7.2) [79]. The VBC–Hif1α structure showed that the Hif1α peptide binds the β-domain of VHL in an extended conformation. The hydroxyproline inserts into a gap in the VHL hydrophobic core, precisely at the protein-binding site predicted by tumor-derived mutations (Figure 7.6). The 4-hydroxyl group of the hydroxyproline is recognized by a pair of buried serine and histidine residues of VHL [79].

ElonginC adopts an α/β structure similar to the BTP/POZ fold [67]. ElonginB does not interact with VHL, and appears to have a structural role in stabilizing ElonginC. Based on the sequence homology between ElonginC and Skp1, it was expected that Skp1 would also have a similar BTP/POZ fold structure, except for a ~40-residue C-terminal Skp1 region extending beyond the homology region [67]. Since the functional relationship between the F-box and Skp1 is highly analogous to that between the SOCS-box and ElonginC, even before the structure of F-box has been determined it had been predicted that the F-box might have a similar structure to that of the SOCS-box. This was supported by threading analysis showing that among a library of 1925 folds, the fold most consistent with the F-box sequence was the three-helix cluster structure of the VHL α-domain [67]. The structural similarity between the SOCS box and F-box proteins became even more obvious when the Skp1–Skp2 structure became available.

7.6.2
Skp1–Skp2 Complex

One of the best-studied F-box proteins is Skp2, which contains an LRR substrate-binding domain. Skp2 is involved in regulating the G1–S transition in mammalian cells by controlling the levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ [80]. In quiescent cells, $p27^{Kip1}$ binds and inhibits Cdk2/cyclinA/E kinase complexes, whose activities are necessary for DNA replication in S phase. Upon stimulation by mitogenic signals, $p27^{Kip1}$ is phosphorylated and targeted to the SCF^{Skp2} for ubiquitination and subsequent degradation. Since $p27^{Kip1}$ functions as a tumor suppressor, Skp2 can be classified as an oncogene. In fact, Skp2 is over-expressed in many tumors and has transforming capacity [81]. Unlike canonical F-box proteins, however, Skp2 is essential but not sufficient for recruiting p27 to the ligase complex. It has been shown that an additional subunit, the 79-amino acid Cks1 protein, is also required for p27 ubiquitination by the SCF^{Skp2} E3 [61, 62]. Other studies have expanded the substrates of SCF^{Skp2} to include the pRb family member p130 and c-Myc [82–84]. The recognition and ubiquitination of p130 by SCF^{Skp2}, however, does not require Cks1.

The crystal structure of a nearly full length Skp1 bound to Skp2 without an N-terminal 100-residue region of unknown function has been determined. Confirming the prediction from the VHL structure, the structure of the complex showed

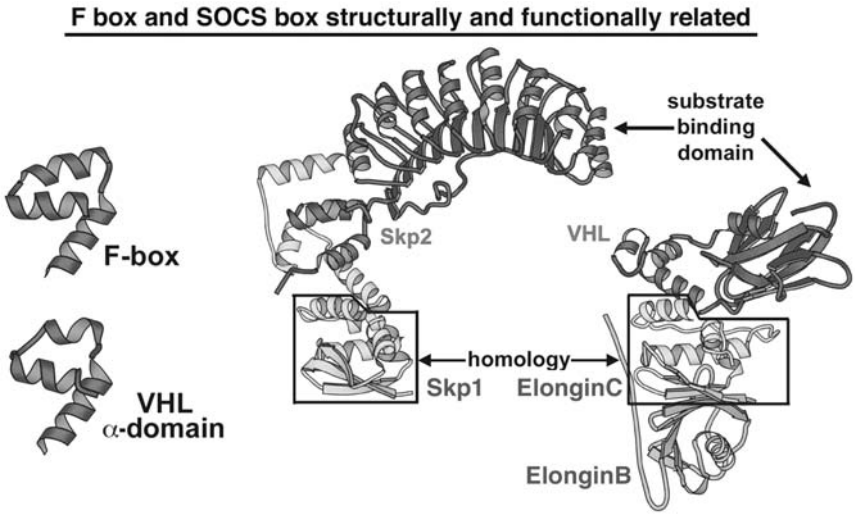

Fig. 7.7. Comparison of the Skp2–Skp1 and VHL–ElonginC–ElonginB complexes reveals the structural similarity between the F-box and SOCS-box (VHL α-domain) and similarity of the assembly of the intact Skp2–Skp1 and VHL–ElonginC–ElonginB complexes.

that the F-box has a three-helix cluster structure very similar to the SOCS box domain of VHL [60] (Figures 7.2 and 7.7). Different from the SOCS box of VHL, which interacts predominantly with the BTB/POZ fold of ElonginC, the F-box of Skp2 mainly interacts with the C-terminal extension of Skp1. This makes the relative orientation between the F-box and the Skp1 BTB/POZ fold different from that of the SOCS-box and the ElonginC BTB/POZ fold [60]. However, when the BTB/POZ folds of Skp1 and ElonginC are aligned, the substrate-binding LRR domain of Skp1 and the β-domain of VHL end up pointing in the same direction (Figure 7.7). This common structural arrangement is functionally relevant as the BTB/POZ fold is the cullin-binding domain of these adapter proteins, as discussed later.

Another striking similarity between the Skp1–Skp2 and ElonginC–VHL complexes is the rigid structural coupling between the adapter-binding motif and the substrate-binding domain in both cases. In the Skp1–Skp2 structure, the linker sequence connecting the F-box and LRRs adopts the structure of three non-canonical LRRs, which together with seven predicted LRRs form a single structural domain packing directly against the F-box motif. In addition, a portion of Skp1 also participates in the F-box–LRR interface (Figure 7.7). The functional importance of this rigid coupling has been further demonstrated by mutation studies showing that altering an Skp1 residue involved in this interface rendered a non-functional mutant in a yeast complement assay without abolishing its Skp2-binding activity [60].

The Skp1–Skp2 structure thus recapitulated the rigidity of the intermolecular

and inter-domain interfaces recurrently observed with the RING E3 structures. Together with mutational analyses in all cases including the c-Cbl and VHL, this common structural feature strongly suggests that the rigid structural architecture of these RING E3 components is functionally important. Therefore, the ubiquitin ligase activity of the RING E3s is best explained by the model that RING E3s catalyze ubiquitination by optimally positioning the substrate for ubiquitin transfer. These concepts were further confirmed by the structure of the SCF^{Skp2} complex.

7.6.3
SCF^{Skp2} Structure

A nearly complete picture of an SCF complex became available when two crystal structures were reported, one of the full length Cul1 bound to full length Rbx1, and the other of the quaternary complex consisting of Cul1, Rbx1, Skp1, and the F-box domain of Skp2 (hereafter referred to SCF^{Skp2}). SCF^{Skp2} has an overall highly elongated structure with Rbx1 and Skp1 segregated to opposite ends (Figure 7.2) [85]. This structural arrangement is organized by Cul1, which interacts with both Rbx1 and Skp1 and serves as the scaffold of the complex. Cul1 consists of an ~400-amino acid N-terminal helical region (hereafter NTD) that adopts a long stalk-like structure, and a ~350-amino acid C-terminal globular α/β domain (hereafter CTD). The two domains pack across an extensive interface that is invariant in the two different crystals [85].

The Cul1 NTD comprises three novel helical repeats, each is formed by five α-helices. The three repeats pack consecutively in a regular manner with extensive hydrophobic interfaces, overall adopting an arc-shaped structure with an ~110-Å span. There is little difference in the NTD in the two crystal structures. One side of the first repeat at the tip of the NTD stalk binds Skp1, interacting with its BTB/POZ fold. The Cul1 NTD residues that contact Skp1 are highly conserved in Cul1 orthologs but not in the cullin paralogs, Cul2 through Cul5, explaining the specificity of Skp1 to Cul1 but not the other cullins. Interestingly, when the sequences of the orthologs of individual cullins are compared, the residues that correspond to the Skp1-interacting residues of Cul1 are evolutionarily conserved and distinct among different cullins (Figure 7.8). This led to the prediction that each cullin would recruit a different protein-binding partner similar to the way that Cul1 binds Skp1. Since Cul2 is known to bind the ElonginC adapter, it is conceivable that the binding of Cul2 to the BTB/POZ domain of ElonginC should structurally mimic the Cul1–Skp1 binding (Figure 7.8). In agreement with the prediction, studies have shown that Cul3 interacts with a family of proteins containing both a BTB/POZ domain and a protein–protein interaction domain [55, 56]. Therefore, interaction between the BTB/POZ fold and the cullin NTD is likely to be a common mechanism involved in the recruitment of adapter/substrate-binding partners by most if not all cullins. (No BTB/POZ fold has been found in any of the protein subunits of the more recently found Cul4-containing E3 ligase complex [86].)

The major function of the Cul1 CTD is to recruit the RING-finger protein Rbx1. The CTD binds Rbx1 through two distinct interfaces (Figure 7.2). One involves an

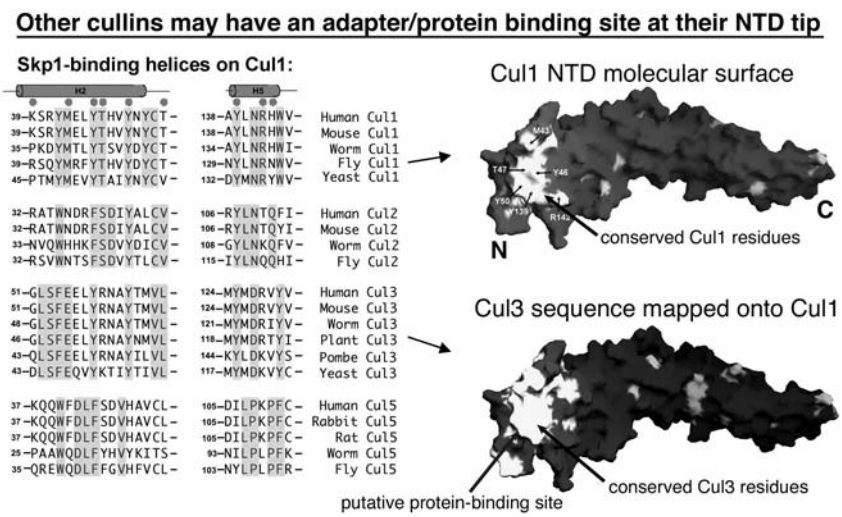

Fig. 7.8. Analysis of the Skp1-binding site of Cul1 and its comparison with other cullins indicate that other cullins all have a protein-binding site at their NTD tip. Left, sequence alignment of orthologs of individual cullins, showing ortholog-restricted conservation of residues that correspond to the Skp1-interacting residues of Cul1 (indicated by dots). Right, surface representation of the Cul1 NTD (top) and Cul3 NTD model (bottom) highlighting surface residues invariant in the respective orthologs.

intermolecular β-sheet, where a five-stranded β-sheet is formed by four β-strands from Cul1 and one central strand from Rbx1. Immediately following the Rbx1 β-strand, the Rbx1 RING domain is embedded in a wide Cul1 CTD cleft, where the second Cul1–Rbx1 interface is formed. The unusual intermolecular β-sheet between Cul1 and Rbx1 likely accounts for the extremely high affinity between the two proteins, as it involves both an extensive hydrogen bond network and hydrophobic packing [53]. The functional reason for this bipartite interacting mode between the two proteins remains to be clarified.

The Rbx1 RING domain is unique among the RING motifs as it contains three coordinated zinc ions instead of two. A 20-residue insertion sequence of Rbx1 provides the extra zinc-binding site. The major part of the Rbx1 RING, however, adopts a structure highly similar to the canonical RING motif, with a hydrophobic groove on its surface analogous to the c-Cbl E2-binding groove. Mutagenesis studies have confirmed that this groove is involved in E2 binding by Rbx1, whereas the insertion region is not essential for its E3 ligase activity [85].

In the cell, a sub-population of all cullins is covalently modified by the ubiquitin-like protein, Nedd8/Rub1, on a specific lysine residue at their C-terminal domain [87]. To date, cullins are the only known substrates of Nedd8 modification (neddylation). It is generally believed that neddylation regulates the cullin-based ubiquitin ligases, although the precise mechanism remains elusive. Nedd8 is essential for cell viability in fission yeast and for early development of *C. elegans*, *Arabidopsis*, and mice [88–91]. In *in vitro* systems, neddylation of Cul1 in the SCF complexes

has been shown to enhance their E3 activities toward natural substrates [92–94]. The crystal structure of the SCF complex reveals that the target lysine residue in cullins for neddylation is in close vicinity to the Rbx1 RING domain, suggesting that Nedd8 might be able to modulate the functions of Rbx1 and/or its associated E2 [85]. Other studies have identified a cellular protein, Cand1, which can bind the Cul1–Rbx1 complex and inhibits its association with Skp1 and F-box proteins [95, 96]. It has been shown that neddylation of Cul1 is able to release the inhibition and promote the assembly of the SCF. The structural basis of the Cand1–Nedd8-mediated assembly and disassembly of the SCF complex remains to be determined. Noticeably, the Cul1 lysine residue targeted for neddylation is surrounded by a number of highly conserved and surface-exposed cullin residues, which could potentially interact with Nedd8 or Cand1.

Consistent with the structural observations of c-Cbl, VHL, and Skp1–Skp2, the SCF^{Skp2} structure suggests that the whole SCF ubiquitin ligase is a rigid assembly. The functional importance of the rigidity of the SCF has been further tested with a Cul1 mutant, whose NTD and CTD were engineered to be linked by a flexible linker sequence (Figure 7.9) [85]. This mutant was made in two steps. First, the hydrophobic packing interface between the NTD and the CTD was disrupted by mutating two residues on the NTD and three on the CTD to polar amino acids. Second, the two domains were connected with 12- or 18-residue polar linkers. The

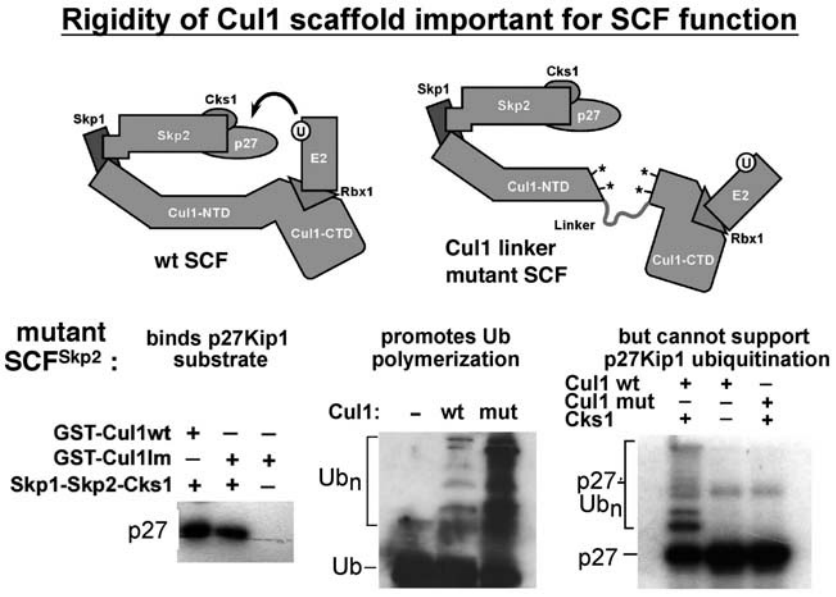

Fig. 7.9. The rigidity of the Cul1 scaffold is important for SCF E3 activity. Top, schematic diagram of the flexible linker Cul1 mutant. Bottom, the SCF containing the Cul1 linker mutant retains the ability to bind phosphorylated $p27^{Kip1}$ and to promote ubiquitin polymerization, but fails to ubiquitinate $p27^{Kip1}$ in an *in vitro* assay reconstituted with purified components.

mutations of the NTD and CTD made the two otherwise insoluble domains completely soluble when expressed separately in *Escherichia coli* (the CTD requires co-expression with Rbx1), indicating that they retain their structural integrity. In fact, the two slightly altered domains also remain functional as the flexibly linked Cul1 mutants are able to bind phosphorylated $p27^{Kip1}$ in an Skp1–Skp2–Cks1-dependent manner and also to promote the substrate-independent polymerization of ubiquitin. Therefore, the substrate- and E2-binding activities of the engineered Cul1 are intact (Figure 7.9). Strikingly, the flexible Cul1 mutants fail to ubiquitinate $p27^{Kip1}$ *in vitro*, indicating that the rigidity of the Cul1 scaffold is indeed crucial for its E3 activity (Figure 7.9) [85].

These findings, together with the structural and mutational analyses of the c-Cbl, VHL and Skp1–Skp2 complexes, supported the model that the RING E3s catalyze ubiquitination by optimally positioning the substrate for E2-mediated ubiquitin transfer. What is the extent of this positioning effect? It could in principle range from just raising the effective concentration of the lysine-containing substrate segment to the precise positioning of the ε-amino group of the lysine side chain at the E2 active site [85]. Answers to this question require a model of a RING E3 bound to both a substrate and an E2. While an SCF–E2 complex model can be constructed based on the c-Cbl–E2 structure and the similarity between Rbx1 and c-Cbl RING domains, structural and biochemical studies of Skp1–βTrCP1 bound to a substrate peptide helped provide structural insights into the substrate-E3 relationship.

7.6.4
Skp1–βTrCP1–β-Catenin Peptide and Skp1–Cdc4–CyclinE Peptide Complexes

βTrCP1 is a WD40-containing F-box protein conserved from *C. elegans* to humans. The two best-characterized substrates of the $SCF^{\beta TrCP1}$ complex are β-catenin and IκBα [97, 98]. These $SCF^{\beta TrCP1}$ substrates contain the DSGΦXS destruction motif (Φ representing a hydrophobic and X any amino acid), which is recognized by βTrCP1 when both serine residues in the motif are phosphorylated. In the Wnt signaling pathway, the destruction motif of β-catenin (Ser33 and Ser37) is constitutively phosphorylated by the GSK3β–APC-Axin complex in the absence of extracellular signals, resulting in $SCF^{\beta TrCP1}$-mediated polyubiquitination of β-catenin. The subsequent degradation of β-catenin maintains a relatively low homeostatic level of the protein. In response to the Wnt signals, phosphorylation of β-catenin is inhibited, leading to its stabilization, translocation to the nucleus, and transcriptional activation of proliferation-associated genes [99]. Loss of β-catenin ubiquitination, through mutations either in the destruction motif of β-catenin or in upstream components of the pathway, is among the most commonly observed alterations in colon cancer [100]. $SCF^{\beta TrCP1}$-mediated ubiquitination of IκBα functions in the opposite manner in the NF-κB signal transduction pathways. IκBα normally inhibits the NF-κB transcription activator by binding and sequestering it in the cytoplasm. Stimuli from extracellular signals or viral infection activate signaling pathways that lead to the phosphorylation of the two serines in IκBα and its ubiquitination.

Upon degradation of IκBα, NF-κB is released and translocates into the nucleus, where it will activate transcription [101].

Cdc4 is another conserved WD40-containing F-box protein, which plays an important role in regulation of cell cycle. In yeast, SCF^{Cdc4} is responsible for ubiquitinating the Cdk inhibitor Sic1 [102], whereas in higher eukaryotes, the same SCF complex ($SCF^{hCdc4/Fbw7/Ago}$) recognizes Cyclin E and promotes its degradation [63–65]. Mutations and alterations of *hCDC4* have been found in numerous cancer cell lines and have been associated with abnormally high levels of Cyclin E.

The crystal structures of both an Skp1–βTrCP1 complex bound to a 26-amino acid human β-catenin peptide that contains the doubly phosphorylated destruction motif and a yeast Skp1–Cdc4 complex bound to a phosphorylated Cyclin E model peptide have been determined [66, 103]. The Skp1–βTrCP1 complex has a relatively elongated structure, with Skp1 and the β-catenin peptide located at opposite ends (Figure 7.2). The seven WD40 repeats of βTrCP1 form a torus-like structure that is characteristic of this fold (commonly referred to as a β-propeller). As seen in other β-propeller structures, the βTrCP1 β-propeller has a narrow channel running through the middle of the torus-like structure, presenting the β-catenin peptide-binding site. Of the 26 β-catenin residues in the crystals, only an 11-residue segment (residues 30 to 40) centered on the doubly phosphorylated destruction motif (residues 32 to 37), is ordered in the structure. The phosphoserine, aspartic acid, and hydrophobic residues of the destruction motif are recognized directly by contacts from βTrCP1. The structure of the Skp1–Cdc4 complex resembles closely that of Skp1–βTrCP1, except that the β-propeller of Cdc4 contains eight instead of seven structural repeats [103]. The substrate peptide is also specifically recognized by the conserved residues lining up the channel in the middle of the Cdc4 propeller.

The rigid structural coupling of the F-box and substrate-binding domains was once again revealed in both structures. The βTrCP1 F-box is linked to the WD40 domain through an ~65-amino acid α-helical domain (linker domain), which interacts extensively with both the F-box and with one face of the WD40 β-propeller (Figure 7.2). A similar structured helical linker is also found in the structure of yeast Cdc4. It has been further shown that mutations in the linker helix of Cdc4 designed to affect the rigid coupling between the F-box and WD40 domains disrupted Cdc4 function *in vivo* [103].

7.6.5
Model of the SCF in Complex With E2 and Substrates

With the handful of known structures of several SCF sub-complexes and c-Cbl–E2, models of the SCF bound to E2 and substrate have been constructed (Figure 7.10). A model of the SCF^{Skp2}–E2 complex was built by superimposing Cul1–Rbx1–Skp1–F-box^{Skp2} on Skp1–Skp2 and docking the UbcH7 E2 onto the Rbx1 RING domain based on the way it binds c-Cbl. In addition, superimposing the Skp1–F-box portions of the Skp1–βTrCP1 and Skp1–Cdc4 complex with the SCF^{Skp2}–E2 model has made available the models of the $SCF^{\beta TrCP1}$–β-catenin–E2 and the

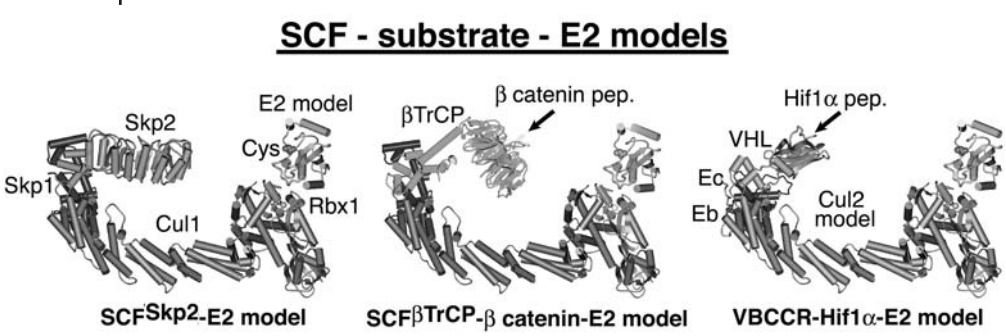

Fig. 7.10. Models of the SCF^{Skp2} complex (left), the $SCF^{\beta TrCP1}$–β-catenin peptide complex (middle), and the VBC–CR–Hif1α peptide complex (right) bound to E2. The E2 active site cysteine and the β-catenin and Hif1α peptides are labeled.

SCF^{Cdc4}–CyclinE–E2 complexes [66, 85, 103]. In all cases, no intermolecular collision was found in the final models. The substrate-binding domains of all three F-box proteins are positioned on the same side of the SCF complex as the E2. In addition, these domains are all oriented toward the E2 active site. Remarkably, the positions of the WD40 domain in the $SCF^{\beta TrCP1}$ and SCF^{Cdc4} models are strikingly similar to the position of the LRR domain in the SCF^{Skp2} structure. In the superimposition of all three SCFs, roughly half of the WD40 domain structure overlaps the LRR domain [103]. Finally, in the $SCF^{\beta TrCP1}$–β-catenin–E2 complex, the β-catenin peptide faces the E2, with both the N- and C-termini of the peptide pointing toward the E2 active-site cysteine (Figure 7.10). Based on the structural similarity between the recognition of F-box by Skp1 and SOCS-box by ElonginC, a model of the VBC–Hif1α peptide complex bound to a model of the Cul2–Rbx1 complex has also been constructed by superimposing the structurally conserved portions of Skp1 and ElonginC from the Cul1–Rbx1–Skp1–Skp2 and VHL–ElonginB–ElonginC–Hif1α peptide complexes (Figure 7.10) [79]. In this model, the position of the VHL β domain relative to the rest of the complex is again very similar to the βTrCP1 WD40 and Skp2 LRR domains. Even though the VHL β-domain does not extend as far towards the E2 as the latter two, the N-terminus of the Hif1α peptide is in clear sight of the E2 active site.

Although the substrate-binding domains are aligned with the E2 in these models, a large gap is always found in between. For example, the N- and C-terminal ends of the structured portions of the β-catenin peptide are $\sim$50 Å away from the E2 active-site cysteine (Figure 7.10), while the Hif1α peptide is $\sim$75 Å away from the E2 cysteine. It should be noticed that these peptide–E2 distances have considerable uncertainty as the E2 is modeled onto the Cul1–Rbx1 complex based on the c-Cbl–E2 structure and the similarity between the RING domains of Rbx1 and c-Cbl. In addition to the RING domain, c-Cbl also uses the linker helix to bind the E2. Yet a counterpart to this is not apparent in the SCF. Pivoting about the E2–Rbx1 interface of the model could affect the final position of the E2 active site,

which is ~20 Å away from the E2–Rbx1 contacting point. For example, a 45° tilt of the E2 can result in an estimated +/− ~20-Å error in the position of the E2 active site [66]. Further structural studies of the SCF complex bound to an E2 will be necessary to clarify this uncertainty.

Even with the uncertainty in E2 active-site position, the models have suggested that there would be no E3 residues near the E2 active site, in agreement with the observations made in the c-Cbl–E2 structure. This again ruled out the possibility that the SCF E3 provides acid/base catalysis and the possibility that the SCF positions the ε-amino group of the lysine at the E2 active site [66]. The only plausible mechanism left accounting for the catalysis mediated by the SCF in substrate ubiquitination is that the E3 complex helps increase the effective concentration of a portion of the substrate that contains the physiological ubiquitination-site lysine at the E2 active site. This model made the testable prediction that the distance between the destruction motif and the ubiquitinated lysine is a determinant of the ubiquitination efficiency.

7.6.6
Mechanism of RING E3-mediated Catalysis

The prediction of the effective concentration model has been tested in an *in vitro* system reconstituted with E1, E2 (UbcH5), and $SCF^{\beta TrCP1}$, all purified to >90% homogeneity [66]. To be able to measure the ubiquitination of the substrate lysine independently of the ubiquitination of lysine(s) on ubiquitin, a ubiquitin mutant that lacks lysines and thus does not form polyubiquitin chains was used. The natural substrates of the $SCF^{\beta TrCP1}$ E3, β-catenin and IκBα were chosen for the detailed analyses. To make the correlations between functional and structural observations straightforward, the biochemical studies have focused on β-catenin, the substrate co-crystallized with Skp1–βTrCP1.

Although the ubiquitination site(s) of β-catenin was previously unknown, two lysines of IκBα located ten and nine residues upstream of the destruction motif had been shown to be necessary and sufficient for ubiquitination and degradation [18, 19]. By analogy, a β-catenin lysine (Lys19) 13 residues upstream of the destruction motif was predicted to be the site where β-catenin is ubiquitinated. This was confirmed by the *in vitro* ubiquitination assay, in which a 26-amino acid β-catenin peptide that contains Lys19 and the doubly phosphorylated destruction motif was ubiquitinated in an $SCF^{\beta TrCP1}$-dependent manner to an overall level comparable to an IκBα substrate peptide (Figure 7.11). These isolated β-catenin and IκBα peptides should accurately reflect the context of these destruction motifs in their respective full-length proteins, since Lys19 and the destruction motif of β-catenin are both in a 133-residue N-terminal region that has been previously shown to have a disordered structure by proteolytic digestion analysis [104]. The destruction motif of IκBα similarly resides outside the structured ankyrin-repeat domain.

With the system established, a series of mutant β-catenin peptides where the spacing between Lys19 and the destruction motif was increased or decreased in four-residue steps (wt−8, wt−4, wt+4 and wt+8 peptides in Figure 7.11) was

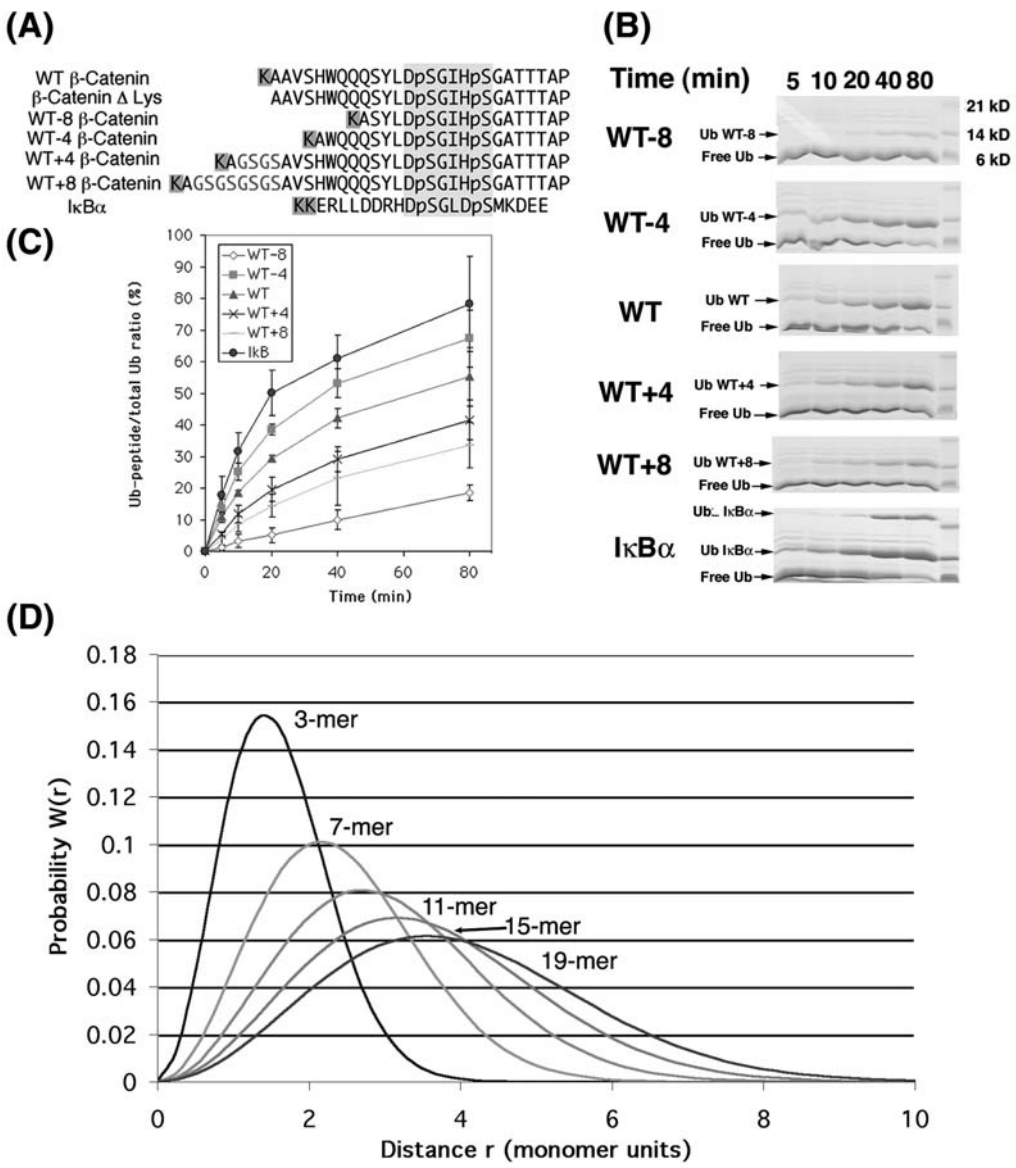

Fig. 7.11. The rate of ubiquitination by the $SCF^{\beta TrCP1}$ is dependent on the lysine-destruction motif spacing. (A) Sequences of the wild type and mutant β-catenin and IκBα peptides, with the destruction motif and ubiquitinated lysine(s) highlighted. (B) Time courses of ubiquitination of the wild-type and mutant peptides, visualized by Coomassie staining. (C) The reaction yields plotted with error bars from four experiments. (D) The radial distribution functions (W(r)) calculated for random unperturbed polymers of length 3, 7, 11, 15, and 19 monomer units [66]. These correspond to the number of residues between the lysine and the first destruction motif residue ordered in the structure, for the wt−8, wt−4, wild type, wt+4 and wt+8 peptides. The distance plotted on the x-axis is expressed in dimensionless units as a multiple of the monomer unit length (r/l).

tested for *in vitro* ubiquitination. The deleted residues are unlikely to be involved in βTrCP1 binding as they are present in the crystallized β-catenin peptide but are disordered in the structure. This is confirmed by experiments showing that the mutant peptides have affinities for βTrCP1 undistinguishable from that of the wild-type peptide [66].

Strikingly, changing the Lys19-destruction motif spacing by four to eight residues had two- to three-fold effects on the rate of ubiquitination. Increasing the spacing reduced ubiquitination, with the wt+8 peptide being ubiquitinated at an ~three-fold lower rate. Surprisingly, reducing the spacing by four residues increased the ubiquitination rate slightly but consistently, although reducing it further by eight residues (wt−8) resulted in a very poor substrate with only trace amounts of product at the longest reaction times. These findings indicated that $SCF^{\beta TrCP1}$ substrates have an optimal destruction-motif lysine spacing of 9 to 13 residues [66]. Interestingly, the 9-residue lysine-destruction-motif spacing of the wt−4 mutant is comparable to the 9 and 10-residue spacing found in IκBα (for Lys20 and Lys19, respectively). IκBα is ubiquitinated at a rate closer to the wt−4 peptide than the wt β-catenin peptide (compare early time points in Figure 7.11, when the majority of the IκBα peptide is mono-ubiquitinated), suggesting that the optimal lysine-destruction-motif spacing is closer to 9 than to 13 residues. If the spacing is an important determinant, it is expected to be conserved in the β-catenin and IκBα orthologs and paralogs. Indeed, all of these proteins contain a lysine located 9 to 14 residues upstream of the destruction motif (Table 1).

The apparent effect of the spacing between the lysine residue and the destruction motif on the ubiquitination rates is intuitively consistent with the model that the SCF catalyzes ubiquitination by increasing the effective concentration of specific lysine(s) at the E2 active site [85]. According to this model, a specific SCF will catalyze ubiquitination maximally when the distance between its substrate-binding site and the E2 active site, a parameter likely unique to a particular F-box protein, closely matches the spatial distance between the substrate's SCF-binding motif and its lysine residue(s). Unstructured polypeptides do not have a single, well-defined length in solution, but rather they sample a distribution of lengths, each associated with a probability. Assuming that the reaction rate is proportional

Tab. 7.1. Closest lysine upstream of destruction motif.

Substrate	*Residues*
IκBα (human)	9, 10
IκBβ (human)	9
IκBε (human)	11
Cactus (fly IκB)	10, 12
β-catenin (human)	13
Armadillo (fly β-catenin)	14
Bar-1 (worm β-catenin)	10
Plakoglobin (human)	11

to the probability that the lysine side chain will hit the E2 active site, the effective concentration model predicts that the relative ubiquitination rates of our wild-type and mutant peptides would be directly proportional to the relative probabilities that the lysine-destruction-motif distances match the optimal distance.

Except for very short polypeptides, it is not yet possible to calculate *ab initio* the length distribution for a given polypeptide sequence, especially as the length distribution also depends on the nature of the amino acids and the distribution of charged and hydrophobic groups. However, approaches based on random polymer theory with empirical corrections have been shown to agree qualitatively with experimental data [105]. Therefore, polymer theory can be used to analyze and compare the length distribution and its associated probability of a polypeptide. Figure 7.11 shows the length distributions for the polypeptide segments between the lysines and the destruction motifs of the wild-type and mutant β-catenin peptides. Assuming that the optimal distance corresponds to that of the wt−4 peptide, which had the highest ubiquitination rate in the *in vitro* ubiquitination assay, the probability that the wt+8 peptide will sample this distance is 2.5-fold lower. This is comparable to the 3-fold lower ubiquitination rate of the wt+8 peptide relative to the wt−4 peptide. These experimental results are thus consistent with the predictions of the effective concentration model. Overall, these structural and biochemical studies have provided a solid body of evidence supporting the model that the SCF E3, and likely other RING E3s, catalyze ubiquitination by increasing the effective concentration of specific substrate lysine(s) at the E2 active site [66].

7.7 The Mms2–Ubc13 Complex

The topology of the polyubiquitin chains determines the nature of the signals they encode. Whereas K48-linked polyubiquitin chains signal for proteasome-dependent degradation, K63-linked chains serves as non-proteolytic signals in cellular processes such as DNA repair and IKK activation. A family of ***u***biquitin ***E***2 ***v***ariant proteins (UEVs) has been identified as mediating the assembly of K63 chains. These E2 variants share sequence homology with the canonical E2s, yet they all lack the active-site cysteine [26]. Yeast *MMS2* gene product, Mms2, is a UEV protein, which functions in the *RAD6/RAD18* post-replication DNA-damage repair pathway [27]. Mms2 performs its biological roles together with a specific E2, yeast Ubc13. It has been shown that Mms2 and Ubc13 are able to form a stable complex and catalyze the assembly of K63 polyubiquitin chains in the presence of E1 only *in vitro*. This suggests that the function of Mms2 might be equivalent to that of an E3, except that the substrate is ubiquitin itself.

The crystal structure of the Mms2–Ubc13 complex has been determined [106]. It shows that the two proteins both adopt the canonical α/β E2 fold and together form an asymmetric heterodimer (Figure 7.12). In the crystal, one end of Mms2 packs against one side of Ubc13, resulting in an overall T-shaped dimer structure. At the junction of the two proteins, a hydrophobic channel is formed, leading toward

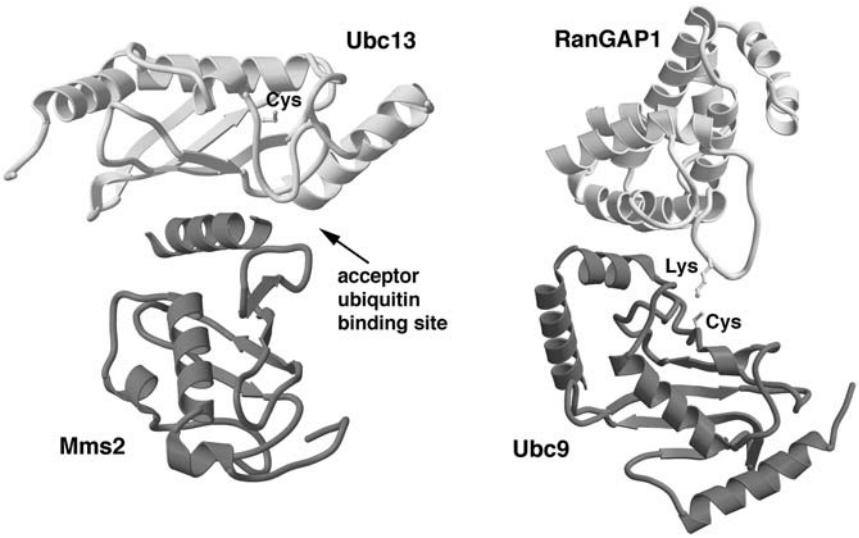

Fig. 7.12. Crystal structures of the Mms2/Ubc13 (left) and the RanGAP1–Ubc9 (right) complexes. The proposed binding site for the acceptor ubiquitin in the Mms2/Ubc13 structure is indicated by arrow. The side chains of the substrate lysine and the Ubc9 active cysteine in the RanGAP1–Ubc9 structure are shown.

the Ubc13 active-site cysteine. Mutations of the surface residues of this channel impaired K63 ubiquitin-chain assembly without disrupting the heterodimerization of the two proteins and thioester formation on Ubc13 [106]. Together, these results suggest that the acceptor ubiquitin likely interacts with this channel during Mms2–Ubc13-mediated K63 chain assembly. In conjunction with previous NMR studies of the ubiquitin–E2 interaction [107], another surface cleft was identified on the other side of Ubc13 as the donor ubiquitin binding site [106].

As repeatedly seen in all the RING E3 ligase structures, there is no residue from Mms2 close enough to the Ubc13 active-site cysteine to mediate acid/base catalysis [106]. Neither does Mms2-binding lead to any significant conformational changes that might activate the Ubc13 E2. Instead, the structure of the Mms2–Ubc13 complex strongly suggests that Mms2 promotes K63 ubiquitin-chain assembly by binding to the Ubc13 E2, forming a ubiquitin-binding site, and positioning the acceptor ubiquitin in an orientation to present its Lys63 residue toward the E2 active site, a mechanism similar to the one derived above for the RING E3s.

7.8
The RanGAP1–Ubc9 Complex

Among the several ubiquitin-like modifiers, SUMO is highly conserved from yeast to human and has been shown to regulate a variety of cellular functions such as nucleocytoplasmic transport, signal transduction, and transcriptional control

[108]. Protein modification by SUMO (sumoylation) requires a similar E1–E2–E3 system as for ubiquitination, although the SUMO E2 Ubc9 can specifically conjugate SUMO to most substrates in the absence of an E3. This is possible partly because the target lysine site in most SUMO-modified proteins is within a consensus sequence motif ΨKxD/E, which is directly recognized by Ubc9. A report of the crystal structure of a RanGAP1–Ubc9 complex marked the first structure of an E2-substrate complex in the ubiquitin and ubiquitin-like conjugation systems [109]. RanGAP1 is the best documented substrate for sumoylation. The structure of RanGAP1–Ubc9 revealed that Ubc9 binds the substrate by both interacting with the consensus sumoylation motif and keeping extensive contacts with two nearby RanGAP1 helices (Figure 7.12). The sumoylation motif of RanGAP1, LKSE, adopts an extended conformation, interacting closely with a rather flat Ubc9 surface with the lysine residue positioned immediately next to the E2 cysteine. Importantly, no residues in the E2 or the substrate surrounding the E2 cysteine and the substrate lysine appear to play a role in deprotonating the lysine, indicating that the SUMO transfer reaction does not involve acid/base catalysis [109]. This was further confirmed by structure-based mutagenesis studies. In agreement with the model described above for the mechanism by which RING E3s mediates ubiquitin transfer, the SUMO E2-substrate structure has implications that SUMO conjugation on the substrate is also catalyzed by proper orientation of the lysine residue toward the E2 active-site cysteine.

7.9 Summary and Perspective

Ubiquitin–protein ligases play a central role in conferring the specificity of protein ubiquitination and promoting ubiquitin transfer from E2 to the substrates. Structural studies of a series of prototypical ubiquitin–protein ligases have significantly advanced our understanding of how these enzymes carry out their biological functions. For the HECT E3s, whose E3 activities involve the formation of a thioester intermediate, structural analyses have revealed a large domain movement within the catalytic domain. For the much larger RING class E3 family, all structural studies support the model that this family of ubiquitin ligases promote protein ubiquitination by optimally orienting and positioning the substrate relative to the E2 active site to raise the effective concentration of the specific lysine(s) for ubiquitin attachment. Further studies will be needed to address the questions of how the extension of the ubiquitin chain is mediated by the E3s and how the E3s are regulated by cellular factors.

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8
The Deubiquitinating Enzymes

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8.1
Introduction

In the mid-1970s ubiquitin was found to be a covalent modifier of proteins [1]. At the time, it was quite surprising to find a protein that covalently modified another protein. Since then, the reversible covalent modification of proteins by other proteins is known to be commonplace and ubiquitin is used to covalently modify hundreds of proteins, often for the purpose of targeting them to the proteasome for degradation.

Protein degradation through the ubiquitin–proteasome system is facilitated by covalently linking ubiquitin to the ε-amino group of a lysine of a substrate protein through its C-terminal glycine [2]. A polyubiquitin (polyUb) chain is formed by linking subsequent ubiquitins to the lysine 48 residue of the preceding ubiquitin in the chain. A chain of four ubiquitins is sufficient for the targeted protein to be recognized and degraded by the proteasome [3]. Conjugation of ubiquitin to other proteins is catalyzed by a three-enzyme cascade [4]. Conjugation begins by activation of ubiquitin by an E1, or Ub-activating enzyme, forming a high-energy thiol ester bond in an ATP-dependent reaction. The ubiquitin is transferred to an E2, or Ub-conjugating enzyme, which then usually pairs with an E3, or Ub-ligase enzyme, to conjugate the ubiquitin to a specific target protein.

The usefulness of ubiquitin conjugation is not limited to the ubiquitin–proteasome pathway. Mono- and polyubiquitin are used as signals in various pathways including endocytosis, DNA repair, apoptosis, and transcriptional regulation [5–8]. Polyubiquitin chains can be formed using lysine residues other than K48, the linkage required for proteasomal degradation [9–11]. In addition, there are a number of other ubiquitin-like proteins that also behave as signaling molecules although they are not involved directly in proteasomal degradation. This group includes SUMO (***s***mall ***u***biquitin-related ***mo***difier), Nedd8 (***n***eural precursor cell ***e***xpressed, ***d***evelopmentally ***d***own-regulated ***8***), ISG15 (***i***nterferon-***s***timulated ***g***ene ***15***), and others [12–14]. These proteins are conjugated to substrates in a similar manner to ubiquitin, using an E1, E2, and E3 cascade of enzymes specific for the particular ubiquitin-like protein involved [15–17].

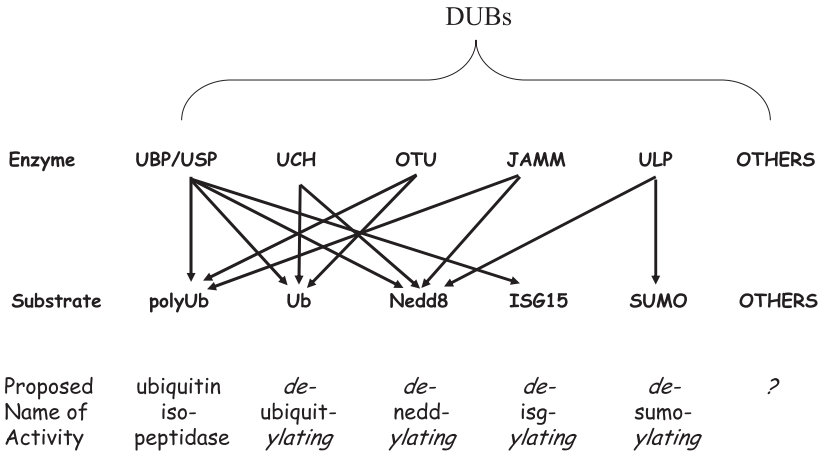

Fig. 8.1. DUB families and substrate specificity. DUBs can be classified by genetic relationships (family) or by substrate specificities (activity). Arrows point towards the substrates that members of each family can process in a physiologically relevant way. Each family is capable of processing multiple substrates and each activity can be catalyzed by members of more than one family.

Soon after it was shown that ubiquitin is conjugated to proteins, it was determined that this was a reversible process and deubiquitinating enzymes, or DUBs, could remove ubiquitin from ubiquitinated proteins [18, 19]. As the genes for ubiquitin and ubiquitin-like proteins were identified it became clear that all ubiquitin family members were synthesized as proproteins and processed to reveal the C-terminal glycylglycine of the active proteins [20]. Based on this information, DUBs were defined as proteases that cleave at the C-terminus of ubiquitin or ubiquitin-like proteins to reverse conjugation to target proteins and also process the proproteins.

Over 100 DUBs have been identified (see Table 8.1 for a list of the DUBs whose roles are known or suspected) and they are used to regulate ubiquitin and ubiquitin-like protein metabolism. Since cells utilize a combination of mono-ubiquitin, polyubiquitin, and ubiquitin-like proteins for a multitude of reasons and conjugate them to thousands of proteins, a regulatory system has evolved that is exceedingly complex and must be exquisitely regulated (see Figure 8.1). DUBs help regulate this system by processing proubiquitin into a mature form, recycling free polyubiquitin chains into monomeric Ub, assisting the degradation of proteasomal substrates, and regulating the ubiquitination levels of proteins in cellular pathways other than proteolysis. Thus, DUBs play crucial roles in determining the cellular fates of many proteins and regulating cellular function.

The study of DUBs has moved at a rapid rate since their initial discovery in the early 1980s. Yet despite all the progress, the total number of DUBs and the substrate specificity of most DUBs are still undetermined. The discovery of novel DUB families including the JAMM isopeptidases and OTU DUBs has highlighted that there may be still more unidentified DUBs. Because of the large number of

Tab. 8.1. Physiological roles of DUBs revealed by deletion or knockdown experiments.

DUB	*Organism*	*Deletion/knockdown phenotype*	*Functional role*
Ubiquitin C-terminal hydrolases (UCHs)			
UCH-L1	mouse	gracile axonal dystrophy	predominant neuronal UCH [112]
UCH-L3	mouse	no detectable phenotype	undetermined neuronal function [113]
UCH37	human	unknown	edits polyubiquitin chains at proteasome [91]
BAP1	human	unknown	tumor suppressor? [22]
YUH1	*S. cerevisiae*	cannot process proRUB1	processes proRUB1, Ub-adducts [83]
Ubiquitin specific processing proteases (UBPs)			
UBP1	*S. cerevisiae*	no phenotype detected	undetermined [97]
UBP2	*S. cerevisiae*	no phenotype detected	undetermined [96]
UBP3/ USP10	*S. cerevisiae*, human	polyubiquitin accumulation	transcriptional silencing inhibitor, regulates membrane transport [66, 118]
UBP6	*S. cerevisiae*	low levels of free ubiquitin	processes polyubiquitin chains at proteasome [94]
UBP8	*S. cerevisiae*	increase in ubiquitinated histone H2B	transcriptional regulator [88]
UBP14/IsoT	*S. cerevisiae*, human	increased polyubiquitin levels, proteasome defects	recycles free polyubiquitin to mono-ubiquitin [33, 86]
UBP16	*S. cerevisiae*	no phenotype detected	undetermined function at mitochondria [70]
DOA4	*S. cerevisiae*	Ub-depletion, defective Ub recycling	recycles Ub and polyubiquitin adducts [71, 84]
Unp/USP4	mouse, human	unknown	undetermined
USP7	human	indirect p53 activation	regulates p53 ubiquitination [36]
UBPy/USP8	human	increase in protein ubiquitination	cell-growth regulation [119]
USP14	mouse (UBP6 homolog)	ataxia	regulating synaptic activity plus proteasome [109]
USP21	human	unknown	process Ub and Nedd8 conjugates, growth regulator? [32]
USP25	human	unknown	over-expression has possible role in Down's Syndrome [120]
UBP41	human	unknown	promotes apoptosis [6]
UBP43	mouse	accumulation of Isg15 conjugates	processes Isg15, regulates Isg15 conjugate levels [31]
CYLD	human	cylindromatosis	regulates K63 polyubiquitination of substrates in NF-κB pathway [7, 103, 104]
fat facets	*Drosophila*	defective germ cell specification, eye formation	regulates specific developmental processes [74, 75]
DUB1, 2, 2A	mouse	unknown	cytokine specific growth regulators

Tab. 8.1. *(continued)*

DUB	*Organism*	*Deletion/knockdown phenotype*	*Functional role*
VDU1	human	unknown	regulation of Ub-proteasome pathway? [64]
JAMM Isopeptidases			
Rpn11	Yeast, human	lethal	processes polyubiquitin chains at proteasome [46, 47]
Csn5	Yeast, *Drosophila*	defects in SCF E3s in yeast, lethal in *Drosophila*	regulation of cullin neddylation levels [45]
OTU DUBs			
cezanne	human	unknown	negative regulation of NF-κB pathway [43]
A20	mouse	severe inflammation, premature death	negative regulation of NF-κB pathway [44, 121]
otubain1	human	unknown	editing DUB? [42, 122]
otubain2	human	unknown	undetermined [42]
VCIP 135	human	unknown	membrane fusion after mitosis [72]
Ubiquitin-like proteases (ULPs)			
Ulp1	*S. cerevisiae*	lethal	regulates cell cycle progression [39]
Ulp2	*S. cerevisiae*	increased SUMO conjugates, DNA repair defective	desumoylating enzyme [123]
Den1/SENP8	mouse	unknown	deneddylates cullins [26, 37]
SENP6	human	unknown	involved in reproductive function? [124]
Others			
Apg4B	mouse	unknown	processes autophagy-related UbLs [116]
ataxin-3	human	unknown	processes polyubiquitin chains? [125]

The organism listed for each DUB refers either to where it was discovered or where the work characterizing the deletion strain and function was performed. DUBs with multiple organism identifiers are either highly similar in sequence in each organism or functional homologs. An unknown deletion phenotype indicates that a deletion, knockout, or knockdown of a particular DUB has yet to be generated. No detectable phenotype indicates that a deletion strain has been made, but no phenotypes were observed.

potential DUB substrates and the exquisite specificity that some individual DUBs exhibit (see Figure 8.1) study of these enzymes has often been challenging. A burgeoning amount of structural data and recent technical advances are being used to address this challenge. The goal of this chapter is to highlight recent developments in the DUB field by giving an overview of DUB families, including DUBs that act on ubiquitin-like proteins, to discuss how DUBs achieve their specificity, and to show how the physiological roles of DUBs and their substrates are being elucidated.

8.2
Structure and *In Vitro* Specificity of DUB Families

8.2.1
Ubiquitin C-terminal Hydrolases (UCH)

The first class of DUBs discovered, the ***u***biquitin ***C***-terminal ***h***ydrolases (UCHs), is a relatively small class with only four members in humans and one in budding yeast. UCHs are cysteine proteases related to the papain family of cysteine proteases. Most UCHs consist entirely of a catalytic core that has a molecular mass of about 25 kDa, although Bap1 and UCH37 have C-terminal extensions [21, 22]. All UCHs have a highly conserved catalytic triad consisting of the active-site cysteine, histidine, and aspartate residues that are absolutely required for function [23].

In vitro studies have determined that UCHs have significant activity in removing small adducts from the C-terminus of ubiquitin, including short peptides, ethyl ester groups, and amides [24]. They are also very efficient at cotranslationally processing the primary gene products (proubiquitin or Ub-ribosomal subunit fusions) to expose the C-terminal gly–gly motif required for conjugation of ubiquitin and ubiquitin-like proteins to substrates. However, UCHs are unable to cleave the isopeptide bond between ubiquitins in a polyubiquitin chain or to act on ubiquitin conjugated to a folded protein. They are similarly inefficient in acting upon small peptide substrates based on the sequence of the ubiquitin C-terminus [25]. As described below, the binding of ubiquitin is required for optimal UCH activity. Nedd8, a closely related ubiquitin-like protein, is also a substrate for human UCH-L3, albeit with three orders of magnitude less efficiency than ubiquitin [26].

The crystal structures of human and yeast UCHs have been solved, the latter in complex with the inhibitor ubiquitin aldehyde [27, 28]. The UCH fold is closely related to that of the papain family of cysteine proteases. Ubiquitin is bound in a cleft on a surface that is highly conserved in all UCHs. NMR studies on the binding of ubiquitin to human UCH-L3 show a similar mode of interaction and define three regions on the surface of ubiquitin involved in this binding [29]. As noted below and in Figure 8.2, the same surface of the ubiquitin fold is also involved in binding to USP7 and ULP1. This is remarkable as these DUBs are from different families and not significantly homologous in sequence or structure.

A second feature of UCHs is the presence of a ''blocking loop'' spanning the active site and limiting the size of substrates that can be accommodated. Yuh1 and other UCH DUBs contain a mobile, ~20-residue loop that is disordered in the unliganded protein, but becomes ordered upon substrate binding. The loop passes directly over the active site and the leaving group attached to the gly–gly at the ubiquitin C-terminus has to pass directly through this loop in order to access the catalytic cysteine. The maximum diameter of this loop was calculated to be ~15 Å, too small for any folded substrate save a single helix [27]. The loop thus allows small substrates to be efficiently cleaved, but excludes larger Ub–protein conjugates. This loop explains, at least in part, the preference of UCHs for small or disordered leaving groups.

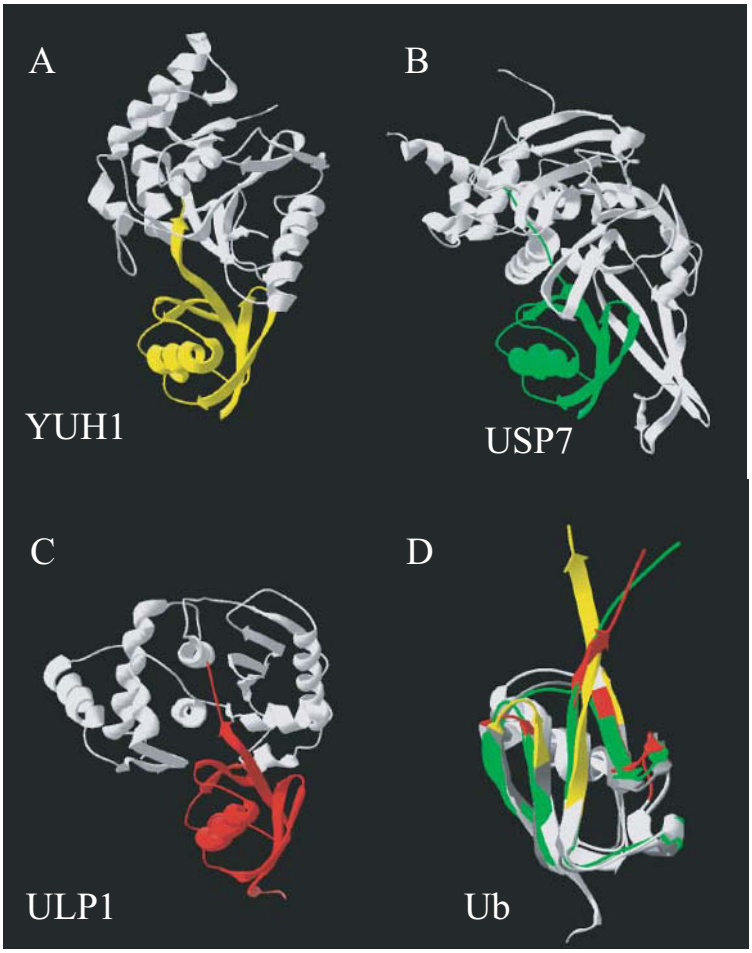

Fig. 8.2. Substrate binding by DUBs revealed by X-ray crystal structures. In the ribbon diagrams, the DUB is represented in white and the substrate in color. The ubiquitin (yellow or green) or SUMO (red) substrates are shown in the same orientation to highlight the similarity of substrate binding by different DUB classes. (A) Ubiquitin (yellow) bound to YUH1. (B) Ubiquitin (green) bound to USP7. (C) SUMO (red) bound to ULP1. (D) Superimposition of substrates from A–C. The regions of each substrate that are within 3.5 Å of the DUB when bound are highlighted in color to demonstrate the conserved regions that are recognized by the different DUB classes.

8.2.2
Ubiquitin-specific Processing Proteases (UBP/USP)

The ubiquitin specific processing proteases (referred to as UBPs in yeast and USPs in human and mouse) were the second class of DUBs discovered. Catalytically, the UBPs are very similar to the UCHs in that they also utilize the catalytic triad of an active-site cysteine and a conserved histidine and aspartate. The UBP catalytic core

of about 400 amino acids contains blocks of conserved sequences (Cys and His boxes) around these catalytic residues [23]. The UBPs are generally larger and more variable in size than the UCH class, ranging from 50 to 300 kDa. N-terminal extensions to the catalytic core account for most of the increased size although a few UBPs have C-terminal extensions. These N-terminal extensions are highly divergent in sequence, unlike the conserved regions of the catalytic core. The sequence and size variations of these extensions are thought to aid in determining UBP localization and substrate specificity.

There are 16 UBPs in yeast and more than 50 USPs identified in humans, making the UBP/USP family much larger than the UCH family [30]. UBPs also process a wider variety of substrates than UCH DUBs, including proubiquitin, free polyubiquitin chains of various linkages, and mono-or polyubiquitin conjugated to target proteins *in vitro* and *in vivo*. In addition, some family members can act on ubiquitin-like proteins. UBP43 has been demonstrated to act on ISG15 while USP21 cleaves conjugated Nedd8 [31, 32].

The diversity of the UBPs and breadth of substrates they act upon, makes them useful in a wide variety of cellular pathways and locations. UBPs regulate apoptosis, DNA repair, endocytosis, and transcription in addition to the ubiquitin–proteasome pathway (see below). The same diversity presents a challenge in determining the specificity of UBPs and with the exception of Isopeptidase T (UBP14/USP5), there have been few quantitative studies of *in vitro* specificity [33, 34]. In general, specificity has been described with qualitative "yes or no" assays that are not particularly useful in suggesting *in vivo* roles.

The structure of one UBP catalytic domain has been solved, that of USP7 complexed to ubiquitin aldehyde [35]. The data may be applicable to the way in which other UBPs function because the catalytic core of many UBPs is highly conserved. USP7 (also called HAUSP) is a human ubiquitin-specific protease that regulates the turnover of p53 [36]. USP7 consists of four structural domains; an N-terminal domain known to bind p53 and EBNA1, a catalytic domain, and two C-terminal domains. The 40-kDa catalytic domain exhibits a three-part architecture comprising Fingers, Palm, and Thumb (see Figure 8.2). The leaving ubiquitin moiety is specifically coordinated by the Fingers, with its C-terminus placed in a deep cleft between the Palm and Thumb where the catalytic residues are located. The domains form a pocket ideal for binding ubiquitin. Residues in the structure important for the above functions are conserved amongst UBPs, indicating that many UBPs may utilize the Fingers, Palm, and Thumb architecture to bind and cleave ubiquitinated substrates.

Another interesting structural observation is that water molecules cushion ubiquitin in the binding pocket. This is necessary because the binding surfaces of ubiquitin are uncharged, and the USP7 binding pocket is made up of predominantly acidic amino acid residues. These water molecules form extensive networks of hydrogen bonds with the bound ubiquitin and USP7. It is possible that they contribute to USP7's substrate specificity by allowing the protein to provide for relatively weak binding of ubiquitin and forcing itself to interact with the target protein to achieve specificity. This seems to be borne out by the fact that ubiquitin does not form a tightly bound complex with USP7 [35].

8.2.3
Ubiquitin-like Specific Proteases (ULP)

The ***u***biquitin-***l***ike specific ***p***roteases (ULPs) are a third class of DUB first thought to act only on SUMO-related ubiquitin-like proteins. There are two yeast ULPs and seven human ULPs (also called sentrin specific proteases, or SENPs). Further analysis determined that ULPs have little or no activity on ubiquitin substrates, but one (SENP8) acts on Nedd8 [26, 37, 38]. Despite acting on non-ubiquitin substrates, ULPs are still classified as DUBs because the function and mechanism of catalysis is so similar to those of the DUBs that act on ubiquitin. ULPs lack significant sequence homology to other DUBs and are more closely related to viral protein-processing proteases [39].

In addition to the lack of sequence homology, ULPs have little structural homology to other DUB classes except in the active site. The structure of ULP1 (see Figure 8.2) in complex with the C-terminal aldehyde of yeast SUMO (SMT3) illustrates that, like most other DUBs, ULPs are thiol proteases, utilizing a conserved catalytic triad consisting of an active-site cysteine, histidine, and aspartate [40]. Also, they require a gly–gly motif at the C-terminus of their UbL substrate for tight binding. The SUMO binding pocket of ULP1 recognizes SUMO through a number of polar and charged-residue interactions, including multiple salt bridges that are not present in the USP7 ubiquitin-binding site, and does not utilize water molecules or a ''blocking loop''.

8.2.4
OTU DUBs

A class of DUBs only identified since 2002 is the OTU (***o***varian ***tu***mor protein) DUB class. The OTU domain was originally identified in an ovarian tumor protein from *Drosophila melanogaster*, and over 100 proteins from organisms ranging from bacteria to humans are annotated as having an OTU domain. The members of this protein superfamily were annotated as cysteine proteases, but no specific function had been demonstrated for any of these proteins. The first hint of a role for OTU proteins in the ubiquitin pathway was afforded by the observation that an OTU-domain-containing protein, HSPC263, reacted with ubiquitin vinyl sulfone (an active-site-directed irreversible inhibitor of DUBs) [41].

Then two groups almost simultaneously discovered that several OTU-containing proteins have DUB activity. Two human OTU DUBs were identified by purification with Ub-aldehyde (a reversible DUB inhibitor) affinity resin [42]. These proteins, named otubain1 and 2 (OTU-domain Ub-aldehyde binding protein) have a mass of approximately 35 kDa and are able to cleave polyubiquitin chains *in vitro*. However, the cleavage mechanism and their true substrates *in vivo* have yet to be determined. The other OTU DUB found was Cezanne, a 100-kDa protein that is similar to the A20 negative regulator of NF-κB [43]. Like A20, Cezanne plays a role in regulating NF-κB signaling pathways and has general DUB activity [44]. These OTU DUBs have highly conserved catalytic cysteine and histidine residues, implying that they utilize a catalytic triad to catalyze cleavage of polyubiquitin. It is unclear

if most proteins containing OTU domains are DUBs, as analysis of the OTU family for DUB activity is only just beginning.

8.2.5 JAMM Isopeptidases

JAMM isopeptidases also constitute a recently identified class of DUBs. The members of this interesting class of DUBs were the first non-cysteine protease DUBs identified. Two JAMM isopeptidases have been confirmed as DUBs: Rpn11, which acts on ubiquitin conjugates, and Csn5, which acts on Nedd8 conjugates [45–47]. A number of other eukaryotic proteins have been annotated as containing the JAMM motif, but whether they have DUB activity has yet to be determined. Instead of cysteine proteases, they are metalloproteases belonging to a family of proteins that contain the Jab1/Csn5 and MPN domains [48]. Their activity depends on the JAMM motif (EX_nHS/THX_7SXXD) in the JAMM domain. The two histidines and an aspartic acid act as ligands to bind a metal ion, presumably zinc although this has not been proven, to achieve catalysis through polarization of a bound water molecule. A glutamic acid serves as a general acid–base catalyst. The crystal structure of a JAMM metalloprotease from *Archaeoglobolus fulgidus* bacteria has been recently been solved, but no structures of a JAMM isopeptidase with DUB activity are yet available [49, 50].

8.3 DUB Specificity

Why are there so many DUBs and how do they achieve specificity? The numerous DUBs identified to date suggest that DUBs have specifically evolved to act on distinct cellular substrates rather than to have general deubiquitinating activity (see Figure 8.1). We can ask what common features of these enzymes define them as DUBs and what differences allow specific DUBs to act on mono- *vs.* polyubiquitin? How have they evolved to cleave only ISG15 or SUMO-modified substrates, for instance? A body of data has been accumulated that at least partially answers these questions.

8.3.1 Recognition of the Ub-like Domain

All DUBs appear to recognize the body of the ubiquitin fold. UCH-L3, for example, makes contact with three regions of ubiquitin; residues 6–12, 41–48, and 69–74 [29]. These surfaces are highly conserved in Nedd8, but divergent in ISG15 and SUMO. Correspondingly, UCH-L3 can cleave ubiquitin and Nedd8 adducts but not those of the other ubiquitin-like proteins [26].

The same regions appear to be important for interactions of ubiquitin with many other DUBs (see Figure 8.2) and Ub-binding proteins. Importantly, all ubiquitin-binding domains examined utilize these same surfaces in binding ubiquitin.

Recognition of a ubiquitin domain can be accomplished by ***ub***iquitin-***a***ssociated domains (UBA), which are present in many proteins, including some DUBs, and interact with polyubiquitin up to 1000-fold better than mono-ubiquitin [51]. However, other binding domains such as UIM (***u***biquitin-***i***nteracting ***m***otif) and CUE (***c***oupling of ***u***biquitin conjugation to ***E***R degradation) domains utilized in endocytic pathways prefer binding mono-ubiquitin [52–54].

Polyubiquitin-binding proteins recognize a subset of this binding surface of ubiquitin, often described as the hydrophobic patch. It is a group of three amino acids, Leu8, Ile44, and Val70, which are oriented in the ubiquitin molecule to form a small hydrophobic patch [55]. Polyubiquitin chains incorporating ubiquitins with mutations at residues 8 and 44 were unable to be disassembled by DUBs present in the 19S subunit of the proteasome [56]. In addition to providing a recognition site for DUBs, the patch is also important in determining the quaternary structure of polyubiquitin, another feature utilized by DUBs in substrate recognition. One UbL protein, ISG15, consists of a fusion of two ubiquitin domains. The crude mimicking of an Ub-dimer could potentially contribute to its specific recognition by deISGylating enzymes.

Polyubiquitin chains linked through all seven lysines in ubiquitin have been detected *in vivo*, and these poorly characterized forms of non-K48-linked polyubiquitin are also likely to have significant roles in the cell [57]. Polyubiquitin chains that are linked through different lysines are expected to be different enough in structure that individual DUBs could distinguish between them. K63-linked polyubiquitin is a well characterized alternative linkage and unlike K48-linked polyubiquitin, is not involved in proteolytic degradation [58, 59]. Structural data confirms the idea that these two types of polyubiquitin can have different structures [60, 61]. The structures of these dimers were solved by NMR analysis and they were found to have quite different conformations. Indicative of this, non-hydrolyzable ubiquitin-dimer analogs containing different linkages have markedly different effectiveness when used to inhibit the enzymatic activity of Isopeptidase T [62]. Isopeptidase T binds and cleaves polyubiquitin linked through at least four of the seven possible chain linkages found *in vivo*, although the catalytic efficiency of these activities is not known [10]. It is interesting to speculate that Isopeptidase T utilizes its two UBA domains to regulate binding of different polyubiquitin substrates. Mutational analysis of the UBA domains and structural data are needed to determine if this is the case and whether it is applicable to other DUBs as well.

8.3.2
Recognition of the Gly–Gly Linkage

The central feature that defines all DUBs is that they recognize and act at the C-terminus of the ubiquitin or ubiquitin-like domain. All mature ubiquitin and ubiquitin-like proteins have a C-terminal gly–gly motif and DUB cleavage releases leaving groups attached to the carboxyl group of the C-terminal glycine. With the exception of the JAMM metalloproteases, DUB catalysis starts with the nucleophilic attack of the catalytic cysteine on the carbonyl carbon of the scissile bond to

form the tetrahedral intermediate. This is converted to an acyl-enzyme intermediate by expelling the C-terminal leaving group. Attack by a water molecule allows regeneration of the free thiol on the catalytic cysteine and releases free ubiquitin. The JAMM isopeptidases appear to use a classical metalloprotease mechanism [50].

DUB structures have evolved to recognize this C-terminal glycylglycine with exquisite specificity. Analysis of ubiquitin-fusion proteins lacking the gly–gly motif has clearly shown that they are not cleaved efficiently by DUBs [63]. All DUBs exclude larger amino acids at the C-terminus of the ubiquitin domain by having a deep cleft in their respective structures that is only large enough to hold two glycines. The narrowest region of USP7's catalytic cleft sterically excludes amino acids with any type of side chain, enforcing specificity for ubiquitin conjugates [35]. However, the end of the cleft is open, which allows USP7 to act on large ubiquitin conjugates like its substrate, ubiquitinated p53. ULP1 uses a similar type of cleft to recognize the gly–gly motif except that it uses a tryptophan residue to restrict access to the catalytic site when a substrate is bound to the enzyme [40]. UCHs have a similarly constrained cleft and also use the previously described "blocking loop" to assist in specifically recognizing the C-terminus of ubiquitin.

8.3.3 Recognition of the Leaving Group

In principle, DUBs might also recognize the leaving group to which ubiquitin is attached. In fact, such a mechanism seems likely as several DUBs have little affinity for ubiquitin and several have been shown to bind the un-ubiquitinated target protein (see Table 8.2). Interactions between DUBs and putative substrates have been shown for the mammalian DUBs VDU1, USP11, and UBPy, as well as UBP3 from yeast and fat facets from *Drosophila* [64–68]. In other cases, DUB-binding proteins may serve as scaffolds or adaptors that localize DUBs (discussed below).

8.3.4 Substrate-induced Conformational Changes

DUBs are not general hydrolases for cleaving after a gly–gly sequence even though they recognize the gly–gly motif at the C-terminus of ubiquitin and ubiquitin-like proteins. What is so special about these particular gly–gly sequences that DUBs will only recognize and act on them and not others? The answer comes from the fact that DUBs interact with the rest of the ubiquitin or ubiquitin-like substrate, and this interaction causes conformational changes in the DUB that are necessary to achieve catalysis. These changes result in rapid and efficient cleavage of only the particular substrate that the DUB is equipped to bind. It also explains why peptides with a gly–gly in them are not susceptible to cleavage by DUBs as they are lacking the substrate-binding domains that cause the DUB conformational change required for cleavage. The different DUB classes utilize a number of conformational

Tab. 8.2. Identification of DUBs and DUB-binding partners through physical and genetic interaction screens.

DUB	*Affinity*	*Characterized by*			*Interaction partner(s)*
		MS	*Yeast two-hybrid*	*Synthetic lethal*	
UCHs					
UCH37			X		S14, UIP1 [21]
UCH-L3			X		Nedd8 [126]
UCH-L1			X		JAB1, p27 [127]
UBPs					
DOA4				X	SLA1, SLA2 [128]
UBP3	X		X	X	SIR4, Bre5, Stu1 [66, 118, 129]
UBP6		X			19S proteasome [130]
UBP8		X			SAGA, SLIK acetyl transferases [88]
USP5	X				ubiquitin [131]
USP7			X		ataxin [132]
USP11			X		RanBPM [67]
CYLD		X			NEMO [7]
fat facets					Vasa [75]
UBPy			X		CDC25(Mm) [133]
OTU DUBs					
cezanne			X		ubiquitin [43]
otubain1 and 2	X				ubiquitin aldehyde [42]
VCIP 135	X				VCP/P47 [72]
JAMM Isopeptidases					
Rpn11				X	UBP6 [92]
Others					
ataxin 3			X		RAD23, HHR23A, HHR23B [134]

changes that are induced upon substrate binding to assist in promoting efficient cleavage.

UCH DUBs have been the most thoroughly analyzed. Comparison of the ubiquitin–UCH complex with unliganded UCH shows two significant conformational differences that contribute to keeping the unliganded enzyme in an inactive state. First, the previously described ''blocking loop'' becomes ordered as it interacts with ubiquitin. Invariant residues form hydrogen bonds with the ubiquitin substrate and other UCH residues, indicating that the loop has functional importance during substrate binding [27]. Second, the side chain of L9 in UCH-L3 intrudes into the substrate-binding cleft, occluding the catalytic cysteine and preventing binding of peptide substrates [29]. When ubiquitin binds to the UCH-L3, an interaction between ubiquitin and UCH-L3 repositions L9, allowing access to the active site cleft. Thus, the energy of ubiquitin binding is required to activate UCH-L3, allowing its cleavage. This type of selectivity (where ubiquitin binding is

required for activity) may be necessary to prevent deleterious cleavage of other protein substrates by UCHs.

A similar situation was observed when the crystal structure of USP7 was solved in the absence of substrate [35]. The catalytic cysteine of the unliganded protein is not in an orientation that would allow catalysis to take place. The histidine residue needed to interact with the active-site cysteine is too distant for a catalytic-triad mechanism to function. Binding of ubiquitin aldehyde induces a significant conformational change that realigns the catalytic triad residues so the hydrogen bonding required for catalysis can take place. Thus, like UCH DUBs, the unliganded protease is inactive and only becomes catalytically active when it is binding substrates.

ULP1 also uses conformational changes to ''clamp down'' on the gly–gly motif when a SUMO substrate is bound. Trp448 lies directly above the active site and interacts with the SUMO C-terminus by Van der Waals interactions, sandwiching the gly–gly motif between Trp448 and the active-site cysteine when SUMO binds [40]. Despite the various methods utilized, all DUBs require a conformational change triggered by binding of a specific substrate to catalyze cleavage. These required conformational changes are driven by the energy of interaction between the DUB and the body of the ubiquitin domain.

8.4 Localization of DUBs

While many DUBs are cytoplasmic, localization of DUBs is also known to be important in regulating DUB specificity. The localization of ULP1, for example, is important in determining its substrates. The N-terminal domain of ULP1 is known to localize the enzyme to the nuclear envelope, and truncation mutations lacking this domain remain in the cytoplasm [69]. When the truncated protein is expressed in ΔULP1 yeast strains, the cells grow at wild-type levels, and the truncated protein is able to cleave SUMO substrates *in vitro*. However, analysis of ΔULP1 cells expressing this truncation shows an accumulation of SUMO conjugates. Apparently, the localization of ULP1 to the nuclear envelope is necessary in order for it to act on specific nuclear-envelope-localized substrates. The localization helps constrain ULP1 isopeptidase activity so ULP1 does not inappropriately act on cytoplasmic substrates. Other examples of DUB activity regulated by localization include UBP6, which is fully active only when bound to the proteasome (see below) and UBP16 residence on the outer membrane of the mitochondria, although its function there is undetermined [70].

Other DUBs have been found to associate with membranes and regulate membrane-associated cellular processes, although they appear not to be membrane anchored like UBP16. The ability of DOA4 to remove ubiquitin from membrane-bound endocytic substrates promotes their degradation in the vacuole or lysosome [71]. DUBs are also important for membrane fusion events as shown by the fact that an OTU domain DUB, VCIP135 (***V***CP/p47 ***c***omplex-***i***nteracting ***p***rotein of

135 kd), is necessary for p97–p47-mediated Golgi cisternae reassembly after mitosis [72]. Also, a neuronal DUB, synUSP, was found to localize to post-synaptic lipid rafts (membrane microdomains involved in membrane trafficking and signal transduction) [73]. However, its function at that location has yet to be characterized.

A well-studied example of a tissue-specific DUB activity is fat facets, a UBP originally found in *Drosophila* [74]. It is important in eye development and germ-cell specification and is active only in specific cell types during certain stages of development [65, 75]. The lack of fat facets results in defective posterior patterning, germ-cell specification, and eye formation. Fat facets activity is required to prevent the inappropriate degradation of vasa and liquid facets. In this case, the role of the DUB appears to be defined by the restricted expression of its known substrates.

Temporal regulation of DUB expression also appears important. D'Andrea and colleagues first described a small family of DUBs that are induced as immediate early gene products of cytokine stimulation [76]. Different cytokines were shown to induce different DUBs and the expression of these enzymes was short-lived [77]. It appears that these DUBs may be involved in down-regulating cytokine receptors, perhaps by removing the ubiquitin involved in sorting of the receptor at the early endosome. Likewise, UBP43, the short-lived processing protein for ISG15, is present at very low levels in normal cells and highly expressed upon interferon induction [78].

8.5
Probable Physiological Roles for DUBs

8.5.1
Proprotein Processing

One important function of DUBs is the processing of ubiquitin or ubiquitin-like proteins to their mature forms. Ubiquitin is expressed in cells as either linear polyubiquitin or N-terminally fused to certain ribosomal proteins [79, 80]. These gene products are processed by DUBs to separate the ubiquitin into monomers and expose the gly–gly motif at the C-terminus. Many DUBs process linear polyubiquitin or Ub-fusion proteins *in vitro*, but this processing appears to take place cotranslationally *in vivo* and is extremely rapid. This makes analysis difficult and leaves unanswered the question of which DUBs actually perform this function *in vivo*. Multiple DUBs may be able to perform this processing at a physiologically relevant level since DUB deletions rarely shows processing defects [81].

Ubiquitin-like proteins are also expressed as proproteins with a short C-terminal extension of a few amino acids that must be removed to make the UbL available for conjugation to target proteins. All ULPs have been shown to metabolize their respective proprotein to an active form *in vitro* although again it is unclear which ULPs are responsible for this activity *in vivo*. An exception to the confusion is the finding that RUB1 (the yeast homolog of Nedd8) is processed by YUH1 in *Saccha-*

romyces cerevisiae. Conjugation of RUB1 to Cdc53 is required for efficient assembly of certain SCF (***s***kp1, ***c***ullin, ***F***-box) E3 ubiquitin ligases [82]. Yuh1 deletion strains do not process Rub1 or modify Cdc53 with Rub1 [83]. Modification of Cdc53 by Rub1 could be reconstituted in a ΔYuh1 strain by expressing a mature Rub1 construct lacking the C-terminal asparagine normally removed by processing. This demonstrated that Yuh1 processes RUB1 proprotein into the mature form *in vivo*. It is not known which DUB performs the processing of proNedd8.

8.5.2
Salvage Pathways: Recovering Mono-ubiquitin Adducts and Recycling Polyubiquitin

It has been speculated that without UCH function, all ubiquitin in the cell would be conjugated with glutathione or other cellular amines and therefore unavailable for conjugation. This would quickly result in the cessation of the ubiquitin–proteasome system function and cell death due to lack of active ubiquitin to conjugate to substrates. The effectiveness of UCH DUBs in liberating ubiquitin from other small adducts makes them likely candidates to act on these particular adducts. In addition, the cell must regenerate mono-ubiquitin from polyubiquitin and various mono-ubiquitinated proteins to maintain levels of mono-ubiquitin for conjugation. Doa4 appears to remove small peptides attached to mono- and di-ubiquitin intermediates resulting from proteasomal degradation as well as removing ubiquitin from proteins targeted for endocytosis [84, 85]. Loss of Doa4 function in yeast results in depleted levels of mono-ubiquitin and increased cell death during stationary phase.

Another function for DUBs is regenerating free ubiquitin from unanchored polyubiquitin chains removed from proteasome substrates or proteins targeted for other pathways. Polyubiquitin inhibits the proteasome and lowers the amount of free ubiquitin available for conjugation to proteins. Thus, these chains need to be processed to mono-ubiquitin to prevent polyubiquitin accumulation and inhibition of the proteasome. This type of DUB activity has been well characterized *in vivo* and *in vitro* and Isopeptidase T appears to be the DUB that is responsible for the majority of this activity. Deletion of UBP14 in yeast is not lethal, although large amounts of polyubiquitin build up in the cell and proteasome function is impaired [86]. Isopeptidase T seems to serve as a general DUB for regenerating mono-ubiquitin as it cleaves polyubiquitin containing various linkages [59].

8.5.3
Regulation of Mono-ubiquitination

DUBs have increasingly been found to be important in regulating the ubiquitination level of proteins not targeted to the proteasome for degradation. Some DUBs are active participants in the regulation of mono-ubiquitin (or mono-UbL) conjugation and others can regulate the conjugation of multiple types of ubiquitin or UbLs to a single substrate. For instance, deneddylating enzymes may regulate the neddylation of cullin proteins both by processing proNedd8 and by removing Nedd8

from neddylated cullins [26, 38]. As a component of the SCF E3 ligase complexes, cullins require neddylation in order for their cognate E3 ligase to be efficiently assembled [82]. Regulation of this modification indirectly regulates the ubiquitination of a subset of proteins. Defects in deneddylation could lead to inappropriate ubiquitination of substrates owing to inappropriate recruitment of E2s to the SCF E3s [87].

Regulating mono-ubiquitination of proteins by DUBs is important in histone modification where ubiquitination is thought to modulate chromatin structure and transcriptional activity. Normally, about 10% of the histone core octomers contain ubiquitinated histones and the ubiquitin is removed at mitosis by DUB activity. UBP8 has been demonstrated to regulate the ubiquitination of histone H2B, which is important in transcriptional activation of many genes [88].

Many cell-surface receptors are ubiquitinated upon internalization and the ubiquitin is removed by DUBs at the early endosome. Properly sorted receptors are then shuttled to the lysosome for degradation. In the absence of Doa4, the ubiquitin is not removed upon sorting and instead is co-degraded in the vacuole, resulting in ubiquitin depletion [84]. Another DUB, UBP3 assists Golgi-ER retrograde transport by deubiquitinating B′-COP, thus preventing its degradation [66]. Mono-ubiquitinated B′-COP cannot be assembled into the COP1 complex without UBP3/Bre5 complex DUB activity. Disruption of the complex in ΔBre5 strains reduces the efficiency of Golgi-ER transport and facilitates the polyubiquitination and degradation of B′-COP by the proteasome.

One fascinating observation is that PCNA (proliferating cell nuclear antigen) can be modified by multiple forms of ubiquitin, demonstrating that DUBs with different specificities can act at the same location on a specific substrate. PCNA can be modified by mono-ubiquitin, 63-linked polyubiquitin, or SUMO at K164 [89]. Modification of PCNA by mono- or polyubiquitin determines whether it is utilized in translesion synthesis or error-free DNA repair, respectively. SUMO modification prevents PCNA function in DNA repair and instead promotes DNA replication. It is probable that multiple DUBs, as yet unidentified, are required to regulate PCNA modification.

8.5.4
Processing of Proteasome-bound Polyubiquitin

DUBs play a crucial role in regulating the function of the proteasome. For a long time it was unclear what happens to polyubiquitin conjugated to a proteasome substrate when that substrate is at the proteasome ready for degradation. Was the conjugated polyubiquitin processed by the proteasome and degraded or was it removed by a DUB and released from the proteasome? The small 13-Å diameter entrance to the 20S catalytic core of the proteasome requires all substrates to be fed through as unfolded polypeptides [90]. A branched polypeptide such as a ubiquitin–protein conjugate apparently has difficulty fitting through the pore, greatly reducing proteasome efficiency [47]. Thus, it seemed likely that DUBs must remove polyubiquitin from proteasome substrates before they enter the 20S catalytic core of the proteasome.

To date, three DUBs are known to perform this function and all are components of the 19S lid of the mammalian proteasome. The first described was UCH37, although its exact function is still unclear [91]. UCH37 is thought to be an editing DUB that assists in clearing the proteasome of ubiquitinated proteins. UCH37 slowly cleaves one ubiquitin at a time from the distal end of the polyubiquitin chain. If chain trimming is faster than the degradation process, loss of the polyubiquitin signal could result in partial degradation or release of proteins from the proteasome. UCH37 activity could also be necessary to recover proteasomes that are having difficulty degrading ubiquitinated proteins.

UCH37 is only found in higher eukaryotes, but the other two proteasome-bound DUBs, RPN11 and UBP6 (USP14), are found in all eukaryotes. RPN11 and UBP6 remove polyubiquitin from substrates that are committed to degradation by the proteasome [92]. The mechanisms for this, and exactly what role each DUB plays in removing ubiquitin, are not fully understood. Interestingly, the Rpn11 DUB activity was first detected over 10 years ago when 26S proteasome DUB activity was inhibited by *o*-phenanthroline, a metal chelator [93]. The metalloprotease DUB activity was not identified until recently [46]. Rpn11 is thought to remove polyubiquitin chains from proteasome substrates before they are degraded, allowing the unfolded substrate to enter the pore of the 20S subunit of the proteasome. It has been proposed that Rpn11 removes most of the polyubiquitin chain attached to a proteasome substrate and then UBP6 acts to remove the remaining one or two ubiquitin residues.

Despite the lack of mechanistic understanding, the DUBs are clearly required for efficient proteasomal degradation to take place. The Rpn11 deletion is lethal in yeast and temperature-sensitive mutants show massive accumulation of polyubiquitin conjugates [46, 47]. UBP6 is approximately 300 times more active when it is associated with the proteasome than in its purified form [94]. The UBP6 deletion is not lethal in yeast, but a large decrease in the cellular pool of monoubiquitin occurs, indicating that ubiquitin is fed into the proteasome and degraded rather than being released from the proteasome and recycled [95].

8.6 Finding Substrates and Roles for DUBs

Surprisingly, little is known about the *in vivo* substrate specificity of DUBs. Difficulty in defining the substrate specificity of individual DUBs often arises from a lack of observable phenotypes in deletion strains. Deletion studies in yeast where up to 4 of the 17 DUBs have been deleted in a single strain have not produced significant phenotypes [96]. It is unclear if this is due to the subtle nature of the phenotypes or if the remaining DUBs compensate for the missing ones. However, several tactics have been fruitful in defining the physiological roles of DUBs. First, definition of *in vitro* specificities can be useful in focusing genetic screens. For example, the first UBPs were cloned and analyzed after it was discovered that they could cleave ubiquitin-fusion proteins [96, 97]. Second, directed screening of dele-

tions or knockdown studies to identify roles for DUBs have also been successful (see Table 8.1 for DUB-deletion phenotypes). Study of DUB deletions, including UCH-L1, UCH-L3, and USP14 (see below), in the mouse have demonstrated their importance in neuronal function. Third, potential roles for DUBs have also been identified by physical and genetic interaction screens. Table 8.2 shows in more detail the interaction screens that have been used in discovering DUBs and characterizing their *in vivo* roles by identifying novel binding partners. For example, Cezanne was suggested to be a DUB after two-hybrid studies demonstrated its interaction with ubiquitin and UBP6 was identified as a component of the 19S subunit of the proteasome by mass spectrometry.

8.7
Roles of DUBs Revealed in Disease

8.7.1
NF-κB Pathway

NF-κB is a transcription factor that can be activated by a number of cellular signals, including stress, inflammation (via tumor necrosis factor) and antigen receptors among others [98, 99]. After receptor stimulation, a cascade ensues that results in the release of NF-κB from its inhibitor IκB. Released NF-κB translocates to the nucleus and activates transcription of a number of genes. Ubiquitin metabolism plays a significant regulatory role in the NF-κB pathway. For NF-κB release from IκB and nuclear translocation to take place, IκB is phosphorylated by IκB kinases, resulting in K48-linked polyubiquitination and proteasomal degradation of IκB [100]. A number of other proteins involved in this pathway such as NEMO, IKKγ, and TRAF6 have K63-linked polyubiquitin chains conjugated to them [101, 102]. It is not clear what purpose the K63-linked chains serve, but they appear to be a regulatory component of the NF-κB pathway.

Most of the DUB activity characterized in the NF-κB pathway appears to act on K63-linked polyubiquitin, suggesting that modulation of K63-linked polyubiquitination by DUBs is important for control of the NF-κB pathway. CYLD, a tumor suppressor gene, has been confirmed as a DUB [7, 103, 104]. Loss of CYLD function leads to cylindromatosis, a syndrome characterized by large benign tumors on the face and neck. This is one of the few examples where a defective DUB has been defined as the direct cause of a specific disease. Preferred *in vivo* substrates of CYLD are believed to be 63-linked polyubiquitin–protein conjugates of NEMO, TRAF6, and TRAF2 components of the NF-κB pathway, but the exact *in vivo* regulatory role of CYLD is still unknown.

OTU family DUBs such as Cezanne and A20 also play significant roles as negative regulators of the NF-κB pathway [43, 44]. A20 can cleave K48- and K63-linked polyubiquitin chains *in vitro* while Cezanne has only been tested on K48-linked chains. Although these DUBs are known to be part of the NF-κB pathway, their *in vivo* substrates are unknown. It is also unclear as to how these DUBs negatively regulate the NF-κB pathway.

8.7.2
Neural Function

DUBs, specifically UCHs, appear to play significant roles in neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's, and others [105]. A mutant form of UCH-L1 with reduced enzymatic activity has been found in a small family of Parkinson's patients and the S18Y allele of UCH-L1 has been associated with a reduced risk of sporadic Parkinson's disease [106, 107]. Many of the inclusion bodies found in patients with Parkinson's are known to contain high amounts of UCH-L1, ubiquitin, and ubiquitinated proteins, as determined by immunostaining [108]. This suggests that defects in some DUBs or their regulation can cause significant harm to the neuronal system, resulting in disease.

DUBs have also been implicated in the formation of other neural inclusion bodies. In addition to the case for their involvement in Parkinson's disease it has been shown that the mutation of USP14 (the mammalian homolog of yeast UBP6) results in Ataxia in the mouse [109]. Many neurological diseases, including Ataxia, result in damaged or mutated proteins aggregating as polyubiquitinated forms at the ***m***icro***t***ubule ***o***rganizing ***c***enter (MTOC) to form inclusions called aggresomes [110]. An adapter, the tubulin deacetylase HDAC6 (***h***istone ***d***e***a***cetylase 6), has recently been shown to bind these polyubiquitinated proteins and tether them to the microtubules where they are then transported to the MTOC [111]. The classic Lewy Body of Parkinson's disease has all the hallmarks of such an aggresome. The formation of an aggresome is thought to be protective and in its absence the aggregated proteins can trigger apoptosis. Thus, the dynamics of ubiquitination and aggregate formation are important responses to this type of cellular stress and several DUBs can modulate this process.

Deletion of UCH-L1 and/or UCH-L3 in mice has demonstrated that they are both involved in neuronal regulation, but have separate functions. The GAD (***g***racile ***a***xonal ***d***ystrophy) mouse has been shown to lack UCH-L1, the predominant neuronal UCH [112]. These mice show a unique neuronal "dying back" phenotype that results in paralysis of the limbs due to death of nerves originating in the gracile nucleus. Mice lacking UCH-L3 have no obvious abnormalities or defects [113]. However, the double deletion mouse shows more severe defects including reduced weight, a more severe gracile axonal dystrophy than the L1 deletion, and earlier lethality caused by a loss of the ability to swallow resulting in starvation [114]. This demonstrates that the two DUBs are not redundant and have separate neuronal functions.

8.8
New Tools for DUB Analysis

Despite all the DUB structures and substrates previously described, in most cases the *in vivo* substrate for a particular DUB is unknown. Structural and localization data can provide clues to determine *in vivo* DUB specificity, especially if one knows

what ubiquitin or ubiquitin-like protein it acts upon. Genomic databases have helped, but many annotated DUBs have never been tested for DUB activity and some DUBs thought to act on one type of substrate (based on their homology) are found to act on another when tested. The characterization of hundreds of potential DUBs is a daunting task and *in vivo* characterization is even more difficult. To make headway, novel tools are needed to conclusively identify potential DUBs and their substrates to help direct appropriate *in vivo* studies.

8.8.1
Active-site-directed Irreversible Inhibitors and Substrates

The most promising tools developed for this sort of analysis are active-site-directed irreversible inhibitors of DUBs. These inhibitors are ubiquitin or ubiquitin-like proteins chemically modified at the C-terminus by an electrophilic moiety such as a Michael acceptor or alkyl halide. The modified ubiquitin can be incubated with a purified DUB or a cell lysate containing DUB activity. ***Ub***iquitin ***v***inyl ***s***ulfone (UbVS) is one such irreversible inhibitor because the vinyl sulfone moiety reacts with the active-site cysteine of the DUB, forming a thioether linkage. The covalent adduct is stable and can be detected in a variety of ways. Labeling of DUBs is specific, as only a DUB active-site cysteine will efficiently react with the vinyl sulfone moiety.

To create these inhibitors, an N-terminally tagged ubiquitin or ubiquitin-like protein (lacking the C-terminal glycine) is expressed using the intein expression system (New England Biolabs). Briefly, in this system a fusion protein consisting of a ubiquitin or a ubiquitin-like protein lacking the C-terminal glycine, an intein linker, and a ***c***hitin-***b***inding ***d***omain (CBD) is expressed in *E. coli*. Clarified cell lysate is incubated with chitin beads to bind the ubiquitin-fusion protein. The ubiquitin is then cleaved from the CBD and intein linker by adding mercaptoethanesulfonic acid (MESNA). After MESNA elution, the resulting truncated ubiquitin C-terminal thioester is reacted with glycine vinyl methyl sulfone to create the Ub or UbL vinyl sulfone derivative. The N-terminal tag on the ubiquitin molecule allows analysis of DUB labeling by immunoprecipitation and Western blotting.

This labeling has been used with success in yeast where 6 of the 17 known DUBs were labeled with UbVS [115]. Incomplete labeling likely results from DUBs that do not act on mono-ubiquitin or where the UbVS could not access the active site. The labeling has also been used with great success in mammalian cell lysates to identify novel ubiquitin DUBs [41]. A novel deneddylating enzyme and a novel DUB that acts on autophagy-related UbL proteins have also been identified using vinyl sulfone labeled probes [26, 37, 116].

This ubiquitin intein system can also be utilized to make a DUB substrate rather than inhibitors by attaching a C-terminal fluorescent tag such as 7-amidomethylcoumarin (AMC) instead of vinyl sulfone. DUBs cleave the ubiquitin derivative and release the fluorescent tag, a process that can be followed fluorometrically. Fluorometric assays can then be used to determine a particular DUB's preferred substrate or to quantitate DUB activity in crude lysates. AMC substrates

have turned out to be excellent tools for identifying the substrates of individual DUBs. Den1, for example, was shown to cleave Nedd8–AMC 60 000-fold faster than it cleaves ubiquitin–AMC, and the ratio was even higher when compared to SUMO–AMC [26]. Clearly, these reagents are powerful tools for identifying novel DUBs and identifying potential DUB substrates.

8.8.2 Non-hydrolyzable Polyubiquitin Analogs

Other modified ubiquitin reagents that are useful in analyzing DUBs are non-hydrolyzable polyubiquitin analogs. These analogs are polyubiquitin chains where the ubiquitins are linked by cross-linking reagents. To synthesize them, one ubiquitin is mutated to cysteine at the C-terminal glycine and another has cysteine introduced at a particular lysine residue. These ubiquitins can then be linked through their cysteine residues with a bifunctional thiol reagent such as dichloroacetone (DCA). As the native ubiquitin sequence contains no cysteines, the ubiquitins will only be linked through the introduced cysteine residues. The result is a ubiquitin dimer analog that mimics physiological dimers. The isopeptide bond is replaced by a DCA linkage, but the ubiquitin subunits retain the appropriate spatial orientation. Thus, DUBs should bind these dimers, but will be unable to cleave them because they cannot hydrolyze the DCA linkage. To make longer polyubiquitin chains, cysteine residues or sulfhydryl groups must be introduced at the desired lysine and the C-terminal glycine on the same ubiquitin molecule.

These chain analogs have been used to characterize DUBs in two fashions. First, they can be used as inhibitors of DUBs [62]. Cleavage of Ub–AMC by Isopeptidase T, a polyubiquitin-binding DUB, was inhibited by the addition of differently linked dimer analogs and kinetic inhibition constants were determined. The K_i values of dimer analogs were all much lower than the K_i for mono-ubiquitin. Further, there was considerable selectivity, as inhibition constants varied depending on the linkage present in the dimer [62]. This demonstrated that the analogs act as faithful mimics of native polyubiquitin. The other way these chain analogs are used is to synthesize them on affinity supports and analyze cell lysates for DUBs that bind a specific type of polyubiquitin chain. These affinity resins have proven useful in identifying a number of binding proteins, including DUBs, from yeast cell lysates [117]. Analogs with different linkages bind a different subset of proteins, perhaps suggesting a way to identify DUBs acting upon polyubiquitin with linkage specificity. Thus, these analogs are excellent tools for characterizing the substrate preferences of known DUBs and discovering novel ones.

8.9 Conclusion

DUBs are clearly an essential cellular component needed for a variety of pathways including protein degradation, DNA repair, apoptosis, membrane trafficking, stress

response, and transcriptional regulation. Not only do they act on various ubiquitin substrates, but they are also needed to process ubiquitin-like substrates. Over 100 DUBs from five major families have been identified and the number is likely to increase. Factors that enhance DUB specificity are the presence of a binding pocket that only accommodates the physiological substrate, the requirement for a substrate-induced conformational change that prevents undesired catalysis, and the recognition of the ubiquitous C-terminal gly–gly motif of all DUB substrates. Subcellular localization to a specific organelle or protein complex and tissue-specific as well as temporal expression are also important components of DUB specificity and function.

In spite of all that has been learned about DUBs and their function, much remains to be discovered. Future studies are likely to focus on identifying *in vivo* DUB substrates, novel DUBs, DUB-binding partners, and phenotypes of DUB deletions. Further development of new reagents, such as the non-hydrolyzable polyubiquitin analogs and active-site-directed inhibitors or substrates will help greatly. Directed proteomics studies should assist in identifying DUBs, loss-of-function phenotypes, and potential binding partners. Despite the major gaps that remain in our understanding of DUBs, our knowledge of their roles and importance has progressed amazingly rapidly. Novel DUB gene families have been identified, new ubiquitin-like DUB substrates have been uncovered, and structural data has been analyzed to elucidate how DUBs perform catalysis and specifically recognize their substrates. *In vivo* substrates of DUBs are beginning to be identified and the tools and techniques needed to search for novel DUBs and analyze known ones for their specificity are rapidly being created. With so much discovered, and yet so much remaining to be found, deubiquitinating enzymes are a vibrant field of study.

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9
The 26S Proteasome

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Abstract

The 26S proteasome is a large ATP-dependent protease composed of more than 30 different polypeptide chains. Like the ribosome, the 26S proteasome is assembled from two ''subunits'', the 19S regulatory complex and the 20S proteasome. The 19S regulatory complex confers the ability to recognize and unfold protein substrates, and the 20S proteasome provides the proteolytic activities needed to degrade the substrates. The 26S proteasome is the only enzyme known to degrade ubiquitylated proteins, and it also degrades intracellular proteins that have not been marked by ubiquitin. The 26S proteasome is located in the nucleus and cytosol of eukaryotic cells, where the enzyme is responsible for the selective degradation of a vast number of important cellular proteins. Because rapid proteolysis is a pervasive regulatory mechanism, the 26S proteasome is essential for the proper functioning of many physiological processes.

9.1
Introduction

It has become apparent since the mid-1990s that the ***u***biquitin–***p***roteasome ***s***ystem (UPS) plays a major regulatory role in eukaryotic cells. The UPS helps to control such important physiological processes as the cell cycle [1, 2], circadian rhythms [3], axon guidance [4], synapse formation [5] and transcription [6–8], to name just a few. In view of the growing family of ubiquitin-like proteins [9, 10], it is possible that covalent attachment of ubiquitin and its relatives will even surpass phosphorylation as a regulatory mechanism. Although ubiquitin serves non-proteolytic roles, such as histone modification [11, 12] the activation of cell signaling components [13], endocytosis [14], or viral budding [15], the protein's principal function appears to be targeting proteins for destruction [16]. To do this, the C-terminus of ubiquitin is activated by an ATP-consuming enzyme (E1) and transferred to one of several dozen or more small carrier proteins (E2s) in the form of a reactive thiol ester. The E2s collaborate with members of several large families of ubiquitin li-

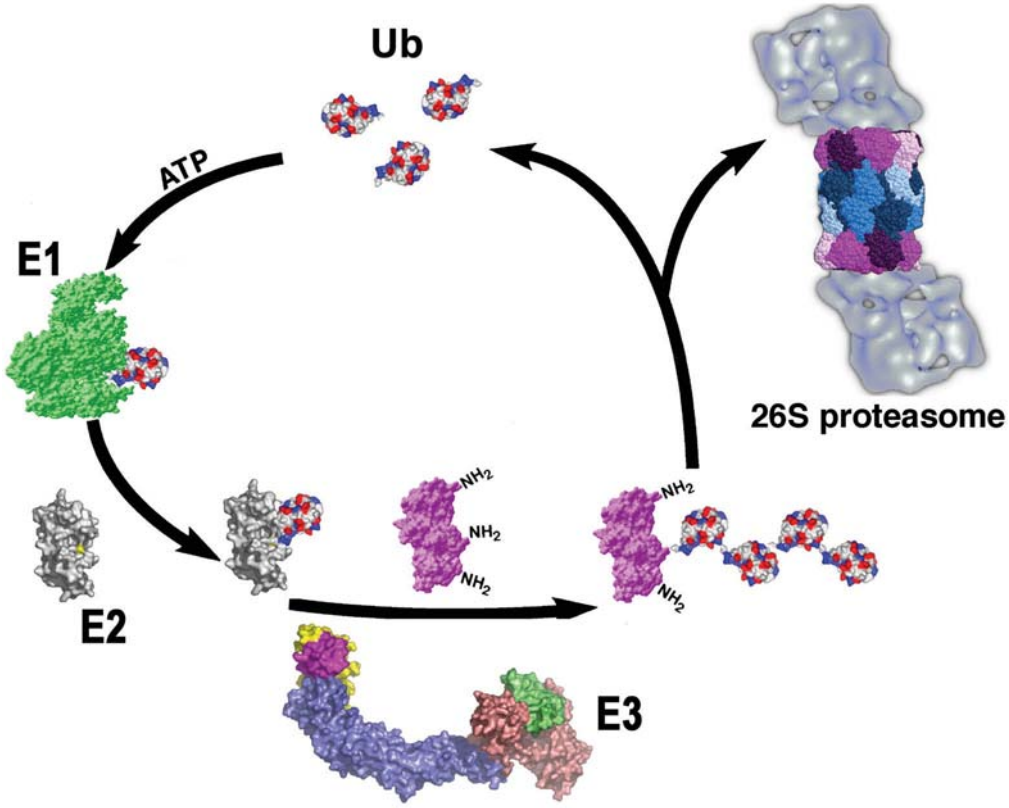

Fig. 9.1. Schematic representation of the ubiquitin–proteasome pathway. Ubiquitin molecules are activated by an E1 enzyme (shown green at 1/3 scale) in an ATP-dependent reaction, transferred to a cysteine residue (yellow) on an E2 or Ub carrier protein and subsequently attached to amino groups (NH_2) on a substrate protein (lysozyme shown in purple) by an E3 or ubiquitin ligase, (the multicolored SCF complex). Note that chains of Ub are generated on the substrate, and these are recognized by the 26S proteasome depicted in the upper right at 1/20 scale.

gases or E3s, and ubiquitin is transferred once again to lysine amino groups on the proteolytic substrates (S) and to itself, thereby generating chains of ubiquitin. The substrate bearing the ubiquitin chains is subsequently recognized and degraded by the 26S proteasome, a large, complex ATP-dependent protease (see Figure 9.1).

Eukaryotic genomes contain information for more than 20 E2s and hundreds of E3s. In contrast to the wealth of components devoted to marking protein substrates for destruction, only one enzyme, the 26S proteasome, has been found to degrade ubiquitylated proteins. However, there is complexity here as well, since the 26S proteasome is an assemblage of at least 30 different subunits. Moreover, there is a growing list of proteins that act as proteasome activators, adapters, or accessory factors. In this chapter I focus on basic biochemical and physiological properties

of the 26S proteasome, drawing occasionally from findings on structurally similar prokaryotic, ATP-dependent proteases [17]. Other chapters will provide greater depth to several aspects of the 26S proteasome.

9.2 The 20S Proteasome

9.2.1 Structure

We know the molecular anatomy of archaebacterial, yeast and bovine proteasomes in great detail since high-resolution crystal structures have been determined for all three enzymes [18–20]. The archaebacterial proteasome is composed of two kinds of subunits, called α and β. Each subunit forms heptameric rings that assemble into the 20S proteasome by stacking four deep on top of one another to form a "hollow" cylinder. Catalytically inactive α rings form the ends of the cylinder while proteolytic β subunits occupy the two central rings. The quaternary structure of the 20S proteasome can therefore be described as $\alpha 7\beta 7\beta 7\alpha 7$. The active sites of the β-subunits face a large central chamber about the size of serum albumin. The α-rings seal off the central proteolytic chamber and two smaller antechambers from the external solvent. Archaebacterial proteasomes, with their fourteen identical β subunits, preferentially hydrolyze peptide bonds following hydrophobic amino acids and are therefore said to have chymotrypsin-like activity [21]. Eukaryotic proteasomes maintain the overall structure of the archaebacterial enzyme, but they exhibit a more complicated subunit composition. There are seven different α-subunits and at least seven distinct β-subunits arranged in a precise order within their respective rings. Although current evidence indicates that only three of its seven β-subunits are catalytically active, the eukaryotic proteasome cleaves a wider range of peptide bonds, containing, as it does, two copies each of trypsin-like, chymotrypsin-like and post-glutamyl-hydrolyzing subunits. For this reason it is capable of cleaving almost any peptide bond, having difficulty only with proline–X, glycine–X and to a lesser extent with glutamine–X bonds [22].

9.2.2 Enzyme Mechanism and Proteasome Inhibitors

Whereas standard proteases use serine, cysteine, aspartate, or metals to cleave peptide bonds, the proteasome employs an unusual catalytic mechanism. N-terminal threonine residues are generated by self-removal of short peptide extensions from the active β-subunits and act as nucleophiles during peptide-bond hydrolysis [23]. Given its unusual catalytic mechanism, it is not surprising that there are highly specific inhibitors of the proteasome. The fungal metabolite lactacystin and the bacterial product epoxomicin covalently modify the active-site threonines and in-

hibit the enzyme [24, 25]. Other inhibitors include vinylsulfones [26] and various peptide aldehydes, which are generally less specific. A peptide boronate inhibitor of the proteasome, Velcade, has been approved for the treatment of multiple myeloma [27].

9.2.3 Immunoproteasomes

Interferon γ (IFNγ) is an immune cytokine that increases expression of a number of cellular components involved in Class I antigen presentation [28]. Among the IFNγ-inducible components are three catalytically active β-subunits of the proteasome, called LMP2, LMP7 and MECL1 [29]. Each replaces its corresponding constitutive subunit resulting in altered peptide-bond cleavage preferences of 20S immunoproteasomes. For example, immunoproteasomes exhibit much reduced cleavage after acidic residues and enhanced hydrolysis of peptide bonds following branch-chain amino acids such as isoleucine or valine. Class I molecules preferentially bind peptides with hydrophobic or positive C-termini, and proteasomes generate the vast majority of Class I peptides [28, 30]. Hence, the observed β-subunit exchanges are well suited for producing peptides able to bind Class I molecules.

9.3 The 26S Proteasome

9.3.1 The Ubiquitin–Proteasome System

Bacteria express as many as five ATP-dependent proteases, all of which contain nucleotide-binding domains that belong to the AAA+ family of ATPases [31]. By contrast, the 26S proteasome is the only ATP-dependent protease discovered so far in the nuclear and cytosolic compartments of eukaryotic cells. Because the 20S proteasome's internal cavities are inaccessible to intact proteins, openings must be generated in the enzyme's outer surface for proteolysis to occur. A number of protein complexes have been found to bind the proteasome and stimulate peptide hydrolysis (see Figure 9.2). The most important of the proteasome-associated components is the 19S ***r***egulatory ***c***omplex (RC) for it is a major part of the 26S ATP-dependent enzyme that degrades ubiquitin-tagged proteins in eukaryotic cells. In Figure 9.2 the 20S proteasome is shown binding only one 19S RC although doubly-capped 26S proteasomes also exist (see Figure 9.3 below). The 20S proteasome also binds activators such as PA28 or PA200. Each of these activators can be present in 26S proteasome complexes forming what are called hybrid proteasomes. Finally a protein called Ecm29p has been found associated with the 26S proteasome. Ecm29p is thought to act as an adapter coupling the 26S enzyme to secretory vesicles.

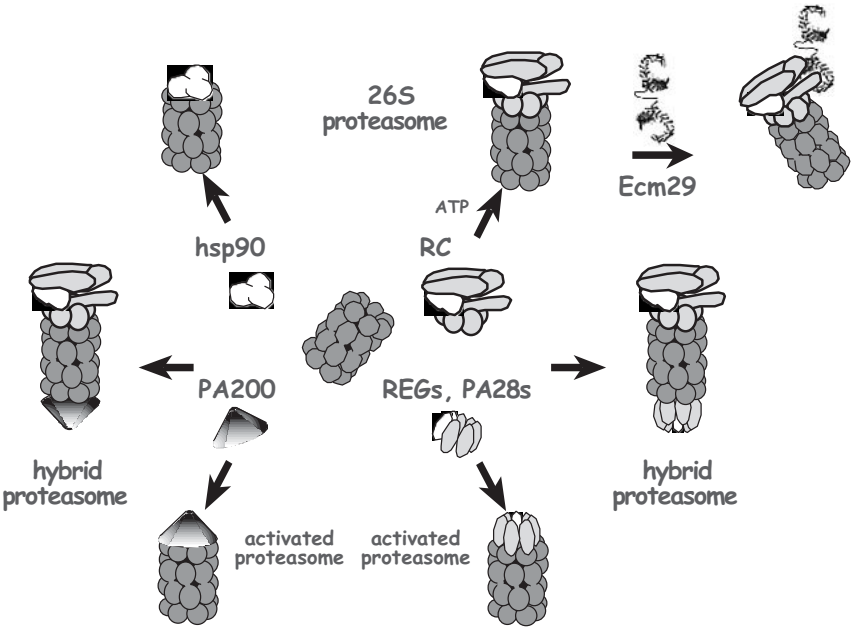

Fig. 9.2. Interaction of the 20S proteasome with other cellular components.

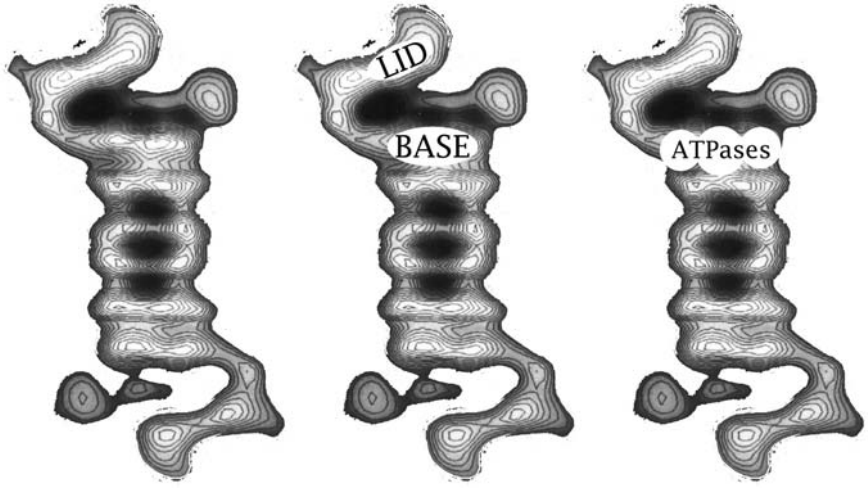

Fig. 9.3. Electron-microscopic reconstructions of the 26S proteasome. Three images of a doubly-capped 26S proteasome are presented to illustrate the positions of the lid and base subcomplexes of the 19S RC and to identify the most probable location of the RC ATPases.

9.3.2
Ultrastructure of the 26S Proteasome and Regulatory Complex

Electron micrographs of purified 26S proteasomes by Baumeister and colleagues [32] reveal a dumbbell-shaped particle approximately 40 nm in length in which the central 20S proteasome cylinder is capped at one or both ends by asymmetric RCs looking much like Chinese dragonheads (Figure 9.3). In doubly capped 26S proteasomes the regulatory complexes face in opposite directions, indicating that contact between the proteasome's α-rings and the RC is highly specific. However, the contacts may not be especially tight since image analysis of Drosophila 26S proteasomes suggests movement of the RCs relative to the 20S proteasome [33]. Electron microscopy (EM) images of the 26S proteasome from several organisms appear similar, indicating that the overall architecture of the enzyme has been conserved in evolution. This conclusion is also supported by sequence conservation among RC subunits (see below).

A yeast mutant lacking the RC subunit Rpn10 contains a salt-labile RC that dissociates into two sub-complexes called the lid and the base [34]. The base contains nine RC subunits, which include six ATPases described below, the two largest RC subunits S1 and S2, and S5a; the lid contains the remaining RC subunits. Thus the RC is composed of two sub-complexes separated on one side by a cavity, i.e. the dragon's mouth (see Figure 9.3). Ultrastructural studies have also been performed on the lid and on a related protein complex called the COP9 signalosome [35]. Both particles lack obvious symmetry. Some particles exhibit a negative stain-filled, central groove; other classes of particles exhibit seven or eight lobes in a disc-like arrangement. Since both the lid and the COP9 signalosome are composed of eight subunits, the lobes may represent individual subunits.

9.3.3
The 19S Regulatory Complex

The regulatory complex is also called the 19S cap, PA700, and the μ particle. As its most commonly used name suggests, the 19S RC is roughly the same size as the 20S proteasome. In fact it is a more complicated protein assembly containing 17 different subunits ranging in size from 25 kDa to about 110 kDa. In animal cells the subunits are designated S1 through S15. Homologs for each of these subunits are present in budding yeast where an alternate nomenclature has been adopted (see Table 9.1). Sequences for the 17 RC subunits permit their classification into a group of 6 ATPases and another group containing the 11 non-ATPases.

9.3.4
ATPases of the RC

The six ATPases belong to the rather large family of AAA ATPases (for ***A***TPases ***A***ssociated with a variety of cellular ***A***ctivities) whose eukaryotic members include the motor protein dynein, the membrane fusion factor NSF, and the chaperone

Tab. 9.1. Subunits of the 19S regulatory complex.

Mammalian nomenclature	*Yeast nomenclature*	*Function*	*Motifs*
S1	Rpn2	Ub/UbL binding	Leu-rich repeats, KEKE
S2	Rpn1	Ub/UbL binding	Leu-rich repeats, KEKE
S3	Rpn3	?	PCI
p55	Rpn5	?	PCI
S4	Rpt2	ATPase	AAA nucleotidase
S5a	Rpn10	polyubiquitin binding	UIM, KEKE
S5b	none	?	
S6	Rpt3	ATPase	AAA nucleotidase
S6′	Rpt5	ATPase	AAA nucleotidase
S7	Rpt1	ATPase	AAA nucleotidase
S8	Rpt6	ATPase	AAA nucleotidase
S9	Rpn6	?	PCI
S10a	Rpn7	?	PCI, KEKE
S10b	Rpt4	ATPase	AAA nucleotidase
S11	Rpn9	?	PCI
S12	Rpn8	?	MPN, KEKE
S13	Rpn11	Isopeptidase	MPN
S14	Rpn12	?	PCI

VCP/Cdc48 and whose prokaryotic members include five ATP-dependent proteases [31]. The six ATPases, denoted S4, S6, S6′, S7, S8, and S10b in mammals, are about 400 amino acids in length and homologous to one another. Based on their sequences, one can distinguish three major regions: (1) A central nucleotide-binding domain of about 200 amino acids, which is roughly 60% identical among members of the RC subfamily; (2) the C-terminal region, approximately 100 amino acids in length and with a lesser, though significant, degree of conservation (~40%); and (3) a highly divergent N-terminal region (< 20% identity) around 120 amino acids in length; this region contains heptad repeats characteristic of coiled-coil proteins. Despite sequence differences among RC ATPases within an organism, each ATPase has been conserved during evolution with specific subunits being almost 75% identical between yeast and humans. The high degree of conservation encompasses the entire sequence, making it likely that even the divergent N-terminal regions play an important role in RC function. Conceivably they are used to select substrates for degradation by the 26S proteasome [36]. Alternatively the variable N-terminal regions in the RC ATPases may promote assembly of the RC by the specific placement of the ATPase subunits within the complex. In this regard, the six ATPases associate with one another in highly specific pairs: S4 binds S7, S6 binds S8, and S6′ binds to S10b. Moreover, the N-terminal regions of RC ATPases are required for partner-specific binding [37].

Staining patterns of two-dimensional gels show the six RC ATPases to be present at comparable levels, and affinity capture of yeast 26S proteasomes indicate the presence of one copy of each in the regulatory complex. Mutational analysis in

yeast demonstrates that the ATPases are not functionally redundant since mutation of yeast S4 has a particularly profound effect on peptide hydrolysis [38]. It is probable that the ATPases form a hexameric ring like other members of the AAA family of ATPases such as NSF or VCP/Cdc48. But this assumption has not been experimentally verified, and pentameric or heptameric arrangements of AAA ATPase complexes have been reported [39, 40]. Finally it is quite likely that the ATPases directly bind the α-ring of the 20S proteasome (see Figure 9.3). Evidence favoring this arrangement comes from chemical cross-linking experiments [41] and the presence of the ATPases in the base subcomplex of the yeast 26S proteasome [34].

9.3.5
The non-ATPase Subunits

Whereas the six RC ATPases are homologous and relatively uniform in size, the non-ATPases are heterogeneous in size and sequence. Nonetheless, they can be grouped on the basis of their location, on the presence or absence of certain sequence motifs, and on their affinity for ubiquitin chains or ubiquitin-like proteins. Eight RC subunits are found in the lid subcomplex, each being homologous to a subunit in a separate protein complex called the COP9 Signalosome [42–44]. One of the lid subunits, S13, is a metalloisopeptidase that removes ubiquitin chains from the tagged substrate prior to its translocation into the proteasome for degradation. Six of the eight lid subunits contain PCI domains, stretches of about 200 residues so named from their occurrence in ***P***roteasome, ***C***op9 signalosome, and the eukaryotic ***I***nitiation factor 3 subunits. The PCI domains are thought to mediate subunit–subunit interactions. Two lid subunits contain 140 amino acid-long MPN domains (***M***pr1p and ***P***ad1p ***N***-terminal regions) with one of these subunits being the S13 isopeptidase. Although several models have been proposed [45–48], the arrangement of subunits within the lid subcomplex is not known. Functionally, only S13 stands out because of its isopeptidase activity. The presence of S13 in the lid explains why the lid is necessary for degradation of ubiquitylated proteins even though the RC base complex supports the ATP-dependent degradation of some small non-ubiquitylated proteins [34]. Interestingly the COP9 signalosome also exhibits isopeptidase activity that removes the ubiquitin-like protein, NEDD8, from certain ubiquitin ligases [49]. Biochemical activity has not been assigned to any of the remaining seven lid subunits, although two lid subunits, S3 and S9, are critical for the degradation of specific substrates [50, 51].

In addition to the six ATPases the base sub-complex contains the two largest RC subunits (S1, S2) and a smaller subunit called S5a [52]. Besides their common location, these three subunits share the property of binding polyubiquitin chains or ***ub***iquitin-***l***ike (UbL) domains. S5a binds polyubiquitin chains even after it has been transferred from SDS-PAGE gels and displays many features that match polyubiquitin recognition by the 26S proteasome [52]. However, S5a cannot be the only ubiquitin-recognition component in the 26S proteasome because deletion of the gene encoding yeast S5a (Rpn10) has only a modest impact on proteolysis

[53]. This strongly suggests that there are other ubiquitin-recognition components in the RC, with S1 and S2 being prime candidates. S1 and S2 display significant homology to each other, and both can be modeled as α-helical toroids [54]. They have been shown to bind the UbL domains of RAD23 and Dsk2, adapter proteins that target ubiquitylated proteins to the 26S proteasome [55, 56]. It has also been found that the S6′ ATPase can be cross-linked to ubiquitin [57]. Currently then it appears that the RC contains three or possibly four subunits able to recognize ubiquitin or UbL proteins. As discussed below there are other ways in which the RC can select proteins for destruction.

9.3.6 Biochemical Properties of the Regulatory Complex

9.3.6.1 Nucleotide Hydrolysis

Both the 26S proteasome and the RC hydrolyze all four nucleotide triphosphates, with ATP and CTP preferred over GTP and UTP [58]. Although ATP hydrolysis is required for conjugate degradation, the two processes are not strictly coupled. Complete inhibition of the peptidase activity of the 26S proteasome by calpain inhibitor I has little effect on the ATPase activity of the enzyme. The nucleotidase activities of the RC and the 26S proteasome closely resemble those of *E. coli* Lon protease, which is composed of identical subunits that possess both proteolytic and nucleotidase activities in the same polypeptide chain. Like the regulatory complex and 26S proteasome, Lon hydrolyzes all four ribonucleotide triphosphates, but not ADP or AMP [18].

9.3.6.2 Chaperone-like Activity

AAA nucleotidases share the common property of altering the conformation or association state of proteins, so it is not surprising that the RC has been shown to prevent aggregation of several denatured proteins including citrate synthase and ribonuclease A [59–61]. The chaperone activity of the RC may explain why the RC plays a role in transcription apparently in the absence of an attached 20S proteasome [62].

9.3.6.3 Proteasome Activation

The 20S proteasome is a latent protease owing to the barrier imposed by the α-subunit rings on peptide entry. Consequently, a readily measured activity of the RC is activation of fluorogenic peptide hydrolysis by the 20S proteasome. The extent of activation is generally found to be in the range 3- to 20-fold [63]. Activation is relatively uniform for all three proteasome catalytic subunits and presumably reflects opening by the attached RC of a channel leading to the proteasome's central chamber.

9.3.6.4 Ubiquitin Isopeptide Hydrolysis

The channel through the proteasome α-ring into the central chamber measures 1.3 nm in diameter, a size too small to permit passage of a folded protein, even one as

small as ubiquitin. This consideration, coupled with the fact that ubiquitin is recycled intact upon substrate degadation, requires an enzyme to remove the polyubiquitin chain prior to or concomitant with proteolysis. Several isopeptidases that remove ubiquitin from substrates have been found associated with the 26S proteasome [64–66]. Of these the ATP-stimulated metalloisopeptidase S13 is an integral component of the enzyme.

9.3.6.5 **Substrate Recognition**

It is clear that the RC plays a predominant role in selecting proteins for degradation. This important topic is covered below in the context of substrate recognition by both 20S and 26S proteasomes.

9.4
Substrate Recognition by Proteasomes

9.4.1
Degradation Signals (Degrons)

The discovery that proteins possess built-in signals targeting them to specific locations within cells was a major success of twentieth-century cell biology [67]. Selective proteolysis can be considered targeting out of existence, and a number of short peptide motifs have been discovered to confer rapid destruction on proteins that bear them. These motifs include PEST sequences [68], the N-terminal amino acid [69], and destruction and KEN boxes [70]. These motifs are recognized by one or more ubiquitin ligases that mark the substrate protein by addition of a polyubiquitin chain. However some proteins are degraded by the 26S proteasome without prior marking by ubiquitin [71]. Denatured proteins are also selectively degraded by both 26S and 20S proteasomes [72]. It is not clear what features of denatured proteins are recognized by proteasomes or by components of the ubiquitin proteolytic system.

9.4.2
Ubiquitin-dependent Recognition of Substrates

Most well characterized substrates of the 26S proteasome are ubiquitylated proteins so our discussion starts with them. Ubiquitin contains seven lysine residues, and proteomic studies in yeast indicate that chains (or dimers) can be formed using any of them [73]. In higher eukaryotes polyubiquitin chains formed via Lys6, Lys27, Lys29, Lys48, and Lys63 have been observed. Lys6 chains are formed by BRCA/BARD heterodimers where they presumably play a role in DNA repair [74]. Ubiquitin monomers linked to each other through Lys63 are involved in endocytosis and DNA repair, but not apparently in targeting proteins to the 26S proteasome [75, 76]. Lys27 chains have been found on the co-chaperone BAG1, and they target degradation of misfolded proteins bound by the Hsp70 chaperone to

the 26S proteasome [77]. Lys29 and Lys48 chains form directly on proteolytic substrates and target them for destruction [78, 79].

Efficient proteolysis of ubiquitylated proteins by the 26S proteasome requires a chain containing at least four ubiquitin monomers [80]. This matches well the ubiquitin-binding characteristics of the RC subunit S5a. It too selectively binds ubiquitin polymers composed of four or more ubiquitin moieties and exhibits increased affinity for longer chains [52]. S5a molecules from a number of higher eukaryotes contain two independent polyubiquitin-binding sites; each is approximately 30 residues long and characterized by five hydrophobic residues that consist of alternating large and small hydrophobic residues, e.g. Leu–Ala–Leu–Ala–Leu [81]. Similar motifs have been found in other proteins of the ubiquitin system and are now called UIMs, (***U***biquitin ***I***nteracting ***M***otifs) [82]. Two recent NMR studies have demonstrated direct interaction between UIMs and the hydrophobic patch on ubiquitin [83–85]. Whereas S5a provides for direct recognition of polyubiquitylated substrates, a second mechanism involves adapter proteins possessing both a UbL domain and one or more UbA (***ub***iquitin ***a***ssociated) domains [86]. UbA domains are polyubiquitin-binding domains found in several presumed adapter proteins of the ubiquitin system. The adapter proteins include RAD23 and Dsk2 in yeast and recruit substrates to the 26S proteasome. The UbL domains of these proteins bind to 26S proteasome subunits while their UbA domains bind substrate-tethered Ub chains. In yeast the RC subunits S1 and S2 serve as UbL-binding components [55, 87]; in mammals S5a serves this purpose [88–90].

The co-chaperone BAG1 illustrates a third way in which polyubiquitin can target substrate proteins to the 26S proteasome. In this case the substrate is not polyubiquitylated; rather it is bound to the chaperone Hsp70. A polyubiquitin chain, linked through Lys27, is attached to the Hsp70-associated co-chaperone BAG1 [77]. Apparently the Lys27 chain promotes association of the chaperone–substrate complex with the 26S proteasome, after which the substrate is degraded while BAG1, Hsp70, and ubiquitin are recycled. Direct interaction between E3 ubiquitin ligases and RC subunits can also deliver ubiquitylated substrates to the protease. The yeast E3 ligase called UFD4 binds RC ATPases [91] and UFD4-mediated delivery of substrates bypasses the requirement for S5a. In what appears to be a similar delivery system, the mammalian E3 Parkin uses a UbL domain to bind the 26S proteasome [89], and the E3 component pVHL binds a 26S proteasome ATPase [92]. Mutational analyses in yeast have shown that whereas deletion of either S5a or Rad23 has a mild impact on proteolysis, loss of both proteins produces a severe phenotype [93]; furthermore, yeasts lacking S5a, RAD23 and Dsk2 are not viable, indicating that direct delivery by E3 ligases cannot compensate for the absence of all three proteins.

9.4.3
Substrate Selection Independent of Ubiquitin

The 26S proteasome also degrades non-ubiquitylated proteins [71]. The short-lived enzyme ornithine decarboxylase (ODC) and the cell-cycle regulator p21Cip provide well documented examples of ubiquitin-independent proteolysis by the 26S en-

zyme [94, 95]. ODC degradation is stimulated by antizyme, a polyamine-induced protein that binds both ODC and the 26S proteasome. Antizyme functions as an adapter much like RAD23 and Dsk2 except that polyubiquitin chains are not involved. However, free ubiquitin chains do compete with antizyme–ODC for degradation [96]. Other potential adapters, such as gankyrin, may target proteins to the 26S proteasome in the absence of ubiquitin marking [97].

The CDK inhibitor p21Cip is degraded in a nonubiquitin-dependent reaction, as clearly demonstrated by substitution of arginines for all the lysine residues in p21Cip. These modifications prevented ubiquitylation of p21Cip, but the lysineless protein was still degraded in human fibroblasts by the proteasome [95]. The C-terminal region of p21Cip binds to the proteasome α-subunit C8, and *in vitro* p21Cip is degraded by the 20S proteasome alone [98, 99]. Direct binding of p21Cip to the 20S proteasome may open a channel through the α-ring allowing the loosely structured protein to enter the central proteolytic chamber. c-Jun, "aged" calmodulin, troponin C, and p53 are other proteins that can be degraded by the 26S proteasome absent marking by ubiquitin [71]. Thus, other 20S proteasome substrates, *in vitro* at least, include oxidized proteins, small, denatured proteins and loosely folded proteins such as casein. Whether the 20S proteasome degrades proteins within cells is an unresolved problem.

9.5
Proteolysis by the 26S Proteasome

9.5.1
Presumed Mechanism

Proteolysis of ubiquitylated proteins by the 26S proteasome can be thought to consist of seven steps: (1) chaperone-mediated substrate presentation; (2) substrate association with RC subunits; (3) substrate unfolding; (4) detachment of polyubiquitin from the substrate; (5) translocation of the substrate into the 20S proteasome central chamber; (6) peptide bond cleavage; and (7) release of peptide products as well as polyubiquitin (see Figure 9.4). Step 1 is optional depending on the substrate, and in principle steps 3 and 4 could occur in either order. Step 4 is unnecessary for substrates like ODC that are not ubiquitylated. The other steps almost have to occur as presented. Although it is easy to conceptualize the reaction sequence, few experimental findings bear directly on any of the proposed subreactions, and virtually nothing is known about molecular movements within the 26S proteasome. However several studies on prokaryotic ATP-dependent proteases permit some informed speculation, and it has been shown that step 6 is not required for sequestration of ODC by the 26S proteasome [100].

9.5.2
Contribution of Chaperones to Proteasome-mediated Degradation

Chaperones are connected to proteasomes in at least four ways. First, chaperones can deliver substrates to the proteasome as described above for the co-chaperone

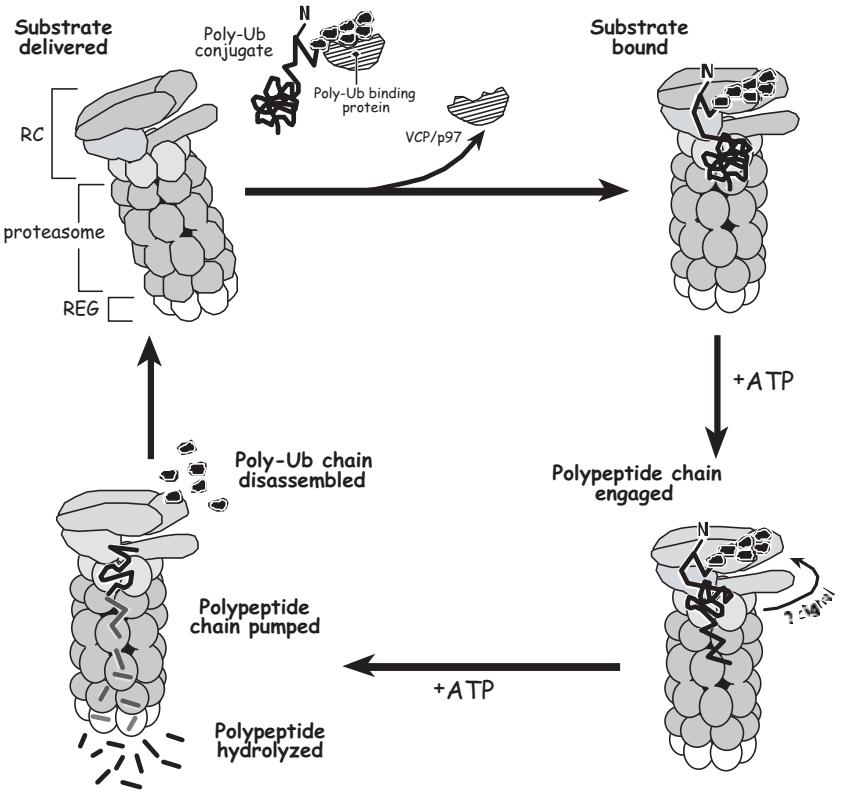

Fig. 9.4. Hypothetical reaction cycle for the 26S proteasome. A polyubiquitylated substrate is delivered to a 26S hybrid proteasome in some cases by chaperones such as VCP/p97 (step 1). Substrate is bound by polyubiquitin recognition components within the regulatory complex (RC) until the polypeptide chain is engaged by the ATPases (step 2). As the polypeptide chain is unfolded and presumably pumped down the central pore of the proteasome, a signal is conveyed to the S13 metallo-isopeptidase to remove the polyubiquitin chain (step 3). The unfolded polypeptide is eventually degraded within the inner chamber of the proteasome (step 4) and peptide fragments exit the enzyme.

BAG1 [77]. In a similar fashion the chaperone VCP/Cdc48 is required for the degradation of several ubiquitin-pathway substrates. VCP, a member of the AAA family of ATPases, is a large hexameric ATPase that appears to function as a protein separase able to remove ubiquitylated monomers from multisubunit complexes [101–104]. In some cases the liberated proteins are degraded by the 26S proteasome; in other cases the separated proteins may change their intracellular location. The proteasome also degrades proteins embedded in the *e*ndoplasmic *r*eticulum (ER) membrane. If these ER membrane proteins possess a large cytoplasmic domain, their proteasomal degradation can require Hsps 40, 70, and 90 as well as VCP [105, 106]. Hsp90 is required to assemble and stabilize the yeast 26S proteasome providing a third connection between chaperones and proteasomes; Hsp90 is

also able to bind and suppress peptide hydrolysis by the 20S proteasome (see below). Finally both chaperones and proteasomes are induced by the accumulation of denatured proteins within eukaryotic cells.

9.5.2.1 Substrate Binding to the 26S Proteasome

Although chaperones may provide for the recognition of some 26S proteasome substrates [107], there is little doubt that the 26S proteasome recognizes substrates directly. As mentioned, ODC is the best-characterized substrate recognized by the 26S proteasome in the absence of a polyubiquitin chain [96]. Which RC subunits actually recognize ODC–antizyme complexes has not been discovered. Presumably one or more subunits in the RC recognize both the C-terminal degron in ODC and some feature of antizyme. The apparent dual recognition of elements in ODC and antizyme may reflect a general mechanism by which ATP-dependent proteases process substrates. For example a number of studies on substrate recognition by *E. coli* ATP-dependent proteases indicate that substrate adapters provide one recognition site while the substrate provides another [108–110]. Similarly, for the 26S proteasome one recognition element, the attached polyubiquitin chains, may be seen by RC subunits S5a or S2, while the substrate's N- or C-termini are recognized by RC ATPases. Interestingly, the location of the polyubiquitin chain on the substrate can affect rates of degradation as much as five-fold [111] perhaps by altering the rates at which the RC ATPases engage substrate termini.

9.5.2.2 Translocation of the Polypeptide Substrate to the Central Proteolytic Chamber

Translocation is thought to proceed by the six ATPases threading the polypeptide through a channel in the 20S proteasome's α-ring. It is also thought that the RC ATPases processively unravel substrates from degrons within the polypeptide chain and are able to "pump" the polypeptide chain in either the N-terminal to C-terminal direction or the opposite [112–114]. Several studies have estimated that hundreds of ATP molecules are needed to degrade small to medium-sized proteins [115, 116]. Rates of translocation range between 10 and 30 amino acids per second [116, 117]. These values are similar to DNA helicases where rates of 50 bases per second and 1ATP per base have been reported [118]. Current models would suggest that the RC ATPases hydrolyze ATP in a sequential rotary fashion essentially screwing the polypeptide chain into the 20S proteasome [119, 120], but the possibility that convulsive movements transfer the substrate has not been ruled out. The ATPases may even be capable of transferring loops into the 20S enzyme. However, a proteomic screen for ClpXP substrates revealed that degrons were either C-terminal or N-terminal [121], so it is likely that the RC ATPases usually engage polypeptide termini.

The peptide fragments generated in the central chamber are generally 5 to 10 residues in length, but fragments as long as 35 amino acids can be present [64]. How these fragments exit the central chamber is not known. They could diffuse back through the RC, through small side panels in the 20S proteasome or in the case of hybrid proteasomes (see below) through the end capped by a proteasome activator.

9.5.3
Processing by the 26S Proteasome

In some cases the 26S proteasome partially degrades the substrate protein, releasing processed functional domains. The best-studied example of processing involves the transcriptional activator NFκB. The C-terminal half of a 105-kDa precursor is degraded by the 26S proteasome to yield a 50-kDa N-terminal domain that is the active transcription component [122]. A glycine-rich stretch of amino acids at the C-terminal boundary of p50 is an important factor in limiting proteolysis [123]. It is possible that polypeptide translocation by the RC starts at the Gly-rich region and proceeds in only one direction owing to the presence of the tightly folded N-terminal domain. Or the RC may start translocation at the C-terminus and stop when the ATPases encounter the Gly-rich region. Insertion of a Gly–Ala stretch as small as seven residues into the C-terminal degron of ODC is sufficient to prevent complete destruction of ODC leading instead to partial processing of the enzyme. This has led Coffino and colleagues to suggest that the Gly–Ala stretch impairs substrate transfer by the RC ATPases [124]. Another example of partial processing involves SPT23, a yeast protein embedded in the endoplasmic reticulum membrane [125]. SPT23 controls unsaturated fatty acid levels, and membrane fluidity regulates 26S proteasomal generation of a freely diffusible transcription factor from the SPT23 precursor. Partial processing may be a more widespread regulatory mechanism than is currently thought.

9.6
Proteasome Biogenesis

9.6.1
Subunit Synthesis

The synthesis of proteasome subunits is markedly affected by proteasome function. For example, inhibition of proteasome activity by lactacystin induces coordinate expression of both RC and 20S proteasome subunits [66]. Likewise, impaired synthesis of a given RC subunit results in over-expression of all RC subunits [126, 127]. Proteasome subunit synthesis in yeast is controlled by Rpn4p, a short-lived positive transcription factor that binds PACE elements upstream of proteasome genes [128]. Rpn4p is a substrate of the 26S proteasome suggesting that the transcription factor functions in a feedback loop in which proteasome activity limits its concentration thereby regulating proteasome levels [129]. To date an Rpn4-like factor has not been identified in higher eukaryotes, but such a factor is likely to exist.

9.6.2
Biogenesis of the 20S Proteasome

Proteasome β-subunits are synthesized with N-terminal extensions and are inactive because a free N-terminal threonine is required for peptide-bond hydrolysis [130]. The precursor β-subunits assemble with α-subunits to form half proteasomes com-

posed of one α- and one β-ring, which then dimerize to form the 20S particle [131]. The N-terminal extensions are removed thereby generating a new unblocked N-terminal threonine in the catalytically active β-subunits. A small accessory protein called Ump1 in yeast or proteassemblin in mammalian cells assists in the final assembly of the 20S proteasome [132]. Interestingly Ump1/POMP is apparently trapped in the proteasome's central chamber and degraded upon maturation of the enzyme [133].

9.6.3 Biogenesis of the RC

Assembly pathways for the RC are virtually unknown. As mentioned above, the ATPases interact with one another and complexes containing all six S4 subfamily members have been observed following *in vitro* synthesis. Impaired synthesis of the yeast lid subunit Rpn6 results in the absence of the entire lid [134], so presumably lid and base subcomplexes assemble independently and associate in the final stages of RC formation cells. In mammalian cells, 26S proteasomes assemble from preformed regulatory complexes and 20S proteasomes [135].

9.6.4 Post-translational Modification of Proteasome Subunits

Proteasome and RC subunits are subjected to a variety of post-translational modifications including phosphorylation, acetylation, myristoylation, and even *O*-glycosylation [136–140]. In yeast all seven α-subunits are acetylated as well as two β-subunits. Since acetylation of the N-terminal threonine in an active β-subunit would poison catalysis, it has been suggested that the propeptide extensions function to prevent acetylation [130]. Three members of the S4 ATPase subfamily (S4, S6, and S10b) and two 20S α-subunits (C8 and C9) are known to be phosphorylated. Phosphorylation appears to be particularly important for 26S proteasome assembly and stability. The kinase inhibitor staurosporine reduces 26S proteasome levels in mouse lymphoma cells [135] and interferon γ results in reduced phosphorylation of 20S proteasome α-subunits and decreased 26S proteasome levels [141].

9.6.5 Assembly of the 26S Proteasome

The RC and 20S proteasome associate to form the 26S proteasome in the presence of ATP [63]. Comparison of the cross-linking patterns of RC and assembled 26S proteasomes indicates that this association is accompanied by subunit rearrangement [142]. In yeast two proteins play a special role in 26S proteasome assembly or stability. Nob1p is a nuclear protein required for biogenesis of the 26S proteasome and is degraded following assembly of the 26S enzyme. Thus Nob1p suffers the same fate as Ump1 does following 20S maturation [143]. In fission yeast the protein Yin6 regulates both the nuclear localization and the stability of the 26S

proteasome [144]. In budding yeast the chaperone Hsp90 also plays a role in the assembly and maintenance of yeast 26S proteasomes, since functional loss of Hsp90 results in 26S proteasome dissociation, indeed even dissociation of the lid subcomplex [145]. The 26S proteasome also dissociates into RC and 20S proteasomes when budding yeast is subjected to long periods of starvation [146].

9.7 Proteasome Activators

In addition to the RC there are two protein complexes, REG$\alpha\beta$ and REGγ, and a single polypeptide chain, PA200, that bind the 20S proteasome and stimulate peptide hydrolysis but not protein degradation. Like the RC, proteasome activators bind the ends of the 20S proteasome and, importantly, they can form mixed or hybrid 26S proteasomes in which one end of the 20S proteasome is associated with a 19S RC and the other is bound to a proteasome activator [147–150]. This latter property raises the possibility that proteasome activators serve to localize the 26S proteasome within eukaryotic cells.

9.7.1 REGs or PA28s

9.7.1.1 REGs

There are three distinct REG subunits called $\alpha\beta\gamma$ [151, 152]. REGsα and β form donut-shaped heteroheptamers found principally in the cytoplasm, whereas REGγ forms a homoheptamer located in the nucleus. REG$\alpha\beta$ is abundantly expressed in immune tissues, while REGγ expression is highest in brain. The REGs also differ in their activation properties. REG$\alpha\beta$ activates all three proteasome active sites; REGγ only activates the trypsin-like subunit. There is reasonably solid evidence that REG$\alpha\beta$ plays a role in Class I antigen presentation [153], but we have little knowledge concerning REGγ function since REGγ knockout mice have almost no phenotype [154]. The crystal structure of REGα reveals that the seven subunits form a donut-shaped structure with a central aqueous channel, and the structure of a REG–proteasome complex provides important insight into the mechanism by which REGα activates the proteasome. The carboxyl tail on each REG subunit fits into a corresponding cavity on the α-ring of the proteasome. Loops on the REG subunits displace N-terminal strands on several proteasome α-subunits reorienting them upward into the aqueous channel of the REG heptamer thereby opening a continuous channel from the exterior solvent to the proteasome central chamber [155].

9.7.1.2 PA200

A new proteasome activator called PA200 was recently purified from bovine testis [156]. Human PA200 is a nuclear protein of 1843 amino acids that activates all three catalytic subunits with some preference for the PGPH active site. Homologs

of PA200 are present in budding yeast, worms, and plants. A single chain of PA200 can bind each end of the proteasome and, when bound, PA200 molecules look like volcanos in negatively stained EM images. PA200 is thought to play a role in DNA repair, perhaps by recruiting proteasomes to DNA double-strand breaks.

9.7.1.3 Hybrid Proteasomes

As the α-rings at each end of the 20S proteasome are equivalent, the 20S proteasome is capable of binding two RCs, two PA28s, two PA200s, or combinations of these components. In fact 20S proteasomes simultaneously bound to RC and PA28 or PA200 have been observed, and are called hybrid proteasomes [147, 148, 150]. In HeLa cells the levels of hybrid proteasomes containing PA28 at one end and an RC at the other are two-fold higher than 26S proteasomes capped at both ends by 19S RCs [149]. Hybrid 26S proteasomes containing PA200 appear to be much less abundant in HeLa cells [156].

9.7.2 ECM29

Another proteasome-associated protein, called Ecm29, has been identified in several proteomic screens [157, 158]. Ecm29p clearly associates with 26S proteasomes; whether it activates proteasomal peptide hydrolysis is currently unknown. Ecm29p is reported to stabilize the yeast 26S proteasome by clamping the RC to the 20S cylinder [159]. However, in mammalian cells Ecm29p is found mainly associated to secretory and endocytic organelles, a location suggesting a role in secretion rather than 26S proteasome stability. Moreover, the levels of Ecm29p vary markedly among mouse organs, so if mammalian Ecm29p serves as a clamp, some tissues either do not require a clamped 26S proteasome or other proteins function as RC-20S clamps.

9.8 Protein Inhibitors of the Proteasome

A number of proteins have been found to suppress proteolysis by the proteasome. One of these is PI31 [160, 161]; another is the abundant cytosolic chaperone Hsp90 [162], and a third is a proline/arginine-rich 39-residue peptide called PR39 [163]. Both PI31 and Hsp90 may affect how the proteasome functions in Class I antigen presentation. PI31 is a 30-kDa proline-rich protein that inhibits peptide hydrolysis by the 20S proteasome and can block activation by both RC and REG$\alpha\beta$. Although surveys of various cell lines show PI31 to be considerably less abundant than RC or REG$\alpha\beta$, when over-expressed, PI31 is reported to inhibit Class I antigen presentation by interfering with the assembly of immuno-proteasomes [161]. A number of studies have shown that Hsp90 can bind the 20S proteasome and inhibit its chymotrypsin-like and PGPH activities. Interestingly inhibition by Hsp90 is observed with constitutive but not with immuno-proteasomes, a finding consis-

tent with proposals that Hsp90 shuttles immuno-proteasome-generated peptides to the endoplasmic reticulum for Class I presentation [164]. PR39 was originally isolated from bone marrow as a factor able to induce angiogenesis and inhibit inflammation. Two hybrid screens showed that PR39 binds the 20S proteasome. Apparently PR39 affects angiogenesis and inflammation by inhibiting respectively the degradation of HIF1 or IκBα, the latter being an inhibitor of NFκB. Finally HIV's Tat protein inhibits the 20S proteasome's peptidase activity [165]. Tat also competes with REGαβ for proteasome binding and, by doing so, Tat can inhibit Class I presentation of certain epitopes [166].

9.9 Physiological Aspects

9.9.1 Tissue and Subcellular Distribution of Proteasomes

Proteasomes are found in all organs of higher eukaryotes, but the degree to which the composition of proteasomes and its activators varies among tissues is largely unexplored territory. Proteasomes are very abundant in testis which contains almost five-fold more 20S subunits than skeletal muscle [167]. At the cellular level there are about 800 000 proteasomes in a HeLa cell and roughly 20 000 proteasomes in a yeast cell. At the subcellular level, 26S proteasomes are present in cytosol and nucleus where they appear to be freely diffusible [168, 169]. They are not usually found in the nucleolus [170] and have not been reported in membrane-bound organelles other than the nucleus. When large amounts of misfolded proteins are synthesized by a cell, the aberrant polypeptides often accumulate around the centrosome in what are called "aggresomes" [171]. Under these conditions 26S proteasomes, chaperones, and proteasome activators also redistribute to the aggresomes, presumably to refold and/or degrade the misfolded polypeptides [172].

9.9.2 Physiological Importance

Deletion of yeast genes encoding 20S proteasome and 19S RC subunits is usually lethal, indicating that the 26S proteasome is required for eukaryotic cell viability. Known substrates of the 26S proteasome include transcription factors, cell-cycle regulators, protein kinases, etc., essentially most of the cell's important regulatory proteins. Even proteins secreted into the endoplasmic reticulum are returned to the cytosol for degradation by the 26S proteasome [173, 174]. Given the scope of its substrates it is hardly surprising that in higher eukaryotes the ubiquitin–proteasome system contributes to the regulation of a vast array of physiological processes ranging from cell-cycle traverse to circadian rhythms to learning. Discussion of these fascinating regulatory mechanisms is covered in other chapters of this volume.

Summary

The 20S proteasome was discovered in 1980 and the 26S proteasome six years later. Research since the mid-1980s has made it abundantly clear that the ubiquitin–proteasome system is of central importance in eukaryotic cell physiology. Yet there is much more to discover. A crystal structure of the 19S RC, or better still the 26S proteasome, would surely provide insight into the mechanism by which the 26S proteasome degrades its substrates. How the 26S proteasome is itself regulated and the extent to which proteasomal components vary among tissues in higher eukaryotes are other important unresolved problems. Hopefully, these and other unanswered questions will spark further interest in the proteasome among readers of this book on Intracellular Proteolysis.

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10
Molecular Machines for Protein Degradation

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10.1
Introduction

The action of intracellular proteolytic enzymes is tightly controlled to avoid destruction of properly folded and functional proteins essential for cell viability and to restrict their activity towards sick molecules or/and those marked for destruction. The four (five) proteases discussed in this chapter display different regulatory mechanisms but show sequestration of their active sites inside molecular cages as a common structural principle, albeit being assembled from different building blocks in different shapes and with varying symmetries. The chapter focuses on structural, functional, and mutational studies from our laboratory. We are aware of other cage-forming proteases and their regulatory components that have been structurally characterized and which are mentioned in brief later in the context of our studies. The chapter is arranged in four main sections focused on the proteases HslVU, proteasome, tricorn (DPPIV), and DegP.

10.2
The ATP-dependent Protease HslVU

ATP-dependent proteases are complex proteolytic machines. They are present in eubacteria, archaebacteria, in eukaryotic organelles and, as the 20S or 26S proteasome, in the eukaryotic cytosol and nucleoplasm. The activators of all known ATP-dependent proteases are related. They all contain an AAA(+) ATPase domain as a module (Neuwald et al. 1999) and are thought to assemble into hexameric particles or, in the case of 26S proteasomes, are present in six variants in the 19S activators (Glickman et al. 1999). Like the ATPases, the proteolytic components of the ATP-dependent proteases form higher order complexes, but unlike for the ATPases, the symmetry of the protease assemblies varies, and the folds of the subunits need not be related. ClpP is a serine protease, FtsH a metalloprotease, and HslV and the proteasomes from archaebacteria and eubacteria are threonine proteases.

Although extensive biochemical data on both the bacterial and eukaryotic ATP-dependent proteases are available, the characterization of these proteolytic machines at atomic resolution has proven difficult, because of both the large size of these complexes and their lability to proteolysis and dissociation. No structural data at all are currently available for Lon and the mitochondrial ATP-dependent proteases. In the case of the cytosolic, membrane-integrated bacterial protease FtsH, atomic resolution data are available only for the ATPase domain (Krzywda et al. 2002; Niwa et al. 2002). In contrast, the ATP-dependent activators of the ClpAP and ClpXP proteolytic machines have so far resisted crystallization. Atomic resolution data are available only for the proteolytic component ClpP (Wang et al. 1997), and separately for a ClpX monomer (Kim and Kim 2003) and a ClpA monomer (Guo et al. 2002b).

The bacterial protease HslVU is unique in two respects: at present, it is the only ATP-dependent protease to have atomic coordinates of the full complex determined; secondly, and in contrast to all other bacterial ATP-dependent proteases, it contains a proteolytic core that is related to the 20S proteolytic core of archaebacterial and eukaryotic proteasomes. The following sections summarize our understanding of HslVU biochemistry, crystallography, and enzymology and end with some speculation on the implications of these results for other ATP-dependent proteases.

10.2.1 HslVU Physiology and Biochemistry

In *E. coli* and most, but not all, other bacteria, the HslU (ATPase) and HslV (protease) genes are found in one operon under the control of a heat-shock promoter. The operon was first found (Chuang and Blattner 1993) and sequenced (Chuang et al. 1993) in the course of a search for new heat-shock genes. It was later independently isolated again in screens for proteins that could down-regulate the heat-shock response (Missiakis et al. 1996) and for suppressors of the SOS-mediated inhibition of cell division in *E. coli* (Khattar 1997). The observed biological responses in HslVU over-expression or deletion strains result from a decrease or increase in the steady levels of HslVU substrates (Kanemori et al. 1997). HslVU, itself a heat-shock protein, affects the heat-shock response by degradation of the heat-shock factor $\sigma 32$ (Missiakis et al. 1996; Kanemori et al. 1997) and the SOS response via the degradation of the cell-division inhibitor SulA (Kanemori et al. 1999; Seong et al. 1999).

The physiological role of HslVU seems to be limited, probably because of overlapping substrate specificity with other ATP-dependent proteases in bacteria. The *E. coli* HslVU deletion strain has no phenotype at standard growth temperature, and it appears that HslVU is required for normal growth only at very high temperatures (Kanemori et al. 1997). According to the protease database MEROPS, some bacterial species appear to lack an HslVU-type peptidase altogether (Rawlings et al. 2002). Therefore, it came as a surprise that some HslV and HslU homologs were recently found in primordial eukaryotes where they appear to be simultaneously present with genuine 20S proteasomes (Couvreur et al. 2002).

Low expression levels and the lability of the HslVU complex make work with proteins from wild-type strains difficult. Gratifyingly, the active protease can be reconstituted *in vitro* from over-expressed and purified components (Rohrwild et al. 1996). It requires ATP for the degradation of folded substrates and ATP or some of its analogs for the purification of small chromogenic peptides. As expected, ATP-hydrolysis and proteolysis activities are mutually dependent (Seol et al. 1997). In addition, the peptidase activity was found to depend in complex ways on the presence of various cations, especially K^+ in the buffers (Huang and Goldberg 1997).

10.2.2 HslV Peptidase

On the sequence level, HslV shows sequence similarity with the β-subunits of archaebacterial and eukaryotic proteasomes, a fact that was immediately noticed when the *E. coli* gene was sequenced (Chuang et al. 1993) and was later shown to extend to other related eubacterial sequences (Lupas et al. 1994). Electron microscopy (EM) of recombinant HslV subsequently suggested that the particle formed a dimer of hexamers that appeared to enclose only one central cavity without antechambers as in proteasomes (Rohrwild et al. 1997). The unexpected six-fold symmetry of HslV and the similarity in subunit fold with eukaryotic proteasomes were subsequently confirmed by X-ray crystallography (Bochtler et al. 1997). The crystallographic data also showed that the contracted ring compared to proteasomes resulted from small changes to the subunit–subunit interface, not from an entirely new mode of oligomerization (Bochtler et al. 1997) (see Figure 10.1).

All 12 active sites of HslV are located on the inner walls of the hollow particle. In the *E. coli* particle, each active site has neighboring active sites 28 Å away on the same ring and 22 Å and 26 Å away on the opposite ring. The environment of the nucleophilic Thr1 looks similar to that in proteasomes, and the presence of a (putatively protonated) lysine residue near the active site probably helps to lower the pK_a of the N-terminal α-amino group so that it is present in the unprotonated form, which can act as the general base to accept a proton from Thr1.

Since the determination of the HslV crystal structure, two additional crystal structures from other species have been determined. The highest resolution structure available to date is the crystal structure of the *Haemophilus influenzae* enzyme (Sousa and McKay 2001). Intriguingly, this structure showed the presence of cation-binding sites near the active centers (Sousa and McKay 2001), a finding that could subsequently be confirmed for the *Thermotoga maritima* enzyme (Song et al. 2003) and that explains, at least in qualitative terms, the dependence of HslVU activity on various cations in solution. Overall, the crystal structures of the enzymes from *H. influenzae* (Sousa and McKay 2001) and *T. maritima* (Song et al. 2003) are very similar to the original structure of the *E. coli* enzyme. Therefore, it came as a surprise that the HslV homolog known as CodW from *Bacillus subtilis* behaves rather differently. Although the enzyme contains a threonine residue that aligns with the active site threonine of the *E. coli*, *H. influenzae*, and *T. maritima* enzymes, it does not contain the glycine at the C-terminus of the profragment

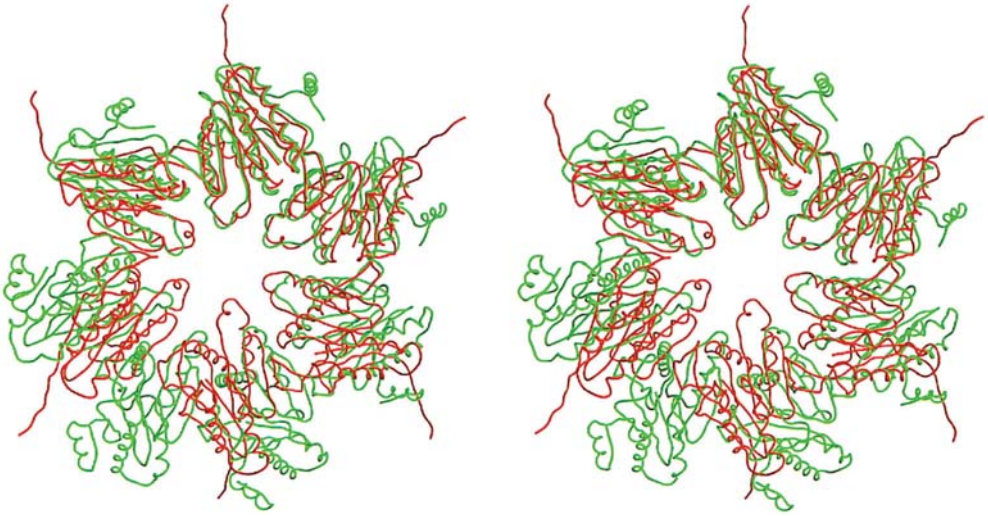


Fig. 10.1. *E. coli* HslV *vs. T. acidophilum* proteasome. Superposition of one hexameric ring of *E. coli* HslV (red) and of one heptameric ring of *T. acidophilum* proteosome β-subunits (green) in stereo representation. The subunits at the "top" of the ring have been overlayed optimally. Note that the "tails" of the HslV subunits that point radially outwards are histidine tags and thus cloning artefacts.

that is believed to be required for efficient autocatalytic processing, and indeed the polypeptide chain is processed five residues upstream of the conserved threonine that is the active-site nucleophile in other species to expose an N-terminal serine residue (Kang et al. 2001). Whether this implies that the serine is in the spatial position normally filled by threonine, implying a discrepancy between the sequence-based and structure-based alignments, or whether it means that the accessory catalytic residues are either dispensable or anchored elsewhere on the sequence is currently not clear.

10.2.3 HslU ATPase

Based on the sequence, HslU can be easily classified as an ATPase by the presence of conventional Walker A (phosphate binding loop or P-loop) and Walker B (magnesium binding) motifs. Beyond this simple classification, two competing models for HslU were proposed, classifying the enzyme either as a PDZ-domain containing ATPase (Levchenko et al. 1997) or alternatively as a AAA(+)-type ATPase (Neuwald et al. 1999). The crystal structure settled the issue in favour of the AAA(+) model (Bochtler et al. 2000). AAA(+)-ATPases consist essentially of two structural domains that are connected through a short linker. A nucleotide binds at the interface of the two domains. As first observed with HslU, the presence or absence of a nucleotide induces different relative orientations between the two domains (Boch-

tler et al. 2000). With the availability of many different nucleotide states of HslU, the model was later refined to include a dependence on the state of hydrolysis of the nucleotide (Wang et al. 2001b).

The nucleotide is in a strategic position both at the interface of the N- and C-domains of one subunit and at the interface of adjacent subunits. A combination of mostly conserved residues from the two subunits around the nucleotide creates a highly polar environment (Bochtler et al. 2000). Two arginine residues have attracted particular attention: R393 of *E. coli* HslU is thought to act as the ''sensor'' that transmits information on the presence or absence of nucleotide, and possibly on its identity, to the C-domain and thus controls the relative orientation of N- and C-domains in HslU. Another conserved arginine residue, R325, is anchored on the subunit that makes fewer contacts with the nucleotide and is the homolog of the proposed ''arginine finger'' in FtsH (Karata et al. 1999). Although the term ''arginine finger'' (taken from small GTP-binding proteins Ras and Rho) implies a direct catalytic role for this residue, its distance from the nucleotide phosphates argues more for an indirect role. A similar conclusion has since been reached for ClpA (Guo et al. 2002b). Experimentally, mutation of either of the two arginine residues abolishes all ATP-dependent proteolysis activity (Song et al. 2000). Loss of subunit interactions plays a major role in the loss of function: The ''arginine sensor'' mutant R325E is fully and the ''arginine finger'' mutant R393 is partially dissociated in gel-filtration experiments in the presence of salt (Song et al. 2000).

A very complex picture has emerged from biochemical and crystallographic studies designed to characterize the substrate-binding sites in HslU. An essential role for the C-terminus of HslU was first suggested on the basis of experimental studies that were designed based on the prediction of PDZ-like domains at the C-terminus of HslU (Levchenko et al. 1997). Although the prediction of PDZ-like domains later turned out to be in error, the conclusion about the role of the C-terminus of HslU in substrate recognition was later corroborated with the definition of a biochemically defined ***s***ensor and ***s***ubstrate ***d***iscrimination domain (SSD) (Smith et al. 1999) that is also present in other AAA(+) ATPases and was suggested to act as the ''hook'' for substrates (Wickner and Maurizi 1999). When the crystal structure of HslU became available, the SSD domain turned out to coincide with its C-domain. This finding is remarkable and not fully understood, since in all crystal structures of HslU the C-domain primarily mediates oligomerization contacts between HslU subunits. Its solvent-accessible regions are found far on the periphery of the HslU ring, far outside the cavity that is formed by the protruding I-domains (see Figure 10.2). From the crystal structure (Bochtler et al. 2000), it would appear likely that the protruding I-domains rather than the SSD domains act as the ''hook'', although the ill-defined tertiary structure of the I-domains makes specific interactions unlikely (see Figure 10.2). Consistent with this model, it was found experimentally that the I-domains are essential for the degradation of the folded substrate MBP-sulA (Song et al. 2000). A recent two-hybrid study is consistent with both points of view. It confirms the essential role of the C-domain (SSD-domain) in oligomerization, but also supports a role for the I-domain and the SSD-domain in substrate degradation (Lee et al. 2003). Presumably, if sub-

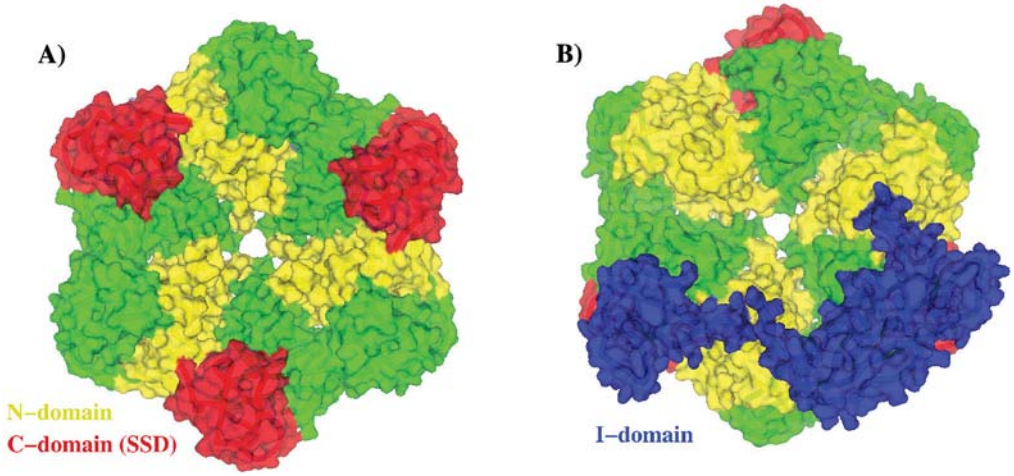


Fig. 10.2. HslU surface colored according to domain. (A) View along the six-fold axis, seen from the side opposite to the I-domains. (B) View along the six-fold axis, seen from the side of the I-domains. Every second subunit of the ring is colored according to domain (N-domain yellow, I-domain blue, C-domain or SSD-domain red), the other subunits are colored in green. The diagram is based on the trigonal crystals of *E. coli* HslU that contain nucleotide in every other subunit. This asymmetry and crystallographic packing effects are responsible for the broken six-fold symmetry of the I-domains. Note that the I-domains of three subunits at the top of the figure have been cut away in (B) to allow a view on the globular N- and C-domains.

strates are translocated through the central pore in HslU as EM data suggest for ClpXP (Ortega et al. 2000), both the I-domains and the globular part of the ring would come into contact with substrate during substrate translocation, although the location of the C-domains (SSD-domains) on the periphery of the HslU ring then still needs to be reconciled with this model.

The precise mode of recognition of substrates is even less clear for CodX, the HslU homolog from *B. subtilis*. In the absence of detergent, the I-domains of two hexameric CodX rings contact each other, leading to a head-to-head stacking of CodX rings and presumably the formation of a central cavity loosely surrounded by I-domains. As the dimer of CodX rings can associate with CodW protease on either side, repetitive, chain-type structures with alternating double rings of the peptidase CodW and the ATPase CodX can be formed (Kang et al. 2003). The physiological significance of these high molecular weight assemblies is currently not clear.

10.2.4
The HslVU–Protease Complex

Over the years, a key theme in ATP-dependent proteolysis has been the issue of ''symmetry-matched'' *vs.* ''symmetry-mismatched'' complexes. In the light of the

clearly established symmetry mismatch of the ClpAP (Kessel et al. 1995) and ClpXP (Grimaud et al. 1998) complexes, the very clear EM data on the six-fold symmetry of HslV (Kessel et al. 1996; Rohrwild et al. 1997) and reports about a predominant species of HslU with six-fold symmetry (Kessel et al. 1996; Rohrwild et al. 1997) came as a surprise because they implied that a ''ratcheting mechanism'' of ATP-dependent proteolysis, if it existed at all, could not be operating in the HslVU system. This conclusion has since been confirmed by all HslU and HslVU crystal structures (Bochtler et al. 2000; Sousa et al. 2000; Wang et al. 2001a; Kwon et al. 2003). In all cases, HslU is hexameric and matches the oligomerization state of HslV. For the first crystal structure of an HslU–HslV co-crystal, a controversial I-domain-mediated contact between HslU and HslV was reported (Bochtler et al. 2001; Wang 2001; 2003). The contact was suspicious from the very beginning because of poor contact area, but seemed compatible with the known low affinity between HslV and HslU and appeared to explain how the symmetry mismatched ClpXP and ClpAP complexes could be formed. Although a crystallographic reinterpretation of our original data that attributed this docking mode to overlooked twinning (Wang 2001; 2003) turned out to be itself in error (Bochtler et al. 2001), it is now clear from the combined results of cryoelectron microscopy (Ishikawa et al. 2000), small-angle scattering (Sousa et al. 2000) and several additional crystal structures of the complex (Sousa et al. 2000; Wang et al. 2001a) that the physiological mode of interaction between HslU and HslV is with HslU I-domains distal to HslV (see Figure 10.3).

10.2.4.1 **Allosteric Activation**

In the absence of a nucleotide, HslVU has residual activity at best, but the presence of several non-hydrolyzable ATP-analogs is sufficient to stimulate HslVU-driven proteolysis activity against substrates that do not require unfolding, suggesting an allosteric effect of nucleotide on HslU and via HslU on HslV. This was further corroborated by the observation that a peptide vinyl sulfone formed a covalent complex with HslV only in the presence of HslU and a nucleotide (Bogyo et al. 1997). The details of this allosteric mechanism emerged from the crystal structure of HslVU from *H. influenzae*. In this case, but not in other crystal structures of the HslVU complex, the normally buried C-termini of HslU distend and insert into active-site clefts in HslV to reach out almost to the HslV active centers (Sousa et al. 2000) (see Figure 10.4). The crystal structure of HslVU in complex with a peptide vinyl sulfone inhibitor (Sousa et al. 2002) and two independent biochemical studies (Ramachandran et al. 2002; Seong et al. 2002) that demonstrated the activatory properties of the C-terminal tails of HslU further corroborated this mechanism. In the light of these data, it is remarkable that wild-type HslU in the presence of ADP does not act as an activator for HslV, not even against unfolded or chromogenic substrates. A possible, but experimentally untested explanation could be that the C-termini of HslU are available for HslV binding only in the presence of activatory nucleotides.

Whatever the details of the allosteric activation mechanism, it is already clear

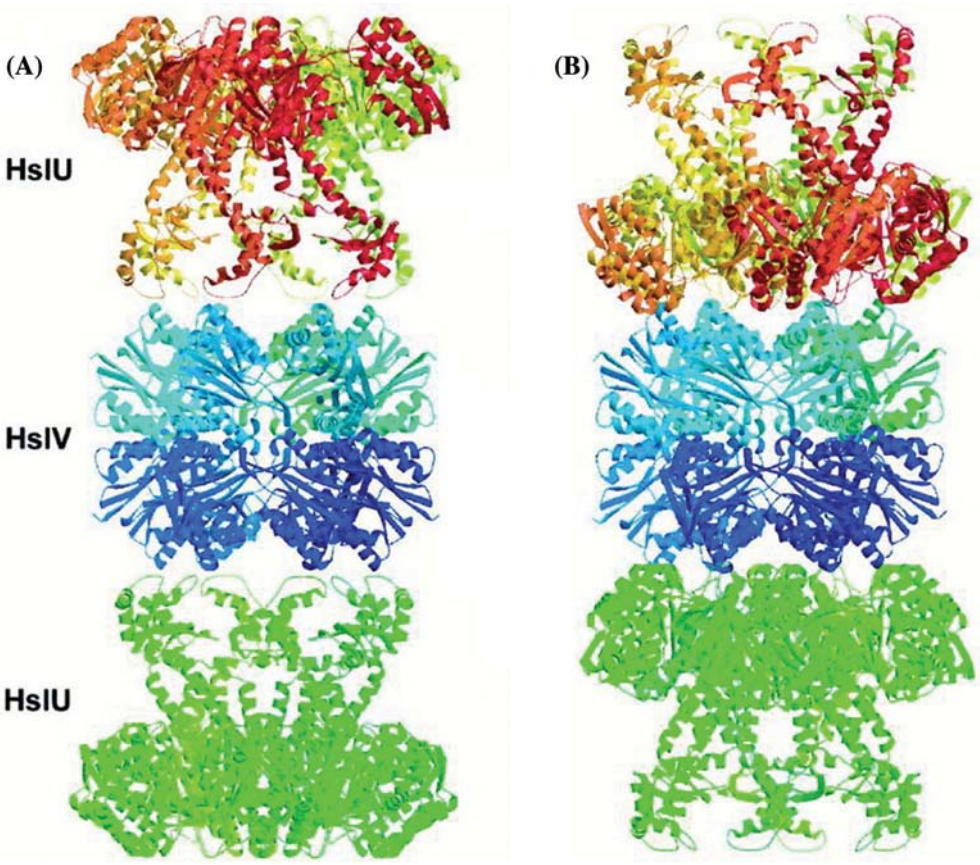


Fig. 10.3. HslVU complex. Originally reported (A) and physiologically relevant (B) docking mode between HslV and HslU. In the physiologically relevant docking mode, the I-domains of HslU point away from the HslV.

that HslU affects primarily the conformation of HslV active sites and not the accessibility of the HslV proteolytic chamber. Two independent lines of *in vitro* evidence support this conclusion. Firstly, an HslV mutant with a widened entrance channel does not show increased proteolytic activity in the absence of HslU, although it can still be activated like wild-type HslV by the presence of HslU (Ramachandran et al. 2002). Secondly, the crystal structure of the *H. influenzae* asymmetric HslVU protease in complex with an inhibitory peptide vinyl sulfone has the inhibitor bound only in HslV subunits that are in contact with HslU (Kwon et al. 2003), strongly arguing against accessibility of the proteolytic chamber as the rate-limiting factor at least under the experimental conditions.

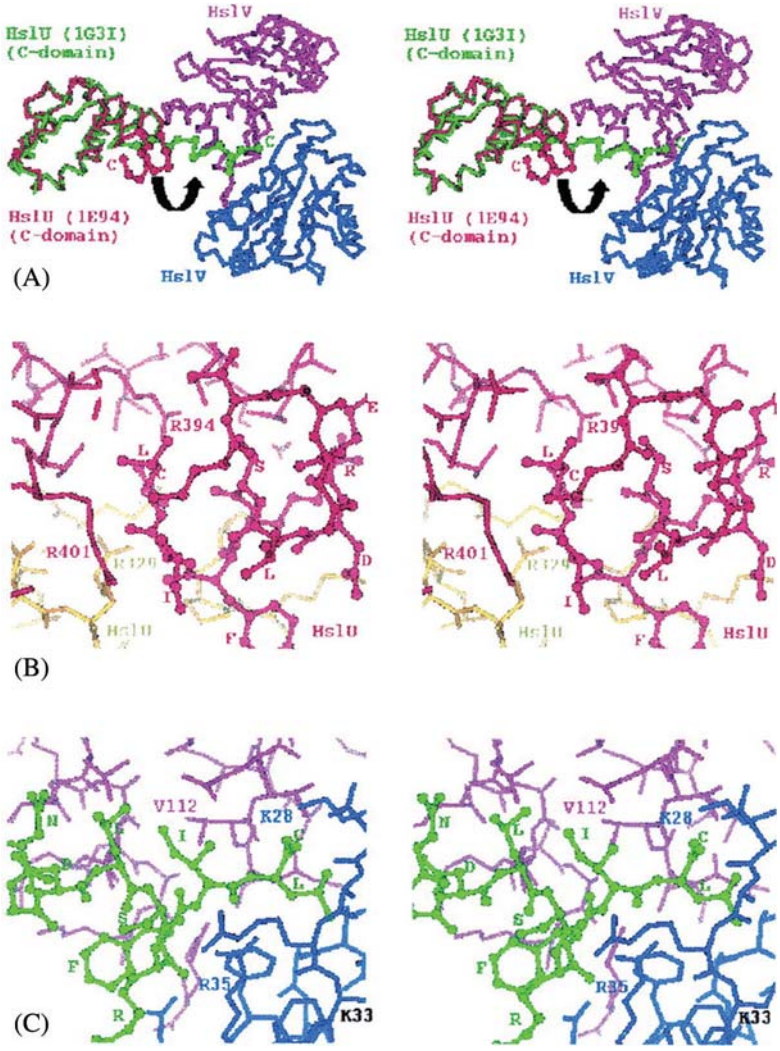


Fig. 10.4. HslVU activation mechanism. (A) Stereo view (C^{α}-trace) of the superposition of the C-domain of an HslU subunit (red) from the original *E. coli* HslVU complex onto that of the *H. influenzae* HslU subunit (green) from its complex Two HslV subunits (pink and blue) from the *H. influenzae* complex are also shown to illustrate the binding of the C-terminal segment of *H. influenzae* HslU to the pocket between the HslV subunits (indicated also by a black curved arrow). (B) Stereo view of the close-up of the C-terminal residues of an *E. coli* HslU subunit (red) from the complex. The carboxylate of the terminal leucine residue forms salt bridges with R394 of the same subunit and with R329 of an adjacent (yellow) HslU subunit that is not illustrated in (A) for clarity. (C) Stereo view of the close-up of the C-terminal residues of an HslU subunit from the *H. influenzae* HslVU complex.

10.2.5
A Comparison of HslVU with ClpXP and ClpAP

The protease core particles HslV and ClpP are assembled from subunits of entirely different fold and catalytic mechanism. ClpP is a serine protease that belongs to the crotonase superfamily of enzymes, a large class of enzymes that catalyze a variety of chemical reactions that all require the stabilization of an intermediate by an oxyanion hole (Babbitt and Gerlt 1997). HslV belongs to the family of Ntn-hydrolases that share the fold and use the N-terminal residue as the nucleophile (Brannigan et al. 1995). Both ClpP (Wang et al. 1997) and HslV subunits assemble into large oligomers that enclose a central proteolytic chamber, although the symmetry is different, since HslV is a dimer of hexamers and ClpP is a dimer of heptamers.

In contrast to the protease components, which have varying symmetry, all known Clp ATPases are assembled from six identical subunits. These subunits contain either one (HslU and ClpX) or two (ClpA) copies of the AAA(+) module. In addition, the Clp ATPases contain additional domains that are unique for each ATPase. In HslU, a mostly helical I-domain is inserted into the AAA(+) module. ClpX contains an N-terminal domain that was shown to bind zinc (Banecki et al. 2001) and act as a dimerization module (Donaldson et al. 2003). Based on the latter result, a model of ClpX as a trimer of dimers was proposed, with N- and C-domains of ClpX forming a regular hexamer and the zinc-binding modules pairing into dimers (Donaldson et al. 2003). In this context, it is remarkable that freshly isolated HslU behaves as a hexamer, but migrates with the apparent molecular weight of a dimer or trimer in gel filtration after a freeze–thaw cycle (Bochtler 1999). Like ClpX, ClpA contains a non-AAA(+) domain at its N-terminus, and like the N-terminal domain in ClpX, this domain also has the capability to bind zinc (Guo et al. 2002a). However, unlike the N-domain of ClpX, the N-domain of ClpA is almost entirely helical and consists of two four-helix tandem motifs.

In both ClpA and ClpX, the N-terminal non-AAA(+) motifs serve as ''docking modules'' for accessory proteolysis factors that modulate or change the activity of the proteolytic complex itself (Dougan et al. 2002a). The N-domain of ClpX interacts specifically with the adapter protein SspB that stimulates the degradation of SsrA-tagged proteins (Dougan et al. 2003). The tag is jointly recognized by the SspB-ClpX complex, where SspB interacts with the N-terminal and central region of the SsrA tag and leaves the C-terminal region for interaction with ClpX (Levchenko et al. 2003; Song and Eck 2003). The C-terminal region of SspB shares considerable homology with the corresponding region in RssB, another ClpX adapter protein (Dougan et al. 2003). It appears that RssB promotes the degradation of a specific substrate, namely a subunit of RNA polymerase known as σ^S (Zhou et al. 2001). It has been shown biochemically that in this case again the adapter protein and the ATPase recognize distinct sites in the substrate (Studemann et al. 2003). ClpA has its own adapter protein, ClpS. At least *in vitro*, ClpS switches ClpAP activity away from SsrA tagged towards heat-aggregated proteins (Dougan et al. 2002b). The independent crystal structures of ClpS in complex with the N-domain

of ClpA are available (Guo et al. 2002a; Zeth et al. 2002). They explain the specificity of ClpS for ClpA over other related Clp proteins, especially ClpB (Zeth et al. 2002). HslU lacks a domain upstream of the AAA(+) module. Consistent with this, no adapter proteins for HslU have been found so far, to the best of our knowledge.

Mechanistically, there are important differences between the protease activation mechanisms of ClpXP/AP and HslVU. Most importantly, HslVU is a symmetry-matched proteolytic machine. In contrast, the definitive seven-fold symmetry of the ClpP protease and the well-established six-fold symmetry of ClpA and ClpX imply that ClpXP is a symmetry-mismatched system. It is hard to imagine how such an arrangement would be compatible with an HslVU-style activation mechanism with insertion of the C-termini of all activator subunits into clefts in protease. Moreover, the C-termini of ClpX particles from various species are very poorly conserved, arguing against their involvement in any allosteric activation mechanism (Ramachandran et al. 2002). Experimental evidence implicates an internal loop of ClpX in ClpP binding (Kim et al. 2001). This loop is required for ClpXP proteolytic activity and may well be the functional equivalent of the C-terminus of HslU. If so, then the symmetry mismatch in ClpXP would suggest that only a subset of ClpX loops could insert into ClpP clefts at any given time.

10.2.6
HslVU Peptidase as a Model for the Eukaryotic 26S Proteasome?

On the sequence level, HslV shows sequence similarity with the β-subunits of archaebacterial and eukaryotic proteasomes. The crystal structure of *E. coli* HslV confirmed that individual subunits share the Ntn-hydrolase fold with Thr1 at the N-terminus as the nucleophile, just as in proteasomes. Despite these similarities, there are substantial differences between bacterial HslVU and archaebacterial and eukaryotic 20S proteasomes. In contrast to HslVU, 20S proteasomes are assembled from four rings of seven subunits each, that build up a central proteolytic chamber and two flanking antechambers.

The essential role of the C-terminus of HslU has its direct counterpart in the essential role of the C-terminus of the ATP-dependent proteasome activator PA28 (Wilk and Chen 1997). A complex of the yeast 20S proteasome with PA26, the *Trypanosoma brucei* homolog of PA28 has been crystallized and shows that the C-termini of PA28 insert into clefts in the 20S core particle (Whitby et al. 2000), leading to an opening of the gates in the antechambers. So far, there is no evidence for allosteric activation in the 20S proteasome–PA26 complex or the 26S proteasome, where channel ''gating'' appears to be important as discussed below in Section 10.3 on the yeast 20S proteaseome.

Currently, high-resolution EM image reconstructions for the 26S proteasome (Walz et al. 1998), but no atomic-resolution crystallographic data are available for any complex of 20S proteasomes with ATP-dependent activators. The expected assembly of PAN, the archaebacterial AAA(+) activator of proteasomes (Zwickl et al. 1999) into hexamers suggests a symmetry-mismatched complex in archaebacteria.

The ATP-dependent proteasome activators of eukaryotic proteasomes known as PA700 and the 19S cap also contain six AAA(+) ATPases in a subcomplex of the 19S complex known as the "base" (Glickman et al. 1999). *A priori*, one would expect the six ATPases to form a ring with pseudo six-fold symmetry similar to the six-fold ring seen in bacterial AAA(+) activators, but two-hybrid experiments have suggested alternative models (Richmond et al. 1997). It is currently not clear whether the C-termini of the AAA(+) ATPases in the 19S cap play a similar role as in the ATP-independent PA28–20S proteasome complex. There is no consensus in the C-terminal sequences of different proteasomal AAA(+) ATPases of any particular species, but consensus sequences for the C-termini of any particular subunit from different species can be defined. Unfortunately, the overall sequence similarity of homologous sequences from different species is too high to infer a functional role of the AAA(+) C-termini from sequence similarity.

As discussed above, the eubacterial HslVU is distantly related in structure to the proteasome found in archaea and eukaryotes. Surprisingly, however, the structural relationship is not reflected in the regulatory properties as will be described in Section 10.3, which focuses on structural studies of the yeast 20S proteasome and its activation, activity, and inhibition.

10.3 The Yeast 20S Proteasome

The most elaborate version of the proteasome ***c***ore ***p***article (CP) is found in eukaryotes as shown by the crystal structure of the yeast 20S proteasome (Groll et al. 1997) (see Figure 10.5B). Here, the α- and β-subunits have diverged into seven different subunits each as compared to the archaeal enzyme which mostly consists of two components (Löwe et al. 1995). The subunits are present in two copies and occupy precisely defined positions within the 20S complex. As in the archaeal proteasome, the α-subunits are inactive and contribute to the antechambers of the particle, whereas the β-subunits set up the inner hydrolytic chamber. Remarkably, four of the seven different eukaryotic β-subunits lack residues that are essential for propeptide autolysis and are therefore proteolytically inactive. As in the archaeal CP, the remaining three subunits mature autoproteolytically to active threonine proteases but with a caspase-like (β1), trypsin-like (β2), and chymotrypsin-like (β5) activity, they exhibit different cleavage potentials (Groll et al. 1997). The crystal structure of the bovine 20S proteasome (Unno et al. 2002) demonstrated that yeast and mammalian CPs are highly homologous in their structural architecture, quaternary assembly, and active-site geometry. However, in mammalian cells, three additional non-essential subunits, β1i, β2i and β5i, respectively, can replace their constitutive counterparts upon induction by the cytokine γ-interferon. The interchange of active subunits modifies the CPs peptidase specificity and is important for the function of the immunoproteasome. The specificity of the S1 pockets of the induced subunits increases the yield of peptides favored for binding to MHC class I molecules and antigen presentation. The bovine proteasome structure provides

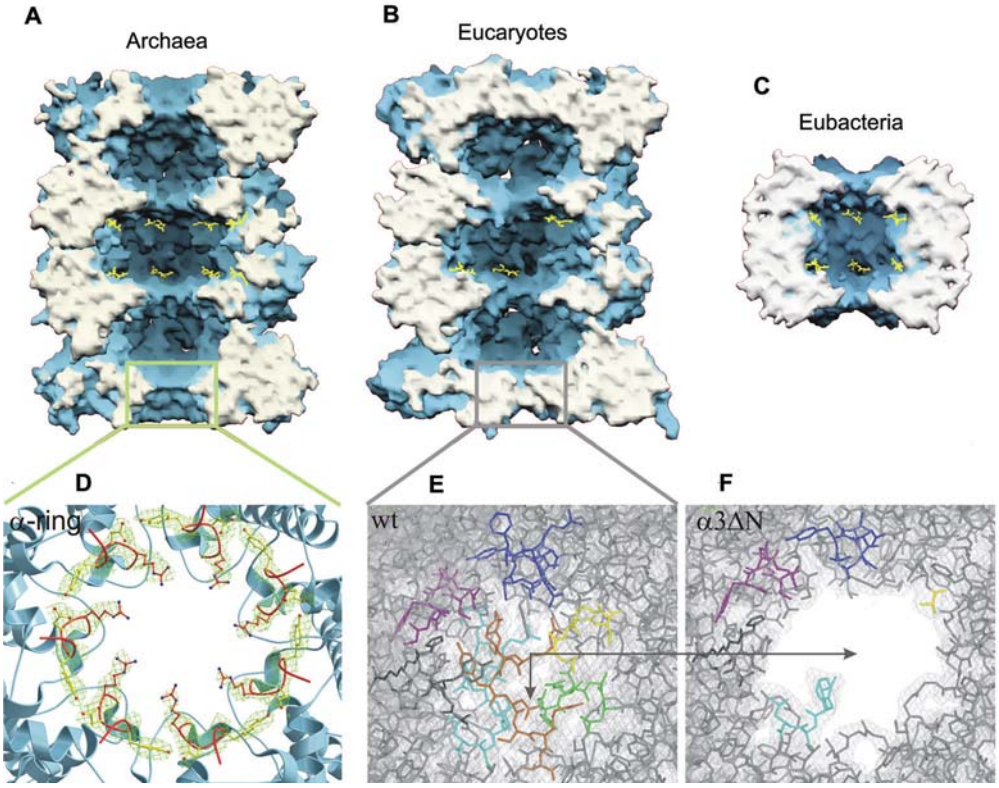


Fig. 10.5. Molecular surface of the archaeal (A), the eukaryotic 20S (B) and the HslV proteasome (C). The accessible surface is colored in blue, the clipped surface (along the cylinder axis) in white. To mark the position of the active sites, the complexes are shown with the bound inhibitor calpain (yellow). (A) The disorder of the first N-terminal residues in the archaeal α-subunits generates a channel in the structure of the CP, (B) whereas the asymmetric but well-defined arrangement of the α N-terminal tails seals the chamber in eukaryotic CPs. (C) The eubacterial "miniproteasome" has an open channel through which unfolded proteins and small peptides can access the proteolytic sites. (D) Ribbon plot of the free α-ring from *A. fulgidus* focusing on the defined N-termini (red). Tyr8 of each N-terminal part makes hydrogen bonds to Asp9 of the adjacent α-subunit (yellow), Arg10 (red) points toward the channel, generating a 13-Å entrance. The final $2F_O - F_C$ electron density map, contoured at 1σ is shown for the YDR-motif. (E, F) Electron density maps of the yeast core particle from wild type and α3ΔN mutant, respectively. The individual N-terminal tails of the α-subunits are drawn in different colours. Asp9 of subunit α3 plays a key role in stabilizing the closed state of the channel and is marked with a black arrow. In the α3ΔN mutant, an open axial channel is visible, whose dimensions are comparable to those of the archaeal CP channel.

an explanation of how the constitutive and inducible β-subunits can be mutually interchanged. In the yeast proteasome, these subunits are held in place by several specific interactions, which are absent in the mammalian homolog (Unno et al. 2002).

10.3.1
The Proteasome, a Threonine Protease

As mentioned in Section 10.3, proteasomes are threonine proteases. Accordingly, a proton acceptor is required to activate the hydroxylic group of Thr1. Although there are several potential acid–base catalysts around Thr1, activation seems to proceed by its own terminal amino group (Arendt and Hochstrasser 1999; Groll et al. 1999). A positively charged side chain of Lys33 lowers the pK_a of the Thr1 amino group (see Figure 10.6). The assignment of the N-terminus as the catalytic base in proteolysis is further supported by the fact that all Ntn-hydrolases share a common fold, but generally do not display any kind of active-site consensus. A second essential factor for proteolysis is a catalytic water molecule that has been observed in the high-resolution structures of CPs (Groll et al. 1997; Sousa and McKay 2001; Unno et al. 2002; Groll et al. 2003). The solvent molecule is ideally positioned to shuttle protons between the Thr1O$^{\gamma}$ and the N-terminus. Furthermore it could act as the base for the cleavage of the acyl ester intermediate, thereby releasing Thr1O$^{\gamma}$ for the next catalytic cycle (Ditzel et al. 1998).

In general, the cleavage products of the proteasome vary in length between 3 and 25 amino acids with an average length of 7 to 8 amino acids. Several models have

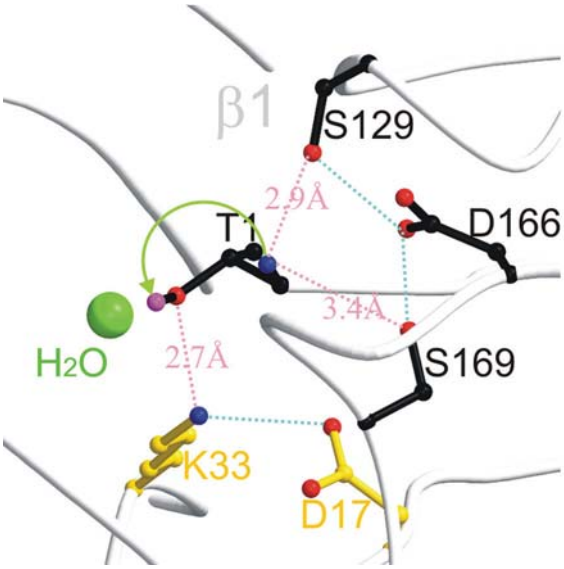


Fig. 10.6. Proteolytic site of the yeast β1 subunit. The protein backbone is drawn as a white coil with the active-site residues Thr1, Asp17, Lys33, Ser129, Asp166 and Ser169 shown in ball-and-stick mode. Owing to the salt-bridge with Asp17, the amino group of Lys33 should be positively charged and thus be able to lower the pK_a of Thr1O$^{\gamma}$ electrostatically. The other active-site residues Ser129, Asp166 and Ser169 define the orientation of Thr1. A water molecule (green sphere) is located properly to shuttle protons between the terminal amino group and Thr1O$^{\gamma}$ during proteolysis.

been suggested for the "molecular ruler" that determines fragment length. On the basis of structural studies of mutants unable to autolyse, we suggest that it is determined by the substrate-binding clefts designed for peptides 7–9 amino acids long (Groll et al. 1999). The likelihood of substrate cleavage depends on the mean residence time at the proteolytic sites, which is maximal if all binding sites are filled (Dick et al. 1998; Nussbaum et al. 1998). The active subunits in eukaryotic CPs differ mainly in their binding pockets, yielding different cleavage specificities. However, it must be emphasized that the proteasome complex does not represent a simple collection of chymotrypsin-like, trypsin-like, and caspase-like enzymes. In fact it is the structural architecture of the proteolytic chamber that determines specificity. The local structure around each active site imposes a physical constraint on the peptide substrates, whereas the selectivity of the S1 pockets is less relevant. The mechanistic importance of the inner chamber can also be seen from the fact that substrate residues other than P1 influence degradation (Cardozo et al. 1994; Bogyo et al. 1998; Groll et al. 2002) and that neighboring subunits interfere with the functions of the catalytic subunits (Heinemeyer et al. 1997; Groll et al. 1999; Jäger et al. 1999). However, inhibitor binding and mutational studies indicate that allosteric interactions between individual subunits are insignificant (Wenzel et al. 1994; Heinemeyer et al. 1997; Groll et al. 1999; Jäger et al. 1999; Groll et al. 2002) contrasting the allosteric activation of HslV by HslU described in the previous chapter.

10.3.2 Inhibiting the Proteasome

Proteasome inhibitors have been instrumental in identifying numerous protein substrates and in elucidating the importance of the proteasome/ubiquitin pathway in many biological processes. Initially, non-specific cell-penetrating peptide aldehydes were used for this purpose. More recently, it became possible to synthesize compounds with increased potency and selectivity (Adams et al. 1998; Elofsson et al. 1999). Furthermore, based on the crystal structure of the yeast and bovine liver CP (Groll et al. 1997; Unno et al. 2002), molecular modeling can now be used to engineer improved inhibitors.

Besides the synthetic inhibitors, a variety of natural compounds is known to inhibit the CP. One of these natural inhibitors, lactacystin, was discovered by its ability to induce neurite outgrowth in a murine neuroblastoma cell line. Incubation of cells in the presence of radioactive lactacystin leads to the labelling of the β5 subunit (Fenteany et al. 1995) and to irreversible inhibition of the CP. As shown by X-ray analysis, the inhibitor is covalently attached to subunit β5 by an ester bond with the N-terminal Thr1O$^{\gamma}$ (Groll et al. 1997) (see Figure 10.7A). The subunit selectivity of lactacystin can be attributed to its dimethyl group, which mimics a valine or a leucine side chain and closely interacts with Met45 in the hydrophobic S1 pocket of subunit β5.

Epoxomicin, an α',β'-epoxyketone peptide, is a natural compound that potently and irreversibly inhibits the catalytic activity of the CP (Meng et al. 1999). Unlike

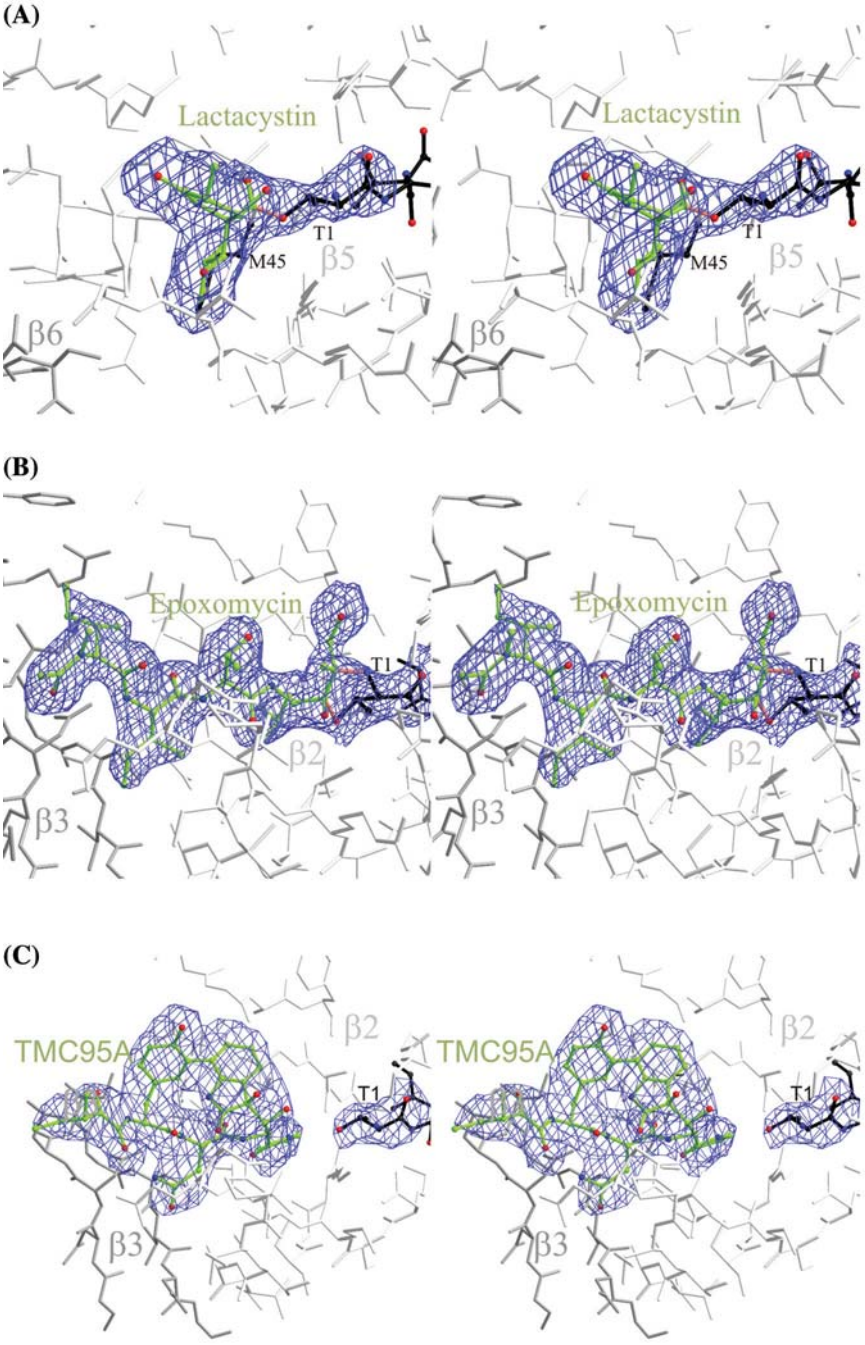
most other proteasome inhibitors, epoxomicin is highly specific for the proteasome and does not inhibit any other protease. The crystal structure of epoxomicin bound to the yeast CP explained the unique selectivity of the inhibitor (see Figure 10.7B). Adduct formation yields an unexpected morpholino ring, which is formed between the Thr1O$^{\gamma}$, the N-terminus, and the epoxy group of the inhibitor (Groll et al. 2000b). However it should be noted that proteasome inhibitors that covalently bind to the active β-subunits, usually cause apoptosis and cell death (Kloetzel 2001). They are therefore cytotoxic and thus may not be pharmaceutically relevant.

Recently, it was shown that certain natural products from *Apiospora montagnei*, TMC-95s, block the proteolytic activity of the CP selectively and reversibly in the low nanomolar range (Koguchi et al. 2000; Kohno et al. 2000). The TMC-95s represent a novel class of proteasome inhibitors consisting of modified amino acids, which form a heterocyclic ring system. The crystal structure of the yeast CP in complex with TMC-95A shows the inhibitor non-covalently bound to all active sites (Groll et al. 2001) (see Figure 10.7C). TMC-95A was anchored by several specific hydrogen bonds, which are formed with main-chain atoms and strictly conserved residues of the β-subunits. The structures of TMC-95s contain a crosslink between a tyrosine and an oxoindol side chain, resulting in a strained conformation that fits ideally to the CP active site. Thus the entropic penalty of binding is lower than for more-flexible ligands, which in turn explains the specificity and high affinity of the TMC-95s. Modeling studies indicate that it is possible to generate a TMC95 scaffold with a variety of functional groups attached to target the proteasomal S1 and S3 pockets and generate subunit specificity (Kaiser et al. 2002; Lin and Danishefsky 2002).

All agents that specifically inhibit the proteasome are potentially of great pharmacological interest (Loidl et al. 1999). As the CP plays a dominant role in generating antigenic peptides, which are subsequently bound by MHC I molecules, compounds that block this activity might serve as a basis for the development of immunosuppressive drugs. Attempts are being made to design synthetic proteasome inhibitors using the discussed natural inhibitors as lead structures. In addition, proteasomal inhibitors represent powerful tools in molecular biology and can be utilized to identify novel cellular roles of the proteasome.

10.3.3
Access to the Proteolytic Chamber

In the *Thermoplasma acidophilum* and *Archaeoglobus fulgidus* CP, two narrow entry ports of ~13-Å diameter exist at both ends of the cylinder, which prevent folded proteins entering (Löwe et al. 1995; Groll et al. 2003) (see Figure 10.5A). Many archaebacteria, such as *Methanococcus jannaschii*, contain a gene named PAN (***p***roteasome-***a***ctivating ***n***ucleotidase), which is highly homologous to the six ATPases in the 19S-component of the eukaryotic 26S proteasome (Zwickl et al. 1999). It was shown that PAN selectively stimulates the degradation of unfolded proteins, whereas the digestion of small peptides is not enhanced. The threading of specific protein substrates into the lumen of the CP requires the action of an


(A)
Lactacystin
T1
M45
β5
β6
Lactacystin
T1
M45
β5
β6
(B)
Epoxomycin
T1
β2
β3
Epoxomycin
T1
β2
β3
(C)
TMC95A
β2
T1
β3
TMC95A
β2
T1
β3

ATPase. The corresponding translocation process catalyzed by PAN follows ATP-dependent unfolding (Navon and Goldberg 2001; Benaroudj et al. 2003). However, it still remains to be clarified whether complex formation is a prerequisite for the cooperation between PAN and CP or whether the two systems work independently. In contrast to the archaeal CPs, the hydrolytic chamber of the eukaryotic 20S proteasome is tightly sealed (see Figure 10.5B). The N-termini of the α-subunits project down and across the axial pore and block the entrances by several layers of interdigitating side chains, which form a lattice-like structure (Groll et al. 1997) (see Figure 10.5E). Thus activation of the eukaryotic CPs requires substantial structural rearrangements of the N-terminal tails to open the molecular gate. This regulatory principle has been confirmed by a yeast CP mutant, in which the first nine amino acids of subunit α3 were deleted (α3ΔN-mutant) (Groll et al. 2000a). The α3-N-terminal tail was chosen for deletion because it traverses the pore of the CP and contacts all other N-termini that are involved in the structural organization of the plug (Groll et al. 1997; Unno et al. 2002). In the crystal structure of the mutant, open axial pores were observed that were equivalent in size to those seen in the archaebacterial CP (see Figure 10.5F). Several points of evidence indicate that opening of the gate is indeed essential for catalytic activation, as all proteolytically active sites are simultaneously activated and no significant structural changes can be seen between mutant and wild-type CP excluding allosteric effects. Furthermore, addition of the synthetic α3-N-terminal peptide to the α3ΔN mutant restores wild-type behavior. An alanine scan of this peptide revealed that α3-Asp9 is essential for stabilizing the closed state of the channel. This aspartate residue closely interacts with Tyr8 and Arg10 of the neighboring subunit α4 (see Figure 10.5E). The strict conservation of this YDR motif and of other α-N-terminal residues suggests a universal mechanism for opening gates in eukaryotic CPs that has been conserved during evolution. We suggest that binding of regulatory proteins to the CP triggers the rearrangement of the α3-tail and thus opens the gate.

This notion was further confirmed by the crystal structure of a 20S/11S heterologous complex between the yeast 20S-proteasome and the Trypanosoma 11S-regulator (Whitby et al. 2000). This approach was justified by the ability of 11S reg-

Fig. 10.7. Inhibitor binding to individual active sites of the yeast 20S proteasome. The inhibitors lactacystin (A), epoxomicin (B) and TMC95A (C) are colored green and are shown in stereo mode together with their unbiased electron densities. The active-site Thr1 is highlighted in black. (A) Covalent binding of the Streptomyces metabolite lactacystin to the active site of β5. The S1 pockets of the active subunits β1 and β2 differ from that of β5 and are not suitably constructed to bind the inhibitor. As discussed in the text, Met45 (black), which is located at the bottom of the β5-S1 pocket, makes the difference for inhibitor binding. (B) Covalent binding of the proteasome inhibitor epoxomycin to β2. The electron density reveals the presence of a unique six-membered ring. The morpholino derivative results from adduct formation between epoxomycin and the proteasomal Thr1O^{γ} and amino terminus (pink sticks) and explains the specificity of the inhibitor towards Ntn-hydrolases. (C) Noncovalent binding of the specific proteasome inhibitor TMC-95A from *Apiospora montagnei* to β2. TMC-95A binds near the proteolytic centre in all active subunits in the extended substrate binding site.

ulators to activate 20S proteasomes from widely divergent species. The structure of the chimeric complex showed that one cylindrical regulator was bound at each end of the 20S barrel structure. Unlike the uncomplexed proteasome, all of the seven α-subunit N-terminal tails extend away from the CP in the complex and project towards the pore of the regulator. This rearrangement provides access to the proteolytic chamber and is basically achieved by two features: Firstly, the 11S-"activation loops" impose a more stringent seven-fold symmetry on the CP thereby straightening out the asymmetrically oriented α-tails and removing them from the entrance/exit gates. Secondly, the high-affinity binding between CP and 11S is accomplished by the C-terminal sequences of the regulator, which insert into pockets formed between the 20S α-subunits. The major contact is observed between the C-terminal main-chain carboxylate of the 11S regulator and the entry of an internal helix of the CP α-subunit. The strength of this interaction is amplified by the heptameric assembly of the 20S/11S complex (Whitby et al. 2000).

Activation of the CP by the 19S-complex is also regulated by controlling access to the proteolytic chamber, but the gating mechanism differs from that seen in the 11S/20S-complex. The 19S RP consists of two subcomplexes termed lid and base (Glickman et al. 1998). The base appears to form a ring like structure, including six conserved ATPase subunits (Rpt1–6), and Rpn1 and Rpn2, which are located proximal to the CP's α-ring. Mutation studies indicated that the ATPase domain of Rpt2 plays a major role in regulating peptidase activity and that the 19S RP opens the gate to the protease in an ATP-dependent manner (Köhler et al. 2001). No detailed structural information is as yet available that could provide further insight into how the 19S RP controls proteasomal activity.

In some but not all archaea the proteasome is accompanied by another large cage-forming protease, the tricorn protease. Tricorn functionally interacts with the proteasome by cleaving the proteasomal peptide products into smaller peptides, which are further degraded into single amino acids by associated factors. Structural and functional aspects of tricorn are described in Section 10.4. The unexpected relationship between tricorn and the eukaryotic dipeptidyl peptidase IV revealed by these structural studies is also discussed in brief.

10.4
The Tricorn Protease and its Structural and Functional Relationship with Dipeptidyl Peptidase IV

Each living cell is a complex system and needs to continuously clear unnecessary or defective components. Within this context, the importance of the proteasome is well established (see Section 10.2). It predominantly carries out the degradation of cytosolic proteins and generates peptides varying in length between 3 and 25 amino acids. In order to be useful resources to the cell, these products need to be further degraded to eventually yield single amino acids. In the model organism *Thermoplasma acidophilum* a proteolytic system has been identified that does indeed perform this processing (Tamura et al. 1996a). Based on the crystal structure

of the tricorn protease (Brandstetter et al. 2001), we provide evidence of how the tricorn protease accomplishes efficient turnover of the proteasome-generated peptides. The structure of tricorn reveals a complex mosaic protein whereby five domains combine to form one of six subunits, which further assemble to form the *D*3 symmetric core protein. The structure shows how the individual domains coordinate the specific steps of substrate processing, including channeling of both the substrate to and the product from the catalytic site. Moreover, the structure shows how accessory protein components might additionally contribute to an even more complex protein machinery that efficiently collects the tricorn-released products.

10.4.1 Architecture of the Tricorn Protease

The hexameric *D*3-symmetric tricorn protein is assembled by two perfectly staggered and interdigitating trimeric rings with every subunit of one ring forming contacts almost exclusively with the two subunits of the other ring related by the molecular diads. The toroid structure has the shape of a distorted hexagon formed by a trimer of dimers (see Figure 10.8). The overall dimensions of the molecule are 160 Å within the plane normal to the three-fold axis and 88 Å parallel to it. The conically shaped central pore connects with additional cavities formed by the indi-

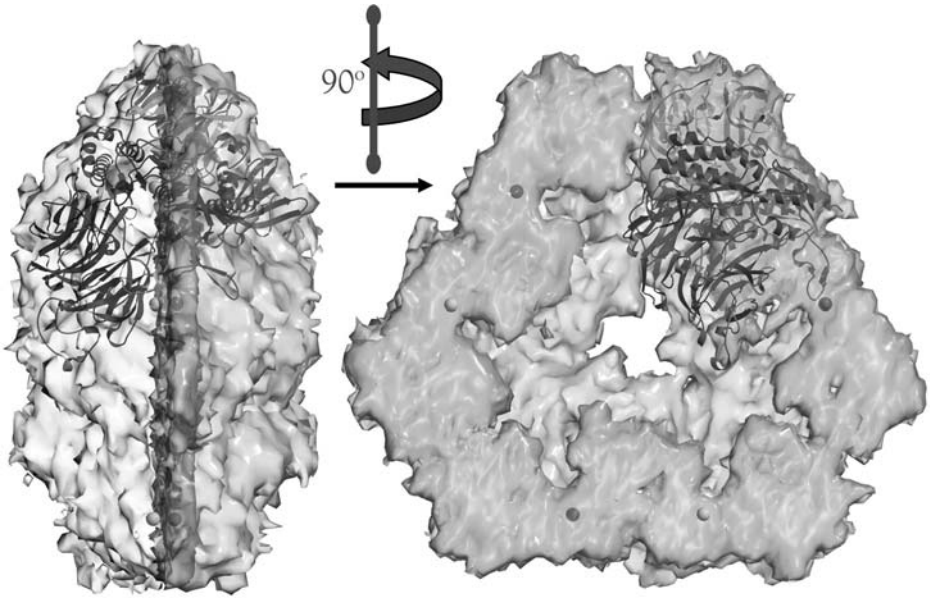


Fig. 10.8. Surface representation of the tricorn protease with the ribbon model of one subunit superimposed. The two orthogonal views are along the molecular two-fold and three-fold axis, respectively. The six solid spheres indicate the active-site positions.

vidual subunits like spokes of a wheel (see Figure 10.8). A single subunit is further divided into five sequential sub-domains, namely the N-terminal six-bladed β-propeller (β6) followed sequentially by a seven-bladed β-propeller (β7). Both β6 and β7 are topologically unclosed, an extremely rare feature observed only in the prolyl oligopeptidase (POP) (Fülöp et al. 1998) and DPIV protease (Engel et al. 2003; Rasmussen et al. 2003). A PDZ-like domain (R761-D855) is interspersed between the two C-terminal mixed α-β domains. These C-terminal domains harbor the catalytic residues and exhibit the α-β hydrolase fold again underlining the relationship of tricorn with DPIV and POP.

10.4.2 Catalytic Residues and Mechanism

To elucidate the amino acids crucial for its catalytic activity, we have co-crystallized tricorn with a series of chloromethyl ketone-based inhibitors, including TLCK and TPCK for which we have confirmed inhibitory efficacy. For all of these inhibitors, we have observed continuous electron density connecting to the side chain of S965 which was unambiguously fitted by the respective inhibitor. S965 is positioned at the entrance to helix H3 within sub-domain C2. The uncapped amino group of D966 forms, together with that of G918, the oxyanion hole, which is occupied by a water molecule in the uninhibited structure. H746 is ideally positioned to activate the catalytic S965 at a hydrogen-bonding distance of 2.7 Å. However, in none of the inhibitor complexes could we observe a covalent linkage between H746 and the inhibitor, as observed in the trypsin-like serine proteases (Bode et al. 1989). Tricorn is related to the cysteine proteinases in this respect (Eichinger et al. 1999). We confirmed that both residues are crucial for catalysis by constructing the single-site mutants S965A and H746A, both of which are amidolytically inactive. The H746 is correctly oriented by the O^{γ} of S745 which in turn is polarized by E1023.

The arrangement of S965, H746, and the oxyanion hole suggests that the classical steps of peptide-bond hydrolysis follow the sequence of the trypsin-like serine proteases, namely the formation of the tetrahedral adduct, the acyl–enzyme complex, and hydrolysis. Tricorn has been shown to exhibit both tryptic and chymotryptic specificities (Tamura et al. 1996a). The X-ray structure reveals that specificity for basic P1 residues is conferred by D936 which is provided by the diad-related subunit (see Figures 10.9 and 10.10).

In this way, the previously described structural linkage (trimer of dimers) is translated into functional cooperativity within the dimers. Intriguingly, in the uninhibited high-resolution crystal structure, the acidic S1 specificity-determinant residue D936 was mobile. Consistent with this, the side chain of D936 in the TPCK complex structure adopts an alternative rotamer to allow the TPCK phenyl ring to freely access the hydrophobic niche formed by Y946, I969, V991, and F1013. D936 thus serves as a substrate-specificity switch accommodating both hydrophobic and basic P1 residues. The SO_2 group of TPCK and TLCK interacts with the NH moiety of I994, thereby already suggesting the strand E993–P996 as the unprimed-substrate docking site. These substrate-recognition sites are rather un-

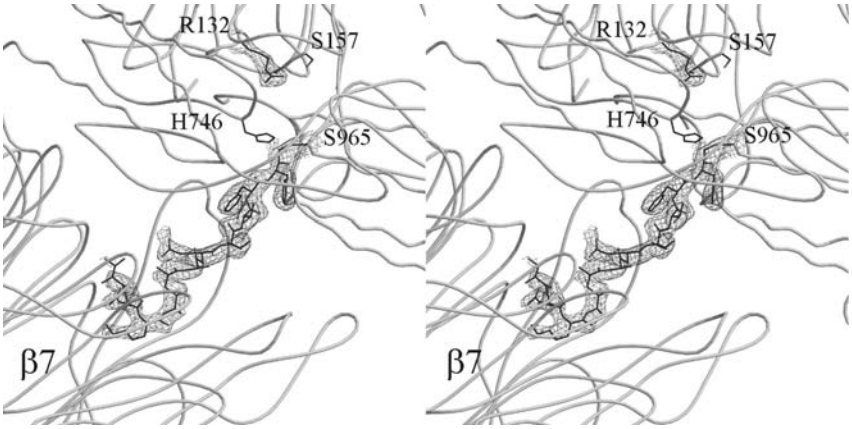


Fig. 10.9. Stereo view of a 13-mer chloromethyl ketone bound to the active site. The electron density of the peptide directs to the β7 propeller.

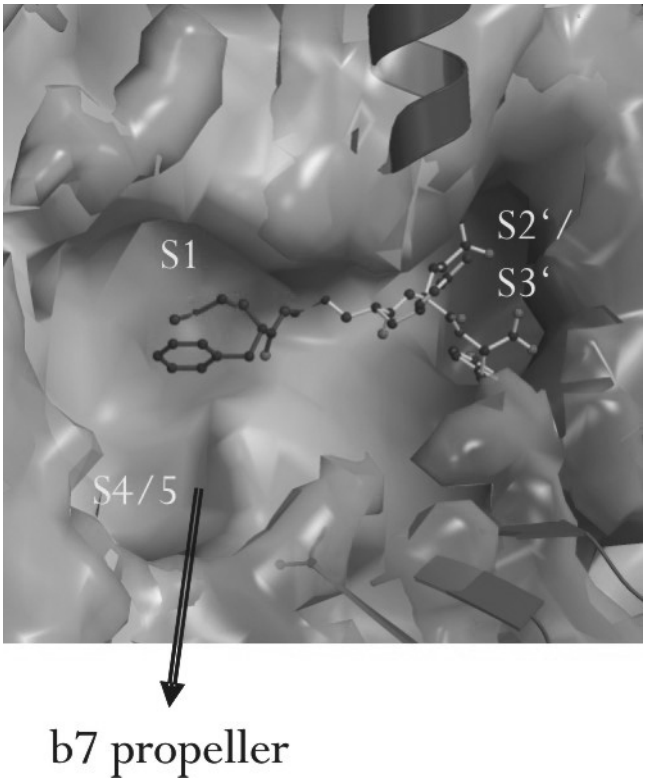


Fig. 10.10. Detailed active-site view and substrate recognition as deduced from experimental complex structures. The substrate C-terminus is anchored by R131 and R132.

restricted in accord with tricorn's broad substrate specificity (Tamura et al. 1996a; Tamura et al. 1996b).

This situation is contrasted by the length restriction of the primed-substrate recognition site. A prominent cluster of basic residues (R131, R132) delineates the binding site of the substrate C-terminus. These basic residues, positioned on a flexible loop as discussed in detail below, together with the primed-site topology, clearly mark tricorn as a carboxypeptidase. The geometric dimensions explain tricorn's preferential di- and tri-carboxypeptidase activity, while the cleavage of longer peptides will require some conformational rearrangement and is energetically less favorable. By contrast, single amino acids cannot be cleaved off a substrate, because the P1′ residue is unable to anchor its carboxylate-group on the basic backstop residues (see Figures 10.9 and 10.10).

A negative charge was not tolerated at positions P3, P4, and P5 of a synthetic fluorogenic AMC-substrate (Tamura et al. 1996b). The crystal structure did not indicate any steric or electrostatic conflicts, if a canonical binding mode of these substrates was assumed. Owing to their lack of a free C-terminus, the charge polarity of N-terminally succinylated fluorogenic substrates is inverted with respect to an unmodified peptide substrate and may lead to unproductive binding with inverted strand polarity.

Each of the three C-terminal domains (C1, PDZ, C2) is remarkably similar to the respective domains (A, B, and C) found in the D1-processing protease (D1P) of photosystem II. The rms deviations between the Cα positions of these domains are 2.2, 2.3, and 2.7 Å with 84, 86, and 135 matching amino acids, respectively. A weak homology between these domains is recognizable in the primary sequences (11, 19, and 20% identities). The relative arrangement of these domains, however, differs very much between tricorn and D1P. With the C2 domain aligned to the C domain of D1P, the orientation of the C1 domain differs from that of the D1P A-domain by 35°. Analogously, the required transformation to align the PDZ-like domains includes a 96° rotation. The rotation axes of these transformations are unrelated to each other. In addition, proper alignment of the PDZ domain requires a 30-Å translation. The catalytic serine residues (S965 and S372, respectively) are positioned on topologically equivalent positions at the helix entrance in the C2 (C) domain (D1P). Further, the amides forming the oxyanion hole (G918, D966, and G318, A373 in tricorn and D1P, respectively) superimpose to within 1 Å. As in other Tsp-like proteases, the residue serving as general base in D1P is a lysine (K372) residing within the C domain of D1P, while it is a histidine in tricorn (H746) which resides on tricorn's C1 domain. The relative arrangement of the C1 and C2 domains in tricorn must, for that reason, remain very restricted to allow for proper catalysis.

One role of the PDZ domain in substrate recognition has been shown for Tsp (Beebe et al. 2000) and was analogously suggested for the tricorn protease (Ponting and Pallen 1999). While the GLGF substrate recognition element is structurally conserved ($R^{764}IAC^{767}$ in tricorn), as pointed out earlier, it appears for a number of reasons unlikely that the tricorn PDZ will participate in substrate recognition in the same way as suggested for D1P (Liao et al. 2000): (1) The putative substrate-

binding site as defined by the crystal structures of the C-terminal peptides complexed with PDZ domains (Cabral et al. 1996; Doyle et al. 1996) is partly occupied by outer strands of blade 3 of β6 within the same subunit; (2) the generally conserved arginine (R247) involved in recognition of the carboxylate of the peptide C-terminus corresponds to a hydrophobic residue in tricorn (I851); (3) the orientation and position of tricorn PDZ differs so strongly from that seen in D1P that any analogy based on the sequential domain arrangement is invalidated on the basis of their respective three-dimensional domain arrangement. Instead, the PDZ domain mainly serves to scaffold the sub-domains as described earlier and, in addition, might be involved in recognition of associating component proteins.

10.4.3
Substrate Access and Product Egress Through β-propellers

The comparison with POP, including the open Velcro-topology (Fülöp et al. 1998), suggests an important role of the β-propellers for substrate access to and product exit from the active site (Engel et al. 2003)(see Figure 10.11). Both the β6 and β7 propeller axes are directed towards the active site of the protein, almost intersecting near S965. The arginine anchor (R131, R132) obstructs the otherwise direct connection from the active-site chamber to the exterior through the β6 propeller. Given these observations, we propose that the β6-propeller channel represents one, if not the major, rear exit from the catalytic chamber. This is consistent with the point mutation L184C, positioned within the β6 propeller. The introduced thiol group was modified with maleimide, partially blocking the β6 propeller. The activity of this mutant enzyme towards fluorogenic substrates is significantly reduced ($<$ 50%) compared with the wild-type protein (Brandstetter et al. 2001; Kim et al. 2002). The substrate entrance and product exit paths are indicated in Figure 10.11.

The chloromethyl ketone-based inhibitor-complex crystal structures suggested the strand E993–P996 as a recognition strand for the unprimed-substrate residues.

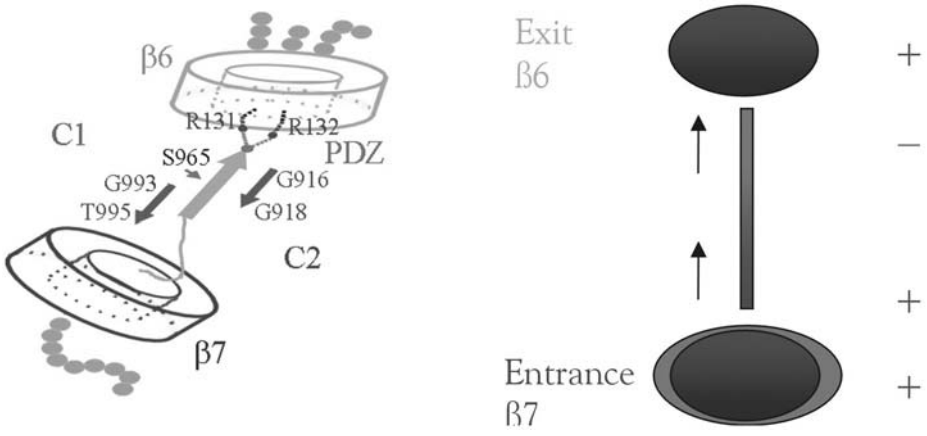


Fig. 10.11. Cartoon of the electrostatically driven processive substrate turnover.

This strand extrapolates towards the β7 channel (see Figure 10.9). The channel through the β7 propeller provides a significantly shorter route from the catalytic chamber to the outside of the protein (60 Å) as compared to the alternative route through the central pore (83 Å). The latter path to the active site has multiple branchings and dead ends. Therefore, the β7 channel might be utilized by the enzyme for the preferred substrate passage to the active site. It is wide open but capped on its outside by four basic residues (R369, R414, R645, K646) which are only partially charge-compensated by one acidic residue (D456). This locally positive lid to the β7 propeller channel is encircled by acidic residues (D333, D335, D372, D456, D506, D508, E592, and E663). Except for E663, which is located on the hairpin connecting strand 3 and 4 of blade 7, all these charged amino acids are positioned between strands 1 and 2 of the respective β7 blades. The resulting charge distribution mimics an electrostatic lens, whereby peptides are pre-oriented with their C-termini towards the central basic propeller lid. Once the entrance to the β7 channel is opened by a concerted side-chain movement of R369, R414, R645, and K646, and possibly assisted by main-chain movements of A643–K646 (blade 7), a peptide is able to enter the channel in an extended conformation where it will find multiple docking sites at the unsaturated inner strands of the β7 propeller blades. A similar substrate-gating filter mechanism through a seven-bladed β propeller has been suggested for the prolyl oligopeptidase (Fülöp et al. 1998; Fülöp et al. 2000), and there is precedent for a β-hairpin binding into a seven-bladed propeller (Ito et al. 1991). The preferred substrate entry through the β7 propeller channel is in line with the point mutation R414C, located in the β7 channel. Derivatization of this introduced thiol group with maleimide markedly reduced the fluorogenic activity of this mutant to about 50% of the wild-type activity (Brandstetter et al. 2001).

Tricorn cleaves substrates in a processive mode (Kim et al. 2002), indicating that only completely digested products will leave the inner protein chambers while larger products will be retained and processed as preferred substrates. The structure suggests several mechanisms to maintain "one way" processing. Basic lids (R414, R645, K646 and R131, R132) are present at the entrances to the β6 and β7 channels. The topology and size of the inner cavities favor an extended conformation of the substrate and the C-terminus of the substrate will be attracted to the basic β6 lid, thereby presenting the substrate's scissile bond at the active site S965 for proteolyis. In one possible scenario, the primed product residues are released by the enzyme through the "rear exit" to the active site formed by the β6 propeller, which is gated by R131–R132. The arginine gate is located on a helical loop containing three glycines (G126, G130, G139) and not restrained to its position via any protein contacts. These glycines might function as hinge residues allowing the gate to move into a sufficiently voluminous cavity of mixed polarity (see Figure 10.10).

The unprimed side of the substrate is held in place by a series of interactions with the protein. In addition to the observed ionic (D936) or hydrophobic S1 interaction site (Y946, I969, V991, F1013), the P1 main chain is held by its interaction with the oxyanion hole (G918, D966). P2–P4 residues will presumably utilize un-

saturated main-chain hydrogen bonds at the strand I994–P996 (see Figure 10.10) and further interactions might occur in the β7 propeller channel as described in galactose oxidase (Ito et al. 1991). The modeling studies and suggested substrate binding at the primed and unprimed sides are fully experimentally confirmed by crystal-structural studies using C- and N-terminally extended covalently bound inhibitors (Kim et al. 2002).

Tricorn reportedly cooperates with three additional proteins, termed interacting factors F1, F2, and F3, to degrade oligopeptides sequentially to yield free amino acids (Goettig et al. 2002) . F1 is a prolyl iminopeptidase with 14% sequence identity to the catalytic domain of prolyl oligopeptidase POP, which has an additional propeller domain (Fülöp et al. 1998; Goettig et al. 2002). Guided by this structural scaffold of the latter structure, we speculate that F1 docks onto the six-bladed β-propeller of the tricorn core protein. As in POP, substrate would enter F1 through the propeller channel in this model. While a physical interaction of F1 with tricorn has been suggested (Tamura et al. 1998), the exact mode of interaction of tricorn with F1, F2, and F3 has not been detailed so far

Similarly, there is evidence for functional but not physical interaction of tricorn with the proteasome (Tamura et al. 1998) A physical interaction between these molecules by aligning their respective central pores would imply a symmetry mismatch. While such a physical interaction would be consistent with the geometric dimensions of both molecules, its existence needs to be experimentally confirmed and characterized.

10.4.4
Structural and Functional Relationship of Tricorn and DPIV

The situation in the tricorn protease is closely resembled by ***d***ipeptidyl ***p***eptidase IV (DPIV) where an eight-bladed topologically open β propeller and a side opening provide entrance to and exit from the active site (see Figure 10.12). Similar to tricorn, DPIV is a serine protease with low but significant structural homology to the family of α/β-hydrolases. We superimposed the catalytic core elements, including the active-site serine and histidine, the strictly conserved helix following the active-site serine (Ser630–Ala642 and Ser965–Leu977, respectively), and tricorn's five-stranded parallel β-sheet onto the equivalent strands of the eight-stranded DPIV-sheet. Both sheets have identical polarity. Significantly, both tricorn propellers come to superimpose onto the two DPIV-openings, the tricorn β7 propeller onto the DPIV β8 propeller, and the tricorn β6 propeller onto the side exit, as schematically indicated in Figure 10.13. This similarity suggests that the β8 propeller provides substrate access to, and the side opening product release from, the DPIV active site. This tricorn-derived model is able to explain the high substrate selectivity critical for DPIV-function to activate or inactivate regulatory peptides. Passage through the β propeller tunnel requires the substrates to unfold thereby providing their ''fingerprint'' to DPIV. Once the amino terminus of the peptide approaches the active site, it is still held in place by its C-terminus interacting with the β propeller which may contribute to conformationally activate the substrate for cleav-

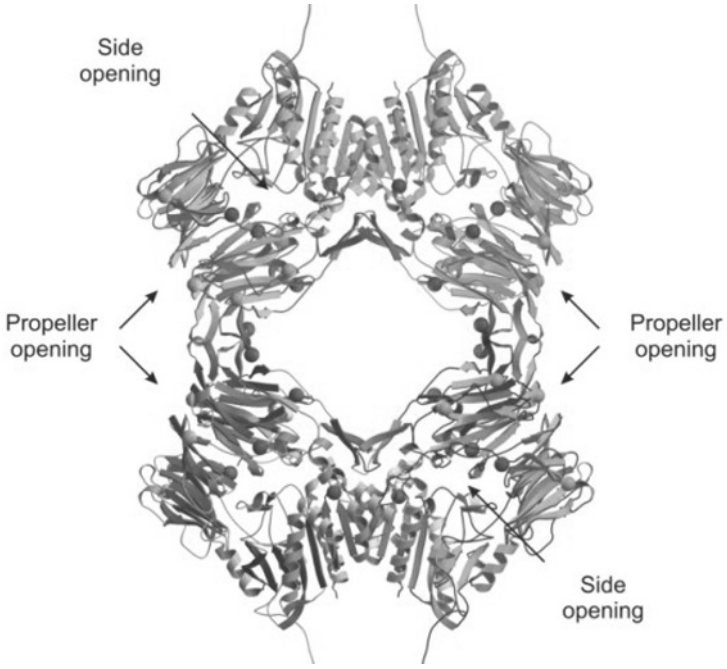


Fig. 10.12. Ribbon representation of the tetrameric DPIV.

age. After the nucleophilic attack the acyl–enzyme intermediate forms, while the primed product is directly released through the side exit. This explains why degradation of glucagon by DPIV is not processive, but occurs sequentially in two independent steps (glucagon 3–29, glucagon 5–29) (Pospisilik et al. 2001). Clearly, the

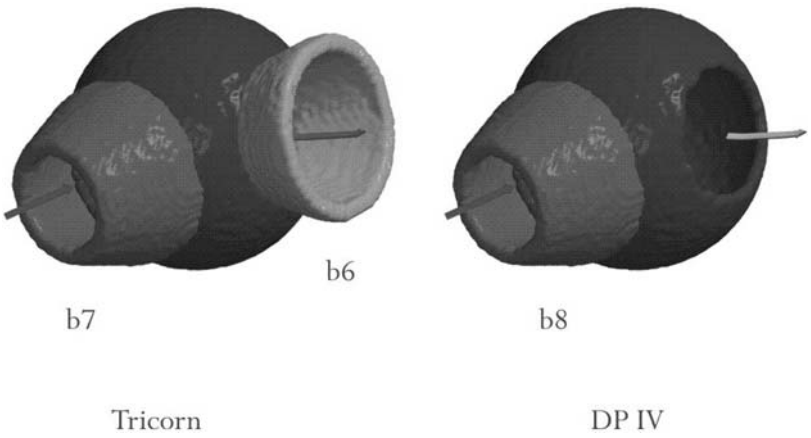


Fig. 10.13. Schematic representation of the active-site access and product egress in tricorn and DP IV.

final determination of the functional roles of the DPIV openings awaits further experiments.

10.5
The DegP Protease Chaperone: A Molecular Cage with Bouncers

In this section we describe DegP, a bacterial cage-forming protease, which has homologs in all kingdoms of life. It is distinguished in essential ways from the previous systems by exhibiting extreme flexibility and potential to change its overall shape and its internal structure. Structural flexibility is translated into function and the unique property of DegP to act predominantly as a chaperone or as a protease dependent on temperature. DegP is a Janus-faced molecule appearing as helper or killer as cells need it.

Cells have developed a sophisticated system of molecular chaperones and proteases to reduce the amount of unfolded or aggregated proteins (Wickner et al. 1999). Chaperones recognize hydrophobic stretches of polypeptides that become surface exposed as a consequence of misfolding or unfolding. If refolding attempts fail, irreversibly damaged polypeptides are removed by proteases.

E. coli contains several intracellular proteases that recognize and degrade abnormally folded proteins. The biochemical and structural features of these ATP-dependent proteases have been studied extensively (see Section 10.2). However, relatively little is known about proteases that are responsible for the degradation of non-native proteins in the periplasmic compartment of gram-negative bacteria. Such function has been attributed to the heat-shock protein DegP, also commonly referred to as HtrA or Protease Do. While most factors involved in protein quality control are ATP-dependent heat-shock proteins (Gottesman et al. 1997), DegP fulfills this role without consuming chemical energy (Lipinska et al. 1990). DegP homologs are found in bacteria, fungi, plants, and mammals. Some, but not all, are classical heat-shock proteins. They are localized in extracytoplasmic compartments and have a modular architecture composed of an N-terminal segment believed to have regulatory functions, a conserved trypsin-like protease domain and one or two PDZ domains at the C-terminus (Clausen et al. 2002). PDZ domains are protein modules that mediate specific protein–protein interactions and bind preferentially to the C-terminal 3–4 residues of the target protein (Sheng and Sala 2001). Prokaryotic DegPs have been attributed to the tolerance against thermal, osmotic, oxidative, and pH stress as well as to pathogenicity (Pallen and Wren 1997). A number of DegP substrates are known. These are either largely unstructured proteins such as casein, small proteins that tend to denature, hybrid proteins, or proteins that entered a non-productive folding pathway (Lipinska et al. 1990; Kolmar et al. 1996; Spiess et al. 1999). Stably folded proteins are normally not degraded. In addition to its protease activity, DegP has a general chaperone function. The dual functions switch in a temperature-dependent manner, the protease activity being most apparent at elevated temperatures (Spiess et al. 1999). The ability to switch

between refolding and degradation activity and the large variety of known substrates make DegP a key factor controlling protein stability and turnover.

10.5.1 The DegP Protomer, a PDZ Protease

DegP from *E. coli* was crystallized at low temperatures in its chaperone conformation and analyzed (Krojer et al. 2002). The protomer can be divided into three functionally distinct domains, namely a protease and two PDZ domains, PDZ1 and PDZ2 (see Figure 10.14). Like other members of the trypsin family, the protease domain of DegP has two perpendicular β-barrel lobes with a C-terminal helix. The catalytic triad is located in the crevice between the two lobes. While the core of the protease domain is highly conserved, there are striking differences in the surface loops L1, L2, and L3 (for nomenclature see Perona and Craik 1995), which are important for the adjustment of the catalytic triad (Asp105, His135, Ser210) and the

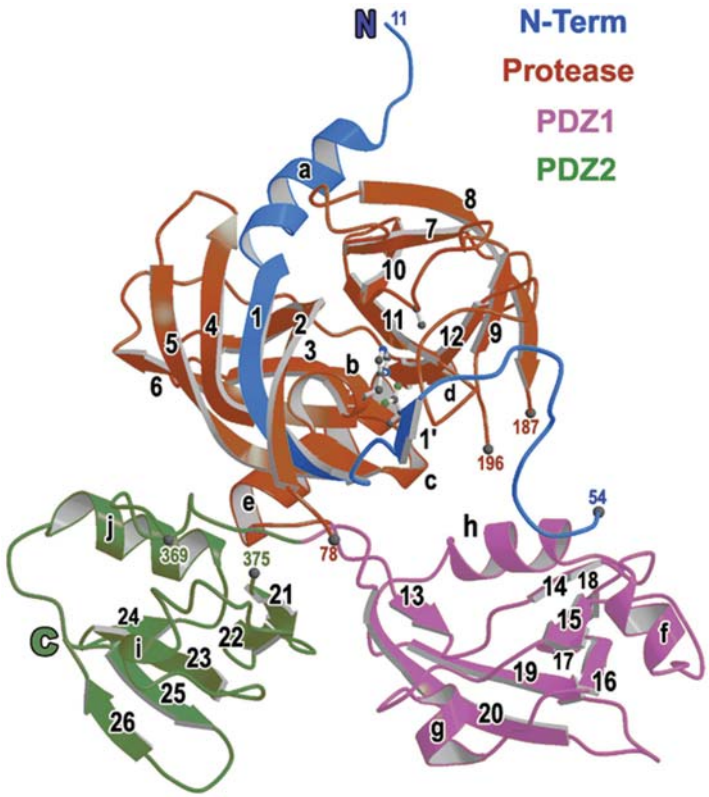


Fig. 10.14. Structure of DegP protomer. Ribbon presentation of the monomer, in which the individual domains are colored differently. Residues of the catalytic triad are shown in a ball-and-stick model. The nomenclature of secondary structure elements, the termini of the protein and regions that were not defined by electron density are indicated.

specificity pocket S1. The enlarged loop LA protrudes into the active site of a molecular neighbor, where it intimately interacts with loops L1 and L2. The resulting loop triad LA*–L1–L2 completely blocks the substrate-binding cleft and results in a severe deformation of the proteolytic site abolishing formation of the catalytic triad, the oxyanion hole, and the S1 specificity pocket. Thus the protease domain of the DegP chaperone is present in an inactive state, in which substrate binding as well as catalysis is prevented. (Krojer et al. 2002).

The structure of the PDZ domains of DegP is similar to PDZ domains of bacterial origin (Liao et al. 2000). Compared to the canonical 4+2 PDZ β-sandwich (Cabral et al. 1996), the DegP PDZ domains show a circularly permuted secondary structure, in which the N- and C-terminal strands are exchanged. Furthermore, they contain a 20-residue insertion following the first β-strand (including helix f) that is important for inter- and intramolecular contacts within the oligomer. In analogy to other PDZ domains, PDZ1 and/or PDZ2 should be involved in substrate binding. PDZ1 contains a deep binding cleft for substrate, which is mainly constructed by strand 14, its N-terminal loop (the so-called carboxylate-binding loop) and helix h. The carboxylate-binding loop is located in a highly positively charged region and is formed by an E–L–G–I motif, which is similar to the frequently observed G–L–G–F motif (Cabral et al. 1996). Binding specificity is mainly conferred by the specific configuration of the 0, -2, and -3 binding pockets (Songyang et al. 1997), where pocket 0 anchors the side chain of the C-terminal residue. In PDZ1, all pockets are built by mainly hydrophobic residues. The thermal motion factors point to the flexibility of strand 14 and its associated carboxylate-binding loop, indicating the plasticity of the binding site. Thus PDZ1 seems to be well adapted to bind various stretches of hydrophobic peptide ligands. Different from PDZ1, the occluded binding site of PDZ2 is unlikely to be involved in substrate recognition.

10.5.2 The Two Forms of the DegP Hexamer

In the crystallographic asymmetric unit, two DegP molecules (A and B) were observed, which build up two distinct hexamers (see Figure 10.15). Both hexamers are formed by staggered association of two trimeric rings. Hexamer A is a largely open structure with a wide lateral passage penetrating the entire complex (see Figure 10.15A), whereas hexamer B corresponds to the closed form, in which a cylindrical 45-Å cavity containing the proteolytic sites is completely shielded from solvent (see Figure 10.15B). In both cases, the top and bottom of the DegP cage are constructed by the six protease domains, whereas the twelve PDZ domains generate the mobile sidewalls. The height of the cavity is determined by three molecular pillars, which are formed by the enlarged LA loops of the protease domain. The PDZ domains are able to adopt different conformations and represent side doors that may open. This en-bloc mobility enables the PDZ domains to function as tentacular arms capturing substrates and delivering them to the inner cavity. This structural organization is strikingly different from the other cage-forming

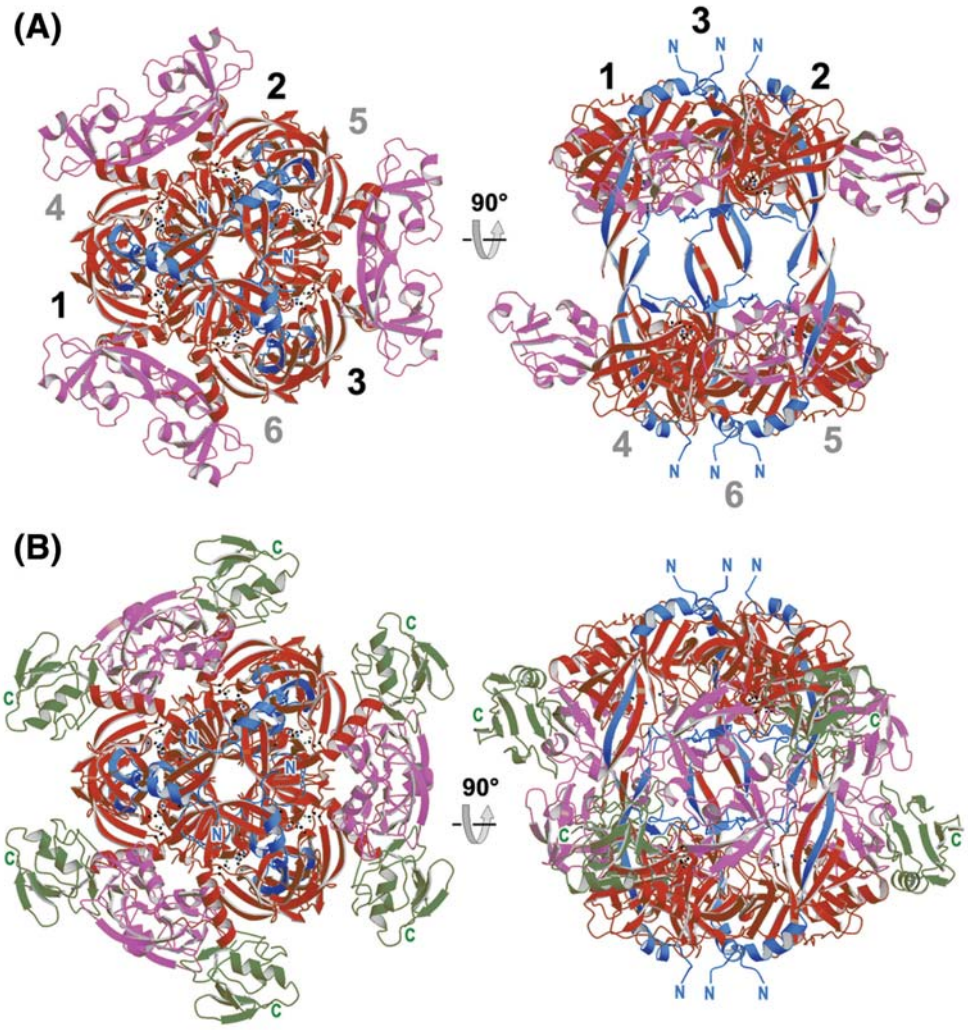


Fig. 10.15. Structure of the DegP hexamer. Ribbon presentation of the monomer, in which the individual domains are colored differently. Residues of the catalytic triad are shown in a ball-and-stick model. The nomenclature of secondary structure elements, the termini of the protein and regions that were not defined by electron density are indicated.

proteases, where substrates enter the central cavity through narrow axial or lateral pores as described in Sections 10.2 to 10.4.

10.5.3 DegP, a Chaperone

E. coli DegP has the ability to stabilize and support the refolding of several non-native proteins *in vivo* and *in vitro* (Spiess et al. 1999; Misra et al. 2000). Possible

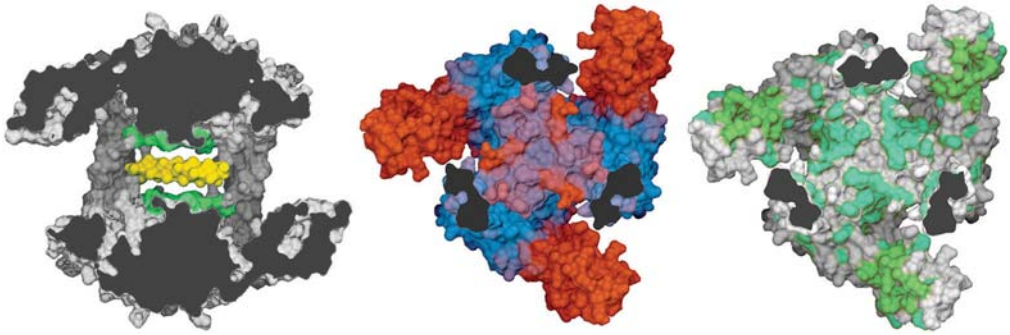


Fig. 10.16. Properties of the inner cavity. Half cut presentations of molecule A (left: side view, center and right: top views) with cut regions shown in dark gray. (Left) Surface representation of the internal tunnel illustrating its molecular-sieve character. Access is restricted to single secondary structure elements as shown by the modeled polyalanine helix, which is colored yellow. (Center) Top view on the ceiling of the inner cavity with mapped thermal motion factors to show its plasticity. Flexible regions are colored red, rigid regions are blue. (Right) Formation of the hydrophobic binding patches within the cavity. Hydrophobic residues of the protease domain are shown in cyan, and the non-polar peptide-binding groove of PDZ1 in green.

binding sites for misfolded proteins are located within the inner cavity (see Figure 10.16). The solvent-accessible height of this chamber is 15 Å at its center and increases to 18 Å near the outer entrance. Owing to these geometric constrictions, substrates must be partially unfolded to reach the active site (see Figure 10.15). As in other chaperones of known structure, the DegP cavity is lined by hydrophobic residues. Two major hydrophobic grooves can be distinguished, which are mainly constructed by residues of loop LA and L2. Notably, the hydrophobic binding sites of the PDZ1 domains are properly oriented to augment the number of potential binding patches. The alternating arrangement of polar and hydrophobic surfaces, both within one trimeric ring and between trimeric rings, should allow the binding of exposed hydrophobic side chains as well as of the peptide backbone of substrates. Taken together, the ceilings of the DegP cavity represent docking platforms for partially misfolded proteins. Both platforms are structurally flexible and should thus allow binding of diverse polypeptides.

10.5.4 The Protease Form

The protease conformation of DegP is still elusive as crystallization of a substrate-like inhibitor complex has failed and maintenance of a stably folded protein precludes long-term experimentation at elevated temperatures where it displays protease activity. We propose a profound rearrangement of the LA*–L1–L2 loop triad into the canonical conformation of active serine proteases competent for substrate binding. This may be initiated by a collapse of the hydrophobic LA platforms and an enlargement of the hydrophobic contacts caused at high temperature.

10.5.5
Working Model for an ATP-independent Heat-shock Protein

Cage-forming proteases and chaperones can be ATP-dependent or -independent. In the former group, ATPase activity is important for recognition of target proteins, their dissociation and unfolding, their translocation within the complex, and for various gating mechanisms. The present crystal structure indicates why these functions are not relevant for DegP. DegP preferably degrades substrates, which are *per se* partially unfolded and which might accumulate under extreme conditions (Swamy et al. 1983; Strauch et al. 1989; Lipinska et al. 1990). Alternatively, threading of substrate through the inner chamber could promote unfolding into an extended conformation. Removal of higher-order structural elements may reinitiate substrate folding after exit from DegP. By binding to the C-terminus or a β-hairpin loop of a protein, the PDZ domains could properly position the substrate for threading it into the central cavity. After accessing this chamber, the fate of the unfolded protein depends on the interplay and structural organization of loops LA, L1, and L2. Recruitment of PDZ domains for the gating mechanism should permit a direct coupling of substrate binding and translocation within the DegP particle. This two-step binding process is similar to that of other cage-forming proteins such as the proteasome or the Clp proteases. Here, two binding sites (chambers) exist, the first of which primarily determines substrate specificity.

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11
Proteasome Regulator, PA700 (19S Regulatory Particle)

George N. DeMartino and Cezary Wojcik

11.1
Overview

The proteasome is responsible for the degradation of most intracellular proteins in eukaryotic cells [1, 2]. It functions as part of a modular system whereby a protease module, the 20S proteasome, forms larger complexes with one or more of a group of regulatory protein modules [3, 4]. The general function of these regulatory proteins is to impart specific catalytic and regulatory features to the resulting proteasome complexes. This chapter describes PA700 (***P***roteasome ***A***ctivator of ***700*** kDa), also known as the 19S RP (***19S R***egulatory ***P***article), a multisubunit ATPase regulatory complex, that binds to one or both ends of the cylinder-shaped 20S proteasome [4, 5–10]. The resulting ''singly capped'' or ''doubly capped'' complexes are both referred to as 26S proteasomes (see Figure 11.1), although it is unclear whether these forms differ in function and/or abundance in cells [3, 11]. The 26S proteasome is responsible for the selective degradation of polyubiquitin-modified

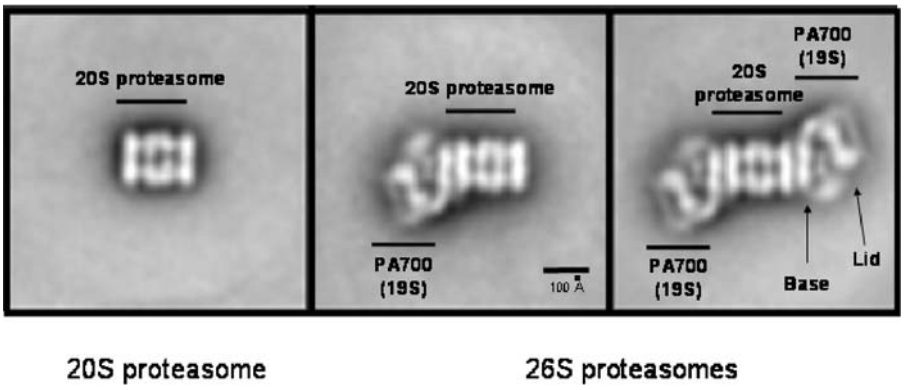

Fig. 11.1. 20S and 26S proteasomes. Image-averaged electron micrographs of 20S and 26S proteasomes from bovine red blood cells. 20S proteasome capped on one or both ends by PA700/19S RP [11].

proteins, the most extensively studied and, in our current understanding, the most physiologically important pathway for proteasome-dependent proteolysis [12]. Our presentation will focus on the structure and function of PA700/19S RP and the mechanisms by which this regulatory complex mediates selective degradation of ubiquitinated proteins. We also will present emerging data about ''non-canonical'' functions of PA700, including its role in ubiquitin-independent proteolysis by the proteasome, and its participation in non-proteolytic processes. Space limitations prohibit a complete or inclusive presentation of many interesting and important topics related to PA700/19S RP biochemistry and physiology. For detailed information about the 20S proteasome and for a specific discussion of the 26S proteasome, readers are referred to Chapters 10 and 9, respectively.

11.2 Structure

11.2.1 Component Subunits of PA700/19S RP

PA700/19S RP is a 700-kDa complex composed of approximately 18 distinct gene products. The best evidence indicates that each gene product is present in a single copy per complex [5, 6]. The overall structure and function of PA700/19S RP are highly conserved in eukaryotes, and only minor differences in subunit composition appear to exist among species (see below). Numerous proteins have been shown to associate physically with PA700/19S RP. Although many of these interactions are of unknown or questionable biological significance, it is likely that many others are physiologically meaningful. As described in detail in later sections, the functions of some of these interacting proteins raise questions about whether they should be classified as authentic PA700 subunits. In this chapter, we distinguish arbitrarily between PA700 subunits and PA700-associated proteins, but recognize that this distinction may be artificial and could be altered as a more complete understanding is obtained about the function and regulation of PA700/19S RP.

PA700/19S RP has been studied in many different species, and many individual subunits were identified prior to realization that they were components of PA700. These factors have resulted in a diverse and confusing subunit nomenclature (Table 11.1). The rational and increasingly accepted ''Rpt/Rpn'' and ''S'' nomenclatures (see below) will be used in the current presentation. An introductory description of PA700/19S PR subunits follows immediately below. Additional details of subunit functions and regulatory features in proteolysis by the 26S proteasome are presented in later sections.

The component subunits of PA700 range in size from 112 to 28 kDa. The ''S'' nomenclature identifies subunits on the basis of their relative mobility during SDS polyacrylamide-gel electrophoresis, whereas the Rpt/Rpn nomenclature distinguishes between the AAA ATPase subunits (**R**egulatory **p**article **t**riple-A protein) and the non-AAA ATPase subunits (**R**egulatory **p**article **n**on-ATPase) (see below).

Tab. 11.1. Component Subunits of PA700/19S RP.

Rpn	*S*	*Other common names*	*Subcomplex*	*Approximate MW (Daltons)*	*Reported Post-translation modifications*	*Structural Features*	*Function*
Rpt1	S7	CIM5	Base	48,500	O-Glc-NAc	AAA domain	ATPase
Rpt2	S4	YTA5, p56	Base	49,000	Phosphorylation, O-Glc-NAc	AAA domain	ATPase; gate regulation
Rpt3	S6b	TBP7, p48	Base	47,000	Phosphorylation	AAA domain	ATPase
Rpt4	S10b	SUG2, p42	Base	44,000	Phosphorylation	AAA domain	ATPase
Rpt5	S6a	TBP1, p50	Base	49,000		AAA domain	ATPase; polybubiquitn binding
Rpt6	S8	SUG1, CIM3, p45	Base	45,500	Phosphorylation, O-Glc-NAc	AAA domain	ATPase
Rpn1	S2	HRD2, NAS1, p97	Base	100,000		LRR, KEKE motifs	UBL binding
Rpn2	S1	SEN3, p112	Base	106,000		LRR, KEKE motifs	UBL binding
Rpn3	S3	SUN2, p58	Lid	61,000		PCI domain	
Rpn4		SON1		60,000			Tanscriptional regulation
	S5b	p50.5	Lid	55,000			
		p42E	Lid	42,000			
Rpn5	–	p55	Lid	53,000		PCI domain	
Rnp6	S9	p44.5	Lid	47,500		PCI domain	
Rpn7	S10a	p44	Lid	45,500		PCI domain	
Rpn8	S12	Mov34, p40	Lid	37,000	Phosphorylation		
Rpn9	S11	Nas7, p40.5	Lid	43,000		PCI domain	
Rpn10	S5a	Mcb1, Mbp1, p54	Base-lid interface	41,000		UIM motif	Polybubiquitin binding
Rpn11	S13	Poh1, Pad1	Lid	35,000		JAMM/MPN domain	Deubiquitinating metalloprotease
Rpn12	S14	NIN1, p31	Lid	31,000			
Rpn13		Daq1					
		UCH37, p37	Lid	37,000			Deubiquitining
		NAS6, p28		28,000		Ankyrin repeats	
	S15					PDZ domain	

The primary structures of all subunits have been determined, but most reveal little detailed insight about their roles. The notable exceptions are six homologous subunits of the AAA (***A***TPases ***A***ssociated with various cellular ***A***ctivities, [13–16] ATPase family (Rpt1/S7, Rpt2/S4, Rpt3/S6, Rpt4/S10b, Rpt5/S6a, and Rpt6/S8). These proteins have similar molecular weights (49–44 kDa) and contain a 200-amino acid domain characteristic of the AAA protein family [14]. These homologous domains contain Walker A and Walker B nucleotide-binding motifs of P-loop ATPases [16]. The remaining portions of the AAA PA700 subunits are divergent. Intact PA700 is an ATPase, but the relative contributions of individual ATP-

ase subunits to this overall activity and to ATP-dependent functions of the 26S proteasome remain incompletely defined [17, 18]. Nevertheless, current evidence indicates that they play distinct and non-redundant roles (see below).

The non-ATPase subunits of PA700 (Rpn1–Rpn13) represent a diverse group of proteins. Rpn1/S2 and Rpn2/S1 share a low degree of sequence similarity and are probably evolved from a common protein; they contain leucine-rich repeats likely involved in protein–protein interactions [17, 19–21]. The remaining subunits have little similarity to one another, and their primary structures generally provide little specific information about their functions. Rpn10/S5a binds polyubiquitin chains, and features of this property helped to establish a motif for this function [22, 23]. Rpn11/S13 contains a JAMM domain characteristic of metalloproteases, and this subunit has been shown to display deubiquitinating activity [24, 25]. Uch37, a non-universal subunit, also functions as a deubuiqutinating enzyme and contains a conserved cysteine residue characteristic of the active-site-family enzymes [26–28].

11.2.2
Non-universal Subunits of PA700/19S RP

Several subunits of PA700 have not been identified universally (see Table 11.1). It is unclear if these discrepancies reflect authentic distinctions among species or whether they result from differences in experimental procedures and/or analysis. For example, Rpn4 was identified as a subunit of yeast 26S proteasome, but an ortholog of this protein has not been identified in other species [29]. Subsequent work has shown the special nature of this component, and calls into question its identity as an authentic PA700 subunit. Rpn4 is constitutively short-lived and is a proteasome substrate. Inhibition of the proteasome activity results in accumulation of Rpn4, which functions as a positive transcriptional factor for global expression of proteasome subunits [30, 31]. An unidentified functional counterpart of Rpn4 may exist in higher eukaryotes because inhibition of proteasome function can upregulate expression of proteasome subunits under certain conditions [32, 33]. As described above, Uch37, a subunit with deubiquitinating activity, has been identified in PA700 from all examined sources except *Saccharomyces cerevisiae*. A Drosophila subunit termed p42E has not been identified in yeast or mammals [34], and a mammalian subunit, S5b has not been identified in yeast [6].

11.2.3
General Architecture of PA700/19S RP: The Base and the Lid

Unlike the 20S proteasome, a crystal structure has not been solved for either the 26S proteasome or isolated PA700/19S RP. Nevertheless, the general architecture of PA700/19S RP has been established, including most subunit–subunit interactions [35]. A major advance in understanding the general architecture of PA700/19S RP has been the identification and characterization of two component subcomplexes, termed the ''base'' and the ''lid'' [36]. The base sub-complex contains

eight subunits, including the six AAA ATPases (Rpt1–Rpt6), and two non-ATPases, Rpn1/S2 and Rpn2/S1. The ATPases form a heterologous six-membered ring that directly abuts the terminal α-ring of the 20S proteasome. It seems likely that the center of the ATPase ring is coaxial with the annulus of the proteasome α-ring and that substrates must pass through it to enter the proteasome (see below). The exact orientation of Rpn1/S2 and Rpn2/S1 relative to the ATPase ring is uncertain. One modeling study has suggested that these Rpn subunits form an α-helical toroid with a central pore that extends the axial channel of the proteasome and ATPase ring [37]. We are unaware of any direct experimental evidence to support this attractive model, and interpret other available data to argue against it; additional structural studies should resolve this issue. As described below, the base probably serves multiple roles in degradation of polyubiquitinated proteins and mediates the overall ATP dependence of 26S proteasome function.

The lid sub-complex contains the remaining Rpn subunits, and is linked to the base via Rpn10/S5a. Yeast with a disrupted Rpn10 gene contain proteasomes from which the lid readily dissociates [38]. The precise function of the lid is poorly understood. Rpn10/S5a is a polyubiquitin-chain-binding protein, but curiously, this property is dispensable for most normal proteasome functions [39]. The only established enzymatic activity of the lid is that of deubiquitination, as expressed by Rpn11/S13 and Uch37 subunits. Rpn11 is a Zn^{2+} metalloprotease that cleaves polyubiquitin chains from their attachment points on proteins. Uch37 is a cysteine protease that cleaves ubiquitin monomers from the distal ends of polyubiquitin chains, thereby progressively decreasing the length of the chain [26]. The relative roles of these subunits are described below. The remaining subunits of the lid have uncharacterized functions. Remarkably, however, most cells also contain an eight-membered complex termed the COP9 signalosome (CSN) with subunit-for-subunit homology to the proteasome lid [36]. CSN may play multiple roles in cellular function, and has been proposed to be physically interchangeable with the lid of the proteasome. In our judgment this exciting possibility has not been established conclusively. A critical review of CSN structure and function with respect to PA700 is beyond the scope of this chapter, but future work will clarify the relationship of CSN to proteasome function.

11.3 Post-translational Modifications of PA700

11.3.1 Overview

Given the structural and functional complexity of PA700, it is not surprising that component subunits are subject to various types of post-translational modifications. Several types of post-translational modifications of PA700 subunits have been described, but in general, the functional significance of these modifications is in an early stage of investigation.

11.3.2
Phosphorylation of PA700/19S RP

Several subunits of PA700 including Rpt2/S4, Rpt3/S6′, Rpt4/S10b, Rpt6/S8, and Rpn8/S12 have been shown to be phosphorylated [40]. The physiological significance of these modifications is poorly understood. One study has shown that treatment of cells with interferon-γ decreased PA700 phosphorylation and increased proteasome binding to PA28 (another proteasome regulator) in favor of PA700. These results suggest that phosphorylation of PA700 might alter its interaction with the 20S proteasome [41].

11.3.3
Glycosylation of PA700/19S RP

Multiple subunits of PA700 including, Rpt1/S7, Rpt2/S4, Rpt6/S8, and Rpn3/S3 are modified by *O*-linked *N*-acetylglucosamine [34, 42]. Modification of at least one PA700 ATPase subunit, Rtp2/S4, inhibits both ATPase activity and certain peptidase activities of the 26S proteasome *in vitro*, and certain proteasome functions in intact cells [42]. These results suggest that glycosylation of PA700 may be a mechanism to regulate its cellular function. Additional work will be required to understand the physiological significance of these early results.

11.4
Function of PA700/19S RP

11.4.1
A Model for PA700 Regulation of Proteasome Function

To focus our discussion of the function of PA700/19S RP in control of proteasome activity, we outline a ''canonical'' model for the ATP-dependent degradation of ubiquitinated proteins by the 26S proteasome, the best studied and most clearly established role of the 26S proteasome (Figure 11.2). PA700/19S RP serves multiple roles in mediating proteasomal degradation of ubiquitinated proteins. First, PA700 relieves the structurally imposed inhibition of proteolytic activity by opening the blocked gates at the α-terminal rings of the 20S proteasome. Second, PA700/19S RP serves as the recognition and binding element for the polyubiquitin degradation tag. Third, PA700/19S RP prepares the protein substrate for degradation by destabilizing its tertiary and/or quaternary structure, and translocating the unfolded polypeptide chain through the proteasome's open ends to the central chamber containing catalytic sites responsible for peptide-bond hydrolysis. Fourth, PA700 removes the polyubiquitin chain from the protein substrate, an essential function for complete proteolysis. PA700-catalyzed ATP hydrolysis is obligatory for overall proteolysis and each of the PA700-mediated processes listed above may

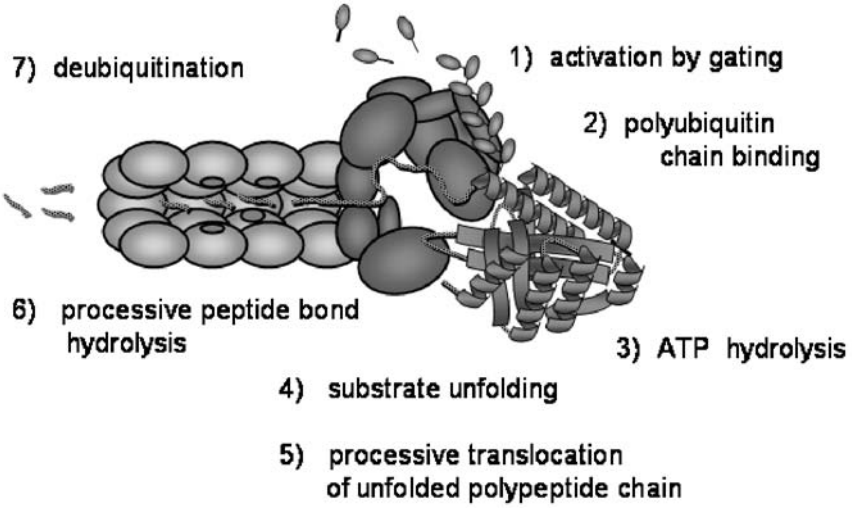

Fig. 11.2. Canonical model for the degradation of polyubiquitinated proteins by the 26S proteasome. The canonical model for the degradation of polyubiquitinated proteins by the 26S proteasome involves multiple functions of PA700 including: 1) PA700 activates the 20S proteasome by opening the gate on the terminal rings of the proteasome; 2) PA700 specifies selectivity degradation of polyubiquitinated proteins by direct recognition and binding of polyubiquitin chains; 3, 4 and 5) PA700 is an ATPase that utilizes ATP hydrolysis to unfold and translocate substrates to the proteasome; 6) PA700 initiates processive proteolysis by translocating the N- or C-terminus of the substrates to the proteasome; and 7) PA700 removes the polyubiquitin chain from the substrate; for the purpose of this illustration, the deubiquitination is shown as a processive process starting at the distal end of the chain, but the deubiquitinating activity most closely linked to substrate degradation probably occurs at the isopeptide bond proximal to the substrate. See text for details on each of these processes.

be coupled to ATPase activity. In the following sections, we present detailed features for each of these PA700 functions. For convenience and clarity, we present these functions separately and in arbitrary order. We recognize, however, that they probably are temporally coordinated and mechanistically linked. Following a description of this canonical model for PA700/19S RP function, we present emerging data about non-canonical functions for PA700/19S RP in the degradation of non-ubiquitinated proteins, and for non-proteolytic roles of PA700/19S RP.

11.4.2 Roles of ATPase Activity in PA700 Function

The degradation of ubiquitinated proteins by the 26S proteasome requires PA700-catalyzed ATP hydrolysis. How is proteolysis mechanistically linked to ATPase activity? Despite the fundamental nature of this question, a detailed answer is unknown. As described below, ATP hydrolysis probably mediates multiple elements

of 26S proteasome function, including assembly of the complex from 20S proteasome and PA700 sub-complexes, proteasome activation, polyubiquitin-chain binding, and substrate unfolding, translocation, and deubiquitination. Although ATPase activity of PA700/19S RP is catalyzed by the AAA ATPase subunits of the base, the relative roles and contributions of the six different ATPase subunits to various functions remain unclear. Available biochemical and genetic evidence suggests that these subunits play distinct non-redundant roles [43], but there are insufficient existing data to be certain that this represents a complete division of labor for these ATPases among different ATP-dependent processes. In any case, the hexameric ring-shaped structure of the ATPases and the position of this ring on the α-ring of the proteasome are almost certainly critical to their functions. Interestingly, the same ring-to-ring orientation of protease and AAA ATPase is found in non-proteasomal ATP-dependent proteases in bacteria, suggesting that this topology is intimately linked to regulation of proteolysis [44].

11.4.3 Proteasome Activation by PA700

The 20S proteasome displays inherently low catalytic activity because of its structure, which excludes substrates from interacting with the catalytic sites. Substrates reach the sequestered catalytic sites only after passing through a narrow 13-Å annulus formed by the terminal α-rings of the proteasome [45–47]. This structural feature is sufficient to prevent entry of substrates with appreciable tertiary structure, but even short or unfolded polypeptides must overcome a second structural impediment posed by the proteasome. Specifically, the annulus is physically occluded by N-terminal peptides of four α-subunits that project across it [46]. Activation of the proteasome by regulatory proteins such as PA700 involves clearing the occlusion by conformational rearrangement of the α-ring peptides. Thus, the terminal rings of the proteasome act as a regulated gate and a critical role of PA700/19S RP is to activate the proteasome by opening the gate. A detailed molecular explanation for PA700-induced proteasome activation is lacking in the absence of the crystal structure of the 26S proteasome, but it seems highly likely that activation is generally analogous to proteasome activation by a distinct proteasome activator, PA28. PA28 is a heptameric ring-shaped protein that activates the proteasome's hydrolysis of short peptides. A co-crystal structure of yeast 20S proteasome and PA26, an ortholog of PA28 from *Trypanosome brucei*, has been determined [48]. Binding of C-termini of PA28 subunits to the α-ring of 20S proteasome promotes an interaction between an ''activation'' domain within individual PA28 subunits and the annulus-occluding peptides of proteasome subunits. This interaction conformationally rearranges the occluding polypeptides from a position roughly perpendicular to the central proteasome channel to one roughly parallel to the channel and projecting into PA28, thereby opening a pore through which substrates may pass. Unlike PA700, PA28 does not promote the proteasome's degradation of ubiquitinated proteins, presumably because PA28 lacks other essential features for processing such substrates (see below). This example highlights the multiple functions that PA700

must conduct for degradation of polyubiquitinated proteins and distinguishes it from simpler regulators such as PA28.

Several lines of biochemical evidence strongly indicate that PA700 also activates the proteasome by relieving occlusion of the proteasome pore, and involves the physical interaction between the heterohexameric AAA ATPase ring of the base and the heteroheptameric α-ring of the proteasome. First, binding of PA700 to the proteasome greatly activates the hydrolysis of short peptide substrates, suggesting that PA700 binding increases access of these substrates to the catalytic sites [8]. This effect can be accomplished entirely by the base sub-complex, indicating that the interaction of the ATPase ring is sufficient for activation [36]. Second, deletion of the pore-occluding peptide of the α3 proteasome subunit results in a constitutively active 20S proteasome, whose activity is not stimulated further by binding to PA700 [49]. The amino terminus of the α3 subunit contacts all other α-subunit peptides that block the pore and seems to be particularly important for the general organization of the structure that obstructs the annulus of the proteasome. Addition of the deleted α3 peptide to the mutant 20S proteasome in *trans* restores the "latent" inhibited state to the enzyme, further demonstrating the critical role of this peptide. Third, mutations in the ATP-binding domain of the Rpt2 ATPase subunit of PA700 have no effect on binding of PA700 to either wild-type or mutant proteasomes with constitutively high activity due to N-terminal deletions of α3 and α7 subunits [50]. Nevertheless, the 26S proteasome composed of mutant Rpt2 and wild-type (i.e. "latent") 20S had low protease activity, whereas 26S proteasome composed of both mutant PA700 and mutant 20S proteasome had high proteasome activity. Thus, a mutant Rpt2 cannot activate wild-type proteasome, whereas a constitutively active mutant 20S proteasome can suppress the inhibitory effect of PA700/19S RP with mutant Rpt2. These results indicate that the Rpt2 subunit of PA700 activates the proteasome by a mechanism involving gating of the annulus [50].

Despite some generally similar features between PA700- and PA28-mediated proteasome activation, detailed mechanisms of these processes are likely to have significant differences. In the case of PA28, binding to the proteasome is necessary and sufficient for activation and neither binding nor activation requires other cofactors such as ATP. In contrast, PA700 binds to the proteasome by an ATP-dependent process whose molecular mechanism is undefined. Moreover, PA700 may utilize ATP for an additional role in proteasome activation *per se*, as suggested by the inhibited states of 26S proteasomes containing Rpt2 mutants [50]. Further work will be required to define the exact role of ATP in proteasome activation by PA700. It is also interesting to note that unlike the proteasome–PA28 interaction, binding and activation of the proteasome by PA700 likely involves an initial symmetry mismatch between the seven-membered proteasome ring and the six-membered ATPase ring of PA700. Such a mismatch also occurs between the homohexamer ATPase rings and the homoheptameric protease rings of bacterial ClpAP protease, and may play a significant role in the mechanism of proteasome activation.

11.4.4 Polyubiquitin-chain Binding

A major physiological role of PA700 is the recognition of polyubiquitinated proteins. This function serves as the principal determinant for the selectivity of ubiquitin-modified proteins for degradation by the 26S proteasome. PA700 binds K48–G76 linked polyubiquitin chains composed of four or more ubiquitin moieties with high affinity, but the exact molecular basis for this property remains poorly understood [51]. Two subunits of PA700, Rpn10/S5a and Rpt5/S6a, have been identified as polyubiquitin-chain-binding proteins, and the features of these properties are described immediately below. Moreover, cells contain other polyubiquitin-binding proteins that interact with the 26S proteasome and may function to deliver substrates to it for degradation (described in later sections).

Rpn10/S5a was the first PA700 subunit to be identified as a polyubiquitin-chain-binding protein [22, 52]. It selectively binds K48–G76 polyubiquitin chains, as demonstrated by "far western" methodology [22, 39, 53–55] or by affinity chromatography with tagged recombinant Rpn10/S5a. Such isolated versions of Rpn10/S5a bind polyubiuquitin chains composed of four or more ubiquitin moieties, thereby mimicking the features of the chain requirements for overall degradation of polyubiquitin-modified proteins. Soluble recombinant Rpn10/S5a inhibits the degradation of ubiquitinated proteins in cell-free extracts, presumably by competing with polyubiquitinated proteins for the 26S proteasome [56, 57]. Structure–function analysis of Rpn10/S5a from various sources has identified a short hydrophobic sequence responsible for polyubiquitin binding. The motif, termed the ***u***biquitin-***i***nteracting ***m***otif (UIM), is found in many proteins, including others involved in various aspects of ubiquitin metabolism [23]. Rpn10/S5a from *Saccharomyces cerevisiae* and *Arabidopsis* contain a C-terminal UIM [58], whereas human and *Drosophila* Rpn10/S5a contain two UIMs [57]. Each isolated site can bind polyubiquitin independently, albeit with very different affinities. The two UIM sites may bind polyubiquitin with some degree of cooperation in intact Rpn10/S5a. In any case, UIMs appear to bind to hydrophobic patches composed of side chains from ubiquitin residues L8, I44, and V70. Structure–function analysis of ubiquitin has established the importance of these residues for ubiquitin's role in targeting proteins for degradation [53]. Therefore, a reasonable model suggests that complementary hydrophobic patches on ubiquitin and the Rpn10/S5a form the interaction site for these proteins [53, 58, 59]. The topology of Rpn10/S5a as a structural link between the base and lid sub-complexes of PA700 further suggests that this subunit would be well positioned to deliver bound polyubiquitinated substrates to the base for ATP-dependent unfolding and translocation into the proteasome.

Despite the initial indication that Rpn10/S5a was the polyubiquitin-chain-binding subunit of the 26S proteasome, subsequent studies showed that it cannot be the principal recognition element for this process. First, disruption of the Rpn10/S5a gene in yeast is not lethal and inhibits the degradation of only a sub-class of ubiquitinated proteins [39]. Likewise, RNA interference-inhibited ex-

pression of Rpn10/S5a in Drosophila cells does not inhibit growth or overall ubiquitin-dependent protein degradation [32]. These features are not expected for a protein with an essential role in ubiquitin-dependent proteolysis. Second, deletion of the single conserved UIM in yeast has no effect on the degradation of Ub–Pro–β-galactosidase, a substrate whose cellular degradation otherwise requires expression of Rpn10/S5a [58]. Remarkably, the degradation of this model substrate of the ubiquitin-fusion-dependent pathway of the ubiquitin–proteasome system does require the N-terminal domain of Rpn10/S5a, demonstrating that this region of the Rpn10/S5a, but not the UIM, is responsible for a critical function in the degradation of at least one cognate substrate. Notably, the N-terminus is highly conserved in all Rpn10/S5a sequences, even though this domain is not required for assembly of Rpn10/S5a into PA700. In sum, these findings indicate that Rpn10/S5a does not serve an obligatory role as an exclusive polyubiquitin-chain-binding component of the 26S proteasome, and the exact significance of the polyubiquitin-chain-binding properties of Rpn10/S5a remains unclear. As described below, Rpn10/S5a can also interact with proteins containing ***ub***iquitin-***l***ike domains (UBLs), and could mediate interactions between PA700 and such proteins. Moreover, Rpn10/S5a appears to exist in a non-proteasome-associated form in some cells. This finding could indicate alternatively that Rpn10/S5a has non-proteolytic roles or that it could function as a polyubiquitin-transfer factor that carries substrates to the proteasome (see below for a discussion of other polyubiquitin-chain-binding proteins that may also serve such a function). In either case, we note that studies demonstrating the binding of polyubiquitin to Rpn10/S5a have been conducted with the isolated protein, a limitation imposed by certain technical constraints of the experiments. In contrast, some evidence indicates that the proteasome-associated Rpn10/S5a may not be competent to bind polyubiquitin [60] (although other data appear to contradict this conclusion [38]); the different results may reflect differences in experimental details. In summary, although most available evidence argues against an obligatory role for Rpn10/S5a in polyubiquitin binding by 26S proteasome, we believe that it is premature to dismiss its role in this process. Additional work will be required to delineate the nature and contribution of Rpn10/S5a to polyubiquitin-chain binding of PA700.

The ''far-western'' methodology that originally identified Rpn10/S5a as a polyubiquitin-chain-binding protein failed to identify other components of the 26S proteasome with this property. This result, however, may reflect unique features of Rpn10/S5a that permit it to retain this function under the harsh conditions required for the far-western binding analysis. In fact, the short UIM responsible for binding is unlikely to have significant tertiary structure [59]. Nevertheless, these results do not exclude the possibility that other PA700 subunits bind polyubiquitin chains under native conditions. Recently, chemical cross-linking has been used to identify Rpt5/S6a as a second polyubiquitin-chain-binding protein of PA700 [60]. Rpt5/S6a is an AAA ATPase of the base and was identified as the cross-linked product of a photoactivatable variant of K48–G76-linked tetra-ubiquitin and purified 26S proteasome. This study produced several important and surprising findings. First, of all 26S proteasome subunits only Rpt5/S6a was labeled specifically by the

tetra-ubiquitin. Purified recombinant Rpn10 was also efficiently labeled by this method, further supporting the view that it is a polyubiquitin-chain-binding protein, but loses this property when it is a constituent of the intact PA700 structure. Second, the interaction between Rpt5/S6′ and polyubiquitin required ATP hydrolysis. This effect could not be attributed to the role of ATP in maintaining the structural integrity of the 26S proteasome. Electron paramagnetic resonance, a direct indicator of binding, confirmed the ATP-dependent physical interaction between tetra-ubiquitin and Rpt5/S6a. Importantly, $(Ub)_5$-dihydrofolate reductase, an established proteolytic substrate of the 26S proteasome, competitively inhibited both the cross-linking and the EPR-monitored binding of tetra-ubiquitin to 26S proteasome. These results establish Rpt5/S6a as a polyubiquitin-chain-binding protein. The molecular basis for the interaction between Rpt5/S6a and polyubiquitin is unknown, and we are unaware of similarities in the structure of this protein with motifs known to interact with polyubiquitin. Unlike Rpt10/S5a, Rpt5/S6a is an essential protein in yeast, and RNAi of Rpt5/S6a significantly reduced growth of Drosophila S2 cells [32, 43]. The selective cross-linking of Rpt5/S6a provides another example of the non-redundant properties of the six ATPases that compose the base structure of PA700. Although these results suggest that polyubiquitin binding at the base of PA700 positions the substrate near to the axial channel of the proteasome, ''lidless'' proteasomes from yeast lacking Rpn10, have defective degradation of model ubiquitinated proteins, suggesting that other features of the lid are important for manifestation of normal degradation of ubiquitinated proteins [36]. These various results highlight that the molecular basis of polyubiquitin-chain binding to PA700 remains poorly understood and is likely to be highly complex.

11.4.5 Unfolding/Modification of Substrates

Many proteins degraded by the proteasome, including those of high regulatory significance, retain most or all of their native tertiary structure after they are ubiquitinated. Because of structural features of the proteasome described above, it is clear that the tertiary structures of these proteins must be destabilized prior to their proteolysis. Moreover, some proteins are ubiquitinated and selectively degraded while they are components of multi-subunit complexes. In such cases, the quaternary structure of the complex must be destabilized to allow the ubiquitinated subunit to be selectively dislodged and degraded. The canonical model of 26S proteasome function posits that PA700/19S RP directly destabilizes substrates under each of these conditions. In other words, once a protein is targeted to PA700/19S RP by its polyubiquitin chain, PA700/19S RP unfolds the protein to allow its transit through the opened annulus of the proteasome for degradation. An excellent example of the ability of PA700/19S RP to accomplish this function directly is the selective degradation of ubiquitinated Sic1 from a Sic1/Cdk/cyclin complex by purified 26S proteasome [61]. An analogous example is the selective degradation of IκB, an inhibitor of the heterodimeric NFκB transcription factor complex, although the direct action of PA700 is this case has not been demonstrated. The exact mech-

anisms by which PA700/19S RP carries out protein unfolding of monomeric or multimeric proteins are unclear. Several studies have demonstrated that PA700/19S RP has chaperone-like properties that would likely participate in substrate unfolding. For example, PA700/19S RP inhibits the aggregation of misfolding proteins and catalyzes the refolding of certain heat- and chemically denatured proteins [62–64]. These properties are manifested by the base sub-complex, indicating a role for ATPase subunits in these functions; at least one study has shown that these features are indeed regulated by ATP [62]. Such results indicate that in addition to polyubiquitin chains, PA700 also recognizes and interacts with certain structural features of non-native proteins. Such features are likely to occur transiently or in specific limited regions of proteins with otherwise high global stability. Thus, proteins initially targeted to PA700/19S RP by a polyubiquitin chain would be subject to this type of secondary interaction. Once such features are recognized by PA700, further destabilization could occur, perhaps linked to cycles of ATP hydrolysis and/or processive proteolysis. Support for this model has been obtained by examining ubiquitin/proteasome-dependent degradation of stable model proteins in reticulocyte extracts [65]. These elegant experiments indicate that 26S proteasome unfolds and degrades proteins processively starting at a point near the polyubiquitination site. Replication of these results in a defined system of purified proteins will confirm PA700/19S RP as the agent that directly unfolds the substrate. Thus, despite considerable overall progress, a detailed molecular mechanism for protein unfolding by PA700 remains very poorly understood. Some of the outstanding issues yet to be resolved include: the exact nature of the interaction between PA700 and the substrate, the role of ATP hydrolysis in substrate unfolding, and details of the likely mechanistic linkage between substrate unfolding, translocation, and proteolysis.

11.4.6
Translocation of Substrates from PA700/19S RP to the Proteasome

With few known exceptions, substrates of the 26S proteasome are degraded completely to short peptides and amino acids once they are engaged by the proteasome. This feature suggests that proteolysis is processive, and examples of processive proteolysis by the proteasome have been demonstrated for several model substrates [65–67]. To satisfy the requirement for unstructured proteins as suitable proteasome substrates, processive proteolysis may often be initiated at a free N- or C-terminus, which could pass easily through the opened annulus of the proteasome. Processive proteolysis could then proceed by a mechanism linked to successive unfolding and translocation of the rest of the substrate. This model is also compatible with the few known examples of limited proteasomal proteolysis, in that degradation could start at one terminus of a protein and proceed processively until reaching a ''stop translocation/degradation'' site dictated by a structural feature of the substrate. Stalled substrates could be released from the proteasome, thereby generating the mature processed protein. The p105 subunit of NFκB is the best example of a protein processed by this mechanism. In this instance, the C-terminal half of

p105 is degraded to yield the mature p50 subunit of NFκB [68]. Although it seems clear that processive proteolysis initiates from a free terminus of many proteins, the proteasome can also catalyze endoproteolysis [69, 70]. This activity requires an unstructured region, presumably to form a loop that can be accommodated by the opened proteasome annulus. Such a structure could be assumed by certain disordered proteins or could be present in certain regions of otherwise well-folded proteins. In any case, once endoproteolysis is achieved, processive proteolysis could proceed from newly generated N- or C-termini, leading either to complete or to limited proteolysis. We assume that PA700/19S RP plays an important role in driving substrate translocation for all processive proteolysis, regardless of its mode of initiation. However, as with unfolding, considerably more work will be required to understand the detailed mechanisms of PA700-mediated substrate translocation.

11.4.7 Deubiquitination of Substrates by PA700/19S RP

Degradation of polyubiquitinated proteins by the 26S proteasome results in the proteolysis of the substrate, but not of ubiquitin moieties that compose the polyubiquitin chain. Instead, the polyubiquitin chain is removed from the substrate during proteolysis and this process appears to be obligatory for, and coupled to, substrate degradation. Two PA700 lid subunits, Rpn11/S13 and Uch37, as well as several PA700-associated proteins, (see below), catalyze deubiquitinating activity. Rpn11/S13 contains a JAMM domain characteristic of a Zn^{2+} metalloprotease catalytic site [24, 25]. Rpn11/S13 occurs in PA700 from all sources, and is an essential protein [24, 25, 71]. Rpn11/S13 cleaves the isopeptide bond linking the polyubiquitin chain to the substrate. This reaction is catalyzed by both free PA700 and 26S proteasome, but curiously depends on ATP hydrolysis only with the 26S proteasome. This feature highlights another likely role of ATPase activity in proteolysis by the 26S proteasome. Because there is no reason to believe that the isopeptide bond catalysis *per se* requires energy, the energy dependence of Rpn11-catalyzed deubiquitination may be linked to translocation or unfolding of the substrate whereby the isopeptide bond is made available for cleavage. Removal of the polyubiquitin chain is probably important for overall substrate degradation on steric considerations because the bulky chain would impede translocation of the attached polypeptide substrate through the opened pore of the proteasome. In fact, inhibition of Rpn11 severely reduces rates of proteolysis by the 26S proteasome [24, 25]. Uch37 is the second deubiquitinating subunit of PA700 [25, 26]. This protein does not exist in budding yeast, but is found in *Schizosaccharomyces pombe*, *Drosophila*, and all mammals [72]. Like Rpn11 it is found in the lid, and immunoelectron microscopy has localized it to a peripheral site on PA700 [73]. Uch37 cleaves ubiquitin from the distant end of polyubiquitin chains [27]. The exact significance of this type of activity is unclear. It is conceivable that it provides an "editing" function whereby tagged proteins that do not become engaged in degradation within a reasonable time are deubiqutinated and released from the proteasome. Unlike Rpn11,

decreased expression of Uch37 has little effect on cell viability, proteasome function, or global ubiquitin-dependent protein degradation [32, 72].

11.5 Interaction of PA700 with Non-proteasomal Proteins

11.5.1 Overview

Numerous studies have identified interactions between individual subunits of PA700 and non-proteasomal proteins. In fact, many subunits of PA700 were first described as interacting proteins of non-proteasomal bait proteins in yeast two-hybrid screens whose purpose was unrelated to direct investigation of the proteasome or protein degradation. Other interactions have been found by various approaches between 26S proteasome, PA700, or individual PA700 subunits and proteins both with and without obvious relationship to the ubiquitin–proteasome system [74, 75]. It is difficult to judge the physiological significance of many of these interactions, which could reflect authentic, but currently unrecognized regulatory interactions between PA700 and proteins of the ubiquitin system or other cellular process, interactions between the 26S proteasome and proteolytic substrates, or spurious interactions with no physiological significance. It is beyond the scope of this chapter to review and evaluate all of these reports. Instead, we will focus on selected examples from several classes of PA700-interacting proteins whose identification seems firmly established and/or that have a rationale for or a promise of physiological significance. Many of these PA700-interacting proteins contain a UBL domain, which appears to serve as a common structural element for the interaction of these proteins with the proteasome (see below). UBL domains have low primary sequence similarity to ubiquitin, but assume a general three-dimensional structure remarkably like that of ubiquitin. UBL domains occur in many proteins, most of which do appear to interact with PA700. As noted above, we arbitrarily classify these proteins as ''non-proteasomal'' but recognize that additional work may alter this classification. (For an overview see Figure 11.3.)

11.5.2 26S Proteasome Assembly/Stability Proteins

The exact cellular process by which the 26S proteasome is assembled remains unknown, but the best evidence suggests that it results from binding of independently assembled 20S proteasome and PA700/19S RP [76]. There is considerable, but incomplete, information about the assembly of the 20S proteasome [77, 78], whereas very little is known about the assembly of PA700. Formation of 26S proteasome from purified 20S proteasome and PA700 can be achieved *in vitro* by an

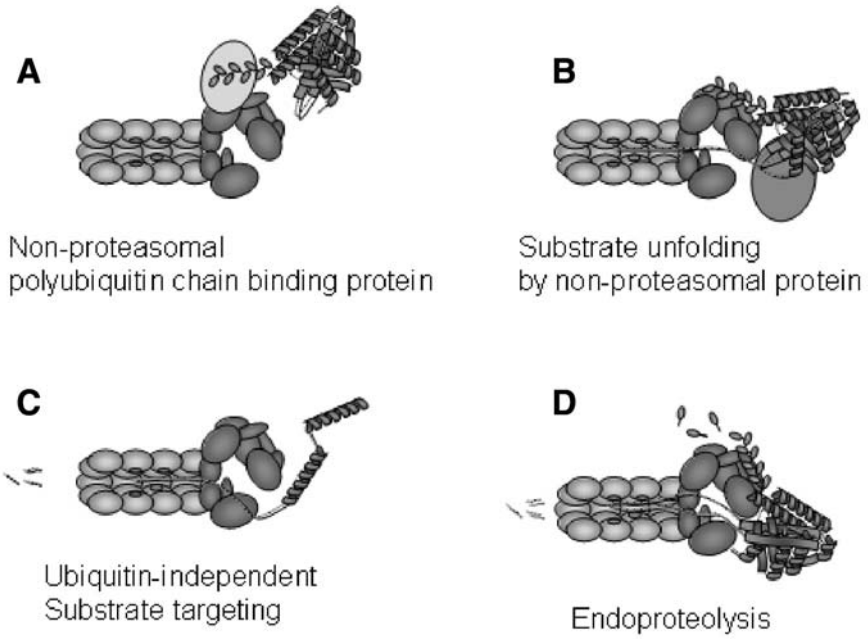

Fig. 11.3. Non-canonical functions of PA700 in the regulation of proteasome activity. A) Certain polyubiquitinated proteins may be recognized by and bound to non-proteasomal proteins that subsequently transfer them to the proteasome after binding to PA700. B) Certain substrates may be unfolded by non-PA700 proteins and then transferred to PA700. C) PA700 may promote degradation of some non-ubiquitinated proteins by recognizing and binding unstructured regions of these proteins. D) PA700 may promote endoproteolysis of certain substrates through recognition of unstructured portions of those substrates. Permutations of these and other functions, not shown explicitly here, likely provide multiple variations of PA700-mediated processes. See text for details.

ATP-dependent process. This suggests that PA700 and 20S proteasome are sufficient for assembly of 26S proteasome [8]. This process, however, is inefficient *in vitro*, and one study has indicated that the Hsp90 mediates 26S proteasome assembly [79]. Recently, a protein termed ecm29 was identified as a stoichiometric component of the 26S proteasome from *Saccharomyces cerevisiae* purified by affinity chromatography without exposure to high salt concentrations [80]. Ecm29 is a 200-kDa protein that binds to both the 20S proteasome and PA700 and has been proposed to tether the two sub-complexes. Electron microscopy reveals a V-shaped protein that may act as "clip" between the α-rings of the 20S proteasome and the base of PA700, but little is known about the molecular basis of such binding. Ecm29 stabilizes 26S proteasome in the absence of ATP, further supporting a role for it in physically linking 20S proteasome to PA700. Orthologs of ecm29 are widely distributed among species, but further study of the protein in yeast and other organism will be required to establish its precise function in 26S proteasome structure and function.

11.5.3 Deubiquitinating Enzymes

Deubiquitinating enzymes constitute a large class of proteins in the ubiquitin system, and play a largely unexplored role in ubiquitin biology (29). Ubp6/Usp14 is a widely distributed deubiquitinating enzyme that appears to be a stoichiometric component of yeast 26S proteasomes isolated without exposure to high salt [80]. This finding suggests that Ubp6 may be an authentic, but easily dissociated, PA700 subunit. Ubp6 contains an N-terminal UBL domain that is responsible for binding to PA700 via the Rpn1/S2 subunit of the base [80]; Rpn1/S2 interacts with several other proteins containing UBL domains (see below). Ubp6 has low catalytic activity as an isolated protein. However, the deubiquitinating activity of Ubp6 is enhanced over 100-fold when the protein is associated with yeast PA700, suggesting that catalytic function is restricted to PA700. Ubiquitin vinyl sulfone, an agent that covalently modifies the active sites of many deubiquitinating enzymes, was used to identify Ubp6/Usp14 as a component of mammalian 26S proteasome [81]. This result also supports the conclusion that Ubp6/Usp14 is active only when associated with the proteasome. Yeast from which Ubp6 has been deleted are viable, but are defective for degradation of some, although not all, model substrates of the ubiquitin system. Interestingly, ubiquitin itself is destabilized in this mutant, suggesting that failure of Ubp6 to deubiquitinate modified substrates at the proteasome results in proteolysis of ubiquitin itself. Inhibition of deubiquitinating activity of PA700 *in vitro* also leads to degradation of ubiquitin attached to protein substrates [25].

In addition to Ubp6, Doa4 is a deubiquitinating protein that associates with the 26S proteasome [82]. Unlike Ubp6, Doa4 is present as a sub-stoichiometric component [83]. The N-terminus of this protein is required for proteasome binding and is sufficient to direct association of otherwise non-interacting proteins to the complex. Yeasts from which Doa4 are deleted are viable, but are defective in degradation of model proteins in several pathways of the ubiquitin–proteasome system. Moreover, over-expression of Doa4 increases rates of degradation of certain substrates, suggesting that it can enhance the function of the proteasome. In contrast, Doa4 also functions in the vacuolar protein-sorting and endocytic pathways, perhaps acting on ubiquitin-modified plasma membrane proteins targeted to the vacuole [84, 85]. Although these later data imply a non-proteasomal function for Doa4, it is possible that Doa4 plays multiple distinct roles, or that the proteasome is involved in aspects of ubiquitin-dependent membrane/vacuolar processes.

11.5.4 Ubiquitin-conjugating Machinery

Numerous components of the ubiquitin-conjugating system, including various E2-conjugating enzymes and E3 ligases, have been identified as PA700-interacting proteins [86]. Such findings raise the intriguing possibility that substrate ubiquitination and degradation are spatially linked. Although available data do not provide

the precise physiological significance or mechanistic details of such linkage, it is easy to imagine that spatial coupling of ubiquitination and degradation could improve the efficiency of substrate targeting to and processing by the proteasome. We briefly list some components of the ubiquitin-conjugating machinery reported as proteasome interacting proteins.

Hul5 is a stoichiometric component of affinity-purified yeast 26S proteasomes purified under low-salt conditions [80]. Hul5 is a HECT-domain E3 ligase known as KIAA10 in mammals. It assembles both K48- and K29-linked polyubiquitin chains and binds to PA700 and to isolated Rpn1/S2 via an N-terminal domain [87].

Ubr1, the E3 ligase of the N-end rule, and Ufd4 a ligase of the Ufd pathway, associate with PA700 [88]. Ubr1 interacts with Rpn2/S1, whereas Ufd4 interacts with the Rpt6/S8 ATPase of the base.

CHIP is a U-box E3 ligase that ubiquitylates misfolded proteins [89]. CHIP forms a complex with Hsp70 and BAG1, a UBL-domain protein. BAG1, like some other UBL-domain proteins, associates with PA700 [90]. These findings suggest an attractive model in which a complex of CHIP, Hsp70, and BAG1 binds to the 26S proteasome to partition misfolded Hsp70 substrates to ubiquitylation and degradation instead of refolding. The physical association of these components might improve the efficiency of degradation. Moreover, because these substrates are misfolded prior to reaching PA700, they might be particularly susceptible to aggregation, and therefore harmful to the cell if left unescorted at each step of degradative process. Formation of a complex to achieve all of the functions of the degradative pathway would likely be of considerable benefit to the cell.

E6-AP, a HECT-domain E3 ligase, and βTrcCP, the F-box component of an SCF-type E3 ligase, associate with hPLIC proteins, the mammalian versions of yeast Dsk2 [91] (see below). PLIC proteins contain both UBL and UBA (***ub***iquitin pathway ***a***ssociated) domains that mediate PLIC interactions with PA700 [92]. This finding raises the possibility that other examples of the many cellular F-box proteins of SCF complexes associate with PA700.

11.5.5 Polyubiquitin-chain-binding Proteins

The canonical model of 26S proteasome function described above invokes the direct recognition of polyubiquitin-modified substrates by polyubiquitin-chain-binding subunits of PA700, such as Rpt5/S6′ and/or Rpn10/S5a. However, cells contain a variety of non-proteasomal proteins that also bind polyubiquitin chains. Many of these proteins have established roles in ubiquitin conjugation and deconjugation, whereas others have unknown functions. Although some of the latter proteins likely mediate non-proteolytic roles of ubiquitin, others also bind to the 26S proteasome via PA700. Such a property suggests a model in which certain polyubiquitinated proteins are initially recognized by and bound to non-proteasomal proteins, and subsequently delivered to the proteasome for degradation [38, 93]. This general mechanism could provide additional regulation and versatility for

substrate selection. Evidence in support of this model has been obtained for several polyubiquitin-chain-binding proteins including, Rad23, Dsk2, and VCP$^{Ufd1/Npl4}$, as described below.

Rad23 and Dsk2 are widely distributed eukaryotic proteins, identified originally in yeast as mediators of DNA repair and spindle-pole duplication, respectively. Rad23 and Dsk2 have similar domain structures; each contains an N-terminal UBL domain, which is responsible for their respective binding to PA700 [38, 93–95] and UBA domains near their C-termini (Rad23 contains two UBA domains, whereas Dsk2 contains only one). UBA domains are found in numerous proteins, and like the UIM of Rpn10/S5a, function as polyubiquitin-chain-binding elements [96, 97]. Thus Rad23 and Dsk2 can bind to both PA700 and polyubiquitin. Several early reports indicated that the UBL of Rad23 binds to the second UIM of human Rpn10/S5a, suggesting that Rpn10/S5a was the Rad23-receptor of PA700 [98]. Although this interaction has been verified [59], 26S proteasomes lacking Rpn10/S5a still bind Rad23, indicating that another subunit also serves this function. Moreover, yeast lacks the second UIM of mammalian Rpn10/S5, making it unlikely that this interaction is of general significance. More recently, Rad23 and Dsk2 have been shown to bind to PA700 via leucine-rich repeats of Rpn1/S2 and Rpn2/S1, the non-ATPase components of the base [38, 94]. This finding has interesting mechanistic implications, because substrates bound to Rad23 or Dsk2 at the base of the 26S proteasome would be positioned for unfolding and translocation into the proteasome by the AAA ATPase subunits. Several lines of evidence involving expression of wild-type and mutant variants of Rad23 and Dsk2 in yeast support the general model of these proteins as carriers of polyubiquitinated substrates to the 26S proteasome [38, 93, 99, 100]. Despite these data, the mechanisms by which substrates would be transferred from carrier proteins to the proteasome remain unclear. Moreover, other results are inconsistent with a carrier model. For example, Rad23 inhibits 26S proteasome-dependent degradation of an otherwise susceptible polyubiquitinated protein *in vitro* [101]. Rad23 also inhibits polyubiquitin-chain formation. Finally, several studies indicate that the interaction between Rad23 and PA700 mediates DNA repair by a mechanism independent of proteolysis [102, 103] (and see below). These various results indicate that Rad23 biology is likely to be complex and may affect PA700 function by multiple mechanisms. Detailed mechanistic information about this relationship will be required to clearly interpret the results of cellular experiments.

The VCP/Npl4/Ufd1 complex represents another prominent example of a polyubiquitin-chain-binding protein that may aid targeting of certain substrates to the 26S proteasome. VCP, known as Cdc48 in yeast, is a ring-shaped homohexamer of 90-kDa AAA ATPase subunits [104]. Thus, VCP assumes an architecture similar to that of the AAA ATPases of the PA700 base and some other AAA ATPases [105]. VCP binds polyubiquitin chains [106, 107], and appears to play diverse cellular roles, determined in part by its differential association with various proteins that modulate its function. For example, VCP forms a complex with two additional polyubiquitin-chain-binding proteins, Npl4 and Ufd1, to mediate ERAD (***e***ndoplasmic ***r***eticulum-***a***ssociated ***d***egradation) [107, 108]. The role of

the ubiquitin–proteasome system in ERAD are covered in detail elsewhere. In brief, ERAD is the process by which endogenous ER proteins, or proteins that transit through the ER, are constitutively or conditionally degraded. ERAD plays a critical role in protein quality control; mutant proteins that fail to fold properly in the ER are retrotranslocated to the cytoplasm, ubiquitylated, and degraded by the proteasome. $VCP^{Npl4/Ufd1}$ is required for degradation of both normal and mutant ER proteins, and may couple its polyubiquitin-chain-binding properties to ATPase activity to assist in the translocation of substrates across the membrane [105, 108]. Certain VCP mutants as well as RNAi-decreased expression of VCP inhibits ubiquitin-dependent proteolysis, suggesting that VCP may effect degradation of both ERAD and non-ERAD substrates [106, 109]. Despite the strong evidence for a critical role of $VCP^{Npl4/Ufd1}$ in ERAD, the exact physical relationship between it and the 26S proteasome remains unclear. At least one report has indicated that VCP binds to the proteasome, but it is unclear whether this interaction is direct [110].

In summary, despite emerging evidence that certain polyubiquitinated proteins are targeted to the 26S proteasome by carrier proteins, additional molecular details will be required for verification of this attractive model. These details include the manner in which specific substrates are selected by different polyubiquitin-chain-binding proteins, and how the substrates are transferred from these proteins to PA700.

11.5.6
Roles of PA700 in Ubiquitin-independent Proteolysis

The 26S proteasome is the only identified protease that selectively degrades polyubiquitinated proteins. Although many, and perhaps most, cellular proteins are degraded by the ubiquitin–proteasome system, certain non-ubiquitinated proteins are also substrates for the 26S proteasome. This latter function suggests that PA700 can recognize and interact with features other than polyubiquitin chains for selection of certain substrate proteins. Ornithine decarboxylase (ODC) is the best example of a non-ubiquitinated protein to be degraded by the 26S proteasome [111, 112]. ODC degradation requires ODC binding to antizyme, an endogenous protein inhibitor. Antizyme, however, does not appear to interact directly with PA700. Instead, antizyme probably induces a conformational state of ODC that permits interaction of its C-terminus with PA700 [113]. Polyubiquitin chains competitively inhibit antizyme-induced ODC degradation, indicating that the same element of PA700 recognizes both features [114]. Once the C-terminus of ODC is engaged by PA700, ODC is inactivated and unfolded prior to degradation, and either or both of these processes require PA700-catalyzed ATP hydrolysis in a mechanism that probably is related to the chaperone-like properties of PA700 [115]. Interestingly, the C-terminus of ODC is probably disordered, a structural feature that may dictate its initial interaction with PA700. Other unstructured non-ubiquitinated proteins also interact with PA700 and are degraded by the 26S proteasome *in vitro* [69]. Thus, it is reasonable to believe that the ability of PA700 to interact with fea-

tures of unstructured proteins (such as might be part of the unfolding or translocation processes) could dictate targeting of certain proteins to the complex for degradation. It is unclear to what extent this type of process occurs in intact cells.

11.6 Roles for PA700 in Non-proteolytic Processes

11.6.1 Overview

The presentation above has focused on the role of PA700 in regulating proteolysis by the proteasome. Although this is undoubtedly a major function of PA700, emerging evidence indicates that PA700, either as part of the intact 26S proteasome or as a separate complex or sub-complex, may mediate non-proteolytic processes. The extent to which PA700 mediates non-proteolytic functions in cells, and the exact mechanisms by which they occur, are unclear at present, but we believe that such functions will prove to be an important part of PA700 biology.

11.6.2 The Role of PA700 in Nucleotide-excision Repair

Rad23 was originally identified as a component of the ***n***ucleotide-***e***xcision-***r***epair (NER) process in yeast; deletion of Rad23 increases UV sensitivity of yeast and extracts of these strains are defective in NER. As noted above, Rad23 is a polyubiquitin-chain-binding protein that also binds directly to PA700. Several lines of evidence indicate that the Rad23–PA700 interaction is required for normal NER. Thus, Rad23 might recruit PA700 to the site of NER through a PA700-UBL domain. The precise role of PA700 in NER is controversial [102, 103, 106]. Some studies indicate that NER requires ubiquitin–proteasome-dependent proteolysis [93, 116]; others indicate that PA700 mediates NER by a mechanism that is independent of proteolysis [102, 103]. In the latter instance, PA700 might provide general chaperone-like properties required for remodeling of proteins of the NER complex.

11.6.3 The Role of PA700 in Transcription

The ubiquitin–proteasome system regulates many aspects of transcription via regulated degradation of specific transcription factors and/or their regulatory proteins [117]. By this traditional mechanism, proteolysis regulates a process, in this case transcription, by determining the content of proteins that mediate it. However, components of the ubiquitin–proteasome system, including PA700, also ap-

pear to serve as fundamental elements of the transcriptional machinery [118, 119]. The exact roles of ubiquitin, 26S proteasome, and PA700 in the mechanism of transcription are not yet clear, but the available evidence indicates that they are multifaceted and include both proteolytic and non-proteolytic aspects. In retrospect, an essential role for the 26S proteasome, and PA700 in particular, is not surprising because several AAA ATPases of PA700 were identified originally as putative transcription factors by genetic analysis in yeast [120]. It is beyond the scope of this chapter to thoroughly and critically review the mechanism by which ubiquitin and the proteasome regulate transcription; we focus here on proposed roles for PA700 and highlight possible non-proteolytic functions.

Activation of transcription by interactions between activator proteins and the general transcriptional machinery is closely linked to proteolytic destruction of the activator [118, 121]. This process is probably triggered by recruitment of ubiquitin ligases to the promoter, followed by destruction of the ubiquitinated activator by the 26S proteasome. Nevertheless, several studies indicate that a sub-complex of PA700, similar or identical to the base, is recruited initially to the GAL promoter of yeast by the Gal4 transactivator, thus forming a PA700–transcription factor complex competent for transcription [122]. This type of process has been found for other transcription factors in yeast and humans [123, 124]. Surprisingly, the PA700 base, perhaps after recruitment of other components of PA700 and proteasome, converts the polymerase to which is it bound to an elongation-competent form [125]. The exact molecular basis for these various non-proteolytic roles of PA700 is unclear, but could be related to PA700's general chaperone-like function, which might be required for restructuring of transcriptional complexes and/or alteration of the chromatin structure. Obviously, these early studies represent only the beginning of our understanding of the role of PA700 in transcription.

11.7 Summary and Perspective

PA700/19S RP is a multifunctional protein complex that plays essential roles in both the birth and death of cellular proteins. The best understood function of PA700 is its regulation of 26S proteasome function in ubiquitin-dependent protein degradation. However, despite remarkable progress, detailed mechanisms for fundamental features of this function, such as how and where polyubiquitin binds to PA700, the role of ATP hydrolysis in substrate degradation, the roles of individual PA700 subunits, and the extent to which PA700 mediates ubiquitin-independent protein degradation, remain largely unknown. Studies showing that PA700 and PA700 sub-complexes may function in various non-proteolytic processes such as transcription open a rich new area of investigation. A crystal structure of PA700 in the presence and absence of a 20S proteasome will be an important advance for understanding the function and regulation of this complex in various cellular processes.

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12
Bioinformatics of Ubiquitin Domains and Their Binding Partners

Kay Hofmann

12.1
Introduction

Since its discovery more than 25 years ago, the small protein ubiquitin has been found to be involved in nearly every important aspect of cell biology. Originally, the covalent attachment of ubiquitin to intracellular proteins was thought to invariably label these proteins for degradation by the proteasome. Since then, there has been a dramatic development in our understanding of both the mechanism and the regulation of protein ubiquitination, at least partially due to the increased application of genomics and bioinformatics techniques. We now known that protein ubiquitination regulates not only proteasomal degradation but also gene expression, chromatin structure, DNA repair, protein sorting, endocytosis, and protein degradation by the lysosome and vacuole. Many of these processes involve a multitude of ubiquitination targets, which have to be recognized with high specificity and whose modification is strictly regulated in space and time. Additional complexity comes from the fact that there are multiple ways to modify a protein: Besides the canonical signal for proteasomal degradation, consisting of a chain of at least four ubiquitin molecules linked via Lys-48, other signals use mono-ubiquitination, multiple mono-ubiquitination, or polyubiquitin chains linked by different isopeptide bonds, e.g. involving Lys-63 or Lys-29. Besides ubiquitin, there are a number of other ubiquitin-related modifiers that appear to work in an analogous fashion but convey signals with a very different meaning.

Our current knowledge of the major protein classes acting in the ubiquitin system, together with the availability of genome-wide sequence data, allows us to appreciate the vast complexity of this signalling network. By using methods of bioinformatical analysis, which will be explained in the following paragraphs, it can be estimated that several hundreds of proteins have a role related to the attachment, removal, or recognition of protein modifications by ubiquitin and its relatives. Only for a small fraction of these proteins do we have experimental data confirming their involvement in the ubiquitin system. For a large number of cellular proteins, their role in ubiquitin-mediated processes can be inferred from their molecular architecture. This prediction is typically based on the presence of ''functional do-

mains'', a concept that will be explained in Section 12.2. Subsequently the section gives a brief introduction to the bioinformatical methods used to identify such functional domains in a given query sequence or in the genome sequence of an organism, and indicates the advantages of this approach.

Every molecular signaling system consists of a number of major components: the signal itself, a mechanism for generating the signal, a mechanism for detecting the signal, and, finally, a mechanism for resetting or destroying the signal. Section 12.3 discusses the signal – ubiquitin and its relatives – and summarizes our current knowledge about the architecture, properties, and evolution of the ubiquitin family. Section 12.4 deals with the various classes of ubiquitin-recognition domains, including UBA, CUE-Ub, UIM, UEV, and GAT domains. Finally, Section 12.5 tries to put the ''parts list'' of the previous sections into context. Several examples demonstrate how nature has used the ''domain-shuffling'' mechanism to generate the vast complexity found in the ubiquitin system, but also show how the bioinformatical detection of homology domains can be useful in understanding the function of complex proteins from their modular architecture.

12.2
The Concept of Functional Domains

The whole domain concept originates from the analysis of three-dimensional protein structures. Typical small proteins have a ''monolithic'' structure that consists of a single fold with several secondary structure elements such as α-helices or β-strands. A structural fold has a hydrophobic core and hydrophilic regions exposed to the solvent. Larger proteins can follow two different architectural principles. Some large proteins just form larger monolithic structures, similar to the situation seen in small proteins. Most large proteins, however, consist of several smaller folding units, the so-called ''domains''. Each of these domains can fold independently of the rest of the protein and has its own hydrophobic core region. Structural domains can be regarded as self-sufficient mini-proteins that are connected to each other by inter-domain linkers. As a consequence of their autonomous folding capabilities, domains can often be excised from their host protein and pasted into a different protein context, without major changes in fold or function. In the course of evolution, such events have happened several times for many domain types. Evolutionary processes, such as exon shuffling and the duplication, fusion and fission of genes and gene regions, have helped to create the multi-domain ''mosaic'' structure found in many extant proteins.

In cases where no structural information is available, the presence of domains can frequently be detected just by analyzing the protein sequences. When comparing two otherwise unrelated sequences that have both acquired a particular domain by shuffling events, this domain appears as a region of localized sequence similarity. Such conserved sequence regions are often called ''homology domains''. A region of localized sequence homology does not always represent a true homology domain. It is also possible that the detected similarity region is just the best-

conserved part of two proteins that are distantly related in their entirety. A true homology domain can be assumed when the boundaries of the similarity region are well defined, e.g. if they are delimited by the N- or C-terminus of the protein or by an adjacent well-characterized domain. Evidently, not all local similarities claimed to be ''homology domains'' in the literature are true domains in the structural sense, and, even if they are, the position of the domain boundaries can deviate. Nevertheless, most homology domains that occur in diverse sets of proteins have been found to correspond nicely to structural domains. Even after multiple rounds of evolutionary shuffling, most domains preserve not only their structure but also the fundamental aspects of their function. Thus, it is frequently possible to attach functional labels to particular domain types. In favorable situations, the property of a novel protein can be predicted from those ''functional domains'' contained in its sequence.

The term ''homology domain'' or ''functional domain'' should be used only for those protein regions that either are known to be domains in the structural sense or that are at least predicted to fulfil that condition. Conserved sequence regions that are too short to fold independently of the rest of the protein should rather be referred to as ''motifs''. A considerable number of those ''functional motifs'' have important roles, e.g. by being responsible for specific domain- or protein-recognition events.

12.2.1 Bioinformatical Methods for Domain Detection

As mentioned above, homology domains can be detected by sequence analysis, where they appear as regions of locally confined sequence similarity embedded into an otherwise dissimilar context. Any tool for local sequence alignment, e.g. those using the Smith and Waterman algorithm [1], is suitable for detecting homology domains, at least if they are moderately well conserved. It is generally assumed that the structure of a protein is much better conserved than its sequence. A similar observation can be made for a protein's function, whose key features are frequently maintained even at evolutionary distances where sequences no longer look similar. Thus, it can be expected that domains exist – in both the structural and the functional sense – that cannot be spotted easily by sequence comparison alone but which are readily visible in a structural comparison. On the other hand, there is only a limited number of energetically favorable protein folds, especially for very short domains. A similarity of two protein folds does not necessarily imply a common evolutionary origin and similar folds can be found in proteins with totally unrelated functions. Evidently, structural comparisons do also have disadvantages and are also hampered by the lack of genome-wide structural data. There are even a number of documented examples where sophisticated methods of sequence comparison are more sensitive than structural comparisons [2].

Over the years there have been considerable improvements in the available sequence analysis techniques. In particular the ''sequence profile'' method [3] with its more recent extension to ''generalized profiles'' [4], and various ''***H***idden ***M***ar-

kov *M*odel'' (HMM) methods [5–7] have proved very useful for detecting very weak sequence similarities. In these methods, the increased sensitivity is made possible by accounting for the fact that not all positions in a protein sequence are equally important and thus equally well conserved. Profile and HMM searches do not start with a single-query sequence but rather with a multiple alignment of established members of a protein family. The relative sequence conservation of the alignment positions is an indication of how important these residues are and how much weight is given to them in the sequence-comparison step. Another important feature of profile-based methods is a sophisticated score statistics that allows a reliable assessment of how trustworthy a newly found sequence similarity really is. The aforementioned properties make profile and HMM methods well suited for domain-detection purposes [8]. A large number of homology domains have been identified by each method, including several of the functional domains discussed below.

When a novel homology domain has been discovered, it is possible to store the corresponding domain descriptor (profile or HMM) in a number of dedicated domain databases, which can be used to analyze newly identified sequences for their domain content [9, 10]. Several competing domain- and motif-databases exist, including PROSITE, PFAM, SMART, and Superfam, which contain descriptors for most, if not all, of the known domains involved in the ubiquitin system [11–14]. Recently, a new meta-database named INTERPRO has been established, which tries to combine the descriptors of several domain databases under a single user interface [15]. Pointers to the very useful search engines of the domain databases are provided in Table 12.1.

12.2.2 Advantages of Studying Domains in the Ubiquitin System

A modular architecture consisting of several functional domains appears to be a hallmark of proteins participating in intracellular signal transduction. The classical signaling paradigm involves protein kinases for generating the signal, a number of specialized domains for recognizing the phospho-Tyr or phospho-Ser/Thr signal, and phosphatases for removing the signal. As the phosphorylating and dephosphorylating enzymes are substrate-specific and stringently regulated, their catalytic domains are frequently associated with specialized targeting or scaffolding do-

Tab. 12.1. WWW-servers for detection of homology domains.

Database	*URL*
PROSITE	http://www.expasy.ch/prosite
PFAM	http://www.sanger.ac.uk/Pfam
SMART	http://smart.embl-heidelberg.de
Superfamily	http://supfam.mrc-lmb.cam.ac.uk
INTERPRO	http://www.ebi.ac.uk/interpro

mains. As we know now, the situation in the ubiquitin system is perfectly analogous, with the tasks of ubiquitination, deubiquitination, and ubiquitin recognition being executed by specialized types of functional domains. Interestingly, this analogy extends to the ubiquitin-related modifiers and their corresponding modification systems.

Most of the domain types used in the ubiquitin system are found exclusively in this pathway. Thus, a newly identified protein containing one of the ubiquitination-specific domains can with high reliability be considered a new component of the ubiquitin system. In some instances, the presence of a particular domain type does not allow prediction of whether the protein is active against ubiquitin or one of its close relatives. Nevertheless, the mining of sequence databases for new proteins containing ubiquitination-specific domains has been a rich source of new components and regulators of the ubiquitin system. Over the recent years, the bioinformatical analysis of those proteins has been instrumental in (i) the discovery of the evolutionary origins of the ubiquitin system, (ii) the transfer of information from better studied model systems to uncharacterized but evolutionary related systems, (iii) the identification of novel components of the ubiquitin system, (iv) the functional elucidation of complex proteins (and protein complexes) by studying their content of functional domains. The following sections will give an overview of the major types of functional domains with a specific role in the ubiquitin system.

12.3 Ubiquitin and Ubiquitin-like Domains

Ubiquitin is a small protein of 76 amino acids that got its name for its ubiquitous distribution in all eukaryotic kingdoms. Owing to its exceptionally high degree of sequence conservation, it is easy to detect even in most remote species – frequently simply by antibody cross-reactivity. Most genomes harbour multiple copies of the ubiquitin gene, which encode identical proteins. Typically, the ubiquitin is not translated in its mature form – most organisms contain genes with multiple ubiquitin copies fused to a single open reading frame. In addition, there are frequently fusion proteins with an N-terminal ubiquitin moiety and a C-terminal ''carrier-protein''. In both cases, ubiquitin must first be cleaved from the remaining protein before it can be activated and attached to target proteins. Interestingly, the nature of the ''carrier proteins'' varies from species to species. Typical examples are ribosomal proteins, but other proteins with a high expression level have also been observed. The genomic organization of ubiquitin genes suggests that cells require high amounts of the protein and the need for ubiquitin seems to be coupled to the amount of protein synthesis.

As will be discussed in more detail elsewhere in this book, ubiquitin attachment to proteins gives rise to a number of different signals. Although it was initially thought to be required only for proteasomal degradation, we now know that there are other ubiquitin-based signals, such as mono-ubiquitination, or

Tab. 12.2. Known ubiquitin-like modification systems.

Modifier	*Substrate*	*Process*
Ubiquitin	Many	Protein degradation, sorting, regulation
Nedd8 (Rub1)	Cullins	SCF regulation
Sumo (Smt3, Sentrin)	Many	Nuclear transport, localization, regulation
ISG15 (UCRP)	Stat1, others	Immune response (interferon)
Hub1	Hbt1, Sbh1	Polarized morphogenesis (yeast)
Fat10	Unknown	Apoptosis, interferon response
MNSF (FUBI, FAU)	Bcl-G, others?	T-cell activation
Urm1	Ahp1	Stress, invasive growth (yeast)
Apg8 (Atg8, Aut7)	Phosphatidylethanolamine	Autophagy, CVT pathway
Apg12 (Atg12)	Apg5	Autophagy, CVT pathway

multi-ubiquitination with different chain architectures, which all signal different events. In addition to ubiquitin, there are a number of related systems for protein labeling that use ubiquitin-related protein modifiers and similar components of the conjugation and deconjugation pathways.

12.3.1 Ubiquitin and Related Modifiers

A survey of the human genome, or that of model organisms, shows that there are multiple proteins with readily detectable similarity to ubiquitin. Even before the advent of genome-wide bioinformatics, we knew about several other ubiquitin-like proteins that become attached to proteins in a similar fashion, yet do not signal proteasomal degradation, the most prominent example being Smt3/Sumo [16]. Currently, 10 different ubiquitin-like protein modifications systems have been described; a complete list is found in Table 12.2. All of the proteins shown in the first column of this table are related to ubiquitin, although for some of them the similarity is quite subtle and either structural comparisons or sophisticated profile-based bioinformatics methods are required to obtain a decent alignment. Structurally, all ubiquitin-like domains adopt the extremely robust "β-grasp" fold [17], and high-resolution structures for a large number of ubiquitin-like molecules are available.

A detailed discussion of the physiological relevance of the ubiquitin-like modification systems is beyond the scope of this chapter, but there is a large body of literature on the pathways involving Sumo [18, 19], Nedd8 [20], ISG15 [21], Hub1 [22], Apg8 and Atg12 [23, 24], Fat10 [25], Urm1 [26], and MNSF [27]. There are also several excellent reviews providing detailed comparisons of the ubiquitin-like modifiers [28–30]. So far, ubiquitin is the only modifier known to form chains. ISG15 (UCRP) consists of two ubiquitin-like domains that do not appear to be cleaved, "ISGylated" proteins are thus modified with the equivalent of two ubiquitin units linked by a true peptide bond.

Besides the characterized modifiers, the genome contains a large number of other proteins with ubiquitin-like domains. It cannot be excluded that there are additional modification systems hidden among them. Thus, it is an important question if there is a way to discriminate these functions purely by bioinformatical methods. Until recently, the answer would have been probably yes: there seemed to be a strict requirement for a C-terminal Gly–Gly motif, whose terminal carboxyl group is involved in forming the isopeptide bond to the substrate. This diglycine does not necessarily form the C-terminus of the open reading frame, as the cleavage of a C-terminal extension by specialized proteases is commonplace. However, the two glycine residues are invariably located at the C-terminal end of the ubiquitin homology domain. Nowadays, it is not clear if the answer is so easy: the small modifier Hub1, a recent addition to the ranks of ubiquitin-like modifiers, completely lacks this diglycine in all species studied [22]. So far, it is not clear if Hub1 is a singular exception to the rule, or if other modification systems have eluded us because of their non-canonical C-terminus. Hub1 appears to be atypical in the sense that it also modifies proteins in a non-covalent fashion [31].

12.3.2 The Evolutionary Origin of the Ubiquitin System

Until recently, it was assumed that ubiquitin and its conjugation system were restricted to eukaryotes. In fact, ubiquitin has even served as a paradigm for gene lineages completely absent in bacteria [32]. Today, we know that ubiquitin and at least some components of the ubiquitination system have evolutionary ancestors that predate the prokaryote/eukaryote separation. It is probably true that the use of ubiquitin as a protein-tagging system is a eukaryotic invention; however, there are clear structural and functional similarities to two bacterial metabolic systems, the biosynthesis of the thiazole moiety of thiamine pyrophosphate [33] and the biosynthesis of molybdopterin [34]. The two bacterial pathways employ a combination of a ubiquitin-related protein (MoaD and ThiS) and an E1-type activating protein (MoeB and ThiF). Similar to ubiquitin, the MoaD and ThiS proteins both end with a glycine residue, which becomes C-terminally adenylated in a typical E1-like reaction by MoeB and ThiF, respectively. In the case of thiazole synthesis, this activation process is followed by the formation of a covalent conjugate between the C-terminus of ThiS and Cys-184 of ThiF [35], underscoring the analogy to the E1 reaction. So far, a similar conjugate has not been detected in the MoaD/MoeB system. However, the crystal structure of the MoaD–MoeB complex shows a striking analogy to the ubiquitin activation system [36]. After this point, the two biosynthesis pathways diverge from the protein-tagging systems, as there is no further conjugation of ThiS or MoaD to a target protein but rather an incorporation of the acyl-bound sulfur into the biosynthesis product.

While the functional analogy between ThiS, MoaD, and ubiquitin-like modifiers is widely accepted, the two protein classes are frequently described as unrelated; sometimes even a convergent evolution to the energetically favorable ubiquitin fold is discussed. Despite these claims, there is a statistically significant sequence

similarity between all of these proteins, which can be detected by standard profile methods described in Section 12.2.1. In particular the Urm1 family serves as a missing link and facilitates the discovery of this distant sequence relationship. Urm1 (*u*biquitin-*r*elated *m*odifier) is an evolutionary conserved protein found in all major eukaryotic lineages [37]. In yeast, Urm1 is activated by Uba4, one of the four E1 enzymes found in that organism. ''Urmylation'' is clearly a protein-modification system; it has been shown to be responsive to stress, and one of the modified targets is the antioxidant protein Ahp1 [26, 38]. When analyzing the sequences of the Urm1 family, it becomes obvious that this protein is more closely related to the bacterial MoaD and ThiS proteins than to the typical ubiquitin-like modifiers. However, a significant similarity to ubiquitin can also be established.

Apart from ThiS, MoaD, and their activating enzymes, no other bacterial homologs of ubiquitination components have been described. There do not seem to be bacterial E2 or E3 enzymes, and also the UCH and USP types of deubiquitinating enzymes appear to be absent. Eubacteria and archaea do have compartmentalizing proteases that resemble the eukaryotic proteasomes to a varying degree. However, the targeting of substrates to the bacterial ''proteasomes'' uses other signals, although one interesting parallel has been reported [39].

12.3.3 Ubiquitin Domains in Complex Proteins

A current census of ubiquitin relatives reveals 18 genes encoded by the budding yeast genome and about 75 in the human genome. These numbers do not include Apg8, Apg12, UBX proteins, or any other protein discussed in Section 12.3.4 below. It does, however, include several proteins of much larger size than the ubiquitin-related modifiers, which contain the ubiquitin similarity region as an integral, non-cleavable part of the protein itself. A small selection of those proteins is shown in Figure 12.1. Here, only proteins of a recognizable modular nature are shown, i.e. proteins that contain other domains in addition to the ubiquitin-like (UbL) domain.

When looking at the examples shown in Figure 12.1, or at the large set of proteins not shown here, two general trends are obvious: (i) the ubiquitin domain tends to be localized at the extreme N-terminus, and (ii) the host protein is typically involved in the ubiquitin system. The first observation has been interpreted as an evolutionary remnant of earlier ubiquitin-fusion proteins [40]. As mentioned above, ubiquitin is typically expressed as a precursor protein, wherein the ubiquitin moiety is localized at the N-terminus and has to be liberated by dedicated ubiquitin hydrolases. It is certainly possible that many extant proteins with ubiquitin-like domains used to be alternative ubiquitin precursors but have lost their cleavability. The second observation will be discussed in more detail in Sections 12.5.1 and 12.5.2.

From the viewpoint of bioinformatics, the second observation has turned out to be most useful. The identification of a ubiquitin-like domain in a protein makes it a good candidate for a new component of the ubiquitin regulatory system. In addi-

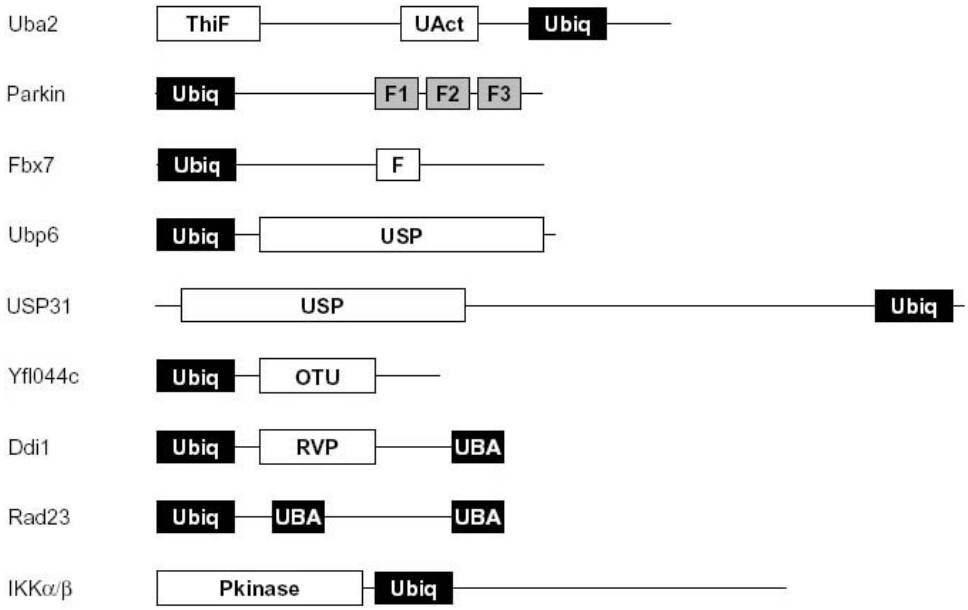

Fig. 12.1. Domain scheme of selected proteins with internal ubiquitin-like domains. Ubiquitin-like domains are indicated by black boxes. Other domains are abbreviated as follows: ThiF, NAD-binding domain in ubiquitin activating enzymes; UAct, 2nd conserved domain in ubiquitin activating enzymes; F1/F2/F3, triad of RING-finger-like domains; F, F-box domain; USP, deubiquitinase catalytic domain; OTU, a particular class of cystein protease domains; RVP, retroviral protease domain; UBA, ubiquitin-associated domain; Pkinase, protein kinase catalytic domain.

tion, other uncharacterized homology domains found in those proteins are good candidates for being ubiquitin-binding proteins or for other functionalities in this pathway. In fact, most of the ubiquitin-recognition domains discussed in Section 12.4 have been discovered by that route.

12.3.4 Other Members of the Ubiquitin Fold

Finally, it should be mentioned that there are a number of protein domains that have some structural resemblance to ubiquitin, although a sequence similarity cannot be established – not even by the most sophisticated methods available today. It cannot be excluded that there are true instances of convergent evolution among these cases. However, it appears more likely that these proteins and domains represent distant members of the ubiquitin superfamily, which have undergone a fundamental change of function and no longer need to conserve sequence positions that are considered hallmarks of ubiquitin-like molecules. In particular three domain classes should be mentioned in this context. The FERM domain (4.1, ezrin,

radixin, moesin) is a widespread module found predominantly in actin-organizing proteins. It consists of several sub-domains, the N-terminal of which has a strong resemblance to the ubiquitin fold [41]. There is no indication that FERM domains have a role in the ubiquitin system. The Ras-binding domain of the Raf-kinase is another module assuming the ubiquitin fold. The structure of this domain in complex with a Ras-like GTPase shows that the binding surface for Ras lies on the opposite face from the surface used by ubiquitin for binding to its recognition molecules, discussed in Section 12.4 [42]. There is a second Ras-binding module, the RA-domain, which also has a ubiquitin-like fold but no detectable sequence similarity to ubiquitin or to the Ras-binding domain of Raf [43].

In addition, there are a number of borderline cases, whose sequence relationship to ubiquitin is hard to establish but most probably is real, as these proteins perform a similar function. One well-known example is the UBX domain [44, 45], which seems to replace an internal ubiquitin domain in a certain class of adapter proteins (see Section 12.5.2). Other examples are the autophagy proteins Apg8 and Apg12 [23, 24] which act as ubiquitin-like modifiers.

12.4 Ubiquitin-recognition Domains

Given the widespread role of protein ubiquitination as a signal, there must be a mechanism to detect whether a protein carries a ubiquitin modification. As poly- and mono-ubiquitination appear to signal different conditions, there ought to be specific detection systems that are sensitive to the chain length and probably also to the mode of polyubiquitin linkage. Since the bioinformatical discovery of the UBA domain as the first ''professional'' ubiquitin-binding domain in 1996, a number of other domains and motifs have been found to bind specifically to ubiquitin and thus to serve as general ubiquitin-recognition modules. Unfortunately, there is still insufficient data to address the important question of whether there really are separate recognition domains for mono- and polyubiquitin, or if ubiquitin-chain recognition requires the cooperation of multiple recognition domains. Several of these domain classes also contain members that bind to ubiquitin-related domains rather than ubiquitin itself. This finding suggests that at least some of the elusive recognition components of ubiquitin-like modifiers might be recruited from the same domain classes. The following sections give a brief overview of the most important ubiquitin recognition domains.

12.4.1 The Ubiquitin-associated (UBA) Domain

The UBA domain was initially identified as a short homology region of about 40 residues, which is found in a multitude of proteins involved in the ubiquitin system [46]. An alignment of some representative UBA domains is shown in Figure 12.2. For a more comprehensive overview, there are a number of excellent reviews

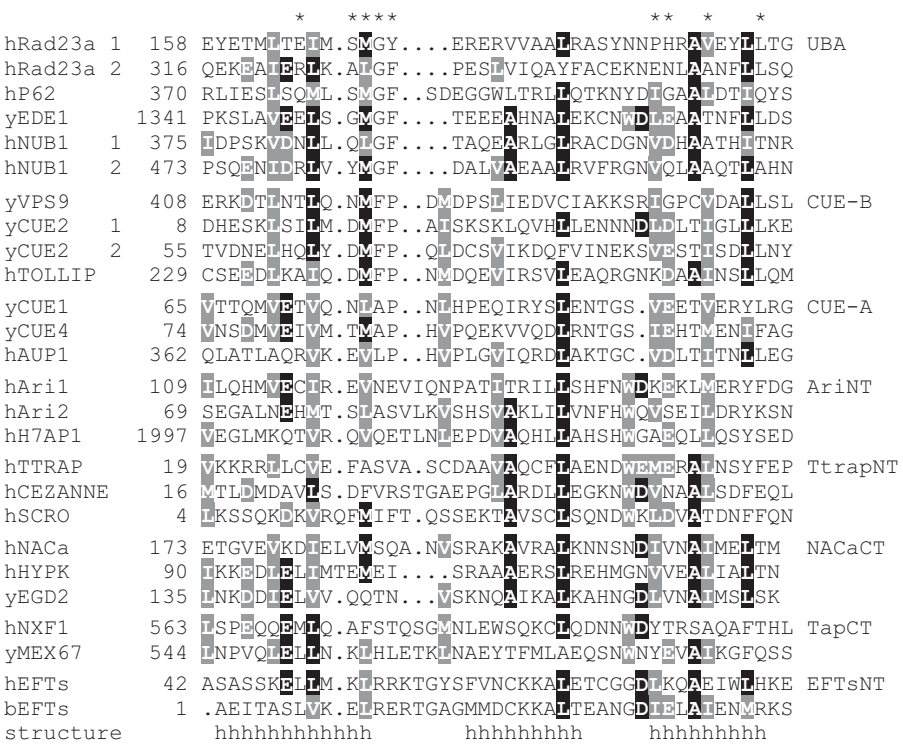

Fig. 12.2. Alignment of some representative members of the various classes of UBA-like domains. Positions invariant or conservatively substituted in at least 40% of the sequences are shown on black and gray background, respectively. The UBA-like domain classes are indicated at the right. In the top line, the positions that interact with ubiquitin in the CUE-domain of Cue2 [64] are labeled by asterisks. The bottom line indicates the position of the α-helices found in that structure.

available, which cover both structural and functional aspects of UBA domains and UBA-containing proteins [45, 47, 48]. UBA domains are found, amongst other examples, in selected ubiquitin-conjugating enzymes (E2), in ubiquitin ligases of both HECT and RING type, and also in several ubiquitin-hydrolyzing enzymes; they are particularly widespread in proteins that also contain ubiquitin-like domains. Based on this observation, a general role of the UBA domain in ubiquitin binding was proposed [46] and this prediction was soon confirmed for the UBA domain of the p62 protein [49]. By now, a large number of UBA domains have been shown to bind to ubiquitin and ubiquitin-like domains. Most data is available on the UBA domains of the Rad23 protein, mainly because of this protein's important role in DNA damage repair.

The three-dimensional structure of both UBA domains of the human Rad23a proteins have been solved and reveal a conserved three-helix bundle fold [50, 51]. So far, no structure of a UBA–ubiquitin complex is known and we can only speculate on their mode of interaction. The original UBA structures revealed two

hydrophobic surface patches as candidate regions for binding to ubiquitin. Recent experiments for mapping the interaction surface have made use of the NMR chemical shift perturbations seen upon binding of UBA domains to ubiquitin or the ubiquitin-like domain of hRad23B [52]. Apparently, UBA domains bind to the Ile-44-containing surface patch of ubiquitin, and to also to a corresponding region of the Rad23-UbL domain. The interaction surface of UBA domains used for ubiquitin binding is more difficult to judge, as even completely buried residues showed a strong chemical shift perturbation [52]. As UBA domains are very small and probably quite flexible, this might be an indication of subtle structural rearrangements during the binding process. A deeper understanding of UBA–ubiquitin binding will have to await the elucidation of the complex structure. Some general ideas, however, can also be derived from looking at the interaction properties of the related CUE domains, which will be discussed in Section 12.4.2.

Typical UBA domains are thought to bind specifically to Lys-48-linked multi-ubiquitin chains, an idea based on experiments using tetra-ubiquitin and a variety of UBA domains, e.g. those of fission yeast Ucp1 and Mud1 [53], Dsk2 [54] or budding yeast Rad23 [55]. There have also been anecdotal reports of UBA domains binding to free mono-ubiquitin in the case of budding yeast Ddi1 [56] and of a specific binding to Lys-29-linked ubiquitin chains [57, 58]. The canonical mode of UBA binding to multi-ubiquitin chains probably involves the "closed" conformation found in Lys-48 linked di-ubiquitin [59]. The recently published structure of a Lys-63-linked di-ubiquitin would allow the simultaneous and semi-independent binding of multiple UBA domains to the different ubiquitin moieties [60]. As there are several proteins containing multiple UBA domains, it can be envisaged that some of those can specifically interact with ubiquitin chains of a particular linkage.

12.4.2 The CUE Domain

The CUE domain was first described in 2000 by bioinformatical means as a short domain conserved in the yeast protein Cue1 and a number of other proteins [61]. Cue1 has a role in the *ER a*ssociated *d*egradation pathway (ERAD), which is also based on ubiquitin signals. As the apparent role of Cue1 is the recruitment of the ubiquitin-conjugating enzyme UBC7, a general role for CUE domains as a UBC-binding module was proposed. Unpublished work from my group (Hartmut Scheel and K.H) showed that the domains originally classified as CUE-domains should be subdivided into two major groups called Cue-A and Cue-B (Figure 12.3), and that both groups are related in sequence to the UBA-domain family. This finding became significant when in 2002 two independent groups reported that the yeast endocytosis regulator Vps9 binds to mono-ubiquitin by its C-terminal CUE domain [62, 63], and that this binding preference also holds for selected other members of the CUE family. Interestingly, there is a good match between the ubiquitin-binding CUE domains and the bioinformatically defined CUE-B subfamily (Figure 12.3). An alignment of selected CUE domains with some UBA representatives is shown in Figure 12.2.

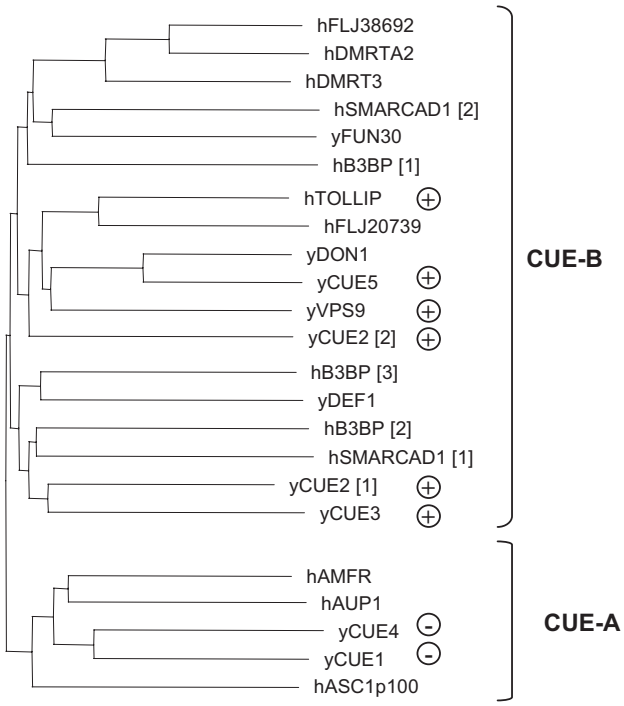

Fig. 12.3. Subclasses of the CUE domain family. All human and budding yeast members of the CUE family have been aligned and subjected to neighbor-joining dendrogram analysis. In proteins having multiple domains, the domain number is indicated in square brackets. The subfamilies CUE-A and CUE-B are indicated at the right border. Domains tested positive or negative for ubiquitin binding [64] are labeled with a circled + or − sign, respectively.

Like the UBA domain, the CUE-A and CUE-B domains occur in a wide variety of seemingly unrelated proteins, although their connection to the ubiquitination pathway is not so obvious, as many CUE-domain proteins still await characterization. Besides the vacuolar sorting protein Vps9 and many uncharacterized ORFs, CUE-B domains are also found in human Tollip, a regulator of interleukin-1 signalling, and in the SWI/SNF helicase SMARCAD1. CUE-A domains are found in the ERAD protein Cue1, in the putative RING-finger ubiquitin ligase AMFR, and in the integrin interactor AUP1.

The CUE domain's propensity to bind ubiquitin was a quite recent discovery, and relatively little is known about its physiological role. Nevertheless, structural work done on this domain type has been instrumental for our understanding of ubiquitin recognition in general. Two independently solved structures of different CUE domains have been reported, both in isolation and in complex with ubiquitin [64, 65]. The NMR structure of the first CUE domain of the uncharacterized budding yeast protein Cue2 shows a three-helix bundle fold resembling that of the

UBA domain [64], nicely confirming the bioinformatical prediction of a common evolutionary history of those two domain classes. Surprisingly, the X-ray structure of the single CUE domain of budding yeast Vps9 is markedly different: here, two CUE domains form a domain-swapped dimer with two bundles of three helices each. One three-helix bundle is formed by $\alpha 1$ and $\alpha 2$ of the first molecule and $\alpha 3'$ of the second one, while the other bundle is formed by $\alpha 1'$, $\alpha 2'$ and $\alpha 3$ [65]. In the complex structure, one molecule of ubiquitin is bound by one CUE dimer. It is still an open question whether this different arrangement reflects a physiological difference between Cue2 and Vps9 CUE domains, or if it is rather caused by the different detection methodologies.

Figure 12.4A shows the interaction of the first CUE domain of Cue2 interacting with ubiquitin, which might serve as a general model for the interaction mode of other UBA-like domains. The CUE domain binds to the Ile-44 patch of ubiquitin, in accordance with the chemical shift perturbation results of the UBA:ubiquitin interaction [52]. On the side of the CUE domain, residues of the first and third helix participate in this interaction surface. These residues include the Phe–Pro and Leu–Leu motifs, which had been predicted to be important for ubiquitin binding, based on comparative sequence analysis of CUE-A and CUE-B domains [62]. Positions in close contact with ubiquitin are also indicated in the alignment of Figure 12.3. The two available structures of the CUE:ubiquitin complexes offer little expla-

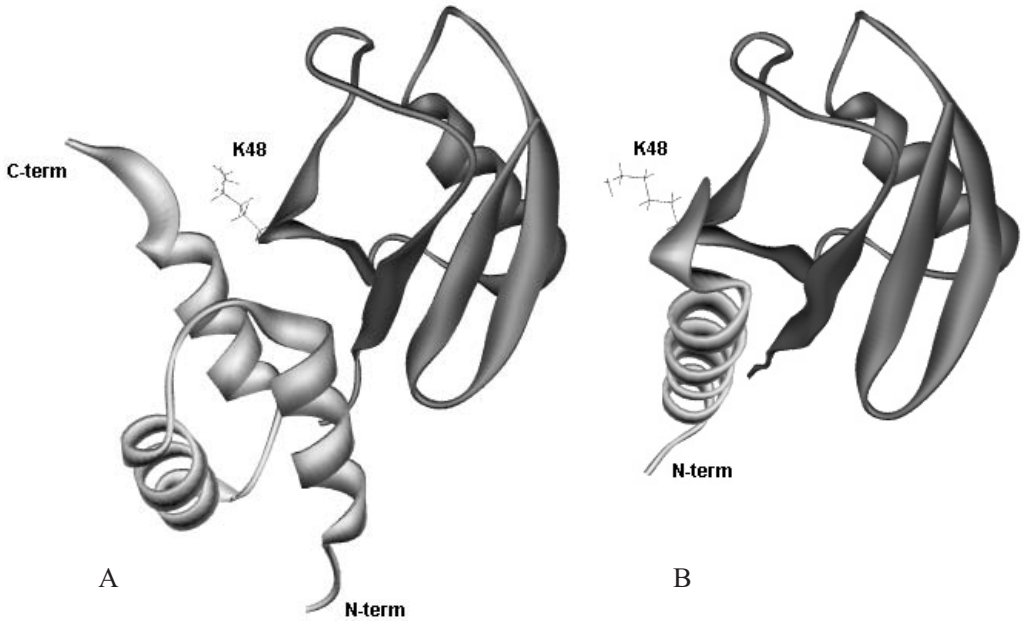

Fig. 12.4. CUE and UIM bind to the same site on ubiquitin. Schematic representation of (A) ubiquitin in complex with the CUE domain of Cue2 [64] and (B) in complex with the N-terminal UIM of Vps27 [79]. In both panels, ubiquitin is rendered in darker colour and the position of Lys-48 is indicated.

nation why CUE domains should prefer mono-ubiquitin. In the Cue2 structure, the C-terminal end of the third helix is in close contact to Lys-48 of ubiquitin, but is unlikely to interfere with the attachment of a further ubiquitin unit to this position. It is, however, conceivable that a CUE domain bound to ubiquitin prevents access of ubiquitin-conjugating enzymes to Lys-48 and thus prevents chain elongation. Other lysine residues used for alternative ubiquitin-chain formation are not within reach of a single monomeric CUE domain. Similar to the situation observed with UBA domains, several proteins contain multiple CUE domains; their relative arrangement might thus be able to specifically recognize different ubiquitin chain topologies.

12.4.3 Other UBA-related Domains

UBA domains are relatively short domains with a high degree of sequence divergence. This combination is quite unfavorable for reliable domain detection with bioinformatical methods, as there is no sharp line between the profile-scores of genuine UBA domains and those of unrelated sequences, particularly if they are of similar amino acid composition. From the bioinformatical point of view, the term ''UBA domain'' is operationally defined: a UBA domain is every sequence region that gives a significant score with a profile or HMM derived from trustworthy UBA domains. In the biological sense, e.g. when looking for the prediction of protein function or structure, this definition is not totally appropriate. The positive cases, i.e. those sequences giving significant UBA scores, will typically behave as ''biological'' UBA domains, at least if the statistics has been handled properly. The converse, however, is not always true: there will be a number of sequences that are functionally and structurally related to UBA domains, but which have diverged by such a degree that they are no longer caught by any UBA profile. A good example for such a situation is the CUE domain mentioned above: no CUE domain protein reaches a significant score with canonical UBA profiles, while some established UBA domains reach a quite convincing score with CUE-derived profiles. Together with secondary-structure prediction methods, this behavior has prompted the prediction of CUE domains as ubiquitin-binding modules, which soon turned out to be correct.

In this respect, the CUE domain is not a isolated case. There are a number of other domain families, each of them only defined in the bioinformatical sense, that have significant matches within established UBA or CUE domain regions. Based on this similarity and on secondary-structure predictions, it can be expected that all of those domain types assume the typical UBA-like three-helix bundle fold. However, it is not clear if all of those domains also bind to ubiquitin, or if they have evolved to different binding properties. Many of the UBA-like domain classes are unpublished. Nevertheless, they should be briefly discussed here, as they are a logical extension of the UBA/CUE paradigm.

AriNT: A novel UBA-like domain is found in certain RING-finger type proteins related to the ariadne protein of the fruit fly. Here, the AriNT domain is invariably

located upstream of the RING-finger triad, while there is a second conserved domain (AriCT, not UBA related) found C-terminally of the Zn fingers. As all known AriNT proteins (four human proteins and the yeast ORF Ykr017c) are putative ubiquitin ligases, a role of this UBA-like domain in ubiquitin binding appears likely.

TtrapNT: A further UBA-like domain is found at the N-terminus of the TNF- and TRAF-associated protein Ttrap, as well as a number of other sequences including eight other human proteins and the yeast ORF Ylr128w. The scope of proteins harboring the TtrapNT domain resembles that of the UBA proteins. The "Cezanne"-like proteins combine the TtrapNT module with an OUT-type protease domain, while other proteins also contain UIM or UBX domains. Most TtrapNT proteins have an established or predicted role in the ubiquitin pathway, making it likely that TtrapNT serves as a recognition module for ubiquitin or ubiquitin-like domains.

NACaCT: Yet another UBA-like domain is found at the C-terminus of the α-subunit of the nascent polypeptide-associated protein complex (NAC-α). This protein has some properties of a chaperone and regulates the attachment of loaded ribosomes to the ER membrane [66], a process that is not known to involve the ubiquitin system. Further NACaCT domains are found in the huntingtin-interactor HYPK, in the human KIAA0363 protein, and in many NAC-α related proteins including the yeast protein Egd2. It is unlikely that the NACaCT domain has a general role in ubiquitin binding, considering that this domain is also found in archaea, which are devoid of ubiquitin.

EFTsNT: A UBA-like domain with a clear role outside of ubiquitin binding is found at the N-terminus of EF-Ts proteins. The relationship of this region to genuine UBA domains is well established as there is a structure of full-length EF-Ts available [67]. Nevertheless, this domain is widespread in bacteria and archaea, which obviously lack a proper ubiquitin system. The physiological role of the EFTsNT domain is rather in the binding to the elongation factor EF-Tu, which has no resemblance to ubiquitin.

TapCT: The C-terminus of the mammalian nuclear RNA export factor NXF1/2 (also known as Tap) contains a sequence region with significant similarity to UBA-like domains. This region is also found in the yeast RNA export factor Mex67. A three-dimensional structure of this domain is available and confirms its similarity to the UBA domain [68]. This UBA-like domain does not appear to bind to ubiquitin but rather to the Phe–Gly repeat motif found in a number of nucleoporins. The interaction surface of the UBA-like TapCT domain with a Phe–Gly-containing loop was mapped by an NMR/X-Ray combination technique and shown to be different from the ubiquitin-binding mode: the Phe–Gly loop binds on the "backside" of the UBA-like domain and is in contact with helices α2 and α3 [68].

12.4.4
The Ubiquitin-interacting Motif (UIM)

The classical receptor for ubiquitinated proteins is the 26S proteasome, although the true nature of the ubiquitin-sensing subunit – at least the physiologically im-

portant one – has always been and is still a matter of discussion. At least three subunits have been suggested to target ubiquitin or UbL domains to the proteasome: S5a/Rpn10 [69], S6′/Rpt5 [70], and Rpn1 [71]. In the case of S5a/Rpn10, the interacting region could be narrowed down to two conserved motifs containing the residues Leu–Ala–Leu–Ala–Leu, termed the ''LALAL-motif'' [69]. Bioinformatical attempts to identify other ubiquitin interactors using a similar sequence motif were initially unsuccessful. During sequence analysis of the ataxin-3 protein mutated in Machado Joseph disease, a repeat motif was identified which later turned out to also include the proteasomal LALAL-motif [72]. The high prevalence of this motif in proteins known to interact with ubiquitin immediately suggested a role of this motif in ubiquitin binding, hence the name UIM for ***u***biquitin-***i***nteracting ***m***otif.

The UIM is a short motif spanning only 16 consecutive residues. An alignment of some representative UIMs is shown in Figure 12.5A. The most prominent fea-

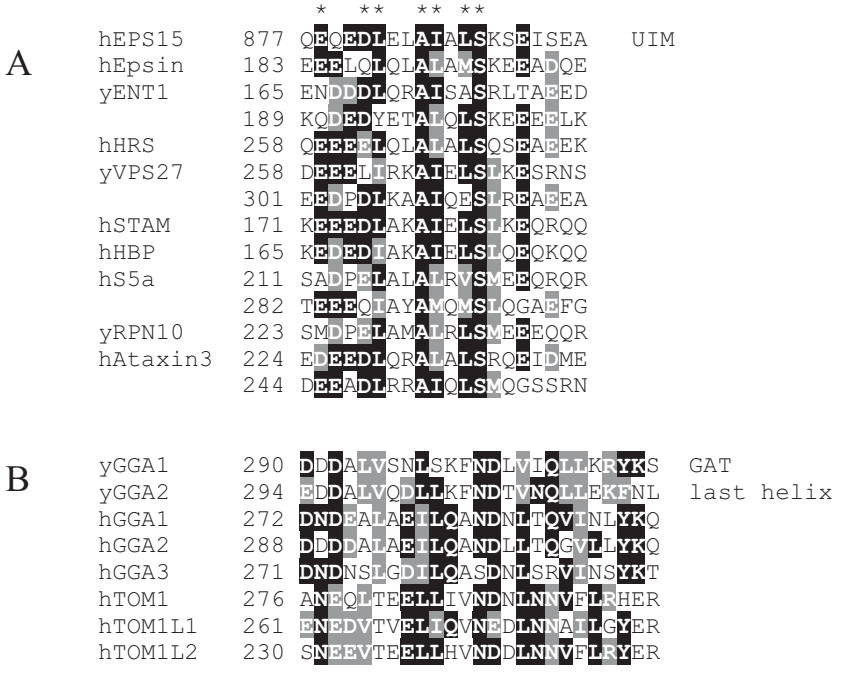

Fig. 12.5. Comparison between the UIM and the ubiquitin-binding part of the GAT domain. Positions invariant or conservatively substituted in at least 50% of the sequences are shown on black and gray background, respectively. (A) The top panel shows some representative members of the UIM family. Positions that in the Vps27 structure [79] have contact with ubiquitin are labelled by asterisks in the top line. (B) The bottom panel shows an alignment of representative GAT-domain members. Only the last helix, which contains the ubiquitin-interaction site, is depicted.

ture of the UIM is the almost invariant Ala–X–X–X–Ser sub-motif, where the conserved Ala corresponds to the second "A" of the original LALAL motif. In addition to this feature, the initial Leu is the only other LALAL residue well conserved in the functional UIMs; the other Leu and Ala residues are more or less specific to the S5a family. This high degree of divergence explains why the UIM was not discovered by motif-searching techniques starting from S5a/Rpn10 but required the more sophisticated profile-HMM techniques.

Probably the most interesting aspect of UIM identification was the presence of four different protein classes working in the receptor endocytosis and protein-sorting pathway. It had been known for a long time that those processes were regulated by mono-ubiquitination of both cargo proteins and signalling components, but the nature of the ubiquitin receptor had been elusive. Immediately after its discovery, a series of studies showed convincingly that the UIM fills this role. A number of reviews cover the field of UIMs in endocytosis, and also highlight the possible mechanisms by which the UIM can contribute to keeping proteins in a mono-ubiquitinated state [73–76]. Besides the proteasomal S5a component and the endocytosis proteins, copies of the UIM can also be found in ubiquitin ligases, UBA-domain-containing adapter proteins, ubiquitin proteases, selected chaperones, and a large collection of uncharacterized proteins. Based on current data, it appears likely that most if not all UIM-containing proteins will turn out to be part of the ubiquitin system. A good example is the identification of the functionally uncharacterized UIM-protein ataxin-3 as a novel ubiquitin protease – another bioinformatical prediction that was followed by experimental confirmation [77, 78].

The sequence properties of the UIM initially suggested the structure of a single helix, which would exclude the UIM from being a "domain" in the strict sense of Section 12.1.1; for that reason the UIM should preferably be called an interaction "motif" instead of an interaction domain. By now, a body of structural data on various UIMs is available, confirming the helical nature of this motif and demonstrating its interaction mode with ubiquitin and UbL domains. Available structures include the UIMs of yeast Vps27 [79, 80] and the complex of the C-terminal UIM of S5a with the UbL of hRad23B [81] and of hRad23A [82]. Also interesting in this respect is the comparison of chemical shift perturbations seen in ubiquitin and UbLs complexed with UIMs and various UBA domains [52]. As shown in Figure 12.4B, the UIM also binds to the Ile-44 patch of ubiquitin, at a similar position to that used by the UBA-type interactors. However, the orientation of the single UIM helix is quite different from the helix-bundle of the UBA-like domains. The UIM:ubiquitin complex structures do not give a clear picture of how UIMs might prevent ubiquitin-chain elongation or how they could discern between different chain topologies. The Lys-48 residue is not part of the interaction surface and a direct interference appears unlikely. It should be noted, however, that UIMs frequently occur in narrowly spaced tandems. Like the multi-UBA and multi-CUE proteins, the UIM tandems might be one way to the specific recognition of certain linkage types.

12.4.5 The UEV Domain

A number of enzyme families are known to contain members that have lost the residues important for catalysis. The maintenance of those catalytically inactive proteins in the translated part of the genome probably means that these proteins have acquired a different function. In some instances this new function is totally unrelated to the original enzymatic activity, e.g. the crystallins of the eye lens contain inactive members of various enzyme families. In other examples, including some inactive kinases and phosphatases, the newly acquired function is related to the original catalysis, e.g. by binding to the substrate or by acting as a heterodimerization partner for a catalytically active version of the enzyme. The latter situation seems to apply for catalytically inactive versions of ubiquitin-conjugating (E2) enzymes, containing a so-called UEV domain (for *u*biquitin-conjugating *e*nzyme *v*ariant). The existence of this homology-domain was first demonstrated bioinformatically in the candidate tumor suppressor TSG101 [83, 84] and a role for this domain as a regulator of ubiquitination was proposed. A second prominent protein with a UEV domain is the DNA-damage-repair protein Mms2, which forms a heterodimer with the active E2 enzyme Ubc13. As this dimer is involved in the creation of the unusual Lys-63-linked multi-ubiquitin chains [85], the presence of a second ubiquitin-binding site in this complex has been proposed, to which the UEV is likely to contribute. Two crystal structures of Mms2/Ubc13 dimers are available [86, 87], but do not give a clear indication where this binding site is located.

More functional and structural information is available on TSG101, whose yeast ortholog, Vps23, is part of the ESCRT-1 complex; both proteins probably play a physiological role in the ubiquitin-dependent sorting of proteins to multivesicular bodies [88]. TSG101 has been shown to bind to ubiquitin *in vitro* and it is also able to bind to certain proline-rich peptides, most importantly the Pro–Thr–Ala–Pro peptide found in the Gag protein of the HIV virus [89]. An NMR structure of TSG101 has been described, which also allowed estimation of the binding sites for the PTAP peptide and for ubiquitin by chemical shift mapping [90]. Somewhat surprisingly, the predicted ubiquitin-binding site does not correspond to the vestigial catalytic site.

12.4.6 The GAT Domain

The GAT domain (GGA and Tom1) has recently joined the ranks of ubiquitin-binding domains [91]. As the name implies, this domain is found in the GGA- and Tom1-like proteins, two regulator classes of clathrin-mediated vesicular traffic. All proteins harboring the GAT domain also contain an N-terminal VHS domain, which is named after the ***V***ps27, ***H***rs, and ***S***TAM proteins. Interestingly, these latter proteins are known to contain a ubiquitin-binding UIM motif, which appears to be replaced by the GAT domain in the GGA and Tom1-like proteins (Figure 12.6).

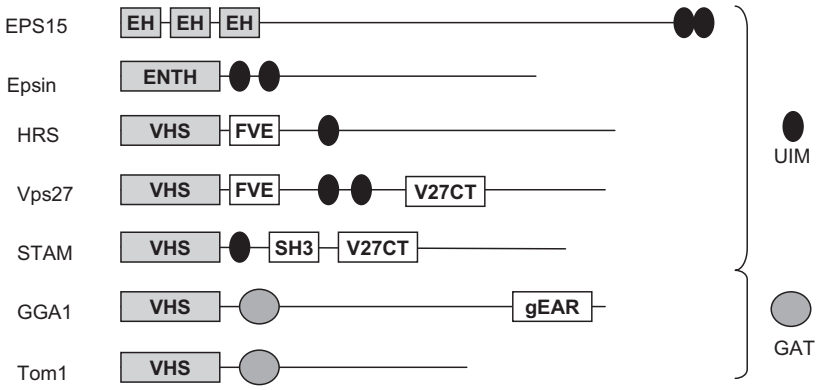

Fig. 12.6. Domain scheme of selected members of the UIM and GAT family. Domain abbreviations are as follows: EH, Eps15-homology domain; ENTH, Epsin N-terminal homology domain; VHS, N-terminal domain of Vps27, HRS, STAM; FVE, FYVE-finger domain; V27CT, C-terminal domain of the Vps27 family; SH3, Src-homology 3 domain; gEAR, γ-adaptin ear domain. The UIM motifs are shown as small black ellipses, the GAT domains as larger gray ones.

The GGA proteins (three in mammals, two in yeast) also contain a so-called "γ-adaptin ear domain" at their C-terminus, which is lacking in the three mammalian Tom1-like proteins.

Initially, the role of the GAT domain in GGA proteins was seen in the binding of small GTPases of the Arf family, a critical step in the recruitment of clathrin to the TGN membrane [92]. However, the GAT domains of the Tom1-like family do not bind to Arf. Recently, GAT domains of both protein classes were found to bind to ubiquitin and it was possible to separate the two binding sites to different subdomains of the GAT domain [91]. A number of X-ray structures of GAT domains are available [93–95], presenting the domain as an elongated three-helix bundle. Unlike the UBA-like structures, the GAT helices are almost parallel and considerably longer. As a prominent feature, the N-terminal helix is much longer than the others; this N-terminal extension contains the Arf interaction site and is not conserved in the Tom1-family [93].

The ubiquitin-interaction site of the GGA3 GAT domain was mapped to the C-terminal helix by deletion and mutation experiments. The Ile-44 patch appears to be the likely interaction site of ubiquitin, as an Ile44Ala mutation abolished the binding [93]. The C-terminal helix bears some resemblance to the UIM: it is an amphipathic helix of similar length that is preceded by a cluster of 3–5 acidic residues (Figure 12.5B). However, the GAT domain lacks the Ala–X–X–X–Ser motif, which is the hallmark of the UIM motif. A conserved Leu–X–X–X–Asp motif that points away from the helix bundle – and thus probably towards the bound ubiquitin – might fill this role in the GAT domain. Taken together, the similarity between GAT and UIM might not be restricted to the scope of the two domains but also include a conserved binding mode.

12.4.7 Other Ubiquitin-binding Domains

In addition to the well-established and widely distributed ubiquitin-interaction domains described above, there are several other domains with a more limited scope or with binding properties that are just beginning to be uncovered. Two interesting candidates are the NZF and ZnF-UBP/PAZ domains.

The NZF domain (***N***pl4 ***Z***n ***f***inger) is a mononucleate Zn-finger domain with four cysteine ligands, which occurs – amongst others – at the C-terminus of the Cdc48/p97 adapter protein Npl4 and in the vacuolar sorting protein Vps36. In both proteins, the region corresponding to the NZF has been shown to bind to ubiquitin [96, 97]. The structure of the NZF domain does not resemble any of the known ubiquitin-interaction domains but rather looks like a typical C4 zinc finger [96]. Bioinformatically, the NZF domain is identical to the Ran-binding Zn finger found in RanBP proteins [98]. It is currently not clear if all members of this quite large family bind to ubiquitin, or if the ubiquitin-binding Zn fingers are just a small subset of this domain class. It should be noted that the two NZF fingers of Npl4 and Vps36 are not particularly closely related.

A second Zn finger with a putative role in ubiquitin binding is found in various ubiquitin proteins of the USP type and in the histone deacetylase HDAC6. Two recent studies have shown that the corresponding region of HDAC6 binds to polyubiquitin [99, 100]. The Zn finger is referred to as Znf-UBP or as PAZ domain (for ***p***olyubiquitin-***a***ssociated ***Z***n finger). The latter acronym is somewhat unfortunate, as there is another unrelated domain that goes by this name (***p***iwi, ***a***rgonaut, ***z***wille). So far, it is not clear if the ubiquitin-binding propensity is specific to HDAC6 or applies to the other family members as well. Since most of the Znf-UBP domains are found in ubiquitin proteases, a general role of this domain in the ubiquitin system is likely.

Obviously, not all proteins known to interact with ubiquitin or ubiquitin-like domains contain one of the "professional" ubiquitin-interaction domains. Rpt5 and Rpn1, two subunits of the proteasome that bind to ubiquitin and UbLs, respectively, do not belong to any of the classes described above. Most probably, a large number of uncharacterized proteins with high affinity and specificity for ubiquitin are still waiting to be discovered. The bioinformatical tools described in the early sections of this chapter will be instrumental for this task.

12.5 Building Complex Systems From Simple Domains

Considering the multitude of ubiquitin domains and their cognate recognition modules, the next question to address is how cells make use of these building blocks to form the highly complex ubiquitination system. Before starting with the discussion of some prominent modular protein architectures, we should bear in mind that there are a number of other functional domains participating in this

pathway. So far, little attention has been paid to the fact that the enzymatic functions of ubiquitin activation, ubiquitin conjugation, ubiquitin ligation, and deubiquitination are also encoded by functional domains. Many enzymes of ubiquitin metabolism are architecturally simple and consist only of a single domain. However, several enzymes do have a modular architecture; those will be discussed in the following section.

12.5.1 Enzymes with Additional Ubiquitin-binding Sites

The first hint that the UBA domain might have a role in the ubiquitin system came from its frequent association with known enzymatic components of protein ubiquitination and deubiquitination, particularly with those that confer specificity to the reaction. The same trend is seen with other ubiquitin-binding domains, such as the UIM motif or the NZF domain. From genome-wide sequence-analysis studies, we know that the multiplicity – and also the architectural complexity – of the ubiquitination system increases from E1 via E2 to E3. The human genome appears to encode only one ubiquitin-activating enzyme (E1), plus three paralogs active against ubiquitin-related modifiers. By contrast, there seem to be 34 active members of the ubiquitin-conjugating enzyme family (E2). Since many of these proteins lack any biochemical characterization, it is not clear how many of them actually conjugate ubiquitin, or which ones are active against ubiquitin relatives. Most E2 enzymes appear to be monolithic, but there is at least one (E2-25K) that harbors a UBA domain. The multiplicity of ubiquitin ligases (E3) is even higher: the human genome encodes 27 proteins with a HECT domain, forming the "classical" E3 superfamily [101]. In addition, there are 259 RING-finger proteins, most (if not all) of which can also be assumed to be ubiquitin ligases. Even more variability comes from the existence of composite ubiquitin ligases, such as the SCF complexes [102], which use a common RING-finger component but a multitude of specificity factors (chosen from a set of 58 F-box proteins, 17 SOCS-box proteins, and perhaps 190 BTB proteins). Many of the E3 components contain additional functional domains, frequently including those with ubiquitin-binding properties. The deubiquitination branch consists of at least 61 ubiquitin proteases belonging to different classes. In particular the enzymes of the USP class frequently harbor ubiquitin-binding domains in addition to their enzymatic function.

Obviously all enzymes of ubiquitin metabolism have to recognize ubiquitin. Why do some – but not all – of them contain dedicated ubiquitin-binding domains? I should like to propose the hypothesis that ubiquitin-binding domains occur only in those enzymes that not only transfer (or remove) ubiquitin but also act on ubiquitin as a *substrate*. These would be ubiquitin ligases with a role in chain elongation, or ubiquitin proteases active in chain trimming, but never those that transfer ubiquitin directly onto a non-ubiquitin substrate. There are some data to support this hypothesis. E2-25K, the only UBC enzyme with a UBA domain, is able to catalyze the unusual formation of unattached polyubiquitin chains in solution [103]. Moreover, human isopeptidase T and yeast UBP14, two UBA-containing

ubiquitin proteases, hydrolyse only polyubiquitin chains that are not attached to a substrate [104, 105]. Unfortunately, the catalytic properties of other enzymes containing ubiquitin-interaction motifs are not characterized in sufficient detail. Nevertheless, the presence of single or multiple ubiquitin recognition modules should allow a ubiquitin ligase or hydrolase to require a certain minimal chain length or a particular chain topology.

12.5.2 The UbL/UBA Adapter Paradigm

Even more mysterious than the large number of ubiquitin-recognition domains is the equally large number of internal UbL domains found in a diverse set of proteins. A genome-wide survey shows that UbL domains are not randomly distributed throughout the proteome but rather are highly enriched in proteins known or suspected to act in the ubiquitin system. Interestingly, this seems to include several kinases whose activity is required for a subsequent ubiquitination, such as, for example, the IκB-Kinase subunits IKKα and IKKβ (Figure 12.1). For most of the cases, we do not know what the UbL domains are doing, although it is tempting to speculate that they are specific interaction partners for selected members of the ubiquitin-binding domain families.

One class of UbL proteins has been the focus of investigation for their crucial role in the targeting of substrates to the proteaseome, and possibly also to the Cdc48/p97 complex. These proteins, with Rad23 being the most prominent member, have a particular architecture with a UbL domain at one end (typically the N-terminus) and a UBA domain in the C-terminal region. These proteins appear to work as ''adapters'' by shuttling ubiquitinated substrates to the proteasome without requiring a direct interaction of the proteasome with the ubiquitin signal [106]. In the Rad23 proteins, the N-terminal UbL domain is able to interact with the proteasome [107–109], while the two UBA domains specifically recognize polyubiquitin signals [53, 54]. Recently, the proteasome component Rpn1 has been identified as a receptor for the Rad23 UbL domain [71].

A related adapter family combines the UBA domain with a UBX domain, which is much more distantly related to ubiquitin than the true UbL domains [44, 45]. In these proteins, the UBA domain is frequently found at the N-terminus while the UBX domain forms the C-terminus of the protein; an example is the yeast Shp1 protein and its mammalian homolog p47. So far, there is limited data on the function of this protein family though they appear to shuttle ubiquitinated proteins to the Cdc48/p97 complex instead of to the proteasome [47, 106].

12.5.3 Non-orthologous Domain Replacement

When analyzing proteins of the ubiquitin system from a genomic perspective, there are a number of interesting examples where in the course of evolution one

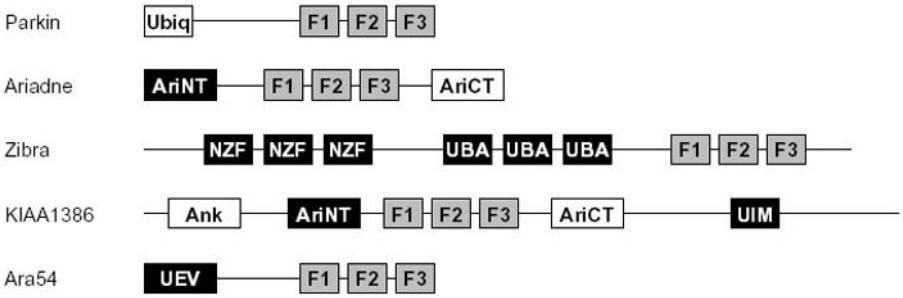

Fig. 12.7. Domain scheme of selected parkin-like ubiquitin ligases. Black boxes represent the ubiquitin-interacting domains discussed in the text. The three gray boxes labeled F1, F2 and F3 represent the triad of RING-finger like domains that define the parkin superfamily. Other domain abbreviations are as follows: Ubiq, ubiquitin-like domain; AriCT, C-terminal domain of the ariadne family; Ank, ankyrin repeats.

domain type has been replaced by a member of a different domain class. A well-known example is the EPS15/Ede1 pair: when analysing the N-terminal region, the human EPS15 and yeast Ede1 proteins appear to be orthologs within the EH-domain family, suggesting a common function. However, the human protein contains two UIM motifs at the C-terminus, while the yeast protein has a UBA domain at an equivalent position [72]. This evolutionary replacement suggests that – at least in this case – the two domain types are functionally equivalent.

A second example, which is quite intriguing although its functional significance is not yet clear, is shown in Figure 12.7. The human genome contains a large number of putative ubiquitin ligases related to the parkin protein. This protein family is characterized by a triad of Zn fingers related to the RING finger. In parkin, the only other domain is a UbL domain at the N-terminus, whose function is unknown. Most other proteins of this family lack the UbL, but several members have a ubiquitin-binding function instead: The Ariadne proteins carry a UBA-like AriNT domain upstream of the Zn-finger triad, the protein KIAA1386 also contains this AriNT domain but has an additional UIM at the C-terminus, the Zibra protein has no AriNT domain but three true UBA domains plus three additional ubiquitin-binding NZF domains. Finally, the Ara54 protein does not have a UBA-like domain but rather a UEV domain filling that position. Apparently, multiple members of the parkin family require the binding of ubiquitin or UbL domains, but evolution has chosen different solutions for that task.

A quite mysterious finding is the occasional positional replacement of ubiquitin-binding domains by ubiquitin-like domains. Parkin in Figure 12.7 is one example, another one is the UBX domain found instead of the UIMs in the ataxin-3 protein from *Plasmodium falciparum* [77]. There is no reason to assume that UbL domains might have a role in ubiquitin binding. A more likely explanation would be the requirement of these proteins to ''look like ubiquitin'', irrespective of whether ubiquitin is part of the protein itself or rather bound to it non-covalently.

12.6 Outlook

The previous paragraphs were meant to give a brief overview of what we know about ubiquitin-like domains and their recognition by specialized ubiquitin-binding modules. Sequence analysis has proven to be a valuable tool for the discovery of such domains and the identification of new components of the ubiquitination pathway. There is still a big gap between the relatively facile identification of those proteins and the tedious functional characterization of their biochemical and physiological properties. Striving to reach a deeper understanding of the ubiquitin system and the intricate interplay of all its components, we are barely scratching the surface. Nevertheless, there are some general principles that can be derived from the existing data, and I have tried to make a case for bioinformatics as a powerful tool to reach that goal.

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13
The COP9 Signalosome: Its Possible Role in the Ubiquitin System

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13.1
Introduction

The COP9 signalosome (CSN) is a multimeric, highly-conserved protein complex [1]. Just like the ubiquitin system it occurs in all studied eukaryotic cells. Following its 1994 discovery in plant cells the complex was postulated to function in signal transduction [2]. Originally described as a regulator of light-dependent growth in plants [3, 4], identification and characterization of the CSN from mammalian cells led to the discovery of sequence homologies between CSN subunits and subunits of the 26S proteasome lid complex [5, 6] as well as subunits of the translation-initiation complex eIF3 [7]. Significant progress has been made towards understanding its structure and function by analyzing different eukaryotic organisms. The complex is involved in developmental processes of plants [8] and *Drosophila* [9] and is essential for embryogenesis in mice [10]. It seems to participate in processes such as DNA repair [11], cell-cycle regulation [12] and angiogenesis [13]. At the moment the pleiotropic effects of the CSN can be explained by its regulatory impact on the ubiquitin system. Here we provide a summary of current knowledge of CSN function in the ubiquitin system.

13.2
Discovery of the CSN

Deng and co-workers discovered the CSN in *Arabidopsis thaliana* when they characterized mutants of light-dependent development, and they called it the COP9 complex [2]. Morphogenesis of germinating seedlings is light-dependent. Light triggers a developmental process called photomorphogenesis. A number of mutations in the *Arabidopsis* COP/DET/FUS loci result in the loss of the COP9 complex accompanied with *cop* phenotypes in which germinating seedlings exhibit light-independent expression of light-induced genes [3]. Therefore the complex was originally hypothesized to be a repressor of photomorphogenesis [14]. The mam-

malian CSN complex was independently isolated during preparations of the 26S proteasome and called the JAB1-containing signalosome [15]. The same studies identified proteins such as JAB1 [15] and TRIP15 [16] as components of the complex and revealed homologies between subunits of the CSN and components of the 26S proteasome lid complex. Purification and analysis of the complex from *Arabidopsis*, pork spleen [6, 17] and human red blood cells [5, 18] led to the conclusion that each subunit of the CSN has its paralog subunit in the 26S proteasome lid complex. These data suggested a common origin for the two complexes during evolution. Because they have similar architectures, the two complexes have been postulated to perform related functions (see below). Unfortunately there is only limited information on the structure or function of the eIF3 complex, and its relationship to the CSN and the lid is not well understood [7].

Studies have revealed that the CSN possesses both intrinsic and extrinsic (associated) activities, which will be reviewed in detail below. Historical gene names of the CSN have been summarized before [1]. In this article we use the unified nomenclature of the CSN [1].

13.3 Architecture of the CSN

13.3.1 CSN Subunit–Subunit Interactions

The CSN is composed of eight subunits called CSN1 to CSN8, which are highly conserved in eukaryotes, although only six of them occur in fission yeast. Two hybrid screens and biochemical methods such as far westerns, pull downs and co-precipitation defined a number of CSN subunit–subunit interactions. Figure 13.1 illustrates known subunit–subunit interactions. Initial insight into the architecture of CSN came from the first 2D electron microscopic analysis of purified CSN from human red blood cells [19] (see also Figure 13.2 below).

The CSN architecture shows similarity to that of the lid. Both complexes have an asymmetric arrangement of their subunits and exhibit a central groove structure [19]. Whether the structural similarity of the two complexes is connected with similar functions remains unclear. The exact arrangement of CSN and lid subunits within their complexes remains uncertain in the absence of high-resolution crystal structures for the two complexes.

Interestingly, the occurrence of smaller CSN sub-complexes apart from the large 500-kDa CSN complex has been described in different species such as *Arabidopsis*, *Drosophila*, *Schizosaccharomyces pombe* and mammalian cells (for a review see Ref. [20]). At the moment the physiological function of CSN sub-complexes is unclear. It can be speculated that a controlled equilibrium exists between the large and small CSN complexes. The small complexes may have a function in shuttling between nucleus and cytoplasma and/or between large multi-subunit complexes such as the 26S proteasome and cullin-based Ub ligases.

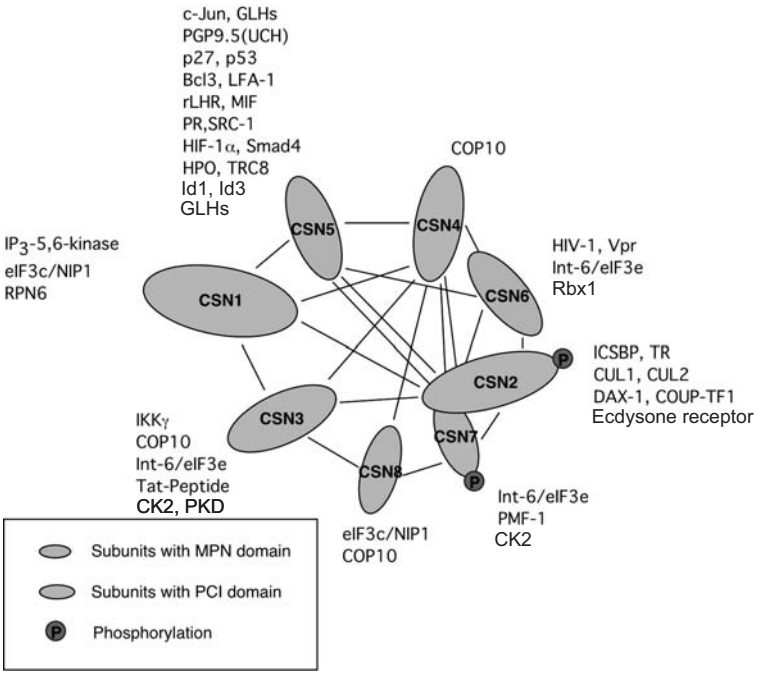

Fig. 13.1. Subunit–subunit interactions of the CSN and interactions of CSN subunits with other proteins. Subunits are numbered according to the unified nomenclature [1]. CSN subunit–subunit interactions have been published before [19]. Darker shading indicates subunits with MPN domains and lighter those with PCI domains. Known phosphorylated subunits are indicated. Details on CSN subunit interactions with other proteins can be found in the text.

13.3.2
CSN-subunit Interactions With Other Proteins

Apart from subunit–subunit interactions within the CSN, a considerable number of cellular proteins interact with CSN subunits (see Figure 13.1). Although the physiological relevance of many of the identified interactions is questionable, most of them might be attributed to a role of the CSN complex in signal transduction and ubiquitin-dependent proteolysis.

CSN1 formerly called Gps1 was first described as a signal transduction repressor in *Arabidopsis* [21]. Over-expression of CSN1 suppresses the activated JNK signaling pathway and also inhibits UV- and serum-induced c-fos expression as well as MEKK-activated AP1-activity in mammalian cells [21, 22]. It remains unclear whether overexpressed CSN1 plays a role as dominant negative regulator when in the CSN complex. Whereas the N-terminal region of CSN1 is sufficient for repression, the C-terminal region is necessary for its integration into the complex and for

the stability of the CSN complex [22]. Curiously, the N-terminal region of CSN1 may be not required for the CSN-associated deneddylation of cullin 1 (CUL1) and cullin 3 (CUL3), components of cullin-based E3 Ub ligases in *Arabidopsis*, although it appears to be one of the binding sites of the CSN for cullin-based complexes [23]. Moreover, CSN1 is the receptor site for the interaction of the CSN with inositol 1,3,4-trisphosphate 5/6 kinase [24]. In addition, CSN1 represents the interactor for a subunit of the translation-initiation factor 3, eIF3c/NIP1 [25], and for the 26S proteasome non-ATPase subunit Rpn6 [26]. Possible functions of these interactions are discussed later.

CSN2 also known as alien [27] is perhaps an important regulatory subunit of the CSN. Firstly, CSN2 was identified as Trip15 (***t***hyroid hormone ***r***eceptor-***i***nteracting ***p***rotein) using a yeast-two-hybrid screen [16]. The binding site of CUL1 and CUL2 is located at the N-terminal region of CSN2. This interaction is important for the CSN-mediated deneddylation of cullin-based complexes that regulate their Ub-ligase activity [28]. Additionally, CSN2 binds to the transcription factor, ICSBP (***i***nterferon ***c***onsensus ***s***equence ***b***inding ***p***rotein), which modulates interferon-directed gene expression [29]. Moreover it interacts with the nuclear receptors DAX-1, COUP1-TF1 and ecdysone receptor [27, 30]. Interestingly, CSN2 is phosphorylated by the CSN-associated kinases CK2 and PKD [19, 31]. However, the phosphorylation sites and their physiological function remain unclear.

The CSN3 subunit interacts with IKKγ, a component of the IκB-kinase complex controlling NF-κB activity [32]. Additionally, it is the binding site for the CSN-associated kinases CK2 and PKD [31]. The subunit of the translation-initiation factor 3 complex, Int6/eIF3e, and the ubiquitin-conjugating enzyme variant, COP10, have been identified as other cellular interactors [33, 34]. Also the HIV-1 Tat protein interacts with CSN3 (our unpublished data).

CSN4 is a poorly studied subunit of the CSN. Only one interactor of CSN4 has been identified, the ubiquitin-conjugating enzyme COP10 [34].

CSN5 appears to be a most important subunit both in terms of interactions with other cellular proteins and because it is a component with intrinsic metalloprotease activity (see below). The binding of CSN5 to cellular proteins including the transcription factors p53 [35] and c-Jun [15], the cell-cycle regulator protein p27 [36], rLHR (lutropin/choriogonadotropin receptor precursor) [37], Smad4 (TGF-β signaling pathway common effector) [38] and HIF1α (***h***ypoxia-***i***nducible ***f***actor 1) [39] appears to regulate their metabolic stability. In many cases the CSN5-interacting proteins are phosphorylated by the CSN-associated kinases, which determines the speed of their destruction [40]. In contrast, Id1 and Id3 binding to the CSN complex via CSN5 leads to their stabilization, not to their phosphorylation [41].

The interaction of CSN5 to the member of the IκB multigene family Bcl3, the progesterone receptor PR, and the steroid receptor co-activator SRC-1, leads to stabilization of Bcl3–p50 and PR–SRC-1 complexes and enhances transcriptional activity [42, 43]. Whereas AP-1 activity is stimulated by interaction of CSN5 with the integrin adhesion receptor LFA-1 [44], the opposite effect was reported in the

case of the cytokine migration inhibitor factor, MIF [45]. Additionally, there are other published interactors of CSN5 including the membrane-associated RING-finger Ub ligase TRC8 [46], hepatopoietin (HPO) [47], germ-line RNA helicases (GLHs) [48], and the ubiquitin C-terminal hydrolase PGP9.5 [49]. However, the exact role of these interactions remains unclear.

Several groups reported the occurrence of a free CSN5 subunit [50] or CSN5 as a component of a smaller complex [51], although the exact physiological function of the different CSN5 forms is so far unclear. It is also unknown whether the occurrence of the different CSN5 forms is regulated. Moreover, little is known about CSN5 interactions *in vivo*, how they are regulated and under what circumstances they take place.

CSN6 like CSN8 exists in eukaryotes except in *S. pombe* [52]. There are only a few published interactions of CSN6 with other cellular proteins. It binds to the HIV-1 Vpr protein affecting cell-cycle-associated signaling [53] and to the RING-finger protein of the SCF-complex, Rbx1 [54, 55]. In addition, CSN6 is another binding site for Int-6/eIF3e [33].

Interestingly, in mammalian cells two homologs of CSN7, CSN7a and CSN7b, have been found [6]. *S. pombe* contains only one form of CSN7 whereas *Arabidopsis* contains two alternative splicing variants, CSN7i and CSN7ii [52, 56]. CSN7 interacts with the ***p***olyamine-***m***odulated ***f***actor PMF-1 [57]. Interestingly, CSN7 also binds the protein kinase CK2, one of the CSN-associated kinases, which phosphorylates CSN7 [31]. Whether the phosphorylated form of CSN7 is necessary for CSN complex assembly or for other regulatory events is unclear.

Little is known of CSN8 interactions. CSN8 like CSN3 and CSN4 binds to COP10 [34].

13.3.3 PCI and MPN

Six of the CSN subunits contain PCI (***p***roteasome, ***C***OP9 signalosome, ***i***nitiation factor 3) domains and two contain MPN (***M***pr-***P***ad1-***N***-terminal) domains [58]. These two characteristic domains have been found in three protein complexes: the CSN, the 26S proteasome lid complex (lid) and the eIF3 complex. The two domains are composed of about 150 to 200 amino acids at the N- or C-terminus of the CSN subunits. Apparently, the PCI domain has been shown to be important for interactions between CSN subunits. Thus, it might have a scaffolding function [22, 59].

The CSN subunit CSN5 has been shown to contain a metalloprotease motif localized on its MPN domain, which is essential for the cleavage of the ubiquitin-like modifier NEDD8 from cullins [60] (see below). Apart from the catalytic activity of the MPN domain of CSN5 it appears to be the receptor for different cellular proteins associated with the CSN complex (see above and Figure 13.1). Interestingly, an MPN domain similar to that of CSN5 is located in the N-terminal region of CSN6. However, this MPN domain has no deneddylation catalytic center like CSN5. The function of the CSN6 MPN domain remains obscure.

13.4 Biochemical Activities Associated With the CSN

13.4.1 Deneddylation Activity

Studies in fission yeast and *Arabidopsis* have revealed that the CSN has a role in the cleavage of NEDD8 from cullins [54, 55, 61]. The MPN domain of CSN5, like its paralog subunit Rpn11 of the 26S proteasome lid complex, possesses a highly conserved pattern of four charged amino acid residues: one glutamate, two histidines and one aspartate. This pattern represents a new type of metalloprotease motif called the JAMM (***Ja***b1/***M***PN domain ***m***etalloenzyme) or MPN+ motif [62, 63]. In CSN5 the catalytic region is important for the cleavage of the ubiquitin-like modifier NEDD8 from its targets. Mutations in the conserved histidine and aspartate residues of CSN5 led to suppression of its deneddylation activity [60]. Crystal-structure analysis obtained with bacterial CSN5/MPN+ domain-containing AF2198 protein confirmed the metal-ion-dependent hydrolytic activity of CSN5, although it was inhibited by the alkylating agent NEM, an inhibitor of cysteine proteases [64].

NEDD8 is activated by a heterodimeric complex of APP-B1 and Uba3 and is conjugated to target proteins by the conjugating enzyme Ubc12. So far, the only known targets are cullin-family proteins (CUL1–5), which are components of the cullin-based E3 ligase complexes. The covalent linkage of NEDD8 to cullins *in vivo* is thought to activate Ub-ligase complex activity by facilitating ubiquitin-conjugating enzyme E2 recruitment [65]. Deneddylation of cullins inactivates ubiquitination *in vitro*, but seems to stimulate the Ub E3 ligase complex activity *in vivo* [66, 67]. In cell lysates only a small fraction of CUL1 is neddylated, but in *csn* deletion cells 100% of CUL1 is modified by NEDD8. The purified CSN complex is able to deneddylate, although recombinant CSN5 protein cannot. Obviously CSN5 deneddylation activity is dependent on its assembly into the CSN complex [28, 54]. The fact that null mutants in most CSN subunits lack the deneddylation activity in the presence of excess CSN5 supports the fact that CSN5 alone is inactive in deneddylation [61]. So far, the exact role of deneddylation is questionable (see below).

13.4.2 Protein Kinases

The CSN is associated with enzymes such as kinases, proteases and Ub ligases, which perhaps, besides the intrinsic deneddylation activity, determine the specific function of the CSN in the Ub system. Here we summarize the associated (extrinsic) activities of the CSN shown in Figure 13.2.

13.4.2.1 Associated Protein Kinases

Originally, a protein kinase was the first enzyme identified with the CSN purified from human erythrocytes. The CSN-associated kinase activity phosphorylated sev-

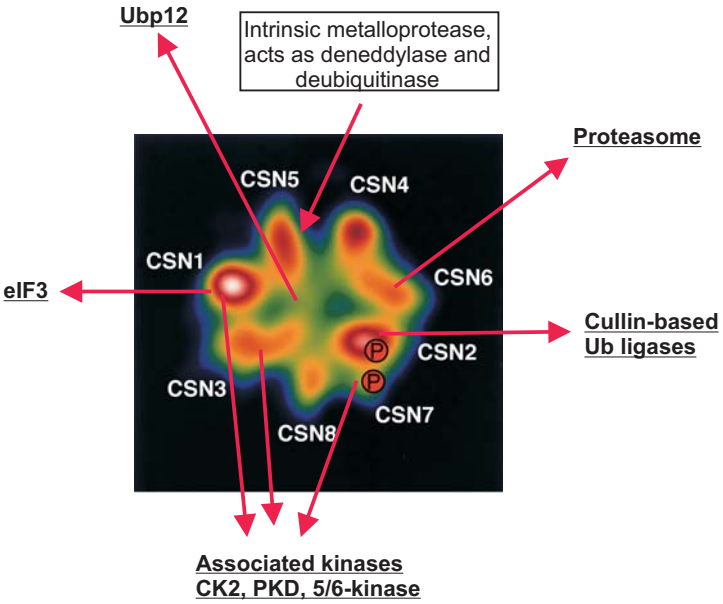

Fig. 13.2. Association of the CSN complex with enzymes. The Figure shows an electron-microscopy image of purified CSN complex from human erythrocytes. As indicated by arrows the CSN is associated with the Ub-specific protease Ubp12, the proteasome, presumably with most of the cullin-based Ub-ligase complexes, with a number of kinases, and with subunits of the translation initiation complex eIF3. In addition, subunit CSN5 has an intrinsic metalloprotease activity, which deneddylates cullins and also removes Ub conjugated to other proteins (for details see text).

eral serine and threonine residues in the N-terminal region of c-Jun [5] resulting in stabilization of c-Jun and increased AP-1 transcriptional activity. The pathway responsible for this c-Jun stabilization/activation was called CSN-directed c-Jun signaling [68]. It was subsequently shown that the CSN-directed c-Jun signaling pathway controls most of the VEGF (***v***ascular ***e***ndothelial ***g***rowth ***f***actor) production in tumor cells [13]. VEGF is essential for tumor angiogenesis (see below).

In contrast to c-Jun, phosphorylation of the tumor suppressor p53 by CSN-associated kinases targets the protein for degradation by the Ub system [35]. For p53 stability, modification on Thr155 is most important as shown by mutational analysis [35] and by using different p53 peptides [31]. Mutation of Thr155 to Val led to stabilization of the transiently expressed p53 mutant in HeLa as well as in HL60 cells [35]. Inhibitors of CSN-associated kinases such as curcumin [18] caused stabilization of cellular p53 followed by massive cell death [35].

In addition to p53 and c-Jun, p27, ICSBP (***i***nterferon ***c***onsensus ***s***equence ***b***inding ***p***rotein) and IκBα were identified as substrates of the CSN-associated kinases (for a review see Ref. [40]). Similar to p53, the phosphorylation of p27 results in

accelerated degradation of the cyclin-dependent kinase inhibitor p27 by the Ub system (our unpublished data). In the case of ICSBP and IκBα, it is still unclear whether CSN-mediated phosphorylation influences their stability. Interestingly, two of the CSN subunits, CSN2 and CSN7, are phosphorylated by the associated kinases [19, 69]. The physiological relevance of these modifications is currently obscure.

Identification of associated protein kinases Based on phosphopeptide analyses it became clear that associated kinases modify principally serine and threonine residues. Moreover, the analysis of putative phosphorylation-specific consensus sequences of p53, c-Jun, p27, ICSBP and IκBα revealed that the protein kinase CK2 and a member of the protein kinase C family might be associated with the CSN. It has been shown by immunoblotting that CK2 and the protein kinase Cμ (also called ***p***rotein ***k***inase ***D***, PKD) co-purify with the CSN from human erythrocytes [31]. In addition, the two kinases co-immunoprecipitated together with the CSN from HeLa cells. Interaction of CK2 as well as PKD with the CSN is mediated by CSN3, as is the interaction between CK2 and the CSN7 subunit [31]. Interestingly, CSN7 itself is phosphorylated.

Majerus and co-workers have published work on the co-purification of inositol 1,3,4-trisphosphate 5/6-kinase (5/6-kinase) with the CSN from bovine brain [24, 70]. Although the 5/6-kinase was not detected in the final preparation of the CSN from human erythrocytes [31], it cannot be excluded that the enzyme is associated with another pool of CSN particles. The enzyme phosphorylates c-Jun, IκBα as well as p53 and is sensitive to curcumin. These characteristics are very similar to those described for CK2 and PKD. It has been shown that the 5/6-kinase interacts with CSN1 and that over-expression of CSN1 inhibits its activity [24]. It might be that it interacts with the N-terminal part of CSN1, which has been shown to suppress activation of an AP-1 promoter [22]. Future studies will show whether additional kinases besides 5/6-kinase, CK2 and PKD can interact with the CSN under certain circumstances. For example, an interaction of CSN3 with IKKγ, a component of the IKK kinase complex, has been published [32].

Functions of associated protein kinases Phosphorylation of a number of Ub-dependent substrates by CSN-associated kinases regulates the stability of the proteins towards the Ub system [40], presumably by promoting substrate ubiquitination. Most of the proteins bind to the CSN via CSN5, are phosphorylated and subsequently channeled to the associated Ub ligase for ubiquitination (see below). Modification of p53 induces a conformational change of the tumor suppressor, which leads to tighter binding to the Ub ligase [35]. In addition, there is evidence that phosphorylation might directly affect Ub-ligase activity. The transcriptional regulator Id3 interacts with the CSN, but is not phosphorylated. Nevertheless, inhibitors of CSN-associated kinases induce ubiquitination and degradation of the Id3 protein [41].

Because of associated kinases and their function in ubiquitination the CSN has

been described as a complex ''at the interface between signal transduction and ubiquitin-dependent proteolysis'' [40]. This becomes even more significant if upstream regulation of the associated kinases is taken into account. Unfortunately at the moment little is known about the receptors or signal-transduction pathways leading to modification of the CSN and its associated kinase activities. It is also unclear whether there are interactions between the kinases and other associated activities of the CSN.

13.4.3 Deubiquitinating Enzymes

To date two deubiquitinating activities associated with the CSN have been identified. By mutational analysis one deubiquitinating activity has been mapped to the metalloprotease motif His–X–His–X10–Asp of the JAMM or MPN+ domain of CSN5 [11]. The conserved Asp residue of that motif was mutated and the mutant Flag-CSN5 was integrated into the CSN. The mutated CSN lost its ability to remove ubiquitin from the isopeptide bond of a mono-ubiquitinated conjugate [11]. Obviously the same intrinsic metalloprotease activity is responsible for deneddylation of mono-neddylated cullins [60], which is not surprising because of the homologies between Ub and NEDD8. It would be interesting to test whether the lid subunit Rpn11, which exhibits a deubiquitinating MPN+ domain [62], is able to deneddylate mono-neddylated proteins.

In addition, another deubiquitinating activity associated with the CSN disassembles poly-Ub chains [11, 71]. This activity is catalyzed in fission yeast by the Ub-specific protease Ubp12, which is a CSN-associated enzyme [71]. The interaction of Ubp12 with the CSN is required for Ubp12 transport to the nucleus. Presumably in the nucleus, S. pombe Pcu1- and Pcu3-based Ub-ligase activities are inhibited by Ubp12 enzyme, since the deubiquitinating enzyme protects a specific adapter protein, Pop1p, from autocatalytic destruction [71]. Thus it seems that the CSN has dual activity in suppressing cullin-based Ub-ligase reactions: one is the intrinsic deneddylation and the other is deubiquitination via associated Ubp12; both reactions serve to inhibit cullin-based Ub ligases *in vitro*.

Data have accumulated showing that CSN-associated deneddylation and deubiquitination are required for Ub-ligase activity *in vivo*. It has been hypothesized, therefore, that CSN-mediated inhibition of cullin-based ubiquitination might be necessary for the assembly of new cullin-based Ub-ligase complexes. After release from the CSN the new cullin-based complex would be active. It has to return to the CSN for re-assembly or is degraded after auto-ubiquitination [71, 72]. For example, p27 has to be degraded at the transition from G1 to S phase. In a first step p27 may bind to the CSN which signals, perhaps by phosphorylation, the assembly of the required SCF complex containing the specific F-box protein Skp2. After formation of the p27-specific SCF complex both p27 and the Ub ligase might be released from the CSN perhaps again by phosphorylation which then results in ubiquitination and complete degradation of p27. Finally the Skp2-containing SCF complex is auto-ubiquitinated and degraded unless additional substrate appears.

13.4.4 Ubiquitin Ligases

Data have been accumulated demonstrating interactions of the CSN with Ub ligases, in particular with the cullin-based Ub ligases. Cullins 1 to 7 (CUL1–CUL7) form a protein family detected in all eukaryotic cells, which is involved in protein ubiquitination. It is known that CUL1 to CUL5 interact with the RING-domain protein Rbx1, the Ub ligase of the cullin-based complexes. So far it has been shown that the CSN interacts with CUL1 to CUL4 [11, 12, 54, 55, 61, 73]. Binding studies with CUL1 and with CUL2 revealed that the two cullin proteins bind via CSN2 to the complex [28, 54, 55]. In addition, Rbx1 seems to interact with CSN6 [54, 55]. Moreover, CUL1 interacts with Skp1, which makes the connection to a substrate-specific F-box protein. Therefore, CUL1-based Ub ligases are called SCF complexes (***S***kp1–***C***DC53/CUL1–***F***-box protein) (for a review see Ref. [74]). CUL2 can be linked to the substrate-adapter protein the von Hippel–Lindau tumor suppressor via elongin C and elongin B forming the so called VCB (***v***on Hippel–Lindau–elongin ***C***–elongin ***B***) complex (for a review see Ref. [74]). BTB/POZ-domain proteins have been identified as possible substrate-specific adaptors of CUL3-based Ub ligases [73, 75, 76]. There are more than 200 putative BTB/POZ-domain proteins expressed in mammalian cells and together with the large number of possible F-box proteins one can estimate that several hundreds of different cullin-based Ub-ligase complexes with different substrate specificities can be formed. The CUL4–Rbx1 complex has been characterized, and seems to be important for checkpoint control [12], DNA repair [11] and ubiquitination of c-Jun [77]. Most likely all cullin-based complexes interact with the CSN. In other words, the CSN is associated with ubiquitinating activity (see Figure 13.2).

There are just a few data on interactions of the CSN with other Ub ligases besides the cullin-based complexes. For example, Mdm2, the RING domain Ub ligase of the tumor suppressor p53, binds to the CSN and is modified by CSN-associated kinases (our unpublished data). Whether Mdm2 is also modified by other CSN-associated activities has to be tested in the future. In addition, COP1, a putative RING-domain Ub ligase, which probably cooperates with the ***C***OP1-***i***nteracting ***p***rotein 8 (CIP8) also binds to the CSN (for a review see Ref. [4]). However, some data indicate that COP1 is associated with a CUL4A complex in which it acts together with DET1 as a heterodimeric substrate adapter [77]. In this complex the CSN interacts with both the CUL4A and the COP1.

13.5 Association of the CSN With Other Protein Complexes

13.5.1 The eIF3 Complex

MPN and PCI domains have been also found in subunits of the eIF3 complex. Because MPN and PCI domains are most likely involved in protein–protein interac-

tions (see above), it is not surprising that there are also cross-interactions between subunits of the CSN, the eIF3 and the lid. It has been reported that eIF3e/INT6 possessing a PCI domain interacts with CSN7 [33, 78]. Another eIF3 subunit eIF3c/p105 co-immunoprecipitated with eIF3e/INT6, eIF3b, CSN1 and CSN8 [78]. eIF3e/INT6 was used as bait in a two-hybrid screen that revealed possible interactions with the 26S proteasome ATPase Rpt4, CSN3 and CSN6 but also with CSN7 [33]. Interestingly, the subunit of the CSN-like complex in *Saccharomyces cerevisiae* Pci8/CSN11 [79] seems also to be a subunit of the budding yeast eIF3 complex and perhaps plays a similar role to eIF3e/INT6 in eukaryotic cells [80]. It has been speculated that these interactions allow the CSN to control translation.

Interactions between eIF3e/INT6 or eIF3i with the 26S proteasome have also been described [33, 81]. It has been shown that eIF3e/INT6 interacts with Rpn5 of the lid complex. This has an impact on 26S proteasome activity/localization, presumably affecting cell division and mitotic fidelity [82]. Perhaps there exists a network of ''PCI complexes'' as suggested [83], which shares polypeptides and communicates via proteins such as eIF3e/INT6.

13.5.2
The Proteasome

In 1998 it was reported that the CSN co-fractionates with the 26S proteasome from human cells [5]. A yeast two-hybrid screen revealed that the C-terminal domain of the Arabidopsis atCSN1 subunit interacts with atRpn6 of the 26S proteasome lid [26]. Recently gel-filtration size-fractionation of material from *Arabidopsis* in the presence of ATP and phosphatase inhibitors indicated that the CSN1 and CSN6 subunits co-elute in the same fractions as subunits of the 26S regulatory complex [84]. Based on these data it has been speculated that the CSN might be an alternative lid of the 26S proteasome [85]. The ''alternative lid hypothesis'', however, makes little sense if the CSN interacts with the 26S proteasome via the lid component Rpn6 [26]. CSN pull-down experiments and subsequent mass-spectrometry analysis of co-precipitated proteins also revealed the presence of proteasome subunits in the precipitate [73]. However, since proteasome subunits are very abundant in cells, one has to be cautious with this type of data. So far there is no systematic binding study showing physical interaction of the CSN with sub-complexes of the 26S proteasome. Moreover, up to now there is no functional evidence for such a CSN/26S proteasome interaction.

13.6
Biological Functions of the CSN

13.6.1
Regulation of Ubiquitin Conjugate Formation

In general, and including all its activities, intrinsic as well as associated, the CSN seems to be a regulator of ubiquitination. Deneddylation, deubiquitination as well

as CSN-mediated phosphorylation (at least with c-Jun and Id3 as substrates) cause inhibition of ubiquitination. It is likely that suppression of ligase activity is an essential step in the dynamic process of specific E3 complex assembly/reassembly. According to the model of Wolf et al. [72] cullin-based Ub-ligase complexes might assemble/reassemble in a protected environment produced by the CSN. In the CSN-associated-state, binding of any E2 to the Ub ligase is prevented, perhaps by deneddylation [65], self-ubiquitination is blocked by continuous deubiquitination [71] and substrate binding could be inhibited by phosphorylation [31]. Only under these conditions can the Ub ligase reassemble without itself being destroyed. For example, an SCF complex might associate with another F-box protein, or a CUL3-Ub ligase with another BTB/POZ-domain protein, as an adaptation to the next phase of cell cycle or signal transduction upon the appearance of a new substrate, which has to be degraded. Following this argument a major question arises. How does the substrate signal the assembly of the required Ub ligase performing its ubiquitination? Is it by binding to the CSN and subsequent signaling via specific kinases?

In the case of the SCF complexes, another protein called CAND1/Tip120A seems to be involved in the dynamic assembly/reassembly process of the E3 [86]. CAND1 binds to the deneddylated CUL1 and inhibits Ub-ligase activity by competing for the Skp1–F-box-protein unit of the SCF complex [87]. After the release of CAND1, a new Skp1–F-box-protein unit can dock to the CUL1–Rbx1 unit to form an SCF complex possessing the necessary substrate specificity. Now the freshly formed Ub ligase has to be released from the CSN to become active. At the moment it is unclear how the Ub ligase might be released from the CSN. The attractive model of CSN-assisted Ub-ligase-complex assembly has to be tested in the future. In this model the CSN would function as a platform for Ub-ligase assembly.

Interestingly, there are no reports of interactions between the 26S proteasome lid complex and Ub ligases. Known E3s directly interacting with the 26S proteasome seem to bind via base ATPases [88, 89]. This is an interesting functional difference between the CSN and the lid developed during evolution.

In an alternative model the CSN might be the platform for complete proteolysis. It forms supercomplexes consisting of both the ubiquitinating and the proteolytic machineries. According to this model, the substrate first binds to the CSN, is then ubiquitinated by the associated Ub ligase and finally directly channeled into the 26S proteasome. Deneddylation, deubiquitination and phosphorylation are necessary to maintain the supercomplex, to protect the intermediates and to stimulate proteolysis.

13.6.1.1 **Cell-cycle and Checkpoint Control**

Initial insight of the role of CSN in cell-cycle control came from the finding that *csn1* and *csn2* deletion *S. pombe* strains have an S-phase delay [52]. Interestingly, this effect did not occur in strains missing other CSN subunits. The S-phase delay was caused by the accumulation of the cell-cycle inhibitor Spd1 (***S-p***hase ***d***elayed 1), which is involved in the misregulation of the ***r***ibo***n***ucleotide ***r***eductase (RNR). RNR catalyzes the production of deoxyribonucleotides for DNA synthesis and

is composed of four subunits including Suc22. Activation of RNR is regulated by nuclear export of Suc22, which is suppressed by Spd1 [12]. Upon DNA damage or during S phase Spd1 is rapidly degraded, presumably leading to the RNR-dependent production of dNTPs. However, in *csn1* and *csn2* deletion mutants, Spd1 accumulates, causing Suc22-dependent suppression of RNR connected with the S-phase delay and DNA-damage sensitivity [12, 15].

In mammalian cells, binding of HIV-1 Vpr-protein to the CSN6 results in cell-cycle arrest at the G2/M phase [53]. Additionally, CSN is involved in the cell cycle via the nuclear export of cell-cycle kinase inhibitor $p27^{kip1}$ (p27). CSN5 binds to p27 and promotes its nuclear export followed by its proteasome-dependent degradation. The over-expression of CSN5 in mouse fibroblasts counteracts cell-cycle arrest induced by serum depletion [36, 51]. Microinjection of the purified CSN complex into synchronized G1 cells blocks the S-phase entry in a deneddylation-dependent manner [28]. Furthermore, the reduction of CSN subunit expression by RNAi in *Caenorhabditis elegans* causes the failure of Mei-1 degradation by regulation of its specific Ub-ligase CUL3-based complex, which leads to severe effects during mitotic cell division [76].

Moreover, the CSN is involved in checkpoint control. The double deletions of *csn1* and *csn2* mutants crossed with checkpoint pathway mutants such as *rad3*, *chk*, and *cds1* are synthetically lethal in *S. pombe* [52]. Cds1 kinase is constitutively activated in *csn1* mutants. Similarly, loss of *csn5* in *Drosophila* results in activation of Mei-41, one of the ATM/ATR family kinases involved in meiotic checkpoint upon DNA damage [90].

13.6.1.2 **DNA Repair**

Two papers have assigned the CSN a function in DNA repair. One study reports on the existence of two different complexes containing human CSN and either one of the two nucleotide-excision-repair proteins, DDB2 or CSA. DDB2 is involved in the global ***g***enome-***r***epair ***p***athway (GGR) and CSA functions in the ***t***ranscription-***c***oupled ***r***epair pathway (TCR). Additionally, these complexes possess Ub-ligase activity and contain cullin-based Ub-ligase components such as CUL4 and Rbx1/Roc1, and DDB1, a UV-damage DNA-binding protein [11]. However, so far their targets remain unclear. CSN differentially regulates the ubiquitin-ligase activity of the DDB2- and CSA-containing complexes in response to UV irradiation. In support of direct involvement of the CSN is the finding that knockdown of CSN5 with RNAi causes a failure in NER mechanisms [11]. Similarly, CSN in combination with the CUL4–Rbx1 complex is involved in Ub-dependent degradation of CDT1, a licensing factor of the pre-replication complex (preRC), after UV- or γ-irradiation. Knockdown of CSN completely suppresses CDT1 degradation, causing a defect G1 checkpoint in response to DNA damage [91].

13.6.1.3 **Developmental Processes**

Although CSN is not essential in yeast, the *csn1* and *csn2 S. pombe* deletion mutants display slow growth and sensitivity to UV- and γ-irradiation [52]. Other csn mutants did not show significant phenotypes apart from the loss of cullin's dened-

dylation activity [61]. In mutants of CSN-like complexes in the budding yeast *S. cerevisiae* the sensitivity to the DNA-damage reagents is not affected [92]. In some *S. cerevisiae* mutants such as *csn5*, *csn9* and *csn12* deletions, increased mating efficiency and enhanced pheromone response has been observed [63].

In *Drosophila*, mutations of CSN causes lethality in early larval stages and defects during oogenesis or photoreceptor R cell differentiation [9, 90, 93, 94]. More specifically, lack of CSN5 leads to the activation of a DNA double-strand-break-dependent checkpoint mediated by Mei-41. This effect is caused by CSN5-dependent inhibition of gurken (Grk) protein translation [90]. In *C. elegans*, knockdown of CSN5 by RNAi resulted in a sterile phenotype, which could be explained by CSN interaction with germ-line RNA helicases [48].

The best studied physiological role of the CSN in developmental processes is derived from studies on *Arabidopsis*. *Csn* mutants can survive embryogenesis, but they die soon after germination. The *csn* mutants exhibit a defect in photomorphogenesis, a light-dependent developmental process of germinating seedlings. Even in total darkness the mutants display a light-dependent morphology and signal-independent expression of light-induced genes [3, 14, 95]. One key mechanism is the CSN-dependent regulation of the stability of the transcription factor HY5, a positive regulator of light-induced genes. In the dark it is degraded by the Ub system [8]. It has been suggested that in darkness the RING-finger protein COP1 ubiquitinates HY5 and triggers its degradation by the 26S proteasome (for a review see Ref. [4]). In the light, COP1 is relocated to the cytoplasm allowing expression of genes through HY5. Although the exact mechanism remains unclear, the CSN may be required for relocation of COP1 from cytoplasm to the nucleus in darkness. Identical phenotypes caused by different *csn* mutants in *Arabidopsis* could be explained by a role of the CSN as a whole complex (for a review see Ref. [20]).

There is accumulating evidence for cooperation of the CSN and cullin-based complexes in specific developmental processes [96]. First insight has been provided by studies on auxin response where the CSN interacts with SCF^{TIR1}, modulating its activity [55]. Similarly, binding of the CSN to other cullin-based complexes regulates their activity in mediating various developmental processes such as flower development, and plant defense responses [97, 98].

13.6.2 Tumor Angiogenesis

Tumor angiogenesis is the vascularization of solid tumors, an essential requirement for tumor growth and metastasis. After a solid tumor has reached a size of approximately 2 mm^3, it needs nutrient supply from blood vessels, otherwise it dies from necrosis. Many tumor cells are able to induce angiogenesis. In an initiation phase the tumor cells produce large amounts of pro-angiogenic factors such as ***v***ascular ***e***ndothelial ***g***rowth ***f***actor, VEGF. During proliferation and invasion VEGF stimulates migration of endothelial cells. Finally, after a maturation phase, vascularization of solid tumors is completed. Now the tumor can grow, and some tumor cells penetrate through vessel membranes and spread via the circulation. There-

fore, inhibition of tumor angiogenesis has become an important strategy in tumor therapy.

There is functional cooperation between the CSN and the Ub system in tumor angiogenesis [13]. It has been known for some time that curcumin is an inhibitor of angiogenesis [99]. However, only in 2001 did it become clear that it acts via inhibition of CSN-associated kinases [13]. It has been demonstrated that overexpression of CSN2 subunit leads to elevated amounts of *de novo* assembled CSN complex connected with increased c-Jun levels and enhanced AP-1 transactivation activity [68]. This c-Jun activation/stabilization is independent of the JNK and the MAP kinase pathway and is called CSN-directed c-Jun signaling [68]. This process can be inhibited by curcumin or other inhibitors of CSN-associated kinases (Figure 13.3). The CSN-directed c-Jun signaling controls up to 75% of VEGF production in tumor cells [13]. In addition, Id1 and Id3 are also essential factors of tumor angiogenesis [100] and are degraded in the presence of CSN-associated kinase inhibitors in an Ub-dependent manner just like c-Jun [41]. Therefore, specific inhibition of CSN-associated kinases might become important for tumor therapy. The application of CSN-associated kinase inhibitors in tumor therapy could be beneficial owing to another effect of curcumin-like compounds, namely they stabilize cellular

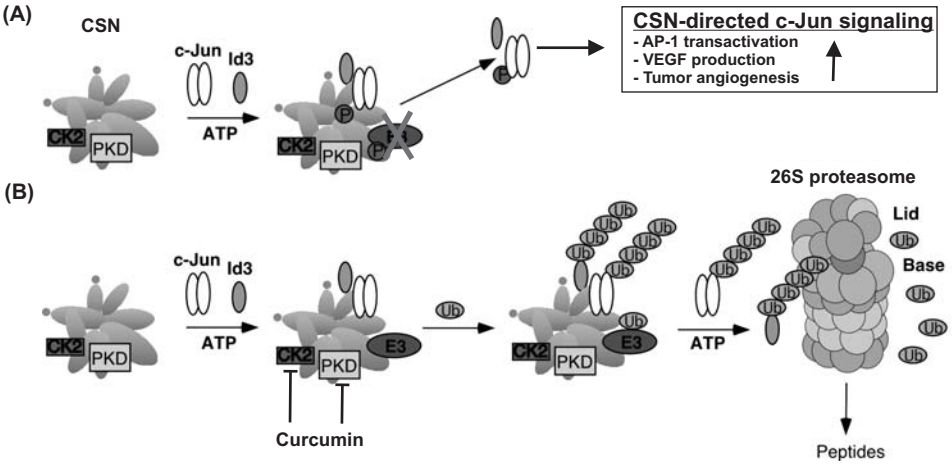

Fig. 13.3. The CSN-directed c-Jun signaling pathway. (A) The active CSN-directed c-Jun signaling pathway is shown. In case of active CSN-associated kinases c-Jun is phosphorylated, which stabilizes the transcription factor towards the Ub system. In addition, phosphorylation of the responsible E3 might inactivate the enzyme. In this situation Id1 and Id3 are also stabilized. Stable/active c-Jun causes enhanced AP-1 transactivation connected with an increase of VEGF production by tumor cells (see text). VEGF is a major pro-angiogenic factor produced by many tumor cells. Id1 and Id3 are transcriptional regulators essential for tumor angiogenesis. (B) In the presence of curcumin or other kinase inhibitors the responsible Ub ligase is most likely active and ubiquitinates both c-Jun and Id3. In addition, unphosphorylated c-Jun might have higher affinity to its Ub ligase. This leads to quick degradation of the proteins by the Ub system.

p53 and, at least in tumors with wild-type p53 protein, massive cell death can be observed [35].

13.7 Concluding Remarks

The CSN is a regulatory complex of the Ub system. Physically it interacts with the proteasome and with Ub ligases. Although the exact mechanism remains obscure, the CSN regulates ubiquitination of important cell-cycle factors and transcriptional regulators. Its intrinsic deneddylating as well as the associated kinase and deubiquitinating activities seem to be required for determining protein stability towards the Ub system. As a major regulator of the Ub system the CSN is involved in processes such as DNA repair, cell-cycle progression and development. Its role in tumor angiogenesis makes the complex attractive for future tumor therapies.

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Protein Degradation, Vol. 2: The Ubiquitin-Proteasome System.
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Preface

There is an incredible amount of current global research activity devoted to understanding the chemistry of life. The genomic revolution means that we now have the basic genetic information in order to understand in full the molecular basis of the life process. However, we are still in the early stages of trying to understand the specific mechanisms and pathways that regulate cellular activities. Occasionally discoveries are made that radically change the way in which we view cellular activities. One of the best examples would be the finding that reversible phosphorylation of proteins is a key regulatory mechanism with a plethora of downstream consequences. Now the seminal discovery of another post-translational modification, protein ubiquitylation, is leading to a radical revision of our understanding of cell physiology. It is becoming ever more clear that protein ubiquitylation is as important as protein phosphorylation in regulating cellular activities. One consequence of protein ubiquitylation is protein degradation by the 26S proteasome. However, we are just beginning to understand the full physiological consequences of covalent modification of proteins, not only by ubiquitin, but also by ubiquitin-related proteins.

Because the Ubiquitin Proteasome System (UPS) is a relatively young field of study, there is ample room to speculate on possible future developments. Today a handful of diseases, particularly neurodegenerative ones, are known to be caused by malfunction of the UPS. With perhaps as many as 1000 human genes encoding components of ubiquitin and ubiquitin-related modification pathways, it is almost certain that many more diseases will be found to arise from genetic errors in the UPS or by pathogen subversion of the system. This opens several avenues for the development of new therapies. Already the proteasome inhibitor Velcade is producing clinical success in the fight against multiple myeloma. Other therapies based on the inhibition or activation of specific ubiquitin ligases, the substrate recognition components of the UPS, are likely to be forthcoming. At the fundamental research level there are a number of possible discoveries especially given the surprising range of biochemical reactions involving ubiquitin and its cousins. Who would have guessed that the small highly conserved protein would be involved in endocytosis or that its relative Atg8 would form covalent bonds to a phospholipid during autophagy? We suspect that few students of ubiquitin will be surprised if it or a

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ubiquitin-like protein is one day found to be covalently attached to a nucleic acid for some biological purpose.

We are regularly informed by the ubiquitin community that the initiation of this series of books on the UPS is extremely timely. Even though the field is young, it has now reached the point at which the biomedical scientific community at large needs reference works in which contributing authors indicate the fundamental roles of the ubiquitin proteasome system in all cellular processes. We have attempted to draw together contributions from experts in the field to illustrate the comprehensive manner in which the ubiquitin proteasome system regulates cell physiology. There is no doubt then when the full implications of protein modification by ubiquitin and ubiquitin-like molecules are fully understood we will have gained fundamental new insights into the life process. We will also have come to understand those pathological processes resulting from UPS malfunction. The medical implications should have considerable impact on the pharmaceutical industry and should open new avenues for therapeutic intervention in human and animal diseases. The extensive physiological ramifications of the ubiquitin proteasome system warrant a series of books of which this is the first one.

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Molecular Chaperones and the Ubiquitin–Proteasome System

Cam Patterson and Jörg Höhfeld

Abstract

A role for the ubiquitin–proteasome system in the removal of misfolded and abnormal proteins is well established. Nevertheless, very little is known about how abnormal proteins are recognized for degradation by the proteasome. Recent advances suggest that substrate recognition and processing require a close cooperation of the ubiquitin–proteasome system with molecular chaperones. Chaperones are defined by their ability to recognize nonnative conformations of other proteins and are therefore ideally suited to distinguish between native and abnormal proteins during substrate selection. Here we discuss molecular mechanisms that underlie the cooperation of molecular chaperones with the ubiquitin–proteasome system. Advancing our knowledge about such mechanisms may open up opportunities to modulate chaperone–proteasome cooperation in human diseases.

1.1
Introduction

The biological activity of a protein is defined by its unique three-dimensional structure. Attaining this structure, however, is a delicate process. A recent study suggests that up to 30% of all newly synthesized proteins never reach their native state [1]. As protein misfolding poses a major threat to cell function and viability, molecular mechanisms must have evolved to prevent the accumulation of misfolded proteins and thus aggregate formation. Two protective strategies appear to be followed. Molecular chaperones are employed to stabilize nonnative protein conformations and to promote folding to the native state whenever possible. Alternatively, misfolded proteins are removed by degradation, involving, for example, the ubiquitin–proteasome system. For a long time molecular chaperones and cellular degradation systems were therefore viewed as opposing forces. However, recent evidence suggests that certain chaperones (in particular members of the 70- and 90-kDa heat shock protein families) are able to cooperate with the ubiquitin–

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proteasome system. Protein fate thus appears to be determined by a tight interplay of cellular protein-folding and protein-degradation systems.

1.2 A Biomedical Perspective

The aggregation and accumulation of misfolded proteins is now recognized as a common characteristic of a number of degenerative disorders, many of which have neurological manifestations [2, 3]. These diseases include prionopathies, Alzheimer's and Parkinson's diseases, and polyglutamine expansion diseases such as Huntington's disease and spinocerebellar ataxia. At the cellular level, these diseases are characterized by the accumulation of aberrant proteins either intracellularly or extracellularly in specific groups of cells that subsequently undergo death. The precise association between protein accumulation and cell death remains incompletely understood and may vary from disease to disease. In some cases, misfolded protein accumulations may themselves be toxic or exert spatial constraints on cells that affect their ability to function normally. In other cases, the sequestering of proteins in aggregates may itself be a protective mechanism, and it is the overwhelming of pathways that consolidate aberrant proteins that is the toxic event. In either case, lessons learned from genetically determined neurodegenerative diseases have helped us to understand the inciting events of protein aggregation that ultimately lead to degenerative diseases.

Mutations resulting in neurodegenerative diseases fall into two broad classes. The first class comprises mutations that affect proteins, irrespective of their native function, and cause them to misfold. The classic example of this is Huntington's disease [4, 5]. The protein encoded by the huntingtin gene contains a stretch of glutamine residues (or polyglutamine repeat), and the genomic DNA sequence that codes for this polyglutamine repeat is subject to misreading and expansion. When the length of the polyglutamine repeat in huntingtin reaches a critical threshold of approximately 35 residues, the protein becomes prone to misfolding and aggregation [6]. This appears to be the proximate cause of neurotoxicity in this invariably fatal disease [7, 8]. A number of other neurodegenerative diseases are caused by polyglutamine expansions [9, 10]. For example, spinocerebellar ataxia is caused by polyglutamine expansions in the protein ataxin-1 [11]. In other diseases, protein misfolding occurs due to other mutations that induce misfolding and aggregation; for example, mutations in superoxide dismutase-1 lead to aggregation and neurotoxicity in amyotrophic lateral sclerosis [12, 13].

Other mutations that result in neurodegenerative diseases are instructive in that they directly implicate the ubiquitin–proteasome system in the pathogenesis of these diseases [14]. For example, mutations in the gene encoding the protein parkin are associated with juvenile-onset Parkinson's disease [15, 16]. Parkin is a RING finger–containing ubiquitin ligase, and mutations in this ubiquitin ligase cause accumulation of target proteins that ultimately result in the neurotoxicity and motor dysfunction associated with Parkinson's disease [17–20].

Repressor screens of neurodegeneration phenotypes in animal models have also linked the molecular chaperone machinery to neurodegeneration [21–24]. Taken together, the pathophysiology of neurodegenerative diseases provides a compelling demonstration of the importance of the regulated metabolism of misfolded proteins and provides direct evidence of the role of both molecular chaperones and the ubiquitin–proteasome system in guarding against protein misfolding and its consequent toxicity.

1.3 Molecular Chaperones: Mode of Action and Cellular Functions

Molecular chaperones are defined by their ability to bind and stabilize nonnative conformations of other proteins [25, 26]. Although they are an amazingly diverse group of conserved and ubiquitous proteins, they are also among the most abundant intracellular proteins. The classical function of chaperones is to facilitate protein folding, inhibit misfolding, and prevent aggregation. These folding events are regulated by interactions between chaperones and ancillary proteins, the co-chaperones, which in general assist in cycling unfolded substrate proteins on and off the active chaperone complex [25, 27, 28]. In agreement with their essential function under normal growth conditions, chaperones are ubiquitously expressed and are found in all cellular compartments of the eukaryotic cell (except for peroxisomes). In addition, cells greatly increase chaperone concentration as a response to diverse stresses, when proteins become unfolded and require protection and stabilization [29]. Accordingly, many chaperones are heat shock proteins (Hsps). Four main families of cytoplasmic chaperones can be distinguished: the Hsp70 family, the Hsp90 family, the small heat shock proteins, and the chaperonins.

1.3.1 The Hsp70 Family

The Hsp70 proteins bind to misfolded proteins promiscuously during translation or after stress-mediated protein damage [26, 30]. Members of this family are highly conserved throughout evolution and are found throughout the prokaryotic and eukaryotic phylogeny. It is common for a single cell to contain multiple homologues, even within a single cellular compartment; for example, mammalian cells express two inducible homologues (Hsp70.1 and Hsp70.3) and a constitutive homologue (Hsc70) in the cytoplasm. These homologues have overlapping but not totally redundant cellular functions. Members of this family are typically in the range of 70 kDa in size and contain three functional domains: an amino-terminal ATPase domain, a central peptide-binding cleft, and a carboxyl terminus that seems to form a lid over the peptide-binding cleft [28] (Figure 1.1). The chaperones recognize short segments of the protein substrate, which are composed of clusters of hydrophobic amino acids flanked by basic residues [31]. Such binding motifs occur frequently within protein sequences and are found exposed on nonnative proteins. In fact,

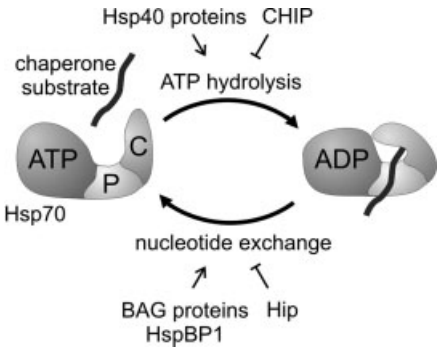

Fig. 1.1. Schematic presentation of the domain architecture and chaperone cycle of Hsp70. Hsp70 proteins display a characteristic domain structure comprising an amino-terminal ATPase domain (ATP), a peptide-binding domain (P), and a carboxyl-terminal domain (C) that is supposed to form a lid over the peptide-binding domain. In the ATP-bound conformation, the binding pocket is open, resulting in a low affinity for the binding of a chaperone substrate. ATP hydrolysis induces stable substrate binding through a closure of the peptide-binding pocket. Substrate release is induced upon nucleotide exchange. ATP hydrolysis and nucleotide exchange are regulated by diverse co-chaperones.

mammalian Hsp70 binds to a wide range of nascent and newly synthesized proteins, comprising about 15–20% of total protein [32]. This percentage is most likely further increased under stress conditions. Hsp70 proteins apparently prevent protein aggregation and promote proper folding by shielding hydrophobic segments of the protein substrate. The hydrophobic segments are recognized by the central peptide-binding domain of Hsp70 proteins (Figure 1.1). The domain is composed of two sheets of β strands that together with connecting loops form a cleft to accommodate extended peptides of about seven amino acids in length, as revealed in crystallographic studies of bacterial Hsp70 [33]. In the obtained crystal structure, the adjacent carboxyl-terminal domain of Hsp70 folds back over the β sandwich, suggesting that the domain may function as a lid in permitting entry and release of protein substrates (Figure 1.1). According to this model, ATP binding and hydrolysis by the amino-terminal ATPase domain of Hsp70 induce conformational changes of the carboxyl terminus, which lead to lid opening and closure [28]. In the ATP-bound conformation of Hsp70, the peptide-binding pocket is open, resulting in rapid binding and release of the substrate and consequently in a low binding affinity (Figure 1.1). Stable holding of the protein substrate requires closing of the binding pocket, which is induced upon ATP hydrolysis and conversion of Hsp70 to the ADP-bound conformation. The dynamic association of Hsp70 with nonnative polypeptide substrates thus depends on ongoing cycles of ATP binding, hydrolysis, and nucleotide exchange. Importantly, ancillary co-chaperones are employed to regulate the ATPase cycle [27, 30]. Co-chaperones of the Hsp40 family (also termed J proteins due to their founding member bacterial DnaJ) stimulate the ATP hydrolysis step within the Hsp70 reaction cycle and in this way promote substrate binding [34] (Figure 1.1). In contrast, the carboxyl terminus of Hsp70-interacting protein CHIP attenuates ATP hydrolysis [35]. Similarly, nucleo-

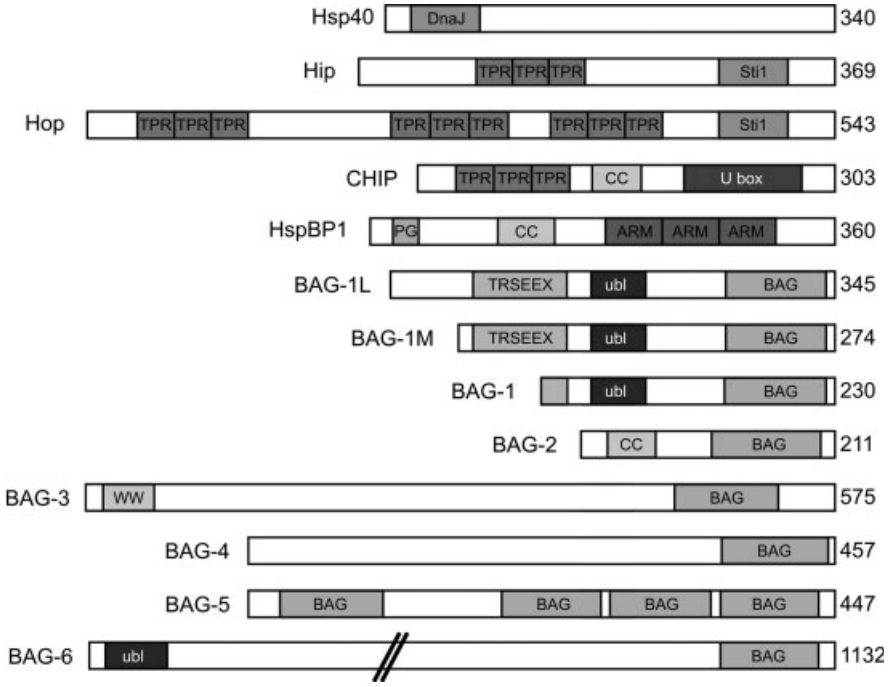

Fig. 1.2. Domain architecture of diverse co-chaperones of Hsp70. DnaJ: domain related to the bacterial co-chaperone DnaJ; TPR: tetratricopeptide repeat; Sti1: domain related to the yeast co-chaperone Sti1; CC: coiled-coil domain; U box: E2-interacting domain present in certain ubiquitin ligases; PG: polyglycine region; ARM: armadillo repeat; TRSEEX: repeat motif found at the amino terminus of BAG-1 isoforms; ubl: ubiquitin-like domain; BAG: Hsp70-binding domain present in BAG proteins; WW: protein interaction domain.

tide exchange on Hsp70 is under the control of stimulating and inhibiting co-chaperones. The Hsp70-interacting protein Hip slows down nucleotide exchange by stabilizing the ADP-bound conformation of the chaperone [36], whereas nucleotide exchange is stimulated by the co-chaperone BAG-1 (Bcl-2-associated athanogene 1), which assists substrate unloading from Hsp70 [37–39]. By altering the ATPase cycle, the co-chaperones directly modulate the folding activity of Hsp70. In addition to chaperone-recognition motifs, co-chaperones often possess other functional domains and therefore link chaperone activity to distinct cellular processes [27, 40] (Figure 1.2). Indeed, as discussed below, the co-chaperones BAG-1 and CHIP apparently modulate Hsp70 function during protein degradation.

1.3.2 The Hsp90 Family

The 90-kDa cytoplasmic chaperones are members of the Hsp90 family, and in mammals two isoforms exist: Hsp90α and Hsp90β. The Hsp70 and Hsp90 families exhibit several common features: both possess ATPase activity and are regulated

by ATP binding and hydrolysis, and both are further regulated by ancillary co-chaperones [41–48]. Unlike Hsp70, however, cytoplasmic Hsp90 is not generally involved in the folding of newly synthesized polypeptide chains. Instead it plays a key role in the regulation of signal transduction networks, as most of the known substrates of Hsp90 are signaling proteins, the classical examples being steroid hormone receptors and signaling kinases. On a molecular level, Hsp90 binds to substrates at a late stage of the folding pathway, when the substrate is poised for activation by ligand binding or associations with other factors. Consequently, Hsp90 accepts partially folded conformations from Hsp70 for further processing. In the case of the chaperone-assisted activation of the glucocorticoid hormone receptor and also of the progesterone receptor, the sequence of events leading to attaining an active conformation is fairly well understood [49–53]. It appears that the receptors are initially recognized by Hsp40 and are then delivered to Hsp70 [54] (Figure 1.3). Subsequent transfer onto Hsp90 requires the Hsp70/Hsp90-organizing protein Hop, which possesses non-overlapping binding sites for Hsp70 and Hsp90 and therefore acts as a coupling factor between the two chaperones [55]. In conjunction with p23 and different cyclophilins, Hsp90 eventually medi-

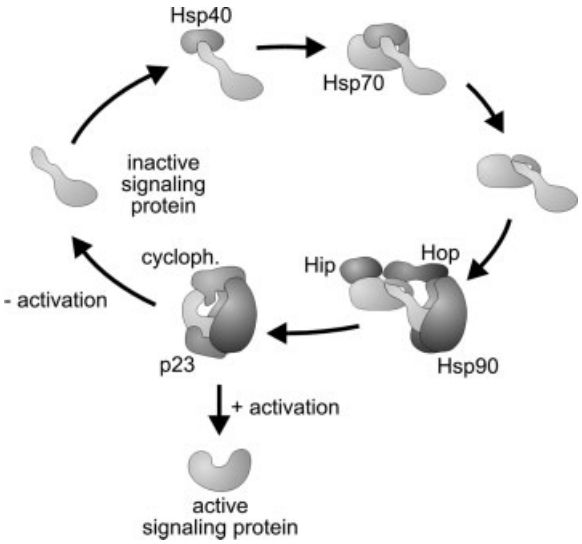

Fig. 1.3. Cooperation of Hsp70 and Hsp90 during the regulation of signal transduction pathways. The inactive signaling protein, e.g., a steroid hormone receptor, is initially recognized by Hsp40 and delivered to Hsp70. Subsequently, a multi-chaperone complex assembles that contains the Hsp70 co-chaperone Hip and the Hsp70/Hsp90-organizing protein Hop. Hop stimulates recruitment of an Hsp90 dimer that accepts the substrate from Hsp70. At the final stage of the chaperone pathway, Hsp90 associates with p23 and diverse cyclophilins (cycloph.) to mediate conformational changes of the signaling protein necessary to reach an activatable state. Upon activation, i.e., hormone binding in the case of the steroid receptor, the signaling protein is released from Hsp90. In the absence of an activating stimulus, the signaling protein folds back to the inactive state when released and enters a new cycle of chaperone binding.

ates conformational changes that enable the receptor to reach a high-affinity state for ligand binding. On other signaling pathways Hsp90 serves as a scaffolding factor to permit interactions between kinases and their substrates, as is the case for Akt kinase and endothelial nitric oxide synthase [56]. Since many of the Hsp90 substrate proteins are involved in regulating cell proliferation and cell death, it is not surprising that the chaperone recently emerged as a drug target in tumor therapy [57–59]. The antibiotics geldanamycin and radicicol specifically bind to Hsp90 in mammalian cells and inhibit the function of the chaperone by occupying its ATP-binding pocket [60–63]. Drugs based on these compounds are now being developed as anticancer agents, as they potentially inactivate multiple signaling pathways that drive carcinogenesis. Remarkably, drug-induced inhibition of Hsp90 blocks the chaperone-assisted activation of signaling proteins and leads to their rapid degradation via the ubiquitin–proteasome pathway [64–69] (Figure 1.4). Hsp90 inhibitors therefore have emerged as helpful tools to study chaperone–proteasome cooperation.

1.3.3 The Small Heat Shock Proteins

The precise functions of small heat shock proteins (sHsps) including Hsp27 and the eye-lens protein αB-crystallin are incompletely understood. However, they

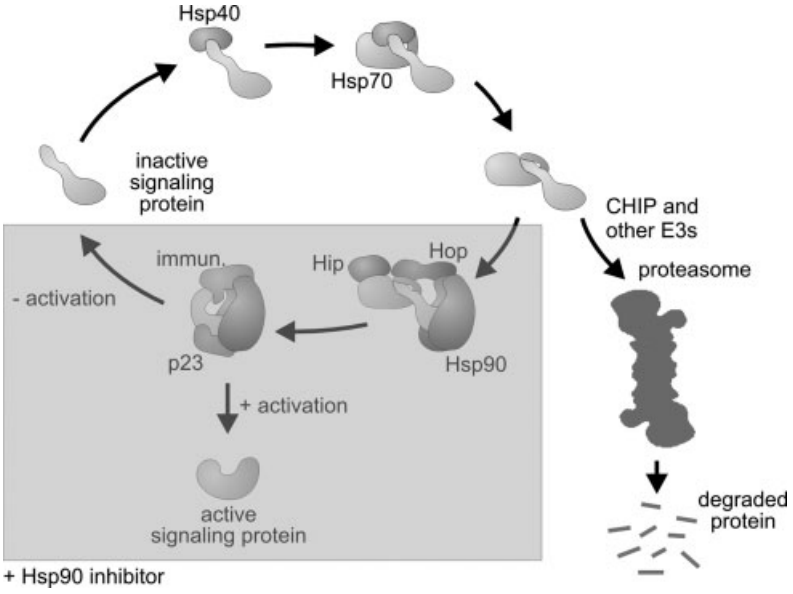

Fig. 1.4. Alteration of chaperone action during signal transduction induced by Hsp90 inhibitors such as geldanamycin and radicicol. In the presence of the inhibitors the activation pathway is blocked, and signaling proteins are targeted to the proteasome for degradation in a process that involves the co-chaperone CHIP and other E3 ubiquitin ligases that remain to be identified.

seem to play a major role in preventing protein aggregation under conditions of cellular stress [70–73]. All members investigated so far form large oligomeric complexes of spherical or cylindrical appearance [74, 75]. Complex formation is independent of ATP binding and hydrolysis, but appears to be regulated by temperature and phosphorylation. The structural analysis of wheat Hsp16.9 suggested that the oligomeric complex acts as a storage form rather than an enclosure for substrates, as the active chaperone appears to be a dimer [75]. In agreement with this notion, dissociation of the oligomeric complex formed by yeast Hsp26 was found to be a prerequisite for efficient chaperone activity [76]. Subsequent refolding may occur spontaneously or may involve cooperation with other chaperones such as Hsp70 [77].

1.3.4 Chaperonins

The chaperone proteins best understood with regard to their mode of action are certainly the so-called chaperonins, which are defined by a barrel-shaped, double-ring structure [25, 28]. Members include bacterial GroEL, Hsp60 of mitochondria and chloroplasts, and the TriC–CCT complex localized in the eukaryotic cytoplasm. Based on their characteristic ring structure, a central cavity is formed, which accommodates nonnative proteins via hydrophobic interactions. Conformational changes of the chaperonin subunits induced through ATP hydrolysis change the inner lining of the cavity from a hydrophobic to a hydrophilic character [78–80]. As a consequence the unfolded polypeptide is released into the central chamber and can proceed on its folding pathway in a protected environment [81]. The chaperonins are therefore capable of folding proteins such as actin that cannot be properly folded via other mechanisms [82].

1.4 Chaperones: Central Players During Protein Quality Control

Due to their ability to recognize nonnative conformations of other proteins, molecular chaperones are of central importance during protein quality control. This was elegantly revealed in studies on the influence of the Hsp70 chaperone system on polyglutamine diseases using the fruit fly *Drosophila melanogaster* as a model organism (reviewed in Refs. [23] and [83]). Hallmarks of the polyglutamine disease spinocerebellar ataxia type 3 (SCA3), for example, were recapitulated in transgenic flies that expressed a pathological polyQ tract of the ataxin-3 protein in the eye disc [84]. Transgene expression caused formation of abnormal protein inclusions and progressive neuronal degeneration. Intriguingly, co-expression of human cytoplasmic Hsp70 suppressed polyQ-induced neurotoxicity. In a similar experimental approach, Hsp40 family members protected neuronal cells against toxic polyQ expression [22]. Enhancing the activity of the Hsp70/Hsp40 chaperone system apparently mitigates cytotoxicity caused by the accumulation of aggregation-prone pro-

teins. These findings obtained in *Drosophila* were confirmed in a mouse model of spinocerebellar ataxia type 1 (SCA1) [85, 86]. Unexpectedly, however, the Hsp70 chaperone system was unable to prevent the formation of protein aggregates in these models of polyglutamine diseases and upon polyQ expression in yeast and mammalian cells [84, 85, 87–89]. Elevating the cellular levels of Hsp70 and of some Hsp40 family members affected the number of protein aggregates and their biochemical properties, but did not inhibit the formation of polyQ aggregates. Notably, Hsp70 and Hsp40 profoundly modulated the aggregation process of polyQ tracts in biochemical experiments; this led to the formation of amorphous, SDS-soluble aggregates, instead of the ordered, SDS-insoluble amyloid fibrils that form in the absence of the chaperone system [88]. These biochemical data were confirmed in yeast and mammalian cells [88, 90]. Although unable to prevent the formation of protein aggregates, the Hsp70 chaperone system apparently prevents the ordered oligomerization and fibril growth that is characteristic of the disease process. In an alternate but not mutually exclusive model to explain their protective role, the chaperones may cover potentially dangerous surfaces exposed by polyQ-containing proteins during the oligomerization process or by the final oligomers. Intriguingly, elevated expression of Hsp70 also suppresses the toxicity of the non-polyQ-containing protein α-synuclein in a *Drosophila* model of Parkinson's disease without inhibiting aggregate formation [24]. Hsp70 may thus exert a rather general function in protecting cells against toxic protein aggregation. This raises the exciting possibility that treatment of diverse forms of human neurodegenerative diseases may be achieved through upregulation of Hsp70 activity.

The mentioned examples illustrate that one does not have to evoke the refolding of an aberrant protein to the native state in order to explain the protective activity of Hsp70 observed in models of amyloid diseases. In some cases it might be sufficient for Hsp70 to modulate the aggregation process or to shield interaction surfaces of the misfolded protein to decrease cytotoxic effects. Another option may involve presentation of the misfolded protein to the ubiquitin–proteasome system for degradation.

1.5 Chaperones and Protein Degradation

Hsp70 and Hsp90 family members as well as small heat shock proteins have all been implicated to participate in protein degradation. For example, the small heat shock protein Hsp27 was recently shown to stimulate the degradation of phosphorylated IκBα via the ubiquitin–proteasome pathway, which may account for the antiapoptotic function of Hsp27 [91]. Similarly, Hsp27 facilitates the proteasomal degradation of phosphorylated tau, a microtubule-binding protein and component of protein deposits in Alzheimer's disease [92]. Hsp70 participates in the degradation of apolipoprotein B100 (apoB), which is essential for the assembly and secretion of very low-density lipoproteins from the liver [93]. Under conditions of limited availability of core lipids, apoB translocation across the ER membrane is

attenuated, resulting in the exposure of some domains of the protein into the cytoplasm and their recognition by Hsp70. This is followed by the degradation of apoB via the ubiquitin–proteasome pathway. Elevating cellular Hsp70 levels stimulated the degradation of the membrane protein, suggesting that the chaperone facilitates sorting to the proteasome. Genetic studies in yeast indicate that cytoplasmic Hsp70 may fulfill a rather general role in the degradation of ER-membrane proteins that display large domains into the cytoplasm [94]. In agreement with this notion, Hsp70 also takes part in the degradation of immaturely glycosylated and aberrantly folded forms of the cystic fibrosis transmembrane conductance regulator (CFTR) [95–98]. CFTR is an ion channel localized at the apical surface of epithelial cells. Its functional absence causes cystic fibrosis, the most common fatal genetic disease in Caucasians [99, 100]. The disease-causing allele, ΔF508, which is expressed in more than 70% of all patients, drastically interferes with the protein's ability to fold, essentially barring it from functional expression in the plasma membrane. However, wild-type CFTR also folds very inefficiently, and less than 30% of the protein reaches the plasma membrane [99]. While trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus, immature forms of CFTR are recognized by quality-control systems and are eventually directed to the proteasome for degradation [101–104]. A critical step during CFTR biogenesis is the inefficient folding of the first of two cytoplasmically exposed nucleotide-binding domains (NBD1) of the membrane protein [105, 106]. The disease-causing ΔF508 mutation localizes to NBD1 and further decreases the folding propensity of this domain. During the co-translational insertion of CFTR into the ER membrane, cytoplasmic Hsp70 and its co-chaperone Hdj-2 bind to NBD1 and facilitate intramolecular interactions between the domain and another cytoplasmic region of CFTR, the regulatory R-domain [96, 107]. However, Hsp70 is also able to present CFTR to the ubiquitin–proteasome system [97], and heterologous expression of CFTR in yeast revealed an essential role of cytoplasmic Hsp70 in CFTR turnover [98]. Hsp70 is thus a key player in the cellular surveillance system that monitors the folded state of CFTR at the ER membrane.

Interestingly, CFTR and the disease form ΔF508 are deposited in distinct pericentriolar structures, termed aggresomes, upon overexpression or proteasome inhibition [108]. Subsequent studies established that aggresomes are induced upon ectopic expression of many different aggregation-prone proteins (reviewed in Refs. [109] and [110]). Aggresomes form near the microtubule-organizing center in a manner dependent on the microtubule-associated motor protein dynein, and are surrounded by a "cage" of filamentous vimentin [108, 111]. Aggresome formation is apparently a specific and active cellular response when production of misfolded proteins exceeds the capacity of the ubiquitin–proteasome system to tag and remove these proteins. They likely serve to protect the cell from toxic "gain-of-function" activities acquired by misfolded proteins. Aggresomes are also of clinical relevance as they share remarkable biochemical and structural features, for example, with Lewy bodies, the cytoplasmic inclusion bodies found in neurons affected by Parkinson's disease [112]. The pathways that regulate aggresome assembly are only now being explicated. Histone deacetylase 6 (HDAC6) appears to be a key reg-

ulator of aggresome assembly [113]. HDAC6 is a microtubule-associated deacetylase that has the capacity to bind both multi-ubiquitinated proteins and dynein motors and is believed to recruit misfolded proteins to the pericentriolar region for aggresome assembly. Deletion of HDAC6 prevents aggresome formation and sensitizes cells to the toxic effects of misfolded proteins, which supports the hypothesis that aggresomes sequester misfolded proteins to protect against their toxic activities. Components of the ubiquitin–proteasome system and chaperones such as Hsp70 are abundantly present in and are actively recruited to aggresomes [114–116]. Furthermore, elevating cellular Hsp70 levels can reduce aggresome formation by stimulating proteasomal degradation [117]. It appears that these subcellular structures are major sites of chaperone–proteasome cooperation to mediate the metabolism of misfolded proteins.

The formation of aggresome-like structures is also observed in dendritic cells that present foreign antigens to other immune cells [118]. Immature dendritic cells are located in tissues throughout the body, including skin and gut. When they encounter invading microbes, the pathogens are endocytosed and processed in a manner that involves the generation of antigenic peptides by the ubiquitin–proteasome system. Upon induction of dendritic cell maturation, ubiquitinated proteins transiently accumulate in large cytosolic structures that resemble aggresomes and were therefore termed DALIS (dendritic cell aggresome-like induced structures). It was speculated that DALIS formation may enable dendritic cells to regulate antigen processing and presentation. DALIS contain components of the ubiquitin–proteasome machinery as well as Hsp70 and the co-chaperone CHIP [118, 119]. Again, an interplay of molecular chaperones and the ubiquitin–proteasome system during regulated protein turnover is suggested.

The cellular function of molecular chaperones is apparently not restricted to mediating protein folding; instead, chaperones emerge also as vital components on protein-degradation pathways. Remarkably, the balance between folding and degradation activities of chaperones can be manipulated. In cells treated with Hsp90 inhibitors, for example, with geldanamycin (see above), the chaperone-assisted activation of signaling proteins is abrogated and chaperone substrates such as the protein kinases Raf-1 and ErbB2 are rapidly degraded by the ubiquitin–proteasome system [64–69, 120]. This appears to be due, in part, to transfer of the substrates back to Hsp70 and progression toward the ubiquitin-dependent degradation pathway.

Substrate interactions with chaperones – and consequently their commitment either toward the folding pathway or to their degradation via the ubiquitin–proteasome machinery – apparently serve as an essential post-translational protein quality-control mechanism within eukaryotic cells. The partitioning of proteins to either one of these mutually exclusive pathways is referred to as "protein triage" [121]. Although some misfolded proteins may be directly recognized by the proteasome [122], specific pathways within the ubiquitin–proteasome system are probably relied on for the degradation of most misfolded and damaged proteins. For example, E2 enzymes of the Ubc4/5 family selectively mediate the ubiquitylation of abnormal proteins as revealed in genetic studies in *Saccharomyces cerevisiae* [123].

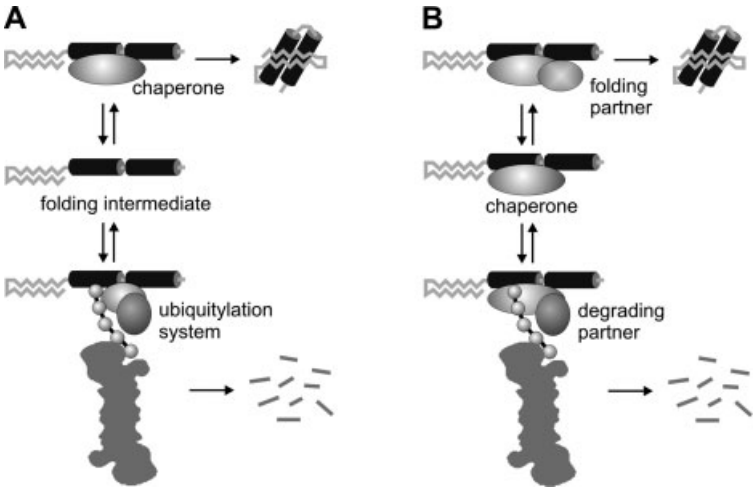

Fig. 1.5. Interplay of molecular chaperones with the ubiquitin–proteasome system. (A) Chaperones and the degradation machinery (i.e., ubiquitylation systems) compete with each other in the recognition of folding intermediates. Interaction with the chaperones directs the substrate towards folding. However, when the protein substrate is unable to attain a folded conformation, the chaperones maintain the folding intermediate in a soluble state that can be recognized by the degradation machinery. (B) The chaperones are actively involved in protein degradation. Through an association with certain components of the ubiquitin conjugation machinery (degrading partner), the chaperones participate in the targeting of protein substrates to the proteasome. A competition between degrading partners and folding partners determines chaperone action and the fate of the protein substrate.

It is well accepted that chaperones play a central role in the triage decision; however, less well understood are the events that lead to the cessation of efforts to fold a substrate, and the diversion of the substrate to the terminal degradative pathway. It is possible that chaperones and components of the ubiquitin–proteasome pathway exist in a state of competition for these substrates and that repeated cycling of a substrate on and off a chaperone maintains the substrate in a soluble state and increases, in a stochastic fashion, its likelihood of interactions with the ubiquitin machinery (Figure 1.5A). However, some data argue for a more direct role of the chaperones in the degradation process. Hsp70 plays an active and necessary role in the ubiquitylation of some substrates [124]; this activity of Hsp70 requires its chaperone function, indicating that conformational changes within substrates may facilitate recognition by the ubiquitylation machinery. Plausible hypotheses to explain these observations include direct associations between the chaperone and ubiquitin–proteasome machinery to facilitate transfer of a substrate from one pathway to the other, or conversion of the chaperone itself to a ubiquitylation complex (Figure 1.5B). It is also entirely possible that several quality-control pathways may exist and that the endogenous triage decision may involve aspects of each of these hypotheses.

1.6
The CHIP Ubiquitin Ligase: A Link Between Folding and Degradation Systems

Major insights into molecular mechanisms that underlie the cooperation of molecular chaperones with the ubiquitin–proteasome system were obtained through the functional characterization of the co-chaperone CHIP (reviewed in Ref. [40]). CHIP was initially identified in a screen for proteins containing tetratricopeptide repeat (TPR) domains, which are found in several co-chaperones – including Hip, Hop, and the cyclophilins – as chaperone-binding domains [27, 55] (Figure 1.2). CHIP contains three TPR domains at its amino terminus, which are used for binding to Hsp70 and Hsp90 [35, 125]. Besides the TPR domains, CHIP possesses a U-box domain at its carboxyl terminus [35] (Figure 1.2). U-box domains are similar to RING finger domains, but they lack the metal-chelating residues and instead are structured by intramolecular interactions [126]. The predicted structural similarity suggests that U boxes, like RING fingers, may also play a role in targeting proteins for ubiquitylation and subsequent proteasome-dependent degradation, and this possibility is borne out in functional analyses of U box–containing proteins [127, 128]. The TPR and U-box domains in CHIP are separated by a central domain rich in charged residues. The charged domain of CHIP is necessary for TPR-dependent interactions with Hsp70 [35] and is also required for homodimerization of CHIP [129].

The tissue distribution of CHIP supports the notion that it participates in protein folding and degradation decisions, as it is most highly expressed in tissues with high metabolic activity and protein turnover: skeletal muscle, heart, and brain. Although it is also present in all other organs, including pancreas, lung, liver, placenta, and kidney, the expression levels are much lower. CHIP is also detectable in most cultured cells, and is particularly abundant in muscle and neuronal cells and in tumor-derived cell lines [35]. Intracellularly, CHIP is primarily localized to the cytoplasm under quiescent conditions [35], although a fraction of CHIP is present in the nucleus [97]. In addition, cytoplasmic CHIP traffics into the nucleus in response to environmental challenge in cultured cells, which may serve as a protective mechanism or to regulate transcriptional responses in the setting of stress [130].

CHIP is distinguished among co-chaperones in that it is a bona fide interaction partner with both of the major cytoplasmic chaperones Hsp90 and Hsp70, based on their interactions with CHIP in the yeast two-hybrid system and *in vivo* binding assays [35, 125]. CHIP interacts with the terminal-terminal EEVD motifs of Hsp70 and Hsp90, similar to other TPR domain–containing co-chaperones such as Hop [55, 131, 132]. When bound to Hsp70, CHIP inhibits ATP hydrolysis and therefore attenuates substrate binding and refolding, resulting in inhibition of the "forward" Hsp70 substrate folding/refolding pathway, at least in *in vitro* assays [35]. The cellular consequences of this "anti-chaperone" function are not yet clear, and in fact CHIP may actually facilitate protein folding under conditions of stress, perhaps by slowing the Hsc70 reaction cycle [130, 133]. CHIP interacts with Hsp90 with approximately equivalent affinity to its interaction with Hsp70 [125]. This interaction

results in remodeling of Hsp90 chaperone complexes, such that the co-chaperone p23, which is required for the appropriate activation of many, if not all, Hsp90 client proteins, is excluded. The mechanism for this activity is unclear – p23 and CHIP bind Hsp90 through different sites – yet the consequence of this action is predictable: CHIP should inhibit the function of proteins that require Hsp90 for conformational activation. The glucocorticoid receptor is an Hsp90 client that undergoes activation through a well-described sequence of events that depend on interactions of the glucocorticoid receptor with Hsp90 and various Hsp90 co-chaperones, including p23, making it an excellent model to test this prediction. Indeed, CHIP inhibits glucocorticoid receptor substrate binding and steroid-dependent transactivation ability [125]. Surprisingly, this effect of CHIP is accompanied by decreased steady-state levels of glucocorticoid receptor, and CHIP induces ubiquitylation of the glucocorticoid receptor *in vivo* and *in vitro*, as well as subsequent proteasome-dependent degradation. This effect is both U-box- and TPR-domain-dependent, suggesting that CHIP's effects on GR require direct interaction with Hsp90 and direct ubiquitylation of GR and delivery to the proteasome.

These observations are not limited to the glucocorticoid receptor. ErbB2, another Hsp90 client, is also degraded by CHIP in a proteasome-dependent fashion [120]. Nor are they limited to Hsp90 clients. For example, CHIP cooperates with Hsp70 during the degradation of immature forms of the CFTR protein at the ER membrane and during the ubiquitylation of phosphorylated forms of the microtubule-binding protein tau, which is of clinical importance due to its role in the pathology of Alzheimer's disease [97, 134]. The effects of CHIP are dependent on both the TPR domain, indicating a necessity for interactions with molecular chaperones, and the U box, which suggests that the U box is most likely the ''business end'' with respect to ubiquitylation. The means by which CHIP-dependent ubiquitylation occurs is not clear. In the case of ErbB2, ubiquitylation depends on a transfer of the client protein from Hsp90 to Hsp70 [120], indicating that the final ubiquitylation complex consists of CHIP, Hsp70 (but not Hsp90), and the client protein. In any event, the studies are consistent in supporting a role for CHIP as a key component of the chaperone-dependent quality-control mechanism. CHIP efficiently targets client proteins, particularly when they are partially unfolded (as is the case for most Hsp90 clients when bound to the chaperone) or frankly misfolded (as is the case for most proteins binding to Hsp70 through exposed hydrophobic residues).

Once the ubiquitylation activity of CHIP was recognized, it was logical to speculate that its U box might function in a manner analogous to that of RING fingers, which have recently been appreciated as key components of the largest family of ubiquitin ligases. If CHIP is a ubiquitin ligase, then its ability to ubiquitylate a substrate should be reconstituted *in vitro* when a substrate is added in the presence of CHIP, E1, an E2, and ubiquitin. Indeed, this is the case [135–137] (Figure 1.6). CHIP is thus the first described chaperone-associated E3 ligase. The ubiquitin ligase activity of CHIP depends on functional and physical interactions with a specific family of E2 enzymes, the Ubc4/Ubc5 family, which in humans comprises the E2s UbcH5a, UbcH5b, and UbcH5c. Of interest is the fact that the Ubc4/Ubc5 E2s

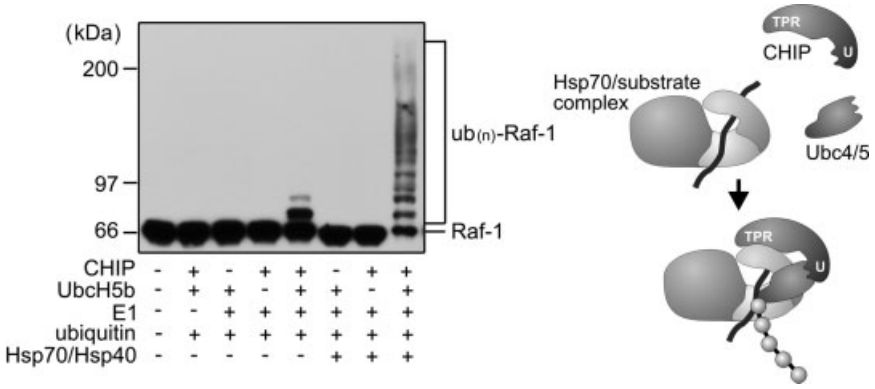

Fig. 1.6. Characterization of CHIP as a chaperone-associated ubiquitin ligase. Purified CHIP, UbcH5b, the ubiquitin-activating enzyme E1, ubiquitin, and the Hsp70–Hsp40 chaperone system were incubated with the bacterially expressed protein kinase Raf-1 (for details, see Ref. [137]). Raf-1 and ubiquitylated forms of the kinase ($ub_{(n)}$-Raf-1) were detected by immunoblotting using a specific anti-Raf-1 antibody. Efficient ubiquitylation of Raf-1 through the CHIP conjugation machinery depends on the recognition of the chaperone substrate by Hsp70, which presents the kinase to the conjugation machinery.

are stress-activated, ubiquitin-conjugating enzymes [123]. CHIP can therefore be seen as a co-chaperone that, in addition to inhibiting traditional chaperone activity, converts chaperone complexes into chaperone-dependent ubiquitin ligases. Indeed, the chaperones themselves seem to act as the main substrate-recognition components of these ubiquitin ligase complexes, as efficient ubiquitylation of chaperone substrates by CHIP depends on the presence of Hsp70 or Hsp90 in reconstituted systems [136, 137] (Figure 1.6). The chaperones apparently function in a manner analogous to F-box proteins, which are required as substrate recognition modules in many RING finger–containing ubiquitin ligase complexes [138–140].

Recently, another surprising function for CHIP has been identified, that of activation of the stress-responsive transcription factor heat shock factor-1 (HSF1) [130]. Through this association, CHIP regulates the expression of chaperones such as Hsp70 independently of its ability to modify their function through direct interactions. The mechanisms through which CHIP activates HSF1 are not entirely clear, but they are dependent in part on the induction of HSF1 trimerization, which is required for nuclear import and DNA binding. In addition, activation of HSF1 by CHIP seems to be independent of CHIP's ubiquitin ligase activity. The consequences of this activation are important for the response to stress, in that cells lacking CHIP are prone to stress-dependent apoptosis and mice deficient in CHIP (through homologous recombination) succumb rapidly to thermal challenge. These data indicate that CHIP plays a heretofore unsuspected role in coordinating the response to stress, not only by serving as a rate-limiting step in the degradation of damaged proteins but also by increasing the buffering capacity of the chaperone system to guard against stress-dependent proteotoxicity.

1.7 Other Proteins That May Influence the Balance Between Chaperone-assisted Folding and Degradation

CHIP is ideally suited to mediate chaperone–proteasome cooperation, as it combines a chaperone-binding motif and a domain that functions in ubiquitin-dependent degradation within its protein structure (Figure 1.2). Some other co-chaperones display a similar structural arrangement [40]. For example, BAG-1 contacts Hsp70 through a BAG-domain located at its carboxyl terminus and, in addition, possesses a central ubiquitin-like domain that is used for binding to the proteasome [141] (Figure 1.2). The co-chaperone thus belongs to a family of ubiquitin domain proteins (UDPs), many of which were shown to be associated with the proteasome [142]. This domain architecture enables BAG-1 to provide a physical link between Hsp70 and the proteasome, and elevating the cellular levels of BAG-1 results in a recruitment of the chaperone to the proteolytic complex. Notably, BAG-1 and CHIP occupy distinct domains on Hsp70 (Figure 1.7). Whereas BAG-1 associates with the amino-terminal ATPase domain, CHIP binds to the carboxyl-terminal EEVD motif of Hsp70 [35, 37]. Ternary complexes that comprise both co-chaperones associated with Hsp70 can be isolated from mammalian cells, suggesting a cooperation of BAG-1 and CHIP in the regulation of Hsp70 activity on certain degradation pathways. In fact, BAG-1 is able to stimulate the CHIP-induced degradation of the glucocorticoid hormone receptor [137]. A cooperation of diverse co-chaperones apparently provides additional levels of regulation to alter chaperone-assisted folding and degradation pathways.

Interestingly, BAG-1 and also Hsp70 and Hsp90 are themselves substrates of the CHIP ubiquitin ligase [135, 143] (J.H. unpublished). Yet, CHIP-mediated ubiquitylation of the chaperones and the co-chaperone does not induce their proteasomal degradation. Instead, it seems to provide additional means to regulate the association of the chaperone systems with the proteasome. In the case of BAG-1, ubiquitylation mediated by CHIP indeed stimulates the binding of the co-chaperone to the proteasome [143]. It remains to be elucidated, however, why Hsp70 and BAG-1 are not degraded when sorted to the proteasome through CHIP-induced ubiqui-

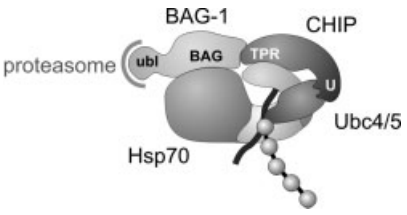

Fig. 1.7. Schematic presentation of the BAG-1–Hsp70–CHIP complex. BAG-1 associates with the ATPase domain of Hsp70, while CHIP is bound to the carboxyl terminus. BAG-1 mediates an association of Hsp70 with the proteasome via its ubiquitin-like domain (ubl), whereas CHIP acts in conjunction with Ubc4/5 as a chaperone-associated ubiquitin ligase to mediate the attachment of a polyubiquitin chain to the chaperone substrate.

tylation, in contrast to chaperone substrates such as the glucocorticoid hormone receptor. Possibly, the folded state of the proteins may serve to distinguish targeting factors and substrates doomed for degradation.

Efficient ubiquitylation of BAG-1 mediated by CHIP is dependent on the formation of the ternary BAG-1–Hsp70–CHIP complex [143]. The formed chaperone complex would thus expose multiple signals for sorting to the proteasome, e.g., the integrated ubiquitin-like domain of BAG-1 and polyubiquitin chains attached to BAG-1, Hsp70, and the bound protein substrate. Such a redundancy of sorting information might be considered unnecessary. Intriguingly, however, several subunits of the regulatory 19S particle of the proteasome are currently thought to act as receptors for polyubiquitin chains and integrated ubiquitin-like domains, including Rpn1, Rpn2, Rpt5, and Rpn10. The Rpn10 subunit was initially identified as a polyubiquitin chain receptor and was later shown to also bind integrated ubiquitin-like domains presented by UDPs [144–146]. Rpn10 possesses two distinct ubiquitin-binding domains, of which only one is used for UDP recognition [145–147]. However, conflicting data exist as to whether the subunit acts as a ubiquitin receptor in the context of the assembled 19S complex [148, 149]. More recently, Rpn1 was identified as a receptor for integrated ubiquitin-like domains [149], and a similar function may be fulfilled by the Rpn1-related subunit Rpn2 [150]. Polyubiquitin chains seem to be recognized by the Rpt5 subunit, one of the AAA ATPases present in the ring-like base of the regulatory 19S complex [151]. Its receptor function was revealed when tetraubiquitin was cross-linked to intact proteasomes [148]. Multiple docking sites for ubiquitin-like domains and polyubiquitin chains are apparently displayed by the regulatory particle of the proteasome. This may provide a structural basis for the recognition of multiple sorting signals exposed by the CHIP–chaperone complex (Figure 1.8). A similar mechanism involving multiple-site binding at the proteasome was recently proposed based on the observation that two unrelated yeast ubiquitin ligases associate with specific subunits of the 19S regulatory complex [152]. In these cases substrate delivery involves interactions of proteasomal subunits with the substrate-bound ubiquitin ligase, with the polyubiquitin chain attached to the substrate, and with the substrate itself. Multiple-site binding may function to slow down dissociation of the substrate from the proteasome and to facilitate transfer into the central proteolytic chamber through ATP-dependent movements of the subunits of the 19S particle.

Human cells contain several BAG-1-related proteins: BAG-2, BAG-3 (CAIR-1; Bis), BAG-4 (SODD), BAG-5, and BAG-6 (Scythe, BAT3) [153] (Figure 1.2). It appears that BAG proteins act as nucleotide-exchange factors to induce substrate unloading from Hsp70 on diverse protein folding, assembly, and degradation pathways. Notably, BAG-6 is another likely candidate for a co-chaperone that regulates protein degradation via the ubiquitin–proteasome pathway. Similar to BAG-1, BAG-6 contains a ubiquitin-like domain that is possibly used for proteasome binding [154]. However, experimental data verifying a role of BAG-6 in protein degradation remain elusive so far.

The cooperation of diverse co-chaperones not only may allow promotion of chaperone-associated degradation but also may provide the means to confine the

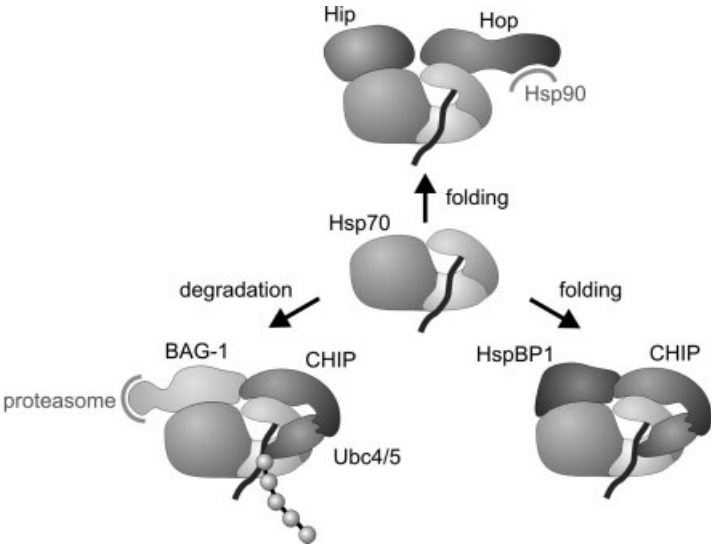

Fig. 1.8. The co-chaperone network that determines folding and degradation activities of Hsp70. BAG-1 and CHIP associate with Hsp70 to induce the proteasomal degradation of a Hsp70-bound protein substrate. When BAG-1 is displaced by binding of HspBP1 to the ATPase domain of Hsp70, the ubiquitin ligase activity of CHIP is attenuated in the formed complex, enabling CHIP to modulate Hsp70 activity without inducing degradation. The ATPase domain can also be occupied by Hip, which stimulates the chaperone activity of Hsp70 and participates in the Hsp70/Hsp90-mediated regulation of signal transduction pathways. At the same time, Hop displaces CHIP from the carboxyl terminus of Hsp70 and recruits Hsp90 to the chaperone complex.

destructive activity of CHIP. The Hsp70-binding protein 1 (HspBP1) seems to fulfill such a regulatory function [155]. HspBP1 was initially identified in a screen for proteins that associate with the ATPase domain of Hsp70 and was shown to stimulate nucleotide release from the chaperone [156, 157]. Notably, association of HspBP1 with the ATPase domain blocks binding of BAG-1 to Hsp70 and at the same time promotes an interaction of CHIP with Hsp70's carboxyl terminus. In the formed ternary HspBP1–Hsp70–CHIP complex, the ubiquitin ligase activity of CHIP is attenuated and Hsp70 as well as a chaperone substrate are no longer efficiently ubiquitylated [155]. By interfering with CHIP-mediated ubiquitylation, HspBP1 stimulates the maturation of CFTR and promotes the sorting of the membrane protein to the cell surface. HspBP1 apparently functions as an antagonist of the CHIP ubiquitin ligase to regulate Hsp70-assisted folding and degradation pathways (Figure 1.8).

The HspBP1-mediated inhibition of the ubiquitin ligase activity may enable CHIP to modulate the Hsp70 ATPase cycle without inducing degradation. In fact, degradation-independent functions of CHIP have recently emerged [130, 133, 158, 159]. CHIP was shown to regulate the chaperone-assisted folding and sorting of

the androgen receptor and of endothelial nitric oxide synthase without inducing degradation [158, 159]. Moreover, CHIP fulfills an essential role in the chaperone-mediated regulation of the heat shock transcription factor, independent of its degradation-inducing activity [130]. It remains to be seen, however, whether HspBP1 cooperates with CHIP in these instances, as HspBP1 displayed a certain specificity with regard to chaperone substrates. The co-chaperone interfered with the degradation of CFTR, but did not influence the CHIP-mediated turnover of the glucocorticoid hormone receptor. Such a client specificity may arise in part from the fact that HspBP1 inhibits the ubiquitin ligase activity of CHIP in a complex with Hsc70, but leaves Hsp90-associated ubiquitylation unaffected [155]. In addition, direct interactions between HspBP1 and a subset of chaperone substrates may contribute to substrate selection. In any case, the cooperation of CHIP with other co-chaperones apparently provides a means to regulate chaperone-assisted protein degradation.

It is likely that there are multiple degradation pathways for misfolded proteins in the eukaryotic cytoplasm. Although CHIP participates in the degradation of chaperone substrates induced by applying Hsp90 inhibitors to cell cultures (see above), drug-induced degradation is not abrogated in cells that lack the CHIP ubiquitin ligase [120]. Furthermore, CHIP cooperates with Hsp70 in the ER-associated degradation of CFTR, but the Hsp70-assisted degradation of apoB at the cytoplasmic face of the ER membrane does not involve CHIP [97]. Taken together, these data strongly argue for the existence of other, yet to be identified, ubiquitin ligases that are able to target chaperone substrates to the proteasome. A likely candidate in this regard is Parkin, a RING finger ubiquitin ligase, whose activity is impaired in juvenile forms of Parkinson's disease [17]. Hsp70 and CHIP were found to be associated with Parkin in neuronal cells, suggesting an involvement of Parkin in the proteasomal degradation of chaperone substrates [160]. Interestingly, α-synuclein, the main component of protein deposits observed in dopaminergic neurons of Parkinson patients, and synphilin, a protein that binds α-synuclein and induces deposit formation, both associate with yet other ubiquitin ligases: Siah-1 and Siah-2 [161, 162]. In the case of Siah-1, a link to cytoplasmic chaperone systems is suggested by the finding that the Hsp70 co-chaperone BAG-1 is a binding partner of the ubiquitin ligase and suppresses some of the cellular activities of Siah-1 [163]. Taken together, it is tempting to speculate about a role of Parkin and Siah on chaperone-assisted degradation pathways; yet, this remains to be explored in detail.

1.8 Further Considerations

Although the appreciation of interplay between molecular chaperones and ubiquitin-dependent proteolysis has greatly expanded over the past decade, a number of critical issues remain to be resolved. It is not entirely clear what determines whether a misfolded protein will undergo repeated attempts at misfolding versus

diversion to the ubiquitin–proteasome pathway. Recruitment of CHIP into chaperone complexes appears to be a critical component of this reaction, which therefore begs the question as to what regulates this step. Since this step in protein quality control must be both rapidly activated and easily reversible, it is likely that regulation occurs at the post-translational level rather than through changes in steady-state protein levels. The precise sorting mechanisms for ubiquitinated proteins are also unclear. BAG-1 is a player, and it is also likely that overlap exists to some extent for sorting of the cytoplasmic and endoplasmic reticulum quality-control pathways. Nevertheless, much remains to be learned about these steps.

From a broader perspective, it is now also imperative to understand the pathophysiological roles of cytoplasmic quality-control mechanisms regulated by chaperone–proteasome interactions. As mentioned previously, there is a strong association between chaperone dysfunction and accumulations of misfolded proteins that characterizes genetic neurodegenerative diseases. An imbalance between protein folding and degradation may also contribute to some features of senescence and organismal aging. The link between chaperone systems and aging is based on increasing appreciation that modified, misfolded, and aggregated proteins accumulate with age [164]. Dysregulation of chaperone expression has been observed with aging and is therefore implicated in aging-related changes [165]; in general, it is accepted that induction of the major chaperones is impaired with aging, a fact confirmed by recent gene-profiling experiments *in vivo* [166], although given the diversity of chaperones it is probably not surprising that age-related changes in expression are fairly complicated [167]. The mechanism underlying this dysregulation is not entirely clear, but seems to be due in part to impaired activation of the stress-responsive transcription factor HSF1. Overexpression of heat shock proteins in yeast, *C. elegans*, and *Drosophila* leads to increased longevity [168–170]. More recently, conclusive genetic evidence from *C. elegans* indicates that mutation of HSF1 causes a dramatic and significant reduction in lifespan [170, 171], further implicating the accumulation of misfolded proteins in age-related phenotypes.

1.9 Conclusions

The associations between molecular chaperones and the ubiquitin–proteasome system represent a critical step in the response to proteotoxic damage. Whether attempts should be made to refold damaged proteins (thus conserving cellular resources) or degrade them instead (to prevent the possibility of protein aggregation and concomitant toxicity) requires a consideration of cellular economy. Defects in the quality-control mechanisms may have enormous consequences even if only slight imbalances occur between protein folding and degradation, as these imbalances can cause accumulated toxicity over time. The relationship between chaperone–proteasome interactions and pathophysiological events is only now being unraveled. Modulation of this system may provide a unique therapeutic target for degenerative diseases and pathologies associated with aging.

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2
Molecular Dissection of Autophagy in the Yeast *Saccharomyces cerevisiae*

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2.1
Introduction

More than half a century has passed since C. de Duve discovered lysosomes using cell fractionation procedures [1]. At that time, intracellular bulk protein degradation was believed to occur mostly within this organelle. Eukaryotic cells must elaborate a strategy to segregate dangerous lytic enzymes from biosynthetic sites and cytosol and to restrict the degradative process to a membrane-bound compartment. The process of degradation of cytoplasmic components in lysosomes is called autophagy, in contrast to heterophagy, which is the degradation of extracellular materials through endocytosis. Electron microscopic studies on lysosomes revealed macroautophagy (hereafter referred to as autophagy) as a major route to deliver the cytoplasmic components to the lytic compartment. The first step of autophagy is sequestration of a portion of the cytoplasm or organelle by a membrane sac, the so-called isolation membrane, resulting a double-membrane structure called the autophagosome. Then the autophagosome fuses with the lysosome, gains lytic enzymes, and turns into an autophagolysosome. Lysosomal enzymes disintegrate the inner membrane of the autophagosome and digest its contents. Digestion products are transported back to the cytosol and reutilized for a new round of protein synthesis.

Autophagy is involved in nonselective and bulk degradation of cellular proteins, while the ubiquitin–proteasome system is responsible for the highly selective degradation of short-lived proteins. Since more than 90% of cellular proteins have long lifetimes, the turnover of long-lived proteins is important to the understanding of cell physiology.

Until recently, autophagy in mammals had been studied mostly using electron microscopy by detecting autophagosomes and autophagolysosomes. Since the lysosomal system consists of very dynamic and complicated membrane structures, it was not easy to analyze lysosomes and their related membrane structures biochemically. Many efforts to detect specific proteins on the autophagosome failed, and genes required for autophagy had not been identified.

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In this chapter, I will focus on the recent progress in the molecular dissection of autophagy in the yeast *Saccharomyces cerevisiae* and its relevance in understanding autophagic protein degradation in higher eukaryotes.

2.2 Vacuoles as a Lytic Compartment in Yeast

The vacuole is the most prominent organelle and is easily visible under light microscopy in the budding yeast *S. cerevisiae*. The inside of the vacuole is kept acidic by a V-type proton-translocating ATPase on the vacuolar membrane. The vacuole plays crucial roles in homeostasis of cellular ions and functions as a reservoir of various metabolites such as amino acids. It contains hydrolytic enzymes, proteinases, peptidases, nucleases, phosphatases, mannosidases, and so on. The vacuolar enzymes and their biogenesis have been intensively studied genetically and biochemically. From these facts the vacuole was postulated to function as a lytic compartment like lysosomes in mammalian cells. Actually, it was reported that bulk protein turnover is induced upon nitrogen starvation, which is dependent upon vacuolar enzyme activities [2, 3], suggesting that the vacuole is responsible for bulk protein degradation. Obvious questions were what kind of intracellular proteins are degraded and how they become accessible to the vacuolar enzymes.

2.3 Discovery of Autophagy in Yeast

In 1988, I started studies on the lytic function of yeast vacuoles and found by light microscopy that the yeast cell induces autophagy under nitrogen-starvation conditions. When vacuolar proteinase-deficient mutants grown in a rich medium were shifted to a nitrogen-deprived medium, spherical structures appeared in the vacuole after a short lag, gradually increased in number, and finally filled the vacuole after 10 hours [4]. These structures, called autophagic bodies, were mostly single membrane-bound, occasionally multilamellar structures containing a portion of cytoplasm [4]. Autophagic bodies contained ribosomes and occasionally various other cellular structures including mitochondria and rER [4]. Subsequently, double-membrane structures of a size equivalent to autophagic bodies, autophagosomes, were found in the cytoplasm of the starved cells. Fusion images between the outer membrane of the autophagosome and the vacuolar membrane were obtained by rapid freezing and freeze substitution, as well as by freeze-fracture electron microscopy [5, 6]. The autophagic body is the final membrane structure of autophagy in yeast, which is derived from the inner membrane of the autophagosome. Autophagic bodies are about 300–900 nm in diameter, about 500 nm on average, and deliver about 0.2% of the cytoplasm via one autophagosome. The rate of

autophagic protein degradation was estimated at 2–3% total cellular protein per hour. Autophagy in a haploid strain linearly proceeds for up to eight hours, gradually slows down, and reaches a plateau at around 20–30% of degradation [7]. Therefore, there must be negative regulation, but its details are not known yet.

Later, we realized that exactly similar membrane phenomena were induced under carbon, sulfate, phosphate, and single auxotrophic amino acid starvation. These observations strongly indicate that yeast cells take up a portion of cytoplasm to the lytic compartment via autophagosomes in conditions adverse for growth. The membrane dynamics of yeast autophagy is topologically the same as macroautophagy in mammals, though the vacuole is much larger than the lysosome. A schematic drawing of autophagy in yeast is shown in Figure 2.1.

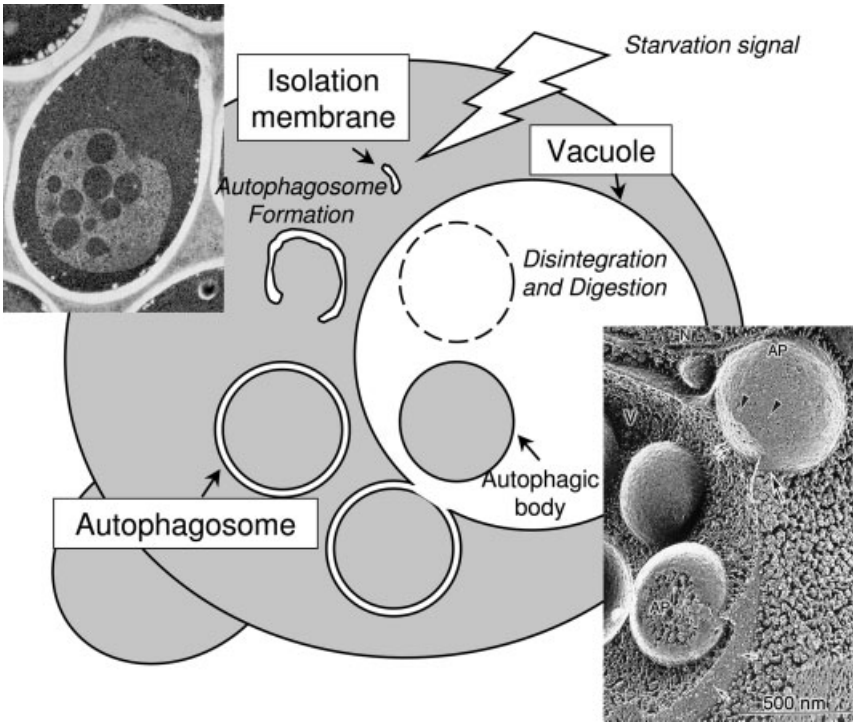

Fig. 2.1. Scheme of autophagy in the yeast *Sac-charomyces cerevisiae*. When yeast cells face various nutrient deficiencies, the isolation membrane encloses a portion of the cytosol and forms a double-membrane structure: the autophagosome (AP). Autophagosomes immediately fuse with the vacuole, and an inner-membrane structure, the autophagic body (AB), is released into the vacuolar lumen. In wild-type cells autophagic bodies are degraded by vacuolar enzymes, but as shown in EM (top left) autophagic bodies are accumulated in vacuolar proteinase-deficient cells. The freeze-fracture image (bottom right) clearly shows fusion of the autophagosome and unique characteristics of the autophagic body.

2.4 Genetic Dissection of Autophagy

To elucidate the molecular mechanism of autophagy, we applied a genetic approach. The most characteristic feature of yeast autophagy is that we are able to monitor the progress of autophagy under the light microscope as the accumulation of autophagic bodies. Taking advantage of this simple technique, we attempted to obtain autophagy-defective mutants. As the first approach, only the morphological changes of the vacuole under starvation were used to screen for mutants. Cells that failed to accumulate autophagic bodies during nitrogen starvation in *pep4* background, deficient of vacuolar enzymes, were selected under light microscopy, and only one mutant, *apg1*, was selected [8]. The *apg1* mutant did not induce bulk protein degradation under starvation, and homozygous *apg1*/*apg1* diploid cells did not sporulate. This mutant grew normally in a rich medium but could not maintain viability under long nitrogen starvation. To obtain further mutants due to defects in autophagy, the loss of viability under starvation was used for the first screen followed by the morphological examination of vacuoles. In this way about 100 autophagy-defective mutants were isolated and divided into 14 groups (*apg2*–*apg15*) by complementation analysis. Another approach taken by Thumm and coworkers was the immunoscreening of cells that retain a cytosolic enzyme, fatty acid synthase, after starvation [9]. By these methods six *aut* mutants were originally obtained. Later, two hybrid screens using Apg proteins as bait identified two more *APG* genes [10, 11]. Klionsky's group isolated mutants defective in maturation of aminopeptidase I (API), one of the vacuolar enzymes. API is first synthesized as a proform in the cytosol and then transported to the vacuole and processed to an active form. Most other vacuolar enzymes are incorporated into the ER lumen and transported to the vacuole through the secretory pathway. Transport of API to the vacuole is mediated by the Cvt (cytoplasm-to-vacuole targeting) pathway [12]. The defective mutants in the Cvt pathway, *cvt*, significantly overlapped with autophagy-defective *apg* and *aut* mutants [7, 12], though the two pathways are apparently different; one is degradative and starvation-induced, and the other is biosynthetic and constitutively active. EM analyses of the Cvt pathway clearly showed that the Cvt pathway is mediated by membrane dynamics that is quite similar to that of autophagy [13, 14]. Small double-membrane structures (the Cvt vesicles) specifically enclose an aggregate of API (Cvt complex) and fuse with the vacuolar membrane, releasing small vesicles into the vacuolar lumen.

Later, many groups isolated autophagy-related genes in *S. cerevisiae* and other yeast species and named them differently. To avoid confusion, recently all groups involved agreed to use a novel nomenclature for the autophagy-related gene: *ATG*. The original *APGx* is now renamed as *ATGx* [15]. The genes presently known to be involved in autophagy are shown in Table 2.1.

Table 2.1. Nomenclature of autophagy-related genes and functions of the Apg, Aut, Cvt, and Gsa proteins and mammalian homologues.

Atg	*Apg*	*Aut*	*Cvt*	*Gsa*	*Mammalian*	*Function/localization*
Atg1	Apg1	Aut3	Cvt10	Gsa10	ULK1	Protein kinase, localizes to the PAS
Atg2	Apg2	Aut8		Gsa11	Apg2	Localizes to the PAS
Atg3	Apg3	Aut1		Gsa20	Apg3	Apg8-conjugating enzyme (E2)
Atg4	Apg4	Aut2			Apg4A, Apg4B	Cysteine protease for processing the C-terminus of Apg8
Atg5	Apg5				Apg5	Substrate of Apg12-conjugating reaction, localizes to the PAS
Atg6	Apg6				Beclin-1	Subunit of the PI3-kinase complex, involved in protein sorting to the vacuole as Vps30
Atg7	Apg7		Cvt2	Gsa7	Apg7	Activating enzyme (E1) of Apg8 and Apg12
Atg8	Apg8	Aut7	Cvt5		LC3, GATE16, GABARAP	Ubiquitin-like protein, conjugates with PE, localizes to the PAS and autophagosomes
Atg9	Apg9	Aut9	Cvt7	Gsa14	Apg9?	Transmembrane protein, required for PAS formation
Atg10	Apg10				Apg10	Apg12-conjugating enzyme (E2)
Atg12	Apg12				Apg12	Ubiquitin-like protein, conjugates with Apg5
Atg13	Apg13				?	Subunit of Apg1 kinase, phosphorylated under growing conditions
Atg14	Apg14		Cvt12		?	Subunit of the autophagy-specific PI3-kinase complex
Atg16	Apg16		Cvt11		Apg16L	Binds with Apg12–Apg5 and forms tetramer, required for Apg12–Apg5 recruitment to the PAS, localizes to the PAS
Atg17	Apg17				?	Member of the Apg1 complex, not required for the Cvt pathway
Atg18		Aut10	Cvt18	Gsa12		WD-repeat protein
Atg22		Aut4				Disintegration of autophagic bodies in the vacuole
Atg15		Aut5	Cvt17			Disintegration of autophagic bodies in the vacuole, putative lipase
Atg11			Cvt9	Gsa9		Required only for the Cvt pathway, localizes to the PAS
Atg19			Cvt19			Receptor of aminopeptidase I for the Cvt vesicle, localizes to the PAS
Atg20			Cvt20			Binds to PI3P, required for the Cvt pathway
Atg21			Mai1			Required for the Cvt pathway but not for macroautophagy

PAS: pre-autophagosomal structure

2.5
Characterization of Autophagy-defective Mutants

Autophagy-defective mutants had been isolated as non-conditional mutants, and we now know that almost all of the original *apg* mutants are null-type mutants. They failed to induce bulk protein degradation under various nutrient-depleted conditions, indicating that autophagy is the major pathway of bulk protein degradation. All *apg* and most *aut* mutants grow normally in a nutrient-rich medium, indicating that autophagy is not essential for vegetative growth in yeast. They showed no significant differences in stress responses against heat, osmotic, and salt stress. Several vacuolar functions tested in these autophagy-defective mutants, including secretion and endocytosis, were almost the same as in wild-type cells. One of characteristic features of autophagy-defective mutants is the loss of viability during nitrogen starvation, which was used as screening marker. Autophagy-defective mutants start to die after two days of starvation and almost completely lose viability after one week [8]. Under starvation conditions the cell needs to synthesize essential proteins to adapt to the conditions; consequently, the supply of amino acids by degradation is essential. In nature, yeast cells must face various forms of nutrient starvation; therefore, autophagy-defective mutants may not survive.

Homozygous diploids with any *apg* mutation have been shown not to sporulate [8]. This cell-differentiation process triggered by nitrogen starvation must require bulk protein degradation via autophagy in order to remodel the intracellular structures. Degradation of preexisting proteins by autophagy must be critical for cell survival.

2.6
Cloning of *ATG* Genes

Recently, we finished cloning all of the original *APG* genes. Most genes were cloned from a chromosomal DNA library by complementation of the loss-of-viability phenotype of *apg* mutants by replica plating on agar medium containing phloxine B, which stains dead cells red, then confirmed by the accumulation of autophagic bodies by light microscopy. Some genes were obtained by complementation of sporulation-negative phenotypes as the first step of the screen. The first *ATG* gene cloned, *ATG1*, turned out to encode a Ser/Thr protein kinase [16]. However, since then almost all *ATG* genes have been unidentified genes with unpredictable functions from their sequence data. Autophagy genes had been neglected because they exhibit specific phenotypes only under starvation conditions. Recent systematic analyses of protein interactions by yeast two-hybrid screens or binding assays also clearly indicate that Atg proteins interact with each other but compose an isolated group of proteins.

Autophagy genes turn out to be mostly novel genes, except for *ATG6*, which is required for the vacuolar protein-sorting (Vps) pathway [17]. In yeast, autophagy

is almost completely shut off under growing conditions and is strictly induced by starvation, but every *ATG* gene is rather constitutively expressed in the growing conditions. Systematic gene expression analyses suggested that several *ATG* genes are transcriptionally upregulated. However, the protein level of most Atg proteins is not dramatically changed by nutrient conditions. It is unclear whether transcriptional regulation plays an important role in the regulation of autophagy in yeast or not.

2.7 Further Genes Required for Autophagy

Screens for autophagy-defective mutants, like the original *apg* mutants, seem to be nearly finished. However, because of the strategies of screens, mutants with aberrant vacuole morphology, partially defective mutants, and mutants of genes shared with other essential functions were eliminated. It has now become obvious that normal levels of autophagy require more genes of known function as well as unknown genes. Most Gcn proteins appear to be necessary for normal autophagy. Several early *SEC* genes such as *SEC12* an *SEC24* are known to be necessary for autophagy [18, 19]. Several mutants such as *vps35*/*vam5* and *ypt7* show accumulation of autophagosomes in the cytoplasm under starvation conditions. These mutant cells contain fragmented vacuoles, suggesting that the fusion machinery of the autophagosome with the vacuole shares SNARE molecules similar to other vacuolar homotypic fusion events [18]. In wild-type cells autophagic bodies effectively disappear within one minute. Atg15/Cut5/Cvt17 and Aut4 are involved in this process [20, 21]. Atg15 contains a putative lipase domain, but lipase activity has not been proved yet. Acidification of the vacuole is a requisite for effective digestion of autophagic bodies, since defects in every subunit of the type-ATPase (Vma) cause an accumulation of autophagic bodies in the vacuole [22]. It is still a mystery why the autophagic body membrane disintegrates so quickly in the vacuoles.

2.8 Selectivity of Proteins Degraded

One of the unresolved problems of autophagy is substrate selectivity for sequestration into autophagosomes. Generally, autophagic protein degradation is believed to be nonselective. We showed that isolated vacuoles containing autophagic bodies exhibit similar rates of sequestration of the cytosolic enzymes ADH, PGK, PK, and GluDH. The density of ribosomes is almost the same in autophagic bodies as in the cytoplasm. Immunoelectron microscopy showed the same signal intensities of ADH or PGK among cytosol, autophagosomes, and autophagic bodies [5]. This suggests that sequestration by autophagosomes is a nonselective process at least for these cytosolic energy-metabolism enzymes.

One biosynthetic pathway for vacuolar proteins, the Cvt pathway, has been studied intensively. Aminopeptidase I (Lap4) and α-mannosidase (Ams1) are delivered

to the vacuoles via the Cvt pathway, which utilizes all the original *APG* genes. Furthermore, under starvation conditions API and α-mannosidase are selectively sequestered into autophagosomes and delivered to the vacuoles. This selective uptake of API to the Cvt vesicle or autophagosome requires the specific factors Atg11/Cvt9 and Atg19/Cvt19, which may specify the recruitment of the cargo to the vesicles. These facts evoke the possibility that autophagy may involve selective transport of certain proteins to be degraded. Recently, we realized that the cytosolic acetaldehyde dehydrogenase Ald6 is preferentially degraded in the vacuoles via autophagy [23]. The mechanism governing this preferential sequestration into the autophagosome is an interesting problem to be unveiled in the near future.

Glycogen granules, the synthesis of which is induced by nitrogen starvation in the presence of glucose, are mostly excluded from the autophagosomes. It is possible that there is a mechanism for excluding glycogen granules from autophagosomes, but an alternative explanation is that the site of autophagosome formation results in this unevenness, since glycogen granules mostly locate to peripheral regions of the cytosol while autophagosomes form next to the vacuole.

We have occasionally detected mitochondria in autophagic bodies, and we can easily estimate the number of mitochondria taken up in the vacuole by counting mitochondrial DNA in the autophagic bodies with fluorescence microscopy after DAPI staining [4]. Under starvation, a significant proportion of the mitochondria are transported to the vacuoles. Rough ER is also frequently detected in the vacuole [4]. Autophagy may provide the most effective system to degrade whole organelles. Therefore, it has been proposed that autophagy regulates the quantity of organelles and is even involved in their quality control. In *S. cerevisiae* it is hard to conclude whether or not organelle degradation has some selectivity.

In *Pichia pastoris*, cells grown in methanol medium develop large numbers of peroxisomes. When the medium is switched to ethanol or glucose, peroxisomes are selectively degraded via microautophagy and macroautophagy, respectively [24]. Microautophagy is the process of direct wrapping of peroxisomes by the vacuolar membrane. These two pathways seem to be quite different membrane phenomena; however, both require many Atg proteins [25, 26]. Recently, it was shown that micropexophagy (degradation of peroxisomes) is not simply the process of invagination of vacuolar membranes but requires formation of a novel membrane, called MIPA [27]. It is known that several genes are required for specific peroxisome degradation by autophagy. These may confer the molecular mechanism of selective sequestration of this organelle.

2.9 Induction of Autophagy

Under growing conditions, the extent of autophagy is negligibly small. Cells growing in a rich medium are adapted to rapid cell proliferation. High cAMP levels block autophagy, and activated A-kinase mutants do not induce autophagy [28], indicating that autophagy is regulated in an opposite manner to cell growth. Autoph-

agy is induced not only by nitrogen starvation but also by other nutrient starvation, including carbon, sulfate, phosphate, and amino acids [4]. So far, there is no mutant identifying a specific starvation signal. Autophagy is a rather general physiological response to nutrient limitation and may be under the control of several general factors.

However, when rapamycin, a specific inhibitor of Tor kinase, is added to the growing cells in a nutrient-rich medium, cells behave as if they were in a starvation medium and autophagy is induced [28]. Thus, Tor kinase negatively regulates autophagy during growing conditions and may be a master regulator. At present, regulation of Tor and downstream events toward autophagy is not fully understood.

2.10
Membrane Dynamics During Autophagy

The most critical step of autophagy is formation of a new compartment in order to sequester a portion of the cytoplasm to be degraded. For a long time the origin of the autophagosome membrane was proposed to be the ER. We also showed that membrane flow from the ER is necessary for autophagy in yeast [29]. Another group reported that post-Golgi transport is involved in its formation. The autophagosomal membrane has a distinct morphology: it is thinner than any other organelle membranes, and the outer and inner membranes stick together with almost no lumenal space [4, 6]. In freeze-fracture images the inner membrane – the autophagic body membrane – completely lacks intramembrane particles, while the outer membrane contains sparse but significant particles [6] that may participate in targeting and fusion of the autophagosome to the vacuole. This indicates that inner and outer membranes, which should be derived from the same isolation membrane, are somehow differentiated and specialized for delivery of cytoplasmic constituents to the lytic compartment. So far, nobody has shown that membrane vesicles are involved in the membrane elongation step of the isolation membrane. Membrane dynamics during autophagy may be quite different from classical vesicular trafficking events. We proposed that autophagosome formation is not a simple enwrapping process by a preexisting large membrane structure such as the ER, but rather assembly of a new membrane from its constituents.

By electron microscopy we could occasionally detect a cup-shaped intermediate membrane structure [5]. The most important outstanding questions are how the isolation membrane is organized, what influences the morphogenesis of this isolation membrane, and how the isolation membrane seals to form a closed autophagosome.

2.11
Monitoring Methods of Autophagy in the Yeast *S. cerevisiae*

It is not easy to precisely estimate protein degradation through autophagy, since measuring the decrease of bulk protein is technically difficult. Isotope-labeling

methods are often used to estimate bulk protein degradation in yeast and mammals. Radioactive valine, leucine, or methionine and cysteine released from prelabeled long-lived proteins are measured. Reutilization of amino acids during starvation may cause underestimation. 3-Methyladenine sensitivity is used for autophagy-dependent degradation. However, in a strict sense it is not clear that 3-methyadenine is a specific inhibitor of autophagy in mammals.

In yeast the vacuole is easily visible under light microscopy. Accumulation of autophagic bodies in the presence of PMSF, a serine protease inhibitor, or in *prb1* or *pep4* strains is the simplest indication of autophagy [4]. In some strains, the accumulation of autophagic bodies is not homogenous among cells, and the number of autophagic bodies is uncountable because of their vigorous Brownian motion: quantitative measurement of autophagy is not straightforward.

Under starvation, aminopeptidase I (Lap4p, API) is mainly sequestered into the autophagosome, delivered to the vacuoles, and processed by the vacuolar enzymes to a mature form [14]. Therefore, the processed form of API during starvation reflects autophagic transport. However, the Cvt complex is transported to the vacuole at once by a single autophagosome, and API maturation is indicative of but not proportional to the extent of autophagy.

The Atg8 protein is entrapped in autophagosomes and delivered to the vacuole via autophagic bodies [30]. When cells expressing GFP-Atg8 cells are shifted to a starvation medium, GFP-Atg8 stains the vacuolar lumen in wild-type cells but not in autophagy-defective mutants, since GFP is fairly resistant to vacuolar enzymes [31]. Therefore, the intensity of fluorescence of GFP in the vacuolar lumen is a good indicator of autophagy. Nobody has analyzed the fluorescence intensity quantitatively.

We have developed a monitoring system for autophagy by genetic manipulation. Alkaline phosphatase (Pho8) is a vacuolar membrane protein with a small N-terminal cytoplasmic tail. We constructed a truncated form of Pho8 lacking a membrane-spanning region at the N-terminal end (Pho8Δ60) that is expressed under the control of a strong constitutive promoter. This proform of Pho8Δ60 is distributed in the cytosol, but under starvation a portion of it is delivered to the vacuoles via autophagy and becomes active in the vacuoles. Since Pho8 is a vacuolar resident protein, it stays stable without further degradation. This assay provides the most reliable estimation of autophagic degradation [32].

2.12 Function of Atg Proteins

As mentioned earlier, the Atg proteins turned out to be mostly novel proteins, but further analyses have revealed that the Atg proteins may be classified into four functional groups: the Atg1 protein kinase complex, the autophagy-specific PI3 kinase complex, the Atg12 protein conjugation system, and the Atg8 lipidation system (Figure 2.2). One of the most remarkable findings is the discovery of two

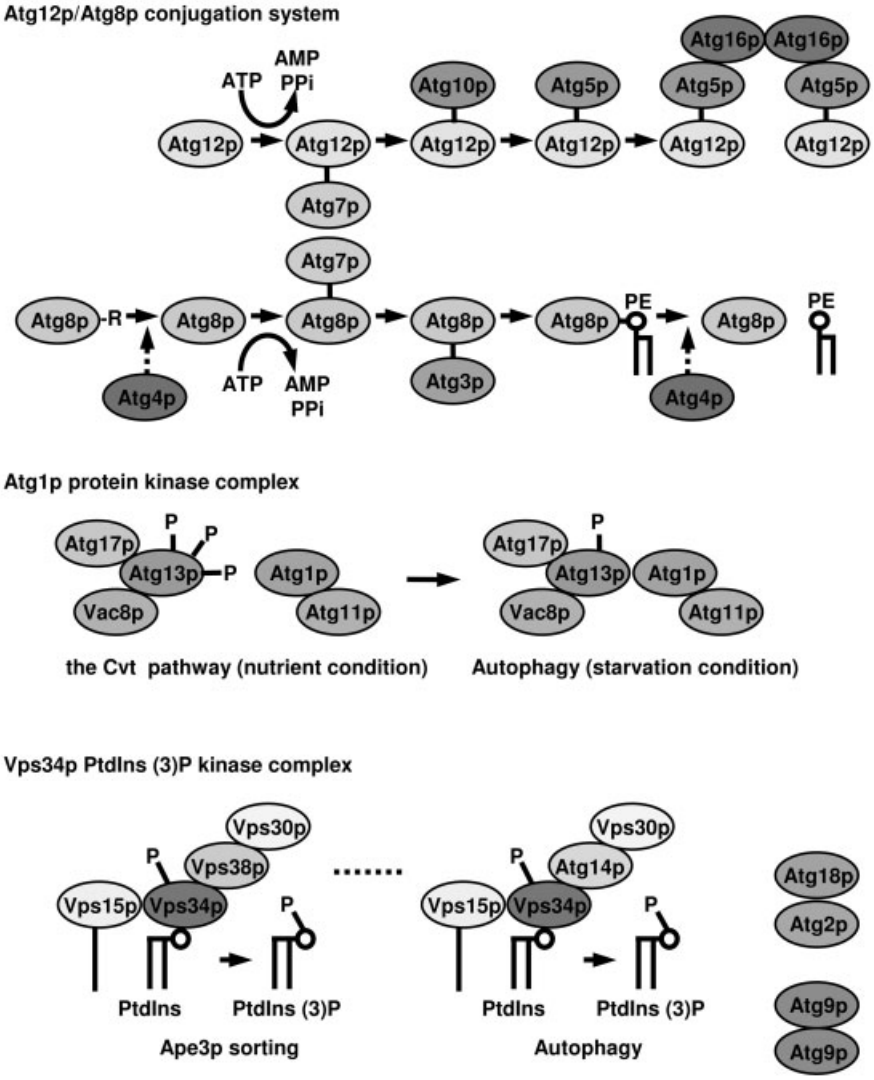

Fig. 2.2. Atg proteins necessary for autophagosome formation. A total of 16 Atg proteins are required for autophagosome formation. The proteins are found in four complexes.

ubiquitin-like conjugation systems for Atg proteins [33]. Half of the original *APG* genes are involved in these novel conjugation systems.

2.12.1 The Atg12 Protein Conjugation System

Atg12 is a small hydrophilic protein of 186 amino acids with no apparent homology to ubiquitin. Western blot analysis of N-terminally HA-tagged Atg12 showed one extra band of about 65 kDa in addition to a band of expected mass of the fusion protein. This high-molecular-mass band did not appear in *atg5*, *atg7*, and *atg10* mutants. HA-tagged Atg5 also showed two bands, and the high-molecular-mass band corresponded exactly to the upper band of Atg12, suggesting a covalent link between Atg12 and Atg5. The C-terminal glycine residue of Atg12 was essential for the Atg12–Atg5 conjugate and also autophagy. Changing the 19 lysine residues of Atg5 to arginine revealed that a lysine residue is the acceptor site of Atg12 [34]. Thus, we concluded that the C-terminal glycine at 149 residue of Atg12 forms an isopeptide bond with the ε-amino group of a lysine residue of Atg5. This conjugate formation was essential for autophagy and was mediated by consecutive reactions such as ubiquitination. The C-terminal glycine residue is first activated by an activating enzyme, Atg7 (E1), and then transferred to a conjugating enzyme, Atg10 (E2), through forming thioester conjugates [35, 36]. Atg7 exhibits a weak but significant homology with E1 enzymes of the ubiquitin system, but Atg10 is a unique E2 enzyme without any homology to known E2 enzymes. Finally, Atg12 is transferred to Atg5 through an isopeptide bond.

The Atg12 conjugation reaction is similar to ubiquitination but has distinct features. Atg12 is synthesized as an active form with a single glycine at the C-terminus, unlike other ubiquitin-like proteins (UBLs), which have C-terminal extensions after double glycine. Atg12 in yeast is much larger than ubiquitin and has no apparent sequence similarity with ubiquitin. However, its secondary structure is predicted to contain a ubiquitin-like domain at the C-terminal region. N-terminal truncation of Atg12 demonstrated that a C-terminal 80-amino-acid fragment is necessary and sufficient for conjugation and also for autophagy, indicating that Atg12 is really a UBL. Atg5 is the only target molecule of the Atg12 modification. Atg12 and Atg5 form a conjugate immediately after synthesis, and free forms of the proteins are hardly detectable in cell lysates. So far, no protease activity to cleave the Atg12–Atg5 linkage has been found, suggesting that this conjugation reaction is irreversible. Conjugate formation is not affected by autophagy-inducing starvation conditions. The Atg12–Atg5 conjugate behaves just like a single polypeptide and functions as part of the machinery of autophagosome formation.

The Atg12–Atg5 conjugate further forms a protein complex with Atg16. Although Atg16 was originally isolated by a two-hybrid screen using Atg12 as bait, it does bind to Atg5 at the N-terminal region and does not bind directly to Atg12 [10]. Atg16 has a coiled-coil region in its C-terminal half and forms an oligomer through this region. Atg12–Atg15 · Atg16 likely forms a tetrameric complex of 350 kDa, which is the functional form essential for autophagy [37]. This large protein

complex is stable and is not affected by nutrient conditions. The minimum essential amount of the Atg12–Atg5 · Atg16 complex in the cell may be small, since several mutations in Atg12 or Atg16 that severely reduce the amount of the complex are still nearly normal in autophagy.

2.12.2 The Atg8 System

The *ATG8* gene encodes a small basic protein of 117 amino acids. Atg8 has many homologues in eukaryotes and is part of a large protein family. By immunostaining, Atg8 was shown to be localized to the autophagosomal and the autophagic body. Immunoelectron microscopy shows that Atg8 is localized not only on the isolation membrane and pre-autophagosome structure but also in the lumen of autophagic bodies, providing a good marker for the intermediate membranes during autophagosome formation [30].

C-terminal myc tagging revealed processing of Atg8 at the very C-terminal end. Another Atg factor, Atg4, was responsible for the process. Atg4 is a member of a novel cysteine proteinase family conserved in all eukaryotes. Mutational analyses of Atg8 indicated that Atg4 cleaves a single arginine residue from the nascent Atg8 and exposes a glycine residue at the C-terminus. Atg12 and Atg8 show significant homology in the C-terminal region. Further analyses indicated that Atg8 is also a ubiquitin-like protein that is activated by Atg7. It is then transferred to Atg3, an E2 enzyme. Thus, Atg7 is a unique E1 enzyme that activates two different UBLs, Atg12 and Atg8, and transfers them to an E2 enzyme, Atg10 and Atg3, respectively [38].

The next apparent question was, what is the target of Atg8? Atg8 shows a single band in SDS-PAGE but was realized to be in two forms; one form is loosely membrane-bound, and the other is tightly membrane-bound. The formation of the tightly membrane-bound form of Atg8 requires Atg7, Atg3, the C-terminal glycine of Atg8, and Atg4, suggesting that it is generated by a conjugation reaction. By SDS-PAGE in the presence of 6 M urea, two forms of Atg8 were found to be separable. The modified form of Atg8 showed faster mobility than Atg8 itself. Mass spectrometry of the modified form of Atg8 clearly showed that it is a covalent conjugate of Atg8 with a membrane phospholipid, phosphatidylethanolamine (PE) [39]. The fatty acids of PE were mostly palmitoyl and oleic acids, quite abundant in yeast membrane.

This indicates that ubiquitin-like modification is not restricted to protein–protein linkages but also occurs in protein–lipid linkages. Importantly, Atg8–PE formation was reversible and the processing enzyme, Atg4, played a role on this process. Thus, Atg4 is a processing enzyme and also a deconjugating enzyme [38]. The cycle of Atg8 lipidation and delipidation is necessary for normal autophagic activity.

To further understand the role of this interesting phospholipid modification, it is necessary to identify the site of Atg8–PE formation and to characterize the structures containing Atg8–PE.

The Atg12 and Atg8 conjugation systems work concertedly; not only do they

share the same E1 enzyme, Atg7, but the proteins also function together because the Atg8–PE level is severely reduced in mutants of the Atg12 system, *atg5*, *atg10*, and *atg12*. Transcription of *ATG8* is known to be highly upregulated during nitrogen starvation. Certainly, Atg8 levels increase under starvation, but not so dramatically [30]. So far it is still not known whether upregulation of Atg8 is necessary for autophagy.

We do not know the precise role of lipidation reactions. Recently we succeeded in reconstituting the *in vitro* lipidation reaction using purified Atg8ΔR, Atg7, Atg3, and PE-containing liposome [40]. Further work will elucidate the molecular details of this interesting reaction system. It is still not clear whether only the modified molecule is essential for its function or whether the unmodified form still has some function. In *Pichia pastoris*, disruption of the Atg8 homologue (Paz2) shows a defect in the early stages of micropexophagy, indicating that the unprocessed form also has a physiological role [26]. In higher eukaryotes there are many homologues of Atg8, but their functions are not clear. Lipidated Atg8 homologues probably have a role during biogenesis of new membranes.

2.12.3 The Atg1 Kinase Complex

The third protein complex required for autophagy is the Atg1 protein kinase complex. Atg1 is a serine/threonine protein kinase [16, 41]. Its N-terminus region contains a protein kinase domain, and kinase activity has been detected *in vitro*. A kinase-negative Atg1 mutant could not induce autophagy, implying that kinase activity is essential for the function of the enzyme [11, 16]. The C-terminal region of Atg1 has no apparent sequence homology to other known proteins and is necessary for autophagy. Atg1 kinase activity is enhanced during induction of autophagy, and thus the level of kinase activity seems to be important for the regulation of autophagosome formation [11].

Atg1 physically interacts with Atg13, Atg17, and Cvt9. The Atg13 protein is a highly phosphorylated protein under nutrient-rich condition. Upon starvation or addition of rapamycin, a specific inhibitor of Tor kinase, it is dephosphorylated by a still-unknown phosphatase [11]. Oppositely, upon addition of nutrients to starved cells, Atg13 is rapidly hyperphosphorylated. The phosphorylation state of Atg13 is controlled by the nutrient conditions through the Tor signaling pathway. A genetic interaction exists between Atg1 and Atg13, since overproduction of Atg1 partially suppresses the autophagy defect of the *apg13* null mutant [42]. In its central region, Atg13 physically binds with Atg1 [11]. Under starvation, Atg13 is tightly associated with Atg1, while under nutrient-rich conditions, the affinity is lowered [11]. In addition, in the *atg13*Δ mutant, the kinase activity of Atg1 becomes low. These results suggest that Atg13 is a positive regulator for the Atg1 protein kinase. Transport of API is completely blocked when the *atg13* null mutant is grown in a nutrient-rich medium, but the block could be partially overcome by incubation in starvation conditions [43]. In an *atg13* mutant that lacks most of its Atg1-binding region, the Cvt pathway was normal but autophagy was completely defective [11].

Thus, Atg13 may regulate autophagy and the Cvt pathway through the Atg1 protein kinase. It is known that Atg13 also associates with Vac8 via its C-terminal region [44].

2.12.4 Autophagy-specific PI3 Kinase Complex

The fourth complex is the autophagy-specific PI3 kinase complex. Cloning and characterization of *ATG6* revealed that it is allelic to *VPS30*. Vps30/Atg6 has dual functions for vacuolar protein sorting and autophagy [45]. Atg14 is a possible coiled-coil protein and is associated with Vps30. Overexpression of Atg14 partially suppressed the autophagic defect of a truncated mutant of Vps30, but it does not suppress the defect of the deletion allele of *VPS30*. This suggests that Atg14 binds to Vps30 to exert its function for autophagy. In contrast with the *vps30* mutant, the *atg14* mutant does not show a defect in vacuolar protein sorting [45].

Later it was found that Vps30 forms two distinct protein complexes [45]. One complex of Vps30, Atg14, Vps34, and Vps15 is necessary for autophagy. The other complex of Vps30, Vps38, Vps34, and Vps15 is required for vacuolar protein sorting. These complexes share three factors. Vps34 is the sole phosphatidylinositol 3-kinase in yeast, and Vps15 is a regulatory protein kinase of Vps30. Vps30 is a possible coiled-coil protein and is peripherally membrane associated. Lack of Vps34 or Vps15 results in solubilization of Vps30. Atg14 is a specific factor in the autophagy-specific PI3-kinase complex; therefore, it may play an important role in determining the specificity of the PI3-kinase complex [46]. Atg14 is peripherally associated to an unknown membrane [45].

2.12.5 Other Atg Proteins

There are three remaining Atg proteins, Atg2, Atg9, and Atg18 that do not form a stable complex with the above factors. Their precise functions are not known yet. However, they may play important roles in linking the four reaction systems. Atg2 is a large, soluble protein [47] and has been shown to interact with Atg18. Atg9 is a putative multi-membrane-spanning protein, but its localization does not fit with known organelle markers [48].

2.13 Site of Atg Protein Functioning: The Pre-autophagosomal Structure

All *atg* mutants do not accumulate autophagosomes in the cytoplasm during starvation, indicating that all genes have functions at or before the formation of autophagosomes. So far, studies on the Atg proteins indicate that all these proteins function at the autophagosome-formation step. There are many fundamental problems to be solved. What is the origin of the autophagosome membrane? How does

the membrane assemble to form the spherical structure? What is the fusion machinery for autophagosome formation and fusion with the vacuolar membrane?

As mentioned, all original Apg proteins function closely together in the autophagosome-formation step. Recently, we showed that many Atg proteins are localized to a small area close to the vacuole, called the pre-autophagosomal structure (PAS) [31]. PAS is detected by GFP-Atg8 and colocalizes with (Atg12-)Atg5, the Atg1 kinase complex and Atg2, and presumably Atg14. Lipidation of PE is a requisite for the recruitment of Atg8 to the PAS. In *atg14* or *atg6* mutants, Atg8 and Atg5 do not form a dot structure in the cytosol, indicating that the autophagy-specific PI3-kinase complex plays an important role in the organization of PAS [31]. In contrast, defects in the Atg1 kinase complex show little effect on PAS structure. *Atg1ts* mutant cells expressing GFP-Atg8 completely block autophagosome formation; instead, they show a bright PAS structure next to the vacuole at the restrictive temperature. Upon shift down to the permissive temperature, a less brightly fluorescent structure is generated from PAS and fused to the vacuole; consequently, the vacuolar lumen is stained brightly. PAS seems to be an organizing center for the autophagosome.

2.14 Atg Proteins in Higher Eukaryotes

As shown in Table 2.1, most of the *ATG* genes are conserved from yeast to mammals and plants, indicating that eukaryotic cells acquired autophagic protein degradation at an early stage of evolution. The two conjugation reactions are especially well conserved. Interestingly, both Atg12 and Atg5 are encoded by single genes, but mammals and plants have many Atg8 homologues. In mammals they are called LC3, GATE16, and GABARAP, LC3 is involved in autophagy, but the other properties of the proteins are not clear yet.

We have shown that in mammalian cells, the Atg12–Atg5 protein conjugate is essential for autophagosome formation. In yeast, the Atg12–Atg5 conjugate interacts with a small coiled-coil protein, Atg16, to form a ~350-kDa multimeric complex [37]. We have demonstrated that the mouse Atg12–Atg5 conjugate forms a ~800-kDa protein complex containing a novel WD-repeat protein [49]. As the N-terminal region of this novel WD-repeat protein shows homology with yeast Atg16, we have designated it mouse Atg16-like protein (Atg16L). Atg16L has a large C-terminal domain containing seven WD repeats and is well conserved in all eukaryotes. The N-terminal region of Atg16L interacts with both Atg5 and Atg16L monomers, but the WD-repeat domain does not. In conjunction with Atg12–Atg5, Atg16L associates with the autophagic isolation membrane for the duration of autophagosome formation, indicating that Atg16L is the functional counterpart of the yeast Atg16. We also found that membrane targeting of Atg16L requires Atg5 but not Atg12 [49]. As WD-repeat proteins provide a platform for protein–protein interactions, the ~800-kDa complex is expected to function in autophagosome formation, further interacting with other proteins in mammalian cells.

Still, several Atg proteins are not identified in mammals or in plants. So far, an Atg1 kinase homologue is reported, but its regulators Atg13 and Atg17 are missing. A requirement of PI3-kinase activity for autophagy is also reported in mammals, but the autophagy-specific component, Atg14, has not been found. Possibly, as in the case of Atg16, sequence homology alone may not sufficient to find their counterparts, or they may be yeast-specific factors.

These proteins provide the most powerful tools for analysis of autophagy in higher eukaryotes.

2.15
Atg Proteins as Markers for Autophagy in Mammalian Cells

In yeast, autophagy is required for cell survival during starvation and is necessary for spore formation. In contrast, the role of autophagy in mammals is still poorly understood. Although the possible involvement of autophagy in development, cell death, and pathogenesis has been repeatedly pointed out, systematic analysis has not been performed, mainly due to limitations of monitoring methods. Moreover, in *S. cerevisiae* autophagy is solely a starvation response, but in multicellular organisms it could be regulated in a different manner. Our recent studies have made available several marker proteins for autophagosomes. To understand where and when autophagy occurs *in vivo*, we have generated transgenic mice systemically expressing GFP fused to LC3, which is a mammalian ortholog of yeast Atg8 [50, 51]. Cryosections of various organs were prepared and the occurrence of autophagy was examined by fluorescence microscopy. Active autophagy was observed in various tissues, such as the skeletal muscle, liver, heart, exocrine glands, thymic epithelial cells, lens epithelial cells, and podocytes. Patterns of induction of autophagy in different tissues are clearly distinct. In brain, autophagosomes were hardly detectable; under starvation conditions, brain cells may not suffer from nutrient limitation. In some tissues, autophagy even occurs spontaneously. Our results suggest that the regulation of autophagy is organ-dependent and that the role of autophagy is not restricted to the starvation response. This transgenic mouse is a useful tool for studying mammalian autophagy.

2.16
Physiological Role of Autophagy in Multicellular Organisms

The elucidation of genes essential for autophagy in yeast has facilitated work on autophagy in various organisms including *Dictyostelium discoideum* (slime mold), *Caenorhabditis elegans* (worm), *Drosophila melanogaster* (fly), *Arabidopsis thaliana* (plants), mouse, and humans [52–57]. Knockout of Atg genes showed severe phenotypes, mostly embryonically lethal at certain stages. These results indicate that autophagy probably has important roles in the development or cell differentiation of multicellular organisms.

2.17 Perspectives

Many researchers now pay attention to autophagy, but it is still a developing field of biology. Further studies on the function of Atg proteins not only will unveil the mystery of autophagosome formation but also may provide new insights into membrane dynamics within cell. Studies using different systems will provide a variety of physiological functions of autophagy in the near future. Finally, further work will define the true meaning of protein and organelle turnover more precisely.

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3
Dissecting Intracellular Proteolysis Using Small Molecule Inhibitors and Molecular Probes*

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Abstract

The ubiquitin–proteasome system has emerged as essential sets of reactions involved in many biological processes in addition to the disposal of misfolded and damaged proteins. Studies in different research areas reveal its role in regulating cell growth, differentiation, apoptosis, signaling, and protein targeting. Small molecule inhibitors against the proteasome have been useful in determining the specific role of this enzyme in these processes. Here we review recent progress made in the development and application of molecules that target proteasomal proteolysis. In addition, an increasing number of other enzymes in this pathway, in particular deubiquitinating enzymes (DUBs) and *N*-glycanases, appear to be attractive alternative targets for developing inhibitors that can be used to interfere with biological processes linked to the ubiquitin–proteasome pathway.

3.1
Introduction

Our knowledge of the ubiquitin–proteasome system as a key player in a wide variety of biological processes rests in part on yeast genetics and on our ability to manipulate it pharmacologically with proteasome inhibitors. Some of these inhibitors are cell-permeable and are active *in vivo*, making it possible to interfere with proteasome function in mammalian cells [1–3]. Proteasome inhibitors have now entered the clinic for the treatment of malignancies such as multiple myeloma and are no longer purely investigational tools [4–7].

Although usually considered a springboard for the analysis of mammalian proteasome structure, the prokaryotic proteasome has also come to the fore as a possi-

* A list of abbreviations used can be found at the end of the chapter.

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ble pharmaceutical target for proteasome inhibitors. *Mycobacterium tuberculosis* apparently requires its intact proteasomes to survive the harsh oxidative conditions inside the lung macrophages in which it usually resides. This observation suggests exciting opportunities to treat mycobacterial disease by rendering proteasome inhibitors specific to the mycobacterial proteasome [8, 9].

Originally viewed mostly as an abundant cytoplasmic protease, the proteasome is now considered central to many different aspects of cellular physiology. To maintain steady-state protein levels, polypeptides are continuously synthesized and destroyed. This process is regulated not only at a transcriptional level but also at the level of post-translational modification. Most cellular proteins are continuously synthesized and degraded within the life span of a cell. Protein turnover serves many critical regulatory roles, including quality control, by ensuring the degradation of proteins with abnormal structures that arise from mutation, metabolic damage, or misfolding. A variety of proteases are responsible for cytosolic protein turnover, but degradation of the vast majority of cellular proteins in mammalian cells is carried out by the proteasome, usually after previous tagging of the substrate with a polyubiquitin chain [10]. With few exceptions, proteasomal proteolysis requires substrates to be conjugated with multiple ubiquitin (Ub) molecular [11, 12]. Proteasomes degrade proteins in a processive fashion, generating peptides ranging in length from three to 22 residues [13].

The proteasome itself (Figure 3.1) can be divided into two distinct portions: a catalytic core and accessory subunits that associate with the proteasome at either end of the catalytic core particle [14, 15]. These associated proteins include polypeptides involved in recognition of Ub-conjugated substrates, proteins capable of unfolding protein substrates, enzymes capable of removing Ub from Ub-modified substrates, and at least one enzyme capable of removing *N*-linked glycans from *N*-glycosylated substrates [16–18]. It is likely that the proteasome is at the nexus of other, yet to be discovered, protein interactions. It follows, then, that the concept of proteasome

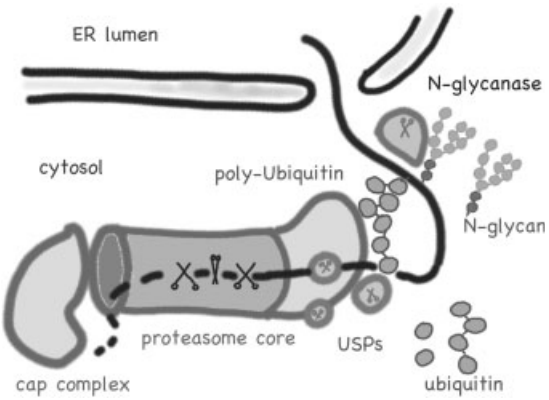

Fig. 3.1. The proteasome and associated factors.

inhibition should be defined to include not only agents that interfere with the catalytic subunits of the core particle but also compounds that target proteasome-associated activities. We consider it likely that many of these activities act in concert and that a pharmacological blockade of any one of them will modulate proteasomal function in a controlled manner. In addition to the protease activities associated with the β1, β2, and β5 subunits of the 20S complex, the full 26S proteasome includes other protease activities, notably the Ub-specific thiol proteases (USP14, UCH37) and the metalloprotease POH1 [19–21].

Modification of proteins with Ub is linked not only to proteolysis but also to targeting of modified proteins to proper intracellular destinations [22]. For instance, internalization of a Ub-modified receptor from the cell surface or the biogenesis of specialized intracellular compartments, such as multivesicular bodies (MVBs) [23–25], is regulated by Ub modification and possibly Ub removal. Even though modification with Ub may be the common theme here, the proteasome is not required for Ub modification to exert its function, nor does it solely degrade ubiquitinated proteins. Whereas internalization of Ub-modified growth hormone receptor requires an intact proteasomal system, this appears not to be the case for MVB formation, even though both processes critically depend on modification of target proteins with Ub [26].

When considering proteasomal proteolysis, it would be a mistake to lump together all aspects of protein degradation. Some proteins are targeted for Ub conjugation and proteasomal proteolysis while still attached to the ribosome: the incorrect incorporation of amino acids, leading to aberrant folding, might be one element that targets the nascent chain for degradation [27, 28]. In other cases, specific signals, such as phosphorylation, are required to polyubiquitinate and destroy the substrate in a carefully timed manner. Proteasomal destruction is usually highly processive and effective, but for some proteins and protein complexes, the proteasome generates the active form from an inactive precursor or protein complex, NF-κB being a case in point [29]. Proteins inserted co-translationally into the lumen of the endoplasmic reticulum (ER) fold in the topological equivalent of extracellular space. In the ER, proteins that fail to fold correctly are recognized and dispatched to the cytoplasm, in a process referred to as dislocation or retrotranslocation [30, 31]. In this process, Ub conjugation of the substrate plays a key role [32]. The Ub-conjugated proteins are then destroyed by the proteasome. The role, if any, of proteasomes in the process of extracting a substrate from the ER membrane is not clear [33]. It is likely that multiple classes of proteasomes can be defined based on their intracellular location and, hence, site of action [34, 35]. Therefore, different classes of proteasomes may differ in their susceptibility to pharmacological inhibition, depending on their interacting partners, their cellular environment, and the pharmacokinetics of the inhibitor used. Furthermore, the function of the proteasomes in the immune system is modulated through the action of cytokines. IFN-γ is a potent inducer of the β1i, β2i, and β5i subunits, which replace the catalytically active β1, β2, and β5 subunits, respectively, in the mammalian proteasome to generate the immunoproteasome [36, 37]. The immu-

noproteasome is structurally and functionally distinct from its constitutive counterpart [38, 39] and may be targeted selectively with appropriate inhibitors.

Importantly, both the proteasome and immunoproteasome are critically involved in the generation of a proper immune response in the context of MHC class I–mediated antigen presentation [40]. Inhibition of proteasomal activity strongly affects a variety of cellular processes. The proteasome is now considered a valid target for cancer therapy and treatment of stroke [41]; selective mycobacterial proteasome inhibitors also hold promise for the treatment of tuberculosis. In addition, proteasome inhibitors have been shown to increase the viability of cells treated with anthrax lethal toxin, inhibiting a proteasome-dependant step that is an early event in intoxication with anthrax lethal toxin. Proteasome inhibitors may thus become important in the defense against chemical warfare [42]. On the other hand, the proteasome inhibitor PS341 (VELCADE, Bortezomib) was recently introduced into the clinic for treatment of multiple myeloma [6] and is in clinical trials for a variety of other malignancies [5, 43–45], underscoring the need for research tools that allow determination of the proteasomal mode of action and its activity *in vivo*.

In this chapter we discuss the presently known classes of proteasome inhibitors and some of their applications. Because it would be difficult to view the proteasome in isolation and to disregard some of the proteasome-associated enzymatic activities as key players, we shall also discuss the identity of some of the proteasome-associated factors and the means to manipulate them where appropriate. There are many questions that remain unanswered, not the least of which is how to get a better understanding of the pharmacodynamics and pharmacokinetics of the various inhibitors, especially those presently in use, or considered for use, in the clinic.

3.2 The Proteasome as an Essential Component of Intracellular Proteolysis

To date, manipulation of the proteasome with the aid of small compounds has mainly been achieved through targeting the actual proteolytic activities of the 20S core. Targeting the individual ATPase and USP activities residing within the 19S caps with inhibitors entails an alternative inroad to the manipulation of proteasomal protein degradation. This also holds true for addressing events up- or downstream of the proteasome. These include the action of *N*-glycanase, which is instrumental in the removal of *N*-linked glycans of proteins that have escaped the secretory pathway and that are degraded by the proteasome [18]. In addition, modifications of components of the proteasome complex, such as phosphorylation/dephosphorylation, *O*-GlcNAcylation by *O*-GlcNac transferase (OGT), and *O*-GlcNac removal by *O*-GlcNacase, also modulate proteasome function [46–48]. The latter carbohydrate modification influences substrate entrance to the 26S proteasome by *O*-GlcNac modification of the Rpt2 ATPase subunit that resides in the 19S cap complex. This dynamic glycosyl modification appears to be under direct metabolic control. GlcNacase inhibitors such as streptozotocin (STZ) open oppor-

tunities to develop targeting strategies upstream of the proteasome. Examples of this type will be discussed in this chapter.

3.3 Proteasome Structure, Function, and Localization

The 20S proteasome, the inner core of the larger 26S particle that comprises the eukaryotic proteasome complex, is highly conserved in nature [49]. Archaebacterial and eukaryotic 20S proteasomes consist of 28 subunits, arranged in four stacked heptagonal rings, forming a hollow, barrel-shaped protein complex [14]. The proteolytic activity of the 20S particle resides within the two inner rings, containing seven β subunits each. The two outer rings, both assembled from seven α subunits, provide stability to the overall $(\alpha_{(1-7)}\beta_{(1-7)}\beta_{(1-7)}\alpha_{(1-7)})$ complex and serve as docking stations for additional protein complexes. These include the 19S cap (to form the 26S complex) and the interferon-γ-inducible PA28 complex in eukaryotic proteasomes, both of which are regulatory components with different functions that influence the activity and substrate specificity of the core particle. The 19S cap activates proteasomal proteolysis by recognition of proteasome substrates through their polyubiquitin chain and then unfolds them, enabling access to the proteolytic chamber. Ubiquitin molecules are recycled through the action of either of at least two proteasome-associated ubiquitin-specific proteases (USPs) or a zinc-dependent ubiquitin-specific metalloprotease that resides within the 19S complex [20, 21, 50–53].

While retaining its overall shape, the nature of individual α and β subunits within the 20S proteasome has diverged among different species. The *Thermoplasma* 20S proteasome is assembled in a fashion similar to that of the mammalian proteasome. The activity of the proteolytic β subunits resides in the N-terminal threonine residues, with the secondary alcohol of the threonine side chain acting as the nucleophilic species. The free N-terminal amine acts as the base in the catalytic cycle, catalyzing nucleophilic attack on the scissile peptide bonds. Importantly, the catalytically active substrate-binding site is formed only upon specific interactions with adjacent β subunits. Therefore, individual subunits do not show catalytic activity in isolation [54, 55].

Within eukaryotic 20S proteasomes, each of the seven subunits in either α or β rings are unique [39, 56]. Eukaryotic 20S proteasomes contain three distinct proteolytic activities, classified based on the use of synthetic substrates, although other proteolytic specificities were also reported [39, 57]. Individual activities have been analyzed with a variety of tools, including inhibitors, protein substrates, and a panel of specific fluorogenic peptide substrates. The main activities are now commonly referred to as the chymotryptic activity (X, β5), which is targeted by most proteasome inhibitors and which cleaves preferentially after hydrophobic residues; the tryptic activity (Y, β2), cleaving after basic residues; and the PGPH or caspase-like activity (Z, β1), responsible for cleaving after acidic residues. The chymotryptic/tryptic/PGPH classification is somewhat ambiguous, since substrate

preference does not accurately reflect catalytic activity, as revealed by studies using longer peptide and protein substrates [58–61]. All three catalytic β subunits show a rather broad substrate tolerance, and we will refer to the individual subunits, responsible for catalytic activity, as β1, β2, and β5 throughout the body of the text.

In higher vertebrates, a distinct 20S proteasome particle, referred to as the immunoproteasome, is expressed in many tissues upon interferon-γ induction [36]. The immunoproteasome contains three unique proteolytically active subunits termed β1i(LMP2), β5i(LMP7), and β2i(MECL1). They are highly homologous to their constitutive counterparts and display similar, yet subtly distinct, substrate specificities [60, 62]. In addition, several hybrid forms of proteasome species that harbor either β5i or β1i subunits, without the other interferon-γ-inducible subunits, have been described in different tissues and cell lines [63, 64]. The role of such proteasome subsets remains to be determined. A recent crystallographic study on eukaryotic proteasomes revealed a possible additional catalytic site associated with the β7 subunit [39].

The 20S core is found in both the cytoplasm and the nucleus [34, 65]. Associated proteins and complexes may dictate activity, or at least distribution-dependent proteasomal activity. The 19S complexes are involved in ubiquitin recognition and unfolding of the targeted polypeptide, and they facilitate translocation into the proteolytic chamber in an ATP-dependent manner, but additional associations and distribution-dependent tasks may well exist. Other studies suggest that cytoplasmic proteasomes co-localize with intermediate filaments and the endoplasmic reticulum (ER) membrane. This would fit the observation that membrane-bound proteins are degraded by the proteasome, perhaps also involving adaptor molecules that direct proteasomes to the ER or other organelles [66].

20S proteasomes are abundant. It has been estimated that the concentration of free 20S proteasomes is twice as high as that of 26S proteasomes in mammalian cells [67]. Since free 20S particles constitute the vast majority of different forms of proteasomes present in cells, a role for them in proteolysis is suggested based on their abundance alone. Proliferating and transformed cell lines usually have both higher proteasomal content and proteasomal activity than quiescent and non-transformed cells. The 20S core particle is capable of destroying highly oxidized proteins, and this may well be an important mechanism to respond to oxidative stress conditions [68].

The tight assembly of the 20S core particle is reflected by the relative ease with which it can be purified. Although a more demanding task, several groups have accomplished the purification of fully assembled 26S proteasome particles [69–72]. The 20S proteasomes from many different sources as well as the eubacterial HsIU/V protease have now been subjected to X-ray structural analysis [39, 56, 73–79]. Crystallized proteasomes retain their enzymatic activity, allowing the structural elucidation of proteasome–inhibitor complexes. In this way the covalent nature of aldehyde inhibitors bound to the catalytic subunits (as a hemiacetal); that of epoxyketones (morpholine adduct), β lactones (ester adduct), and vinyl sulfones (Michael adduct); as well as the noncovalent nature of TCM-95 inhibition have been established unambiguously. Co-crystal structures of inhibitor–proteasome complexes

have been used to determine the effects of occupancy of catalytic sites on structural elements more distal to the proteolytic core [77].

3.4 Proteasome Inhibitors as Tools to Study Proteasome Function

Ever since its discovery as a key player in protein turnover, the proteasome has been subjected to studies involving the use of small molecule inhibitors. The aim of such studies is usually twofold. With specific inhibitors, the nature of the individual subunit-associated activities can be charted. Moreover, the ability to partially disable the proteasome allows a study of its role in biological processes.

The ideal proteasome inhibitor would be both cell-permeable and specific, allowing the study of the proteasome in living cells and in live animals. The cleavage preferences of proteasomes can be assessed using fluorescent substrates, but such substrates can be used only in cell extracts. The accuracy of the fluorescent readout cannot be readily extrapolated to proteasomal activity *in vivo*. Standard, commercially available fluorogenic peptides include Z-LLE-βNA (β1-specific), Boc-LRR-AMC (β2-specific), and Suc-LLVY-AMC (β5-specific). By using such substrates, not only subunit specificity but also the kinetics of subunit inhibition can be monitored [80, 81].

Whereas a chemical knockout approach, disabling all activities, is expected to be lethal, disabling of specific catalytically active β subunits, for instance, an immunoproteasome subunit, may be useful to determine its contribution to the generation of MHC class I antigenic repertoires and its ability to modulate immune responses [82]. Inhibition of the chymotrypsin-like site as achieved by most proteasome inhibitors, or its inactivation by mutation alone, causes a large reduction in the rates of protein breakdown *in vitro* [59]. Potent and selective inactivation of trypsin-like or caspase-like sites is more difficult to achieve by small molecule inhibitors [83]. Compounds that selectively target proteasome particles located in specific subcellular compartments, or that show tissue- or species-dependent specificity, will be valuable both for biological research and therapeutic applications [84], but no such compounds have been identified to date. The same holds true for drugs that inhibit targets up- and downstream of proteasomal degradation.

Progress in inhibitor development has been described extensively in recent reviews [2, 85–87]. In the following sections the main classes of existing proteasome inhibitors and some future directions will be described briefly. Approaches that allow interference with targets up- and downstream of the proteasome will be discussed thereafter.

3.4.1 Peptide Aldehydes

Peptide aldehydes are the most popular class of proteasome inhibitors in biomedical and biological research. The most widely used member, Z-Leu-Leu-Leu-Al (**1**,

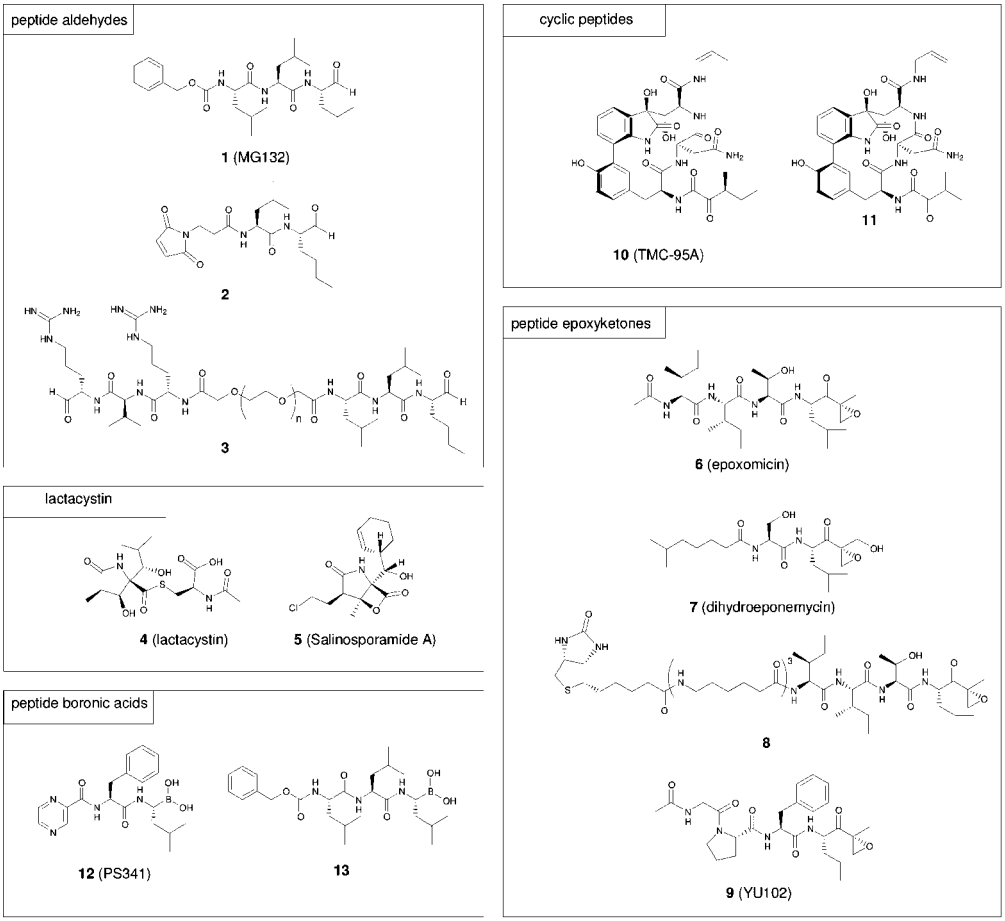

Fig. 3.2. Representative structures of different inhibitors.

MG132, Figure 3.2), is one of the standard tools used to modulate proteasome activity. The catalytic mode of action of peptide aldehydes was first demonstrated by X-ray diffraction of *Thermoplasma* 20S proteasomes in the presence of the peptide aldehyde Ac-Leu-Leu-Nle-Al [55]. The inhibitor's aldehyde moiety presumably forms a hemiacetal linkage with a catalytic threonine residue. Although this linkage is covalent, it can be hydrolyzed under physiological conditions, making peptide aldehydes reversible, competitive inhibitors. Many variations in the peptide backbone have appeared in recent years, including several compounds from natural sources such as tyropeptin A [88, 89]. A major drawback of peptide aldehydes is their propensity to cross-react with other proteolytic activities, primarily cysteine proteases, and their tendency to undergo oxidation to the corresponding acids, rendering the probes inactive under physiological conditions.

Moroder and coworkers developed a set of bifunctional peptide aldehydes, represented by maleimide derivative **2**, with specificity for β_2 over β_1 and β_5 [90]. After initial hemiacetal formation with the N-terminal threonine of β_2, the maleimide moiety undergoes an irreversible Michael reaction with a cysteine residue of the neighboring β_3 subunit, which protrudes into the β_2 active site that then becomes disabled. The reactivity of the maleimido group towards mercaptans in general limits its use, but fine-tuning of the reactivity of the maleimido group may provide a route to β_2-selective inhibitors for broader applications. The development of homo- and heterobifunctional peptide dialdehydes, interspaced with polyethylene glycol (e.g., compound **3**), was reported by the same group, showing a 100-fold increase in potency compared to monovalent counterparts.

3.4.2 Lactacystin

The fungal metabolite lactacystin (**4**), isolated from *Streptomyces*, is a classical proteasome inhibitor and one of the few that does not have a peptoid structure [91, 92]. The β-lactone metabolite of lactacystin, *clasto*-lactacystin-β-lactone (omuralide), is the reactive species. After binding to active sites, the β-lactone reacts with the threonine hydroxyl moiety to result in acylation of the active site. Although this acylation is covalent, hydrolysis of the ester linkage occurs over time, resulting in the loss of inhibition and loss of effective inhibitor. In studies of its mode of action using radiolabeled lactacystin, it was found that at low concentrations it effectively inhibits β_5, whereas β_1 and β_2 active sites are targeted only at higher concentrations [93]. Importantly, hydrolysis of the acylated β_1 and β_2 subunits appears to be faster than that of the corresponding β_5 subunit. Recently, a naturally occurring β-lactone named salinosporamide A (**5**), closely resembling omuralide, was discovered in marine actinomycete bacteria [94]. Based on its resemblance to omuralide, it was tested for its ability to inhibit proteasomes. When tested against purified 20S proteasome, the compound showed an efficient inhibition of the chymotryptic activity, with an IC_{50} value of 1.3 nM. Therefore, this compound is at least 35 times more potent than its structural relatives omuralide and lactacystin. It is likely that the intermediate reaction product, formed upon opening of the β-lactone of salinosporamide A by nucleophilic attack of the threonine hydroxyl moiety, undergoes a second reaction. Recently, Corey and coworkers reported a total synthesis of salinosporamide A [95].

3.4.3 Peptide Epoxyketones

The natural product epoxomicin (**6**) and related structures were isolated and identified based on their anti-tumorigenic properties in pharmacological screens [96]. A very potent proteasome inhibitor, epoxomicin shows strong preference for β5. The analogous natural product eponemycin and its synthetic analogue dihydroeponemycin (**7**) show enhanced affinity for β2. The epoxyketone chemical warhead har-

bors two, not one, reactive groups. Reaction of the threonine hydroxyl with the β-carbonyl results in a ketal linkage, while subsequent reaction of the free proteasomal N-terminus at the γ position results in a rigid morpholine ring system.

Crews et al. have devoted considerable effort to the generation of synthetic epoxomicin derivatives. With biotinylated epoxomicin derivative **8** they showed the proteasome to be the biological target of epoxomicin [97]. Recent efforts include the synthesis of oligopeptide epoxyketone derivatives with varying amino acid functionalities (including non-natural ones) at positions P1–P4 [83]. From these studies stems YU102 (**9**), to date the only compound that comes close to being a selective β inhibitor. Epoxyketones are relatively selective, metabolically quite inert, and in some cases cell-permeable, and they modify the proteasome irreversibly, thereby enabling affinity tagging and target retrieval [98].

3.4.4 Cyclic Peptides

TMC-95A (**10**) is a synthetically challenging cyclic peptide metabolite of *Apiospora montagnei*. It is a potent competitive proteasome inhibitor [99, 100]. Unlike the aforementioned inhibitors, it appears not to form a covalent link with a threonine moiety of catalytically active β subunits upon fitting into the active site. Rather, it blocks access to the active sites by imposing steric constraints. Therefore, it offers excellent opportunities to modulate its inhibitory profile specific for different subunits, not being hampered by covalent bonds. In addition to TMC-95A, several closely related structures with distinct inhibitory profiles, named TMC-95B, -C, and -D, were isolated from the same source [101–105]. At first glance, the structure of **10** appears rather daunting; one would expect it to be difficult to obtain synthetic analogues. A recent study revealed that the structure can be simplified, as in **11**, by omitting a chiral center and by replacing the difficult to obtain *N*-acyl enamine moiety by an *N*-acyl allylamine moiety [106]. These replacements have no detrimental effects on its potential as an inhibitor. TMC-95A and some other structurally similar compounds are the only proteasome inhibitors that do not covalently bind the threonine active sites [100, 107–109].

3.4.5 Peptide Boronates

Peptide boronates are considered to the most potent inhibitors of the proteasome and is the only class which a member has reached the clinic so far [4, 5, 44, 45, 110]. Boronic acids have a high affinity for hydroxyl groups, displaying an empty p-orbital to threonine oxygen lone-pair electrons. Based on the hard-soft acid-base principle, it is assumed that peptide boronates show a general preference for serine and threonine proteases over cysteine proteases (sulfhydryl moieties). Such assumptions may not always apply; for example, vinyl sulfones (see Section 3.4.6) were described originally as cysteine protease inhibitors [111], but they also proved

to be potent and selective proteasome inhibitors [112, 113]. Whatever the exact mechanism, peptide boronic acids fall in the class of covalent, competitive, reversible inhibitors due to the strength of the boron–oxygen bond. The off-rate of the inhibitor, however, is much slower compared to peptide aldehyde inhibitors. Importantly, boronates provide greater metabolic stability. The combination of inhibitory potency, selectivity, and stability makes peptide boronic acids well suited as candidates for clinical use. Indeed, PS341 (**12**) has reached the clinic for the treatment of multiple myeloma and is in clinical trials for treatment of other cancers [114]. Another relevant example of the peptide boronic acid family is derivative **13**. Compound **13**, featuring the ZLLL tripeptidyl core, is far more potent than its analogous peptide aldehyde **1** and peptide vinyl sulfone analogue **15**, demonstrating the potential of the boronic acid moiety as a chemical warhead.

3.4.6
Peptide Vinyl Sulfones

Peptide vinyl sulfones are a prominent class of irreversible proteasome inhibitors [113] (representative structures are given in Figure 3.3). They covalently modify

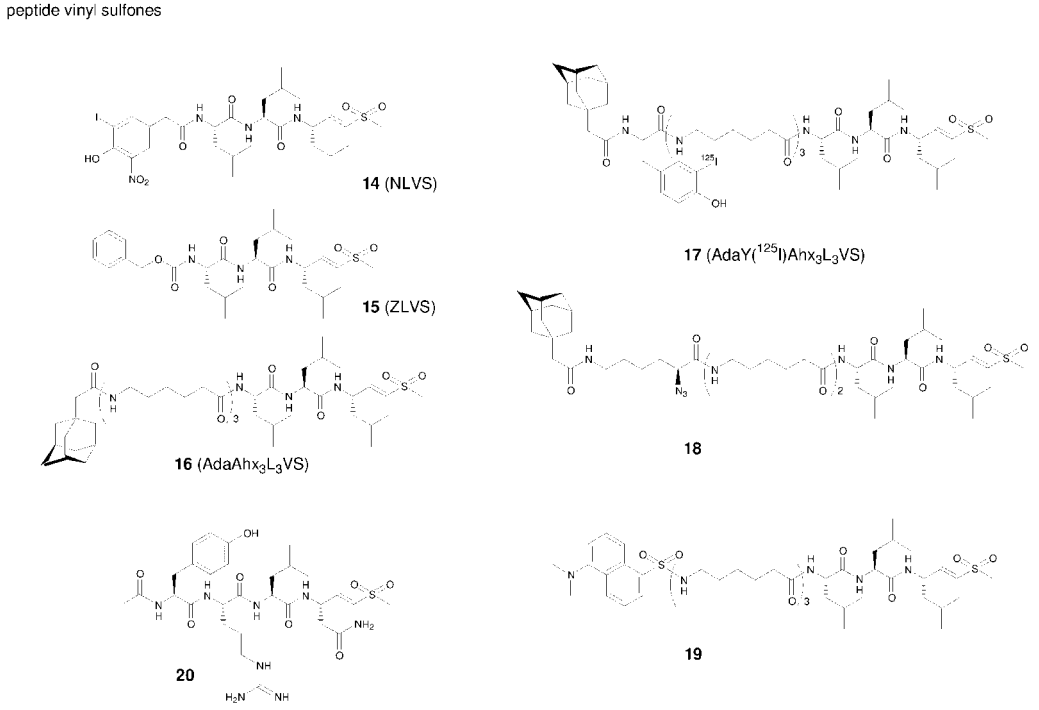

Fig. 3.3. Structures of vinyl sulfone–based probes.

the catalytic subunits through Michael reaction of the threonine hydroxyl with the vinyl sulfone moiety, resulting in the formation of a physiologically stable β-sulfonyl ether linkage. The finding that vinyl sulfones have turned out to be such effective proteasome inhibitors is in itself surprising: "hard-soft" acid-base principles dictate that this pharmacophore should have preference for "soft" nucleophilic thiols over "hard" alcohol nucleophiles. Indeed, peptide vinyl sulfones are widely used as cysteine protease inhibitors [115, 116]. The fact that, depending on the nature of the peptide portion attached to the electrophilic trap, the activity of peptide vinyl sulfones can be directed almost exclusively towards the proteasome underscores the importance of the peptide-based recognition elements in attaining protease specificity. Representative peptide vinyl sulfones are NLVS (**14**) and ZL_3VS (**15**), both of which are cell-permeable and show preferential targeting of β5 [113, 117].

3.4.7 Peptide Vinyl Sulfones as Proteasomal Activity Probes

One limitation of experimental work with proteasome inhibitors is the difficulty in gauging some of the most basic pharmacokinetic and pharmacodynamic parameters. While it is feasible to measure serum half-life, accumulation in various tissues and organs is more difficult to assess. Furthermore, even though the inhibition constant of these inhibitors for isolated proteasomes is well established, the extent to which proteasomal inhibition occurs *in vivo* has been more complicated to estimate.

N-terminal extension of peptide vinyl sulfones, as in $AdaAhx_3L_3VS$ (**16**), has a profound effect on their inhibitory activity. The effective labeling of all proteasomal activities in cultured cells with a single compound remained elusive until recently. Incorporation of three aminohexanoic acid residues and introduction of a large hydrophobic N-terminal cap such as the adamantane acetyl group resulted in a set of compounds capable of inhibiting all catalytically active β subunits of both the constitutive proteasome and the immunoproteasome [61] with comparable efficiency. This is illustrated by the treatment of cell lysates of EL-4 cells (expressing both the constitutive proteasome and the immunoproteasome) with the ^{125}I-labeled $AdaYAhx_3L_3VS$ (**17**, Figure 3.3). Probe **17**, however, is not cell-permeable, due to the presence of the iodotyrosyl residue. To overcome this shortcoming, compound **18** (Figure 3.3), a modification of **16** containing a bio-orthogonal azide moiety, was prepared. The azido group interferes with neither its inhibitory profile nor its cell permeability. Labeling of whole cells with **18** decorates all catalytic activities of the proteasome with an azide as a latent ligation handle. After cell lysis and retrieval and denaturation of the protein content, the azido groups can be addressed by a biotinylated phosphine reagent in a Staudinger ligation reaction, effectively biotinylating active-site subunits [118–120]. Streptavidin-horseradish peroxidase conjugate–mediated Western blot can now reveal proteasomal activity profiles in cultured cells.

Derivatizations such as radioiodination, biotinylation, or introduction of an azide

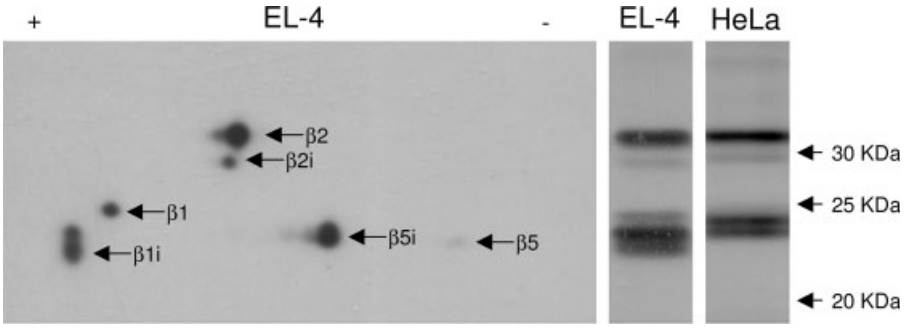

Fig. 3.4. Proteasome labeling in EL4 mouse cell extracts. Incubation of EL4 cell extracts with AdaY(^{125}I)Ahx$_3$L$_3$VS results in the covalent attachment of the radiolabeled probe to the active subunits of the proteasome. Proteins were separated by 2D isoelectric focusing (IEF)-SDS PAGE followed by autoradiography.

moiety, not surprisingly, have both advantages and disadvantages. A biotinyl or tyrosyl moiety, the latter enabling radioiodination, generally interferes with cell permeability. Azide-containing proteasome-specific probes retain cell permeability [121], but the required two-step labeling strategy makes the strategy more demanding for high-throughput applications. Small haptens such as a dansyl moiety may allow retention of cell permeability of probes and may allow detection of labeled enzymes using high-affinity antibodies directed against the incorporated hapten. This approach would allow a high-sensitivity level of detection. For this purpose, cell-permeable dansylated proteasome inhibitor **19** was synthesized. Inhibitor **19** freely reaches cellular targets and modifies covalently and irreversibly all of the proteasome's catalytic subunits (Figure 3.5). The methodology is thus entirely independent of the use of radioisotopes, biotinylation, or secondary chemoselective ligations. This dansylated inhibitor allows accurate assessment of the proteasomal targets hit in living cells by drugs such as **12** (PS341, Velcade, Bortezomib). In principle, this strategy is applicable to other proteases as well.

3.4.8
Future Directions in the Development of Inhibitors of the Proteasome's Proteolytic Activities

Next to broad-spectrum inhibitors, the search for subunit-specific (other than β5) inhibitors remains an important research objective. Several approaches have been made to achieve this goal. Nazif and Bogyo reported an elegant strategy towards peptide vinyl sulfone libraries, based on immobilizing aspartic vinyl sulfone to a matrix through the carboxylic acid side-chain functionality [122]. Positional scanning of amino acids at P2–P4 resulted in the identification of AcYRLNVS (**20**), a selective inhibitor of β2 (Figure 3.3). Reagents like these will help determine the role of individual catalytic subunits in proteasome function, protein degradation, and the generation of antigenic peptides. In this context, it will be of consider-

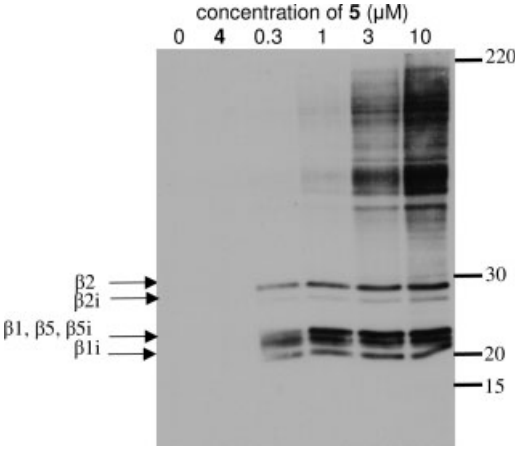

Fig. 3.5. Immunoblot using inhibitor **19**. Labeling pattern observed by immunoblot after incubation of EL4 cell extracts with different concentrations of inhibitor **19** or an inactivated control probe.

able interest to generate inhibitors capable of targeting distinct proteasome species, such as the immunoproteasome or proteasomes from bacterial or fungal origin [81, 83].

3.5 Assessing the Biological Role of the Proteasome With Inhibitors and Probes

Inhibitors of the proteasome have been essential tools in the discovery of many new substrates of the ubiquitin–proteasome pathway and in establishing its role in different biological processes [3].

When treated with otherwise lethal concentrations of NLVS or lactacystin, small cell subpopulations within cultured EL4 cell lines are capable of adapting and proliferating in the presence of this inhibitor. Partial impairment of the proteasome (in the adapted cells, β5 proved to be completely disabled, whereas β1 and β2 remained to a large extent active) can be overcome by a small subpopulation of cells that can outgrow the rest of the culture, resulting in a cell line resistant to inhibition of the β5 subunit. As may be expected, adapted EL4 cells are partially compromised in their ability to generate MHC class I antigenic peptides [93, 117, 123, 124]. Tripeptidyl peptidase II (TPPII) appears to compensate in these cells for the loss of proteasomal activity, a finding that may become very important regarding development of resistance in cancer patients treated with proteasome inhibitors [125–127]. Burkitt's lymphoma cells prove to be quite resistant to apoptosis induced by proteasome inhibitors [128]. Although proteasomal peptidase activities are significantly reduced in these cells, the overall rates of protein breakdown barely change. As in NLVS- and lactacystin-adapted cells, in Burkitt's lymphoma

cells it was found that the activity of TPPII is increased compared with other cells. This effect appears to be related to expression of the constitutively activated c-*myc* oncogene. Moreover, an inhibitor of TPPII activity, AAFcmk (alanyl-alanyl-phenylalanyl chloromethyl ketone) [129–131], in contrast to proteasome inhibitors, was able to inhibit proliferation of these cells, suggesting that upregulation of TPPII may indeed compensate for the decreased overall activity of proteasomes in these cancer cell lines. The inhibitor butabindide [129] and analogues thereof [132] form superior alternatives to the use of AAFcmk.

3.6 Proteasome-associated Components: The Role of *N*-glycanase

Successful maturation of proteins determines the intracellular fate of secretory and membrane proteins in the endoplasmic reticulum (ER). Failure of adaptor molecules such as calnexin and calreticulin to provide assistance in folding and refolding or assembly of glycosylated proteins can lead to retention in the ER and redirection to the cytosol for degradation by the proteasome of these glycoproteins [131, 133]. Many substrates may be subject to this mode of degradation. For instance, MHC class I molecules are assembled and loaded with antigenic peptide in the ER and subsequently displayed at the cell surface. *N*-linked glycosylation of class I nascent chain that enters the secretory pathway contributes to its proper folding, assembly, and trafficking. The identification of a role for peptide-(*N*-acetyl-β-D-glucosaminyl)-asparagine amidase (PNG) activity in the cytosol of mammalian cells emerged from a strategy used by the human cytomegalovirus (HCMV) to evade detection by the immune system of its host by the HCMV gene products US2 and US11 [30, 134–137]. Inhibition of proteasomal proteolysis results in the accumulation of a deglycosylated MHC class I heavy-chain intermediate in the cytosol. This finding is consistent with the action of a peptide:*N*-glycanase (PNGase) on the substrate prior to its destruction by the proteasome [18, 138–144]. Oligosaccharyl transferase [144, 145] and PNG1 play important roles in the degradation of ER proteins. PNG1 is located in the cytosol, where it is thought to assist the proteasome in degradation of ER-derived glycoproteins. PNG1 recognizes glycosylated, preferentially denatured [138] protein substrates and, at least in yeast, may directly associate with the 19S cap subunit mHR23B [143]. It is therefore reasonable to assume that selective *N*-glycanase inhibitors, allosteric *N*-glycanase activators, and oligosaccharyl transferase inhibitors will form useful tools to explore pharmaceutical targets upstream of the proteasome.

A high-throughput screen ($n > 100{,}000$) for small-molecule inhibitors of mammalian PNG revealed the general caspase inhibitor ZVAD(OMe)fmk (benzyloxycarbonyl-valine-alanine-aspartic fluoromethyl ketone) (**21**, Figure 3.6) as an inhibitor of *N*-glycanase. Caspases and PNGases share no obvious structural or functional similarities. At concentrations of ZVAD(OMe)fmk commonly required to block apoptosis, *N*-glycanase is inhibited as well [146]. ZVAD(OMe)fmk inhibits PNG1 with an IC_{50} of about 12 μM in cultured cells. ZVAD(OMe)fmk is *in situ* converted

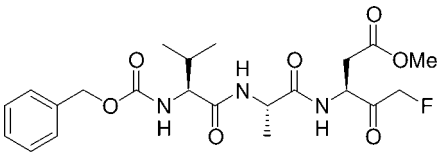

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Fig. 3.6. The *N*-glycanase and caspase inhibitor ZVAD(OMe)fmk.

into the active inhibitor ZVADfmk by saponification catalyzed by esterases. *In vitro* only the product of saponification, ZVADfmk, exhibits inhibitory activity, but it is not cell-permeable. It is unlikely that this would be a unique example of such unexpected cross-targeting. It will therefore be important to explore cross-reactivities of inhibitors in general.

3.7
A Link Between Proteasomal Proteolysis and Deubiquitination

3.7.1
Reversal of Ub Modification

The biological effect of ubiquitin-specific proteases (USPs) is twofold: they either rescue proteins from destruction or condemn proteins to destruction via proteasomal degradation [147–149]. The steady-state level of Ub conjugates is the result of a subtle balance between the action of ubiquitin ligases and USPs in a manner comparable to the opposing actions of kinases and phosphatases.

3.7.2
Ubiquitin-specific Proteases

Four major subfamilies of ubiquitin-specific proteases have been identified to date [150]. The best-studied subfamilies, characterized by the presence of a catalytically active cysteine residue, are known as ubiquitin-specific processing proteases (UBPs) and ubiquitin carboxy-terminal hydrolases (UCHs). Members of these families possess the signature sequence motifs of a cysteine protease and show characteristic patterns of sequence conservation in their catalytic core domains. USPs can remove Ub from large polypeptides and disassemble poly-Ub chains, whereas UCHs normally target Ub derivatives with C-terminal linear extensions [151]. Ovarian tumor domain (OTU)-containing cysteine proteases form a third large family that shares no obvious homologies with either UBP or UCH families [152–155]. A single JAMM family metalloprotease within the 19S cap of the proteasome, RPN11 (POH-1), was shown to cleave ubiquitin moieties [20, 53].

RPN11 lacks a cysteine protease signature and is insensitive to the classical USP inhibitor Ub aldehyde. Other families of USPs may well exist.

3.7.3
USP Reactive Probes Correlate USP Activity With Proteasomal Proteolysis

Several reports have described the association of USPs with the proteasome. Development of radioiodinated Ub-nitrile led to the discovery of UCH37's association with the mammalian proteasome [50], whereas a radiolabeled ubiquitin probe with a C-terminal vinyl sulfone moiety was crucial for the discovery of the association of USP14, the mammalian homologue of Ubp6, with the 19S proteasome cap [51, 52]. In all cases, labeling of these USPs was abolished by pre-incubation with ubiquitin aldehyde.

USP-reactive probes are mechanism-based and thus provide a convenient tool to examine the enzymatic activity of USPs in response to proteasome inhibition [153, 156–158].

Whereas labeling, and hence activity, of UCH37 does not change upon treatment with proteasome inhibitor, the labeling of proteasome-associated USP14 was increased up to 15-fold in a time-dependent manner [51] in EL4 cell extracts (Figure 3.7). The observed increase in probe modification of USP14 is not unique to pro-

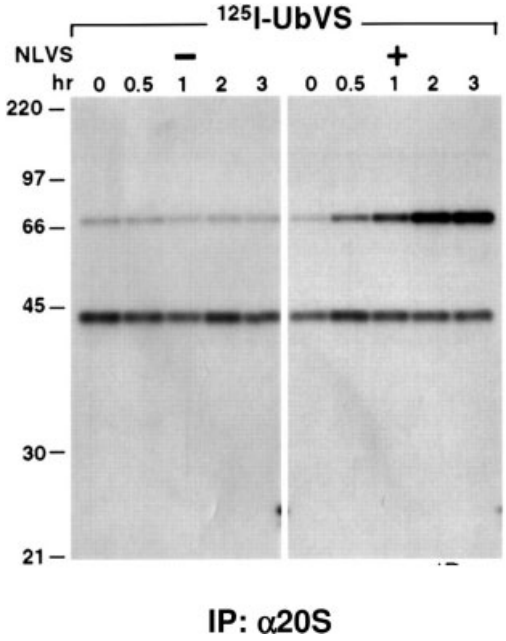

Fig. 3.7. Association of USP14 with the proteasome. Superose-6 fractions were labeled with ^{125}I iodinated ubiquitin vinyl sulfone and 20S complexes were immunoprecipitated with an anti-20S antibody. Fractions containing probe-modified USP14 were detected only in fractions corresponding to 26S proteasome complex and not in fractions with free 20S proteasomes.

teasome inhibition by NLVS, as treatment of cells with other proteasome inhibitors produced similar effects. Complete Ub removal is thought to precede proteasomal proteolysis. When proteasomal proteolysis is blocked, the resultant accumulation of Ub-conjugated substrates may elicit enhanced activity of USPs. In other words, the activities of the proteasome and associated USPs may be interdependent.

The exact reason for this increase in activity upon proteasomal inhibition is not yet fully understood. USP14 was also shown to exist either in a free form or bound to the proteasome. Only the latter is enzymatically active, as demonstrated in labeling experiments with mechanism-based probes. The requirement for USP14 (ubp6 in yeast) to associate with proteasome particles to become active may represent a regulatory mechanism that prevents random deubiquitination of substrates within cells. Removal of ubiquitin from substrates bound to the proteasome prior to their destruction salvages ubiquitin and may be important to maintain a steady-state ubiquitin level [159].

The importance of proper USP14 function is also underscored by the fact that mice deficient in USP14 develop cerebellar ataxia early on in their development [160]. Small molecule inhibitors specific for deubiquitinating enzymes would be an alternative inroad to interfere with targets upstream of proteasome function.

3.8 Future Developments and Final Remarks

Advances in understanding protein degradation and protein-folding pathways have been made possible by inhibitors of distinct activities, directly or indirectly, involved in proteolytic degradation pathways. Development of novel inhibitors may allow a deeper insight into the ubiquitin–proteasome system and will offer new approaches for blockade of up- and downstream events as well as future pharmacological intervention and hence treatment of disease.

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Abbreviations

Ub ubiquitin;
UBL ubiquitin-like protein;
UCH ubiquitin carboxy-terminal hydrolase;

UBP ubiquitin-processing protease;
USP ubiquitin-specific protease;
PNG peptide-*N*-glycanase;
ER endoplasmic reticulum;
MVBs multivesicular bodies;
UPS ubiquitin–proteasome system

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4
MEKK1: Dual Function as a Protein Kinase and a Ubiquitin Protein Ligase

Zhimin Lu and Tony Hunter

4.1
Introduction

Protein kinases are important regulators of intracellular signal transduction pathways, which mediate the development and regulation of diverse eukaryotic cellular activities, including cellular metabolism, transcription, cytoskeletal rearrangement and cell movement, apoptosis, cell-cycle progression, and differentiation. Through phosphorylation of substrates, protein kinases also play an important role in intercellular communication during development, in physiological responses and homeostasis, and in the functioning of the nervous and immune systems [2, 27, 28]. The 518 putative protein kinase genes identified in the human genome sequence comprise approximately 2% of all human genes, making them one of the largest families of eukaryotic genes [28]. In comparing the kinase gene chromosomal map with known disease loci, 164 kinases have been mapped to amplicons that are frequently found in tumors, and 80 kinases have been mapped to loci that are associated with major diseases such as diabetes, obesity, and hypertension [23, 28]. Perturbations of protein kinase function caused by mutation, overexpression, and dysregulation have causal roles in diverse human illnesses [2, 18].

4.2
Types of Protein Kinases

Based on their catalytic specificity, protein kinases can be subdivided into two major categories, tyrosine kinases and serine/threonine kinases. They function primarily by phosphorylating tyrosine or serine/threonine residues, respectively, either their own via autophosphorylation or those of their substrates, whose activity is consequently modulated. The activation of protein kinases and the phosphorylation of their substrates can play a role in regulating protein expression levels via a ubiquitin/proteasome-mediated degradation pathway through which target proteins are covalently tagged with ubiquitin and marked for degradation. The process of conjugating ubiquitin to substrate proteins depends upon three enzymes:

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a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Intriguingly, an increasing number of protein kinases are known to be rapidly degraded via the ubiquitin/proteasome pathway following their activation. Such protein kinase deactivation through downregulation at the protein level provides an additional feedback mechanism, along with phosphorylation and dephosphorylation, that controls protein kinase activity. Protein kinases can also initiate ubiquitin/proteasome-mediated degradation of their protein substrates through direct phosphorylation. For instance, phosphorylation of β-catenin at serines 33 and 37 by GSK 3β creates a binding motif for the β-TrCP/HOS F-box protein, which is the substrate recognition subunit of the $SCF^{\beta\text{-TrCP}}$ E3 ubiquitin ligase [15, 33]. The cyclin-dependent kinase (Cdk) inhibitor p27 is phosphorylated directly by cyclin E/Cdk2 at Thr187 [30, 38], which then interacts and is ubiquitinated by the SCF^{SKP2} E3 ligase [8, 35, 36]. In addition to protein kinase phosphorylation by catalytic domains, 83 different types of domains are found in 258 protein kinases [28]. These domains regulate kinase activity, localize proteins to subcellular compartments, interact with various signaling molecules, and are involved in protein degradation. Recent reports have shown that MEK kinase 1 (MEKK1), a serine/threonine protein kinase that has an important regulatory role in mitogen-activated protein (MAP) kinase cascades, functions both as a serine/threonine kinase through its kinase domain and as an E3 ubiquitin ligase via its N-terminal cysteine-rich domain [26, 40].

E3 ligases interact with both a ubiquitin-charged E2 molecule and the targeted substrate protein, facilitating polyubiquitination and directing substrate specificity. Thus, ubiquitination is primarily controlled by regulating E3 ligase activity and E3-substrate interactions [12]. There are two distinct types of E3 ligases: enzymatic HECT (homologous to E6-AP C-terminus) domain E3s and adaptor E3s containing a RING finger domain [19]. The ~350-residue HECT domain E3s forms a thioester with ubiquitin and transfers ubiquitin to substrates. On the other hand, the ~50-residue RING finger does not form a thioester with ubiquitin. Instead, it functions as an adaptor and facilitates the interaction between substrates and the E2. The RING finger is a zinc-binding domain with an octet of cysteines and histidines with a defined spacing configuration, which function as molecular scaffolds to conjoin proteins [4]. There are two varieties of RING finger E3 ligases. In one case the RING finger is part of a single polypeptide E3 ligase, such as Cbl, whereas in the other the RING finger protein is a subunit of a multi-subunit E3 complex, such as the small RING finger proteins present in the SCF (Skp1, cullin, and F-box) and APC (anaphase-promoting complex) E3 ligases [20, 24].

MEKK1, a 195-kDa protein with a C-terminal protein kinase domain and a large non-catalytic N-terminus, acts as a MAP kinase kinase kinase (MAPKKK or MAP3K) [25, 42]. The N-terminal non-catalytic region of MEKK1 contains a 48-residue region (aa 433–488) that has seven cysteines and a histidine that are linearly arranged in a C4HC3 consensus sequence [26] (Figure 4.1). Based on classification by the order of cysteine and histidine residues arrayed in a domain, this consensus sequence is categorized as a plant homeodomain (PHD) domain (also called a leukemia-associated protein (LAP) domain) rather than a RING finger do-

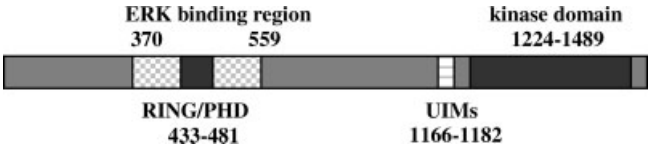

Fig. 4.1. RING/PHD domain (aa 433–481), ERK binding region (aa 370–559), UIMs (aa 1166–1182), and kinase domain (aa 1224–1489) of MEKK1 in a schematic structure.

main [6, 10, 26, 31]. The PHD domain is an approximately 50-residue C4HC3 zinc finger–binding motif, whereas the classical RING finger has a C3HC4 zinc finger–binding motif. The PHD domain structurally resembles the RING finger domain, with eight similarly spaced conserved metal-binding ligands [5, 7, 32]. Based on a different classification, which uses sequence profile or Hidden Markov models, a search of the PROSITE and Pfam databases or the non-redundant database of protein alignment shows that the cysteine-rich domain of MEKK1 retrieves NFX1- and H2-type variant RING domains [1, 34]. Given the close structural similarity between the RING and PHD domains and because the cysteine-rich domain of MEKK1 has features of both the RING and PHD domains, further studies are needed to determine whether the MEKK1 domain properly belongs to the RING finger or PHD domain family. For now, this domain will be called a RING/PHD domain, and this issue will be considered further in the discussion of whether a subset of PHD domains may, like many RING domains, have E3 ligase activity.

MEKK1 is one member of a family of related serine/threonine protein kinases that regulate three-tiered MAP kinase cascades. The three-tiered cascades are composed of a MAP3K, a MAP kinase kinase (MAPKK or MAP2K), and a MAP kinase (MAPK) (Figure 4.2A). MAP3Ks transduce signals received at the cell surface into

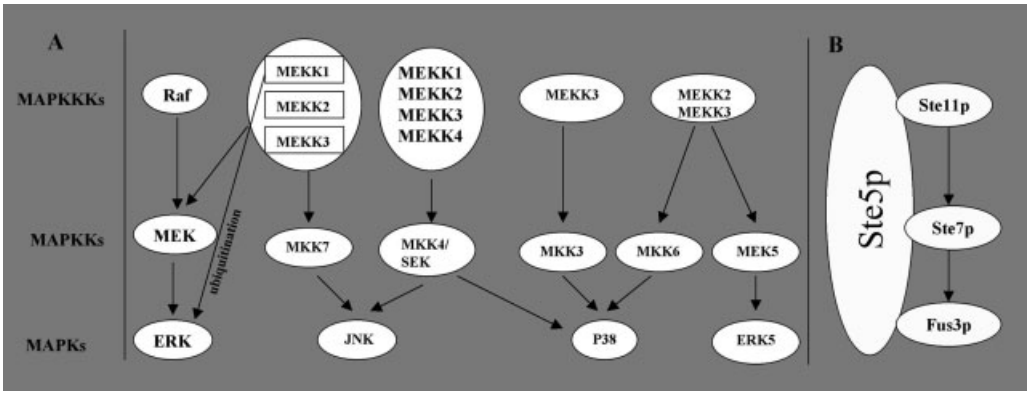

Fig. 4.2. MAP kinase cascade in (A) eukaryotic cells and (B) *Saccharomyces cerevisiae* (modified from [17]).

the nucleus by activating MAPK family members, including extracellular signal-regulated kinases (ERKs), the c-Jun NH2-terminal kinase (JNK), and p38. MAPK activation occurs in response to growth factor stimulation, cellular stress (e.g., UV and irradiation, osmotic stress, heat shock, and protein synthesis inhibitors), inflammatory cytokines (e.g., tumor necrosis factor [TNF] and interleukin-1 [IL1]), and G protein–coupled receptor agonists (e.g., thrombin) [17]. The activation of MAPK cascades has been implicated in cell growth and differentiation, apoptosis, oncogenesis, and inflammatory responses. Upon stimulation, MAP3Ks phosphorylate and activate their substrates, MAP2Ks, which in turn phosphorylate critical threonine and tyrosine residues in the activation loop of MAPK, thereby activating them.

4.3 Functions of Protein Kinases

The MEKK1 kinase domain phosphorylates several different MAP2K/MEKs and can regulate both the ERK and JNK pathways in response to specific stimuli [22, 41, 43] (Figure 4.2A). MEKK1 is activated in response to cellular stresses, including hyperosmolarity. When triggered by a mitogenic stimulus, ERK1/2 MAPKs are phosphorylated and activated by the MEK1/2 MAP2Ks. Nevertheless, this activated state is only transient in response to EGF and serum treatment, as ERK1/2 can be dephosphorylated by MAPK phosphatases (MKPs). In contrast, a hyperosmotic stimulus (sorbitol) results in sustained activation of ERK1/2, which is downregulated by ubiquitin/proteasome-mediated ERK1/2 protein degradation rather than by MKP activity. This illustrates that at least two different mechanisms downregulate MAPK activity after its initial activation: (1) dephosphorylation mediated by MKPs without a decrease in ERK1/2 protein levels upon mitogenic stimuli and (2) ubiquitination-mediated degradation of ERK1/2 without detectable dephosphorylation in response to hyperosmotic stimulation. Interestingly, ERK activity per se is not required for its own degradation since blocking its activation by inhibition of MEK does not block ERK degradation. Given that MEK1/2 can be activated by phosphorylation on serines 218 and 222 either by Raf or MEKK1, and that sorbitol-induced MEKK1-MEK-ERK but not mitogenically induced RAF-MEK-ERK activation involves protein degradation, MEK activation and the interaction between MEK and ERK are probably not important regulatory factors of ERK degradation [26].

MEKK1, like Raf, interacts with MEK via its catalytic domain. In addition, it also binds ERK2 through residues 370–559 in its N-terminal domain [21]. The ability of MEKK1 to interact with ERK and the fact that some RING domains possess E3 ubiquitin ligase activity suggested that the MEKK1 RING/PHD domain might play a role in ERK degradation in response to a hyperosmotic stimulus. Consistent with this idea, the MEKK1 RING/PHD domain exhibits ubiquitin ligase activity toward ERK both *in vivo* and *in vitro* [26]. In the presence of recombinant E1, E2 (Ubc4), and ubiquitin, the GST-MEKK1 RING/PHD fusion protein autoubiquitinates and

also ubiquitinates purified ERK2 protein. In addition, overexpression of MEKK1 in 293T cells, which by elevating MEKK1 activity mimics sorbitol treatment, results in enhanced polyubiquitination of ERK1. As expected, mutation of conserved cysteines in the RING/PHD domain significantly reduces MEKK1-mediated ubiquitination of ERK *in vivo* and *in vitro*. These studies provided the first demonstration that a protein kinase could also act as an E3 ligase and thereby be directly involved in the ubiquitination process. In addition to the requirement for an intact RING/PHD domain, MEKK1 kinase activity is also required for ubiquitination of ERK. The activation and autophosphorylation of MEKK1 may cause a conformational change that facilitates a productive association between ERK1/2 and an E2–ubiquitin conjugate, which might be aided by two overlapping ubiquitin-interacting motifs (UIM) in MEKK1 (residues 1166–1180 EEEALAIAMAMSASQ and 1168–1182 EALAIAMAMSASQVA, where the underlined residues are conserved in UIMs). Consistent with the idea that direct interaction of ERK with MEKK1 is important in ERK degradation, when the interaction between ERK1/2 and MEKK1 is abrogated by mutation of residues in ERK2 that are critical for binding docking motifs in substrates and regulators, sorbitol-induced ERK2 degradation is reduced. This degradation-resistant ERK2 mutant, which continues to be activated by sorbitol treatment, provides survival signals against sorbitol-induced cell apoptosis [26]. Therefore, MEKK1 functions not only as an upstream activator of MAPKs, such as ERK1/2 and JNK, through its kinase domain but also as an E3 ligase to provide a negative regulatory mechanism that decreases ERK1/2 activity [26]. The simultaneous activation of ERK1/2 survival signaling and JNK pro-apoptotic signaling provides cells with two paradoxical effects, which allow them to recover from transient stress stimuli. If, however, the stress stimulus persists for a prolonged period of time, the survival signal pathway is downregulated, allowing the cell to commit to an apoptotic response. Based on the observation that sorbitol-induced ERK1/2 ubiquitination was not dramatically reduced in MEKK1-deficient mouse embryo fibroblasts, it appears that MEKK1 may not be the only E3 involved in ERK1/2 ubiquitination and that an as yet unknown pathway results in the simultaneous activation of both MEK1/2 and ERK1/2 and ubiquitination of ERK1/2 [26].

MEKK1 not only has E3 ligase activity toward other substrates but can also autoubiquitinate *in vitro*. Consistent with this, overexpressed wild-type MEKK1, but not a RING/PHD domain cysteine mutant, polyubiquitinates itself, at least under conditions where exogenous ubiquitin is co-expressed [40]. Moreover, the polyubiquitinated MEKK1 accumulates in cells and is not significantly degraded by proteasomes, perhaps because non-Lys48 ubiquitin branches are generated. Instead, however, *in vivo* polyubiquitination of MEKK1 inhibits its ability to phosphorylate the MKK1 and MKK4 MAP2Ks *in vitro* and also to activate ERK1/2 and JNK *in vivo*, suggesting a novel role for ubiquitination in regulating kinase activity. Further investigation of how autoubiquitination inhibits the catalytic activity of MEKK1 and whether this requires the UIM, which might bind to the ubiquitin chains, and a demonstration that autoubiquitination affects the activity of endogenous MEKK1 are needed to elucidate the cellular function of MEKK1 autoubiquitination.

The finding that the MEKK1 RING/PHD domain has E3 ligase activity raises the issue of whether true PHD domains also have E3 ligase activity. Five other putative PHD domains – present in the Kaposi's sarcoma–associated herpesvirus MIR1 and MIR2 proteins, the murine gamma-herpesvirus MK3 protein, the poxvirus and murine gamma-herpesvirus M153R protein, and the cellular c-MIR protein – have been reported to have E3 ligase activity [3, 9, 11, 16, 29], but these domains have also been suggested to be more RING-like than PHD domain–like [1, 10, 34]. However, a recent report showed that the autoimmune regulator (AIRE) gene, whose mutation is responsible for the development of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), encodes a protein with two classic PHD domains. The first PHD domain of AIRE exhibits E3 ligase activity *in vitro*, and disease-causing missense mutations in the first PHD domain (C311Y and P326Q) abolish its E3 ligase activity [37]. This finding supports the idea that a subset of true PHD domains function as E3 ligases.

The prototypic MAP3K, Ste11p, mediates mating and high-osmolarity glycerol and filamentous growth responses in *Saccharomyces cerevisiae*. Ste11p phosphorylates and activates Ste7p (MAP2K), which in turn phosphorylates and activates two MAPKs, Fus3p and Kss1p (Figure 4.2B). Ste11p, Ste7p, Fus3p, and Kss1p exist in a complex with the Ste5p scaffold protein in the *S. cerevisiae* mating pathway [13]. Pheromone stimulation, but not hyperosmotic stress, results in the degradation of Ste11p through the ubiquitination–proteasome pathway [14]. However, Ste11p does not manifest E3 ligase activity, and although Ste5p, which assembles the kinase components of this pathway, has a RING finger domain that could in principle play a role in the ubiquitination of Ste11p, it now appears that Ste7p ubiquitination is mediated by an SCF E3 ligase complex, which recognizes Ste7p once it is phosphorylated by Ste11p [39].

4.4 Conclusions

MEKK1 is probably not the only protein kinase directly involved in the ubiquitination process. MEKK2–4, which are the MAP3Ks most closely related to MEKK1, do not have a RING/PHD domain, but analyses of the human genome show that at least seven other protein kinases besides MEKK1 contain ubiquitin-binding domains [28]. Whether these protein kinases are also involved in protein ubiquitination and what role their kinase activity plays in these process are issues that will need to be addressed to better understand their unique cellular function.

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5
Proteasome Activators

Andreas Förster and Christopher P. Hill

Abstract

In this chapter we discuss the possible roles and mechanisms of protein complexes that bind and stimulate 20S proteasomes, the primary proteases of the cytosol and nucleus of eukaryotic cells. We review structural and biochemical studies of 11S/PA28 activators and PA200, two protein complexes that are known to activate proteasomes. Our discussion of biological functions will be brief, since these are currently quite speculative and have been addressed elsewhere (Rechsteiner and Hill 2005). Instead our focus will be on structural studies and biochemical mechanisms. We start by briefly reviewing salient features of 20S proteasome architecture and mechanism. We will emphasize the role of N-terminal residues of 20S proteasome α subunits in restricting substrate access and their activator-induced reorganization to an open conformation. We do not discuss the 19S/PA700 activator in detail, since this topic has been discussed in this series (DeMartino and C. Wojcik 2005). Nor do we address reports of protein inhibitors of the 20S proteasome, since these have been discussed elsewhere (Rechsteiner and Hill 2005).

5.1
Introduction

5.1.1
20S Proteasomes

20S proteasomes are abundant proteases in all eukaryotic cells examined, where they are found in the cytosol and nucleus, and appear to perform the majority of proteolysis that occurs in these compartments (Coux et al. 1996). Many proteins have been identified as proteasome substrates, generally as polyubiquitylated substrates of the 26S proteasome, which is comprised of the 20S proteasome and 19S/PA700 (Pickart and Cohen 2004). Substrates include short-lived regulatory proteins (Hershko and Ciechanover 1998) and proteins that are damaged, denatured, or misfolded (Goldberg 2003). Given the fundamental importance of protein turnover,

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it is not surprising that 20S proteasomes are essential in yeast (Emori et al. 1991; Heinemeyer et al. 1994; Velichutina et al. 2004). 20S proteasomes are also found in archaea and in a few prokaryotes (De Mot et al. 1999), although prokaryotes generally make use of mechanistically related but distinct protein complexes (Gottesman 2003).

20S proteasomes are barrel-shaped structures comprised of four rings that each contain seven subunits, with α subunits forming the two end rings and β subunits forming the two central rings (Figure 5.1). For reviews of the structural studies, see Baumeister et al. (1998), Groll and Clausen (2003), and Groll and Huber (2003). Whereas archaeal 20S proteasomes are built from multiple copies of identical α and β subunits, eukaryotic proteasomes have seven different α subunits (α1–α7) and seven different β subunits (β1–β7), with each subunit occupying a precise location in the appropriate ring.

The mechanism by which 20S proteasomes avoid indiscriminate degradation of folded proteins was explained by the crystal structure of the proteasome from *T. acidophilum* (Löwe et al. 1995) and later confirmed with crystal structures of the 20S proteasomes from yeast (Groll et al. 1997) and cow (Unno et al. 2002). The proteolytically active sites (Seemüller et al. 1995) are sequestered within the central catalytic chamber formed by the β subunits. Access to the proteasome interior is through a pore in the middle of the α-subunit ring that permits passage of unfolded substrates (Wenzel and Baumeister 1995). This aperture (α-annulus; green in Figure 5.1) is defined by the main-chain atoms of the short loops in the middle of the α-subunit sequences and appears to have a fixed diameter of ~17 Å between atomic nuclei.

A wealth of structural data on proteasome–inhibitor complexes has illuminated the mechanism of proteolysis at the active sites, which are located at the N-termini of some (eukaryotes) or all (archaea) proteasome β subunits (Groll and Clausen 2003; Seemüller et al. 1995). The 20S proteasome active sites are fully formed in the unliganded proteasome. The naturally repressed state of isolated 20S proteasomes results, therefore, entirely from sequestration of the active sites within the hollow structure, with the α-annulus preventing entrance of folded proteins and a closed-gate structure (see next section) blocking smaller substrates. This mechanism for preventing hydrolysis of inappropriate substrates is in marked contrast to the analogous bacterial HslV protease, for which binding of the HslU activator induces formation of an active conformation at the proteolytic active sites (Ramachandran et al. 2002; Sousa et al. 2002; Sousa et al. 2000; Wang et al. 2001).

5.1.2 The 20S Proteasome Gate

Eukaryotic 20S proteasomes seal their entrance/exit port through the α-annulus by a gate structure formed by the N-terminal residues of their α subunits (Groll et al. 1997; Unno et al. 2002) (Figures 5.1c and 5.1d). In particular, the N-terminal residues of subunits α2, α3, and α4 adopt unique ordered conformations that are stabi-

lized by an extensive network of hydrogen-bonding and van der Waals interactions. The other four subunits make less extensive contributions to the closed conformation. Rather than crossing the central gate area, their N-terminal residues project away from the proteasome surface. This asymmetric arrangement results from the unique amino acid sequences of the proteasome α-subunit N-termini, which are well conserved between equivalent subunits of different species but differ significantly between paralogous subunits. In pairwise comparisons between equivalent yeast and human α subunits, the residues prior to residue 13 (archaeal *T. acidophilum* proteasome numbering) are 56–100% identical between species. In contrast, only Tyr8 and Asp9 are highly conserved between different subunits of the same species.

In contrast to the ordered closed gate of eukaryotic proteasomes, the N-terminal 12 residues of isolated archaeal 20S proteasomes from *T. acidophilum* (Löwe et al. 1995) and *Archaeoglobus fulgidus* (Groll et al. 2003a) are disordered and presumably flexible (Löwe et al. 1995). The inability of archaeal 20S proteasomes to form the ordered, closed-gate conformation is explained by the symmetric configuration in which all seven α subunits have the same sequences and therefore are unable to form the asymmetric closed state. In contrast to the eukaryotic enzymes, the archaeal 20S proteasome degrades small peptides efficiently, since they are apparently able to diffuse through the ''curtain'' of flexible α-subunit N-termini with little hindrance. The flexible tails do, however, provide a significant barrier to passage of unfolded protein substrates, since a variant in which the N-terminal tails have been deleted degrades unfolded proteins, whereas the wild-type *T. acidophilum* 20S proteasome does not (Benaroudj et al. 2003).

Although the two reported crystal structures of intact archaeal 20S proteasomes show the α-subunit N-terminal residues to be unstructured (Groll et al. 2003a; Löwe et al. 1995), there is one example in which these gate residues adopt an ordered, open conformation. The structure of an isolated ring of α subunits from the archaeon *A. fulgidus* reveals a conformation essentially identical to that observed for the yeast 20S proteasome in complex with the activator PA26 (Förster et al. 2003), which is discussed below. The original motivation for determining the α-subunit ring structure was to understand the process of proteasome assembly (Groll et al. 2003a). However, because of the similarity to the activator complex and the apparent absence of a role for the open conformation in assembly, we favor the possibility that crystallization here fortuitously captured a conformation that is functionally important but not highly populated in the absence of an activator. As discussed below, formation of this ordered, open conformation appears to be important for efficient entry and degradation of protein substrates.

It is not entirely clear why eukaryotic 20S proteasomes require an ordered, closed-gate structure, since the α-annulus and flexible N-terminal residues of archaeal 20S proteasomes are sufficient to restrict passage of folded protein substrates. Indeed, the closed-gate conformation does not appear to be important for logarithmic growth of yeast under favorable conditions, although a defect in release from the stationary phase is revealed in yeast when the gate is disrupted by mutagenesis

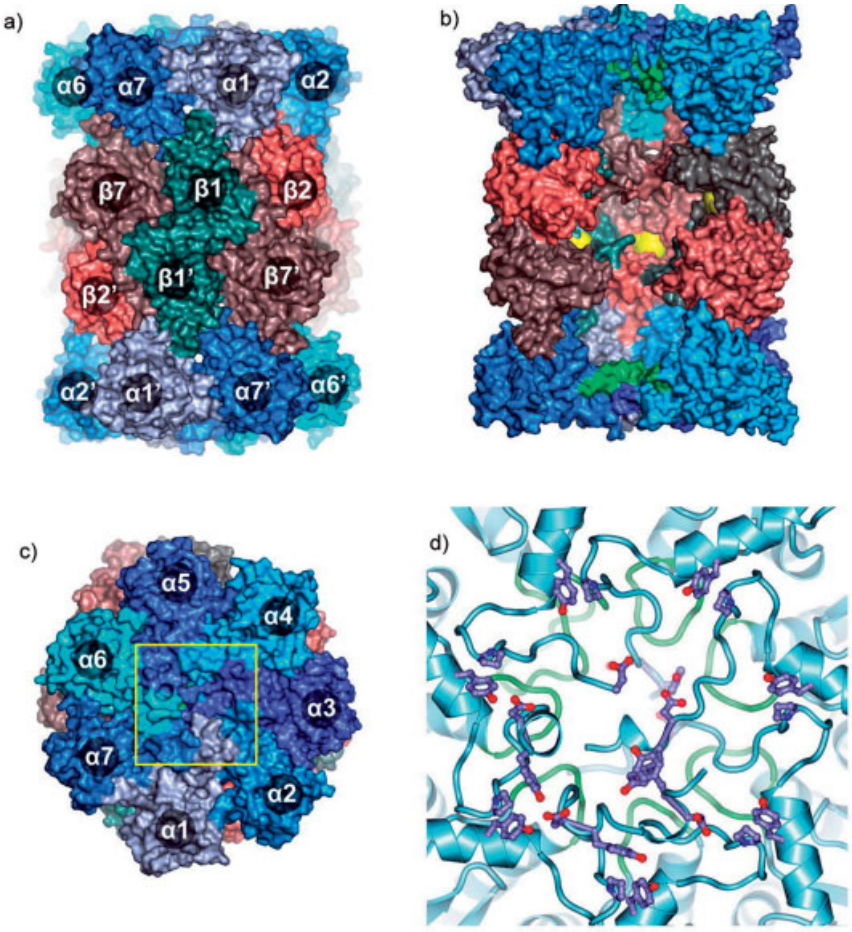

Fig. 5.1. Structure of the yeast 20S proteasome (Groll et al. 1997). (a) Space-filling representation, side view. (b) Same as panel a, with subunits closest to the viewer removed to reveal the hollow interior and proteolytic active sites (yellow). The α-annulus is colored green. (c) Same as panel a, top view. (d) Ribbon representation showing the central boxed region of panel c. Tyr8, Asp9, Pro17, and Tyr26 side chains from each α subunit are shown explicitly. These residues form clusters that stabilize the open conformation (Förster et al. 2003) (Figure 5.5).

(Bajorek et al. 2003). This mutant proteasome also displayed an accelerated rate of protein turnover *in vitro* and *in vivo*. One possibility is that eukaryotes contain more natively unfolded proteins or functionally important oligopeptides that are able to pass a flexible gate. It is clear, however, that disruption of the stable gate structure is necessary for proteolysis to occur, and that even the flexible gate of archaeal proteasomes provides a significant barrier to passage of protein substrates.

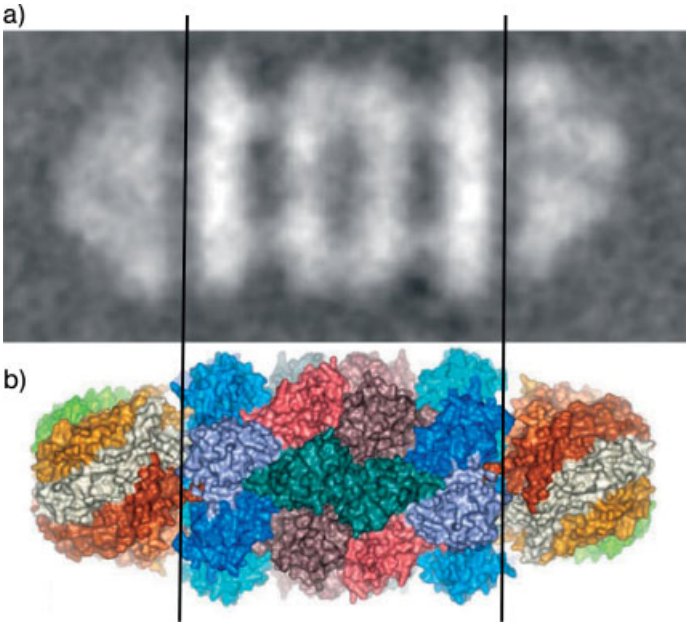

Fig. 5.2. Structure of proteasome–activator complexes. (a) Averaged negative-stain electron micro-graph of bovine 20S proteasome–PA200 complex (A. Steven and J. Ortega, personal communication). (b) Crystal structure of yeast 20S proteasome in complex with *T. brucei* PA26 (yellow) (Förster et al. 2003).

5.1.3 Proteasome Activators

Proteasomes are activated by protein complexes that bind to one or both rings of α subunits (Figure 5.2). The best known of these activators is the 19S activator, also known as proteasome activator MW 700 (PA700) and regulatory complex (RC). PA700 has a well-defined biological role, namely, the degradation of polyubiquitylated protein substrates. It is a remarkable machine, comprised of at least 17 stoichiometric subunits (Glickman et al. 1998) and a number of other transient or weakly associated components (Leggett et al. 2002; Verma et al. 2000). It contains subunits that recognize polyubiquitin chains, edit the chains, remove chains from substrates, unfold the substrate, open the proteasome gate, and translocate substrate into the 20S proteasome interior for degradation. We do not discuss 19S further here, since it is the focus of the chapter in this series by George DeMartino and Cezary Wojcik. Rather, our focus is on the other characterized activators, 11S (also called PA28, REG, PA26; reviewed in DeMartino and Slaughter 1999, Hill et al. 2002, and Kuehn and Dahlmann 1997) and PA200 (Ustrell et al. 2002). Unlike 19S, 11S and PA200 do not recognize ubiquitin or utilize ATP and have unknown *in vivo* substrates but, at least in the case of 11S, are better understood from a biochemical and structural perspective.

5.2
11S Activators: Sequence and Structure

5.2.1
Amino Acid Sequences

Members of the 11S family were first identified as protein complexes that stimulate the peptidase activity of 20S proteasomes (Dubiel et al. 1992; Ma et al. 1992). The three homologues of higher eukaryotes, PA28α, -β, and -γ (REGα, -β, and -γ) each have a subunit mass of ~28 kDa and share 35–50% sequence identity in pairwise comparisons. More primitive species than jawed vertebrates appear to have only one 11S activator, which is most closely related to PA28γ, and yeasts and plants appear to lack an 11S homologue (Masson et al. 2001; Paesen and Nuttall 1996; Murray et al. 2000). Sequence analyses indicate that duplication and divergence of the PA28γ gene produced the PA28α gene, which duplicated in turn to produce PA28β (Kim et al. 2003). A very distantly related homologue, PA26, has been identified in *Trypanosoma brucei* and found to share only ~14% identity with other 11S activators (Yao et al. 1999).

5.2.2
Oligomeric State

Following some initial confusion, 11S activators are now known to be assembled as ~200-kDa heptamers (Johnston et al. 1997; Knowlton et al. 1997; Li et al. 2000, 2001a; Yao et al. 1999; Zhang et al. 1999) (see Hill et al. 2002 for a full discussion). This is an important point because, as discussed later, structural studies have shown that the sevenfold assembly is central to the mechanism of binding and gate opening by 11S (Förster et al. 2003; Whitby et al. 2000). Whereas PA28γ forms a homoheptamer (Li et al. 2001a; Realini et al. 1997; Tanahashi et al. 1997), PA28α and PA28β preferentially assemble as hetero-oligomers with a stochastic distribution of α and β subunits (Zhang et al. 1999). Consistent with this, PA28α and PA28β copurify as a single complex from tissues (Kuehn and Dahlmann 1996a, 1996b, 1997; Mott et al. 1994). PA28α and PA28β can also each assemble into functional heptamers, although PA28β is monomeric and inactive at low concentrations (Realini et al. 1997; Song et al. 1997; Wilk et al. 2000a; Zhang et al. 1998b). The distant relative from *T. brucei*, PA26, is also known to be heptameric (Whitby et al. 2000; Yao et al. 1999).

5.2.3
PA28α Crystal Structure

The crystal structure of human PA28α (Figure 5.3) revealed that each subunit forms an elongated bundle of four helices and that subunits assemble to form a doughnut-shaped heptamer that has a central channel of 20–30 Å diameter (be-

tween atomic nuclei) (Knowlton et al. 1997). The base of the heptamer provides a sevenfold symmetric array of two functionally important motifs; the C-terminal tails, which are important for proteasome binding (Li et al. 2000; Ma et al. 1993; Song et al. 1997), and the activation loops, which are required to stimulate the proteasome's peptidase activity (Zhang et al. 1998a). The C-terminal eight residues of human PA28α are disordered and apparently flexible in the crystal structure. As described below, these residues provide a flexible tether that becomes partially ordered upon binding proteasome.

5.2.4 Activation Loop

The activation loop was identified from a random mutagenesis screen as a segment of nine residues, located in the turn between helices 2 and 3, that is important for proteasome activation (Zhang et al. 1998a). Interestingly, mutations in this region have been identified that bind 20S proteasome with the same affinity as wild type yet fail to stimulate the peptidase activity, thereby indicating that binding and activation are to some extent separable. As discussed below, PA26–proteasome complex crystal structures have revealed contacts with the activation loops that explain how PA26 opens the proteasome entrance/exit gate. Remarkably, however, whereas residues of the activation loop are almost universally conserved among 11S activators, PA26 is an exception that has very different residues in this functionally important part of the structure. A structure-based alignment of PA28α and PA26 sequences is shown in Figure 5.4. The basis for how the different activation loops might stabilize the same activated proteasome conformation is discussed below.

5.2.5 Homologue-specific Inserts

The sequences of PA28α, -β, and -γ are primarily distinguished by 15–30 residue segments known as "homologue-specific inserts" (Song et al. 1997; Zhang et al. 1998a, 1998c). As shown by the structure of human PA28α (Knowlton et al. 1997) (Figure 5.3), the PA28α homologue-specific insert is disordered but located at the end of the activator distant from the proteasome-binding surface. The PA28α insert sequences are rich in lysine and glutamate residues and define the so-called KEKE motif, which has been suggested to mediate protein–protein interactions (Realini et al. 1994). The PA28β insert has a similar amino acid composition but is shorter, whereas the PA28γ insert has a mixed composition with a larger number of hydrophobic residues. Notably, PA26 lacks homologue-specific insert sequences but possesses tight turns of just a few ordered residues between helices 1 and 2 (Whitby et al. 2000). One attractive possibility is that the homologue-specific inserts function by binding specific partner(s), although no such partners have been convincingly demonstrated to date.

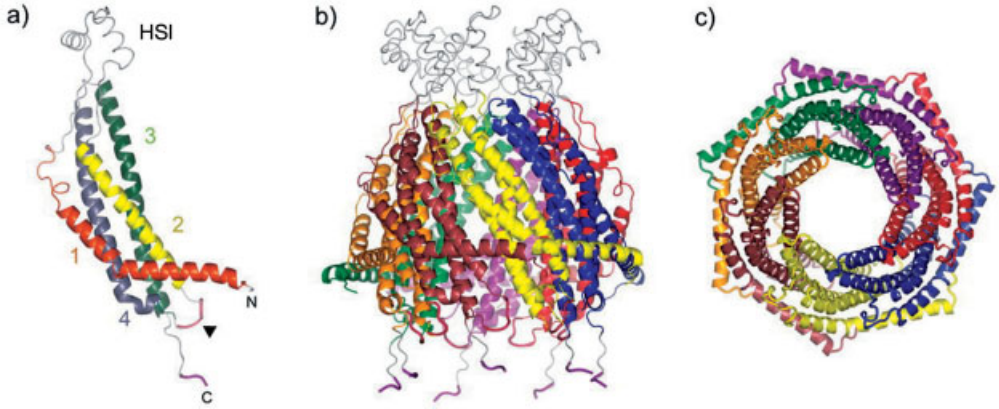

Fig. 5.3. PA28α crystal structure (Knowlton et al. 1997). (a) Structure of an isolated subunit. N- and C-termini are labeled. The homologue-specific insert (HSI) is disordered in the structure and is included here in an arbitrary conformation. The activation loop is colored red and indicated with a triangle. The C-terminal tails, which are disordered in the isolated PA28α structure, have been included in the conformation observed for a high-resolution archaeal 20S proteasome–PA26 structure (Förster and Hill, unpublished), with residues that contact proteasome colored magenta. (b) Side view of the PA28α heptamer colored by subunit. Activation loops and C-terminal tails are colored as in panel a. (c) Top view of panel b. For clarity, the disordered HSI has been omitted.

5.3 PA26–Proteasome Complex Structures

The mechanism of proteasome activation by 11S activators has been explained, in part, by the crystal structure (Förster et al. 2003; Whitby et al. 2000) of a complex

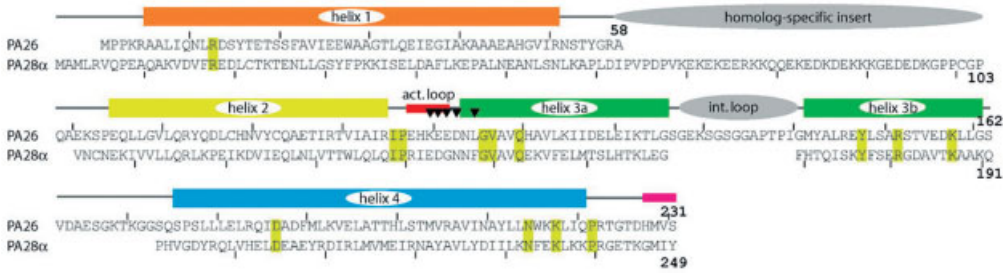

Fig. 5.4. Structure-based sequence alignment of PA26 and PA28α. The structural alignment is clear for residues from the beginning of helix 2 through to the C-terminus, but is ambiguous for helix 1. Residue identities that are conserved in PA26 and all three of the human PA28 homologues ($\alpha\beta\gamma$) are shown on a yellow background. Residues of the PA26 activation loops that are within Van der Waals contact distance of a proteasome atom are indicated with black triangles. Residues at the PA26 C-terminus that contact the proteasome are indicated with a purple box.

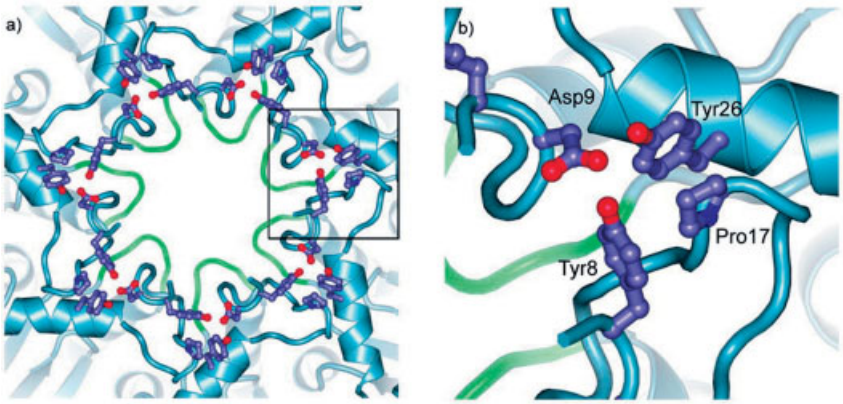

Fig. 5.5. Open conformation of yeast 20S proteasome formed in complex with PA26 (Förster et al. 2003). (a) Same view as closed conformation of Figure 5.1d. (b) Close-up of the cluster of invariant residues (Tyr8, Asp9, Pro17, Tyr26) boxed in panel a.

between the yeast 20S proteasome and PA26 (Figure 5.2b). Binding of PA26 induces the entrance/exit gate that is closed in isolated yeast 20S proteasomes to adopt an open conformation (Figure 5.5). This allows peptide substrates to diffuse freely into the proteasome interior. It is important to note that the structure was solved for a highly non-cognate complex, especially considering that yeast do not appear to posses 11S activators. Nevertheless, 11S activates seem to stimulate essentially any 20S proteasome, regardless of source. For example, PA26 activates 20S proteasome from rat (Yao et al. 1999) and yeast (Whitby et al. 2000), human PA28α activates proteasome from cow (Eugene Masters, personal communication) and yeast (Martin Rechsteiner, personal communication), and cow PA28 activates 20S proteasome from lobster (Mykles 1996). It therefore seems likely that the yeast 20S proteasome–PA26 complex reveals conformational changes that underlie activation of cognate complexes. The structural analysis has recently been advanced by determination of complexes between PA26 and an archaeal 20S proteasome that diffract to relatively high resolution (Förster and Hill, unpublished).

5.3.1 Binding

The mechanisms of binding and activation depend in large part upon the symmetry of the PA26 heptamer. PA26, like human PA28α (Knowlton et al. 1997), is exactly sevenfold symmetric. As indicated by the earlier biochemical observations (Li et al. 2000; Song et al. 1997), binding is mediated by the C-terminal tails of PA28 subunits, which project into pockets that are formed between neighboring α subunits on the 20S proteasome surface. The exact match in spacing of the seven PA26 C-terminal tails with the seven pockets on the proteasome surface explains

how the individual interactions, which are probably quite weak, can sum to provide a significant binding affinity.

Details of this interaction were obscure in the medium-resolution yeast 20S proteasome complex. Recently, a structure of a complex between PA26 and an archaeal 20S proteasome (Förster and Hill, unpublished) has revealed that the interaction is largely mediated by main chain–main chain contacts. This explains why the different 11S homologues can all bind the same proteasome and why most 11S activators bind 20S proteasomes from most species, even though the 11S C-terminal residues are highly variable between the homologues. It also explains why activation is tolerant of many mutations in the C-terminal residues of PA28 (Song et al. 1997).

5.3.2 Symmetry Mismatch Mechanism of Gate Opening

The PA26–proteasome complex structure revealed a symmetry-mismatch mechanism of gate opening. As illustrated in Figure 5.1d, the closed-gate structure of unbound proteasomes is asymmetric, with the N-terminal residues of subunits α2, α3, and α4 adopting unique, ordered conformations that make a large number of specific hydrogen-bonding and van der Waals interactions. As the C-terminal residues of the seven PA26 subunits bind into the appropriately spaced pockets between each of the seven proteasome α subunits, the symmetric surface of activation loops is pressed against the reverse turns containing Pro17 of proteasome α subunits. This induces the proteasome to follow the symmetry of PA26 by moving Pro17 of individual subunits by as much as 2.5 Å. This displacement in a subset of the proteasome Pro17 turns appears to be the trigger that leads to gate opening.

Repositioning of the Pro17 turns appears to induce gate opening for two reasons. Firstly, consequent displacement of more N-terminal residues disrupts the many van der Waals and hydrogen-bonding interactions formed by the N-terminal residues of subunits α2, α3, and α4. This destabilization explains why the closed-gate conformation is no longer maintained, although it does not explain why a specific open-gate conformation is assumed rather than the disordered state observed in crystal structures of isolated archaeal proteasomes. As described below, the second contribution to gate opening provided by displacement of Pro17 turns is to allow formation of stabilizing interactions between conserved proteasome residues.

It is remarkable that only one of the PA26 activation loop residues, Glu102, appears to make significant contact with the proteasome. Surprisingly, this residue is not present in any of the other known 11S activators. The equivalent residue in all PA28 sequences is an invariant glycine. It is not obvious how this Gly145 (PA28α numbering) would contact 20S proteasome to reposition the Pro17 turn. One possibility, suggested from the structural overlap of PA26 and PA28α (Figure 5.6), is that the previous residue, Asp144 of PA28α, which is also invariant among PA28 sequences, might be functionally equivalent to PA26 Glu102. This unusual shift of a functionally critical residue along an amino acid sequence might explain why the Cα trace of the activation loops is relatively divergent between PA28α and PA26.

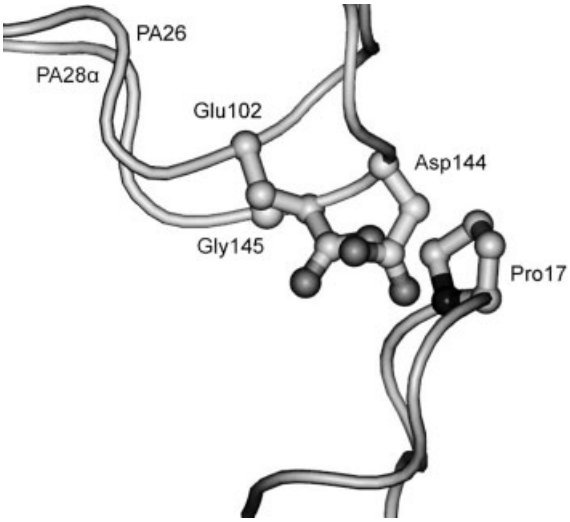

Fig. 5.6. Superposition of PA26 and PA28α activation loops after global overlap. The primary activating contact to proteasome is through Glu102 of PA26. Based simply on structural overlap, the equivalent residue in PA28α is Gly145. We speculate that the adjacent residue, Asp144, might mediate contacts equivalent to those of PA26 Glu102. The Asp144 conformation shown here is a preferred rotamer that differs from the rotamer seen in the crystal structure of isolated PA28α. No other adjustments were made to the crystal structures for these figures.

(Following global overlap of the PA28α and PA26 structures, the six residues N- and C-terminal to the activation loop have equivalent Cα atoms separated by an average of less than 1.0 Å, whereas activation loop (PA26 99–104) Cα atoms show an average deviation of 2.0 Å in this structural alignment.) Resolution of this point will require determination of a crystal structure of 20S proteasome bound to a PA28 activator.

5.3.3
Open-gate Stabilization by Conserved Proteasome Residues

Because of the limited resolution of the yeast 20S proteasome–PA26 complex, it was initially not appreciated that the proteasome's open conformation is ordered, rather than being comprised of disordered α-subunit N-terminal residues (Whitby et al. 2000). Closer inspection revealed that the open conformation is in fact ordered and appears to be stabilized by interactions between four highly conserved proteasome residues; Tyr8, Asp9, Pro17, and Try26 (Förster et al. 2003). Although these residues do not make direct contacts with the activator, they are allowed to form stabilizing clusters between adjacent α subunits mediated by the repositioning of Pro17 induced by PA26 (Figure 5.5). The importance of these residues for stabilizing the symmetric, open conformation explains why they are conserved be-

tween the different α subunits, whereas other residues N-terminal to residue 15 are variable between subunits.

The four cluster residues are not absolutely conserved between all proteasome α subunits. The few deviations from perfect conservation seen for these cluster residues are limited to the cluster between α1 and α2 (based on sequences of man, rat, mouse, *Arabidopsis*, fruit fly, worm, and fission and baker's yeasts). This cluster, which we call the non-canonical cluster, has a slightly different structure from the other six. In particular, residue 9 of α2 is always a serine or smaller side chain, rather than the invariant aspartate of the other α subunits. This substitution is explained by the requirement that the adjacent residue 10 invariably be a phenylalanine for subunit α2, whereas it is never a phenylalanine for the other subunits. Phe10 of α2 occupies a critical buried position in the closed conformation, and its location in the open conformation appears to be incompatible with an aspartate at position 9. As described more fully in Förster et al. (2003), these two substitutions in α2 explain the occasional additional substitution at position 8 of α1 and position 26 of α2.

The model that the cluster residues are required for formation of the ordered, open conformation is supported by the recently determined structures of PA26 with wild-type and mutant (Asp9Ser) archaeal proteasome (Förster and Hill, unpublished). These structures have been refined against ~2-Å data. The wild-type archaeal 20S proteasome complex shows the same ordered, open conformation as seen for the yeast 20S proteasome complex. In contrast, α subunits in the Asp9Ser mutant complex are disordered before residue 12. This indicates that whereas eukaryotic proteasomes can accommodate one non-cognate cluster, the open conformation is not stable when all seven α subunits substitute the aspartate at position 9 for a serine.

5.3.4 Do Other Activators Induce the Same Open Conformation?

Not only are the four cluster residues conserved in the different subunits of the yeast proteasome, they are also conserved in all known proteasome sequences, including eukaryotes from yeast to human and 18 archaeal species. It was initially surprising to realize that conservation of Tyr8, Asp9, Pro17, and Tyr26 extends to species, such as yeast and archaea, that do not appear to possess 11S activators. This observation implies that these residues are important for a function that does not involve 11S activators. Our preferred explanation is that proteasomes have just one open conformation and that different activators, such as 19S, function, in part, by inducing the same open conformation as seen in the PA26–proteasome crystal structure.

Support for this hypothesis has been obtained using a mutagenic/biochemical approach with an archaeal 20S proteasome and PAN, an archaeal analogue of the 19S activator. In this study (Förster et al. 2003), degradation of PAN-dependent model substrates was impaired in a number of mutant proteasomes that had

disruptions in the cluster residues. In particular, the proteasome in which Tyr8 and Asp9 were both mutated to glycine was inactive in this assay. This is especially noteworthy because the model that activators induce a disordered gate structure would predict that this mutant, with inherently much more flexible amino-terminal tails, would be even more active than wild type. The observed impaired activity against protein substrates supports the hypothesis that these residues adopt an ordered, open conformation, which is necessary for efficient degradation of protein substrates.

Further indication that proteasomes have an inherent ability to adopt this open conformation, independent of binding by an 11S activator, is demonstrated by observation of the open conformation in an isolated ring of α subunits from the archaeon *Archaeoglobus fulgidus* (Groll et al. 2003b). Based upon the available structural and biochemical data, we find the hypothesis that other activators induce the same open conformation attractive, although resolution of this point will require structure determination of other proteasome–activator complexes.

5.3.5
Differential Stimulation of Proteasome Peptidase Activities

20S proteasome active sites have been characterized by their ability to stimulate hydrolysis of small fluorogenic peptides. The three distinct active sites at the N-termini of β1, β2, and β5 are referred to, respectively, as peptidyl-glutamyl peptide hydrolytic (PGPH), trypsin-like, and chymotrypsin-like because they preferentially cleave following acidic, basic, and hydrophobic residues (see Bochtler et al. 1999 and references therein). Although other determinants are also important for specificity (Bogyo et al. 1998; Groll et al. 2002; Harris et al. 2001; Wang et al. 2003), the simplest interpretation of the structural data is that stimulation of proteasome peptidase activity by 11S activators results simply from opening of the gate, since no conformational changes are evident at the active sites in the β subunits. Presumably, therefore, the different extents to which hydrolysis of different peptide substrates is enhanced results from the relative rates at which the peptides can pass through the activator channel and into the proteasomes antechamber and catalytic chamber.

Although the structural data support the simple model outlined above, another possibility has been suggested by studies on recombinant human PA28γ (Li et al. 2001a). Whereas PA28α stimulates the hydrolysis of all three fluorogenic peptide substrates that are diagnostic for hydrolysis at the three distinct 20S proteasome active sites, PA28γ stimulates hydrolysis of the ''trypsin site'' peptide but not of the chymotrypsin or PGPH peptides. This difference in specificity exists despite identical activation loop sequences for PA28α and PA28γ, and substitution of residues close to the activation loop does not alter specificities (Li et al. 2000). Curiously, substitution of PA28γ Lys188 with either Asp or Glu changes the specificity of PA28γ to that of PA28α (Li et al. 2001a). Since Lys188 is thought, based upon homology modeling with the PA28α structure, to face the central channel at the

end distant from the proteasome, one possibility is that this residue performs a gating function by restricting passage of the chymotrypsin and PGPH substrates. This explanation is challenged, however, by the observation that positively charged substrates are processed rapidly when residue 188 is lysine, whereas these are the type of substrates that are expected to be excluded by a simple gating mode. Li et al. (2001a) therefore proposed that binding of PA28γ not only opens the substrate entrance gate but also induces long-range conformational changes that repress activity at the chymotryptic and PGPH active sites. Lys188Asp/Glu PA28α forms less stable heptamers, and Li et al. (2001a) proposed this mutant to be more flexible than PA28γ and therefore unable to impose the same conformational changes on the proteasome. PA28γ has also been shown by a second group to activate all three active sites (Wilk et al. 2000b). In that case, however, protein purification included ammonium sulfate precipitation, which yielded PA28γ much less stable than activator purified the traditional way (Gao et al. 2004). Reconciliation of these observations with the currently available structural data will require further study. In particular, it will be important to determine structures at higher resolution and for cognate complexes with mammalian proteasomes and activators.

5.3.6 Hybrid Proteasomes

Immune precipitation with monoclonal antibodies specific for 19S or PA28 demonstrated that both of these activators are present in the same complex, which was presumed to be comprised of 19S and P28 complexes bound to the rings of α subunits at opposite ends of the same 20S proteasome (Hendil et al. 1998). Analysis of HeLa cell extracts determined that these ''hybrid'' proteasomes account for about a fourth of all proteasomes and that induction of PA28 expression and hybrid proteasome formation with γINF appreciably enhanced degradation of a PA700-dependent substrate (Tanahashi et al. 2000).

Two studies have reported reconstitution of hybrid proteasomes from singly capped 19S–20S complexes and recombinant PA28. Kopp et al. (2001) reported that hybrid proteasomes have peptidase activities similar to those of singly capped PA28 or 19S complexes, and also reported negative-stain electron micrographs. The expected complex is formed from its components without apparent structural distortion. Cascio et al. (2002) performed a similar analysis and showed similar electron micrographs. They found enhanced hydrolysis of small peptides in hybrid proteasomes, but no significant acceleration of protein breakdown. They also demonstrated that hybrid proteasomes generate a pattern of peptide products different from those generated by 26S proteasomes, without altering mean product length. This observation suggests that the change in peptides produced accounts for the capacity of PA28 to enhance antigen presentation and argues against the proposal of Whitby et al. (2000) that binding of PA28 facilitates release of larger product peptides.

5.4 Biological Roles of 11S Activators

Our discussion of *in vivo* function will be brief because the biological roles of 11S activators are not yet precisely defined and because potential roles have been recently discussed at more length elsewhere (Rechsteiner and Hill 2005). Several observations suggest that PA28α and PA28β function in the immune system (reviewed in Rechsteiner et al. 2000). These activators appear to have arisen during evolution at roughly the same time as vertebrate cellular immunity. They are particularly enriched in immune tissues and virtually absent in brain. Finally, PA28$\alpha\beta$, but not PA28γ, is induced by interferon-γ and infection (Khan et al. 2001; Maksymowych et al. 1998; Tanahashi et al. 1997), and the presence of PA28$\alpha\beta$ influences production of some class I epitopes. Evidence of the involvement of PA28$\alpha\beta$ in antigen presentation is reviewed in Kloetzel and Ossendorp (2004), Rechsteiner et al. (2000), and Rock et al. (2002).

The role of PA28$\alpha\beta$ in antigen presentation is confused by the contradictory findings of two independent knockout mouse studies. Preckel et al. (1999) found a general impairment in CTL responses and concluded that that PA28 functions in immunoproteasome assembly. (Immunoproteasomes are the same as constitutive 20S proteasomes except that the three catalytic subunits are replaced with inducible counterparts [Rock and Goldberg 1999]). In contrast, Murata et al. (2001) found a phenotype that was almost wild type, including normal immunoproteasome assembly, although these mice were unable to process a specific epitope. Also arguing against the immunoproteasome assembly model is the finding that upregulation of immunoproteasome subunits occurs early in dendritic cell maturation, whereas PA28$\alpha\beta$ subunits are expressed later (Li et al. 2001b). Thus, although PA28$\alpha\beta$ appears to function in antigen presentation, the mechanistic basis for this activity is currently unknown. Alternative proposed mechanisms include substrate channeling, facilitating release of longer products (Whitby et al. 2000), and alteration of cleavage sites (Murata et al. 2001). Currently, the importance of these possible mechanisms is unclear. Several non-immune functions of PA28$\alpha\beta$ have also been proposed (Rechsteiner and Hill 2005), but these also lack validation and a convincing mechanism.

The biological role of PA28γ is even more obscure than that of PA28$\alpha\beta$. The knockout mice have mild phenotypes (Murata et al. 1999), including defects in processing some specific antigens (Barton et al. 2004). It is likely, however, that PA28γ has at least one important role that does not involve antigen presentation by MHC class I molecules, since simple eukaryotes that lack an MHC class I system generally contain a single PA28 molecule that is most closely related to the γ homologue of higher eukaryotes. Unlike PA28$\alpha\beta$, which most reports describe as being mainly cytoplasmic, PA28γ is largely confined to the nucleus (Soza et al. 1997; Wojcik 1999; Wojcik et al. 1998). Also, whereas PA28$\alpha\beta$ is most heavily expressed in immune tissues such as spleen, PA28γ is most heavily expressed in brain. One possibility is that PA28γ functions in apoptosis and/or cell-cycle pro-

gression (Masson et al. 2003). The physiological role of PA26 in *T. brucei* is unknown.

5.5 PA200/Blm10p

PA200 is a large nuclear protein that stimulates the proteasome's peptidase activity (Ustrell et al. 2002). It is a single-chain activator with a molecular weight of ~200 kDa, which is similar to that of the PA28 heptamer. Like PA28, bovine PA200 stimulates proteasomal hydrolysis of peptides, but not proteins, and it does not utilize ATP. The likely homologue of PA200 in yeast is Blm10p, (previously known as Blm3p, Doherty et al. 2004) with which PA200 shares just 13% sequence identity. Blm10p is also a nuclear protein, although, surprisingly, Blm10p does not appear to stimulate the 20S proteasome's peptidase activity (Fehlker et al. 2003).

It was suggested that PA200 functions in DNA repair because gamma irradiation of HeLa cells resulted in alteration of the usual uniform nuclear distribution of PA200 to a punctate pattern, a behavior characteristic of many DNA-repair proteins (Ustrell et al. 2002). Further support for this proposal was provided by earlier work that described Blm10p as complementing the bleomycin hypersensitivity of the blm3-1 mutation, thereby indicating a role in DNA repair (Febres et al. 2001; Moore 1991). This proposal has been weakened, however, by a subsequent analysis that concluded that Blm10p does not in fact contribute to bleomycin resistance in yeast (Aouida et al. 2004).

An alternative function for Blm10p in the assembly of nuclear proteasomes has been suggested based upon the finding that Blm10p associated with nascent proteasomes (Fehlker et al. 2003). Because the Blm10p-deleted strain was found to display an increased rate of processing of a proteasome subunit precursor, it was proposed that Blm10p functions to regulate late stages of proteasome assembly and maturation in the nucleus. Given the fundamental importance of proteasome maturation, however, it is surprising that the phenotypes associated with Blm10p deletion and overexpression are quite mild. At the current time we consider the functional role(s) of PA200/Blm10p to be an open question. The proposed roles in DNA repair and proteasome assembly require further clarification and support. Indeed, in view of their highly diverged amino acid sequences, it is possible that PA200 and Blm10p perform different *in vivo* roles.

Biochemical and structural analysis of PA200 activity is at a relatively preliminary stage (Figure 5.2). Based upon analysis of amino acid sequences, it has been concluded that PA200 is comprised of multiple HEAT/ARM repeats (Kajava et al. 2004). This implies that PA200 is comprised almost entirely of helices, a property shared with 11S activators, although the role of multiple PA28 C-termini in proteasome binding indicates that PA200 must bind via a different arrangement. Similarly, the amino acid sequence does not give obvious clues about the mechanism of gate opening. Because HEAT repeat proteins generally function in protein–protein interactions, it is attractive to speculate that PA200 forms hybrid protea-

somes and functions to localize 26S proteasome activity to specific intracellular locations.

5.6 Concluding Remarks and Future Challenges

In contrast to the 19S activator, whose biological role is well established, it is not clear what physiological roles are performed by 11S activators or by PA200. Numerous publications link PA28$\alpha\beta$ to production of peptide ligands for MHC class I molecules, although, arguably, definitive data are lacking. The two reports of knockout mice, which might have provided conclusive data on this point, are largely contradictory. PA28γ has been linked to apoptosis and cell-cycle progression, although a convincing direct connection is elusive. Studies of PA200/Blm10p are similarly inconclusive, and the true role of PA200/Blm10p is still an open question. We believe that PA28 and PA200 will be found to perform important biological functions, since evolution is unlikely to have preserved 200-kDa proteins/complexes that bind and activate 20S proteasomes unless they provide a functional advantage. It is an urgent priority for the field to firmly establish the physiological roles of these proteasome activators.

Biochemical analysis of PA28 and PA200 is generally more advanced than the biological studies. Important mechanistic questions for the future include whether activation results simply from opening of the entrance/exit gate. A possible role for channels through PA28 and PA200 in defining substrate preference through filtering mechanisms should be tested explicitly. Similarly, the possibility of long-range allosteric changes that selectively repress specific active sites needs to be confirmed or refuted.

Structural analysis of 11S activators (PA26) is relatively advanced. We know how they bind in a manner that is insensitive to a specific amino acid sequence. We also know how PA26 repositions the proteasome Pro17 turn, and thereby induces formation of the open-gate conformation. PA26 is only distantly related to other PA28 activators, however, with no residues conserved in the activation loop. Therefore, questions still remain about the mechanism of activation by other 11S activators, and it would be interesting to visualize the structure of a cognate mammalian PA28–proteasome complex. Obtaining higher-resolution structural information on PA200/Blm10p and 19S remains an important priority.

An attractive possibility is that PA28 and PA200 might primarily function in the context of hybrid proteasomes as adaptors that tether 20S–19S complexes to specific intracellular locations or substrate complexes. In this model, opening of the entrance/exit gate might be of some advantage, but the primary role would be to define a location or association. The idea that PA28 and PA200 are primarily adaptors, rather than activators, is quite general and has a number of possibilities. For example, mediating interactions with components of the ER have been suggested as a possible mechanism for delivery of product peptides to the TAP transporter and hence to nascent MHC class I molecules (Realini et al. 1994; Rechsteiner

et al. 2000). It is also conceivable that PA28 or PA200 interacts with substrate complexes or with chaperones that deliver substrates for degradation. There is still much to be resolved concerning the biology and biochemistry of proteasome activators.

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6
The Proteasome Portal and Regulation of Proteolysis

Monika Bajorek and Michael H. Glickman

Abstract

The proteolytic active sites of the 26S proteasome are sequestered within the central chamber of its 20S catalytic core particle. Access to this chamber is through a narrow channel defined by the outer alpha subunits. An intricate lattice of interactions anchors the N-termini of these alpha subunits, blocking access to the channel in free 20S core particles of eukaryotes. Entry of substrates can be enhanced by attachment of activators or regulatory particles to the proteolytic 20S core. Regulatory particles rearrange the blocking residues to form an open pore and promote substrate entry into the proteolytic chamber. Channel gating is apparently partially rate limiting for proteasome activity, as facilitating substrate entry in the open channel state leads to enhanced overall proteolysis rates. Interestingly, some substrates, particularly hydrophobic ones, can activate gate opening themselves, thus facilitating their own destruction. Properties of channel gating and the interactions required to maintain stable closed and open conformations and their consequences for proteasome function are discussed.

6.1
Background

In eukaryotes, the 26S proteasome hydrolyzes most nuclear, cytoplasmic, and endoreticulum (ER) proteins into peptides of varying lengths. Normally, substrates destined for elimination are first covalently attached to multiple molecules of ubiquitin (Ub) – a process that is executed by a cascade of ubiquitinating enzymes specific for each class of substrate – and then recognized by the 26S proteasome, unfolded, translocated into the proteolytic chamber, and irreversibly degraded [1–3]. The somewhat simpler 20S proteasomes found in archaea and some bacteria (actinomycetes) can degrade and remove non-ubiquitinated proteins in a very similar manner, though recognition of substrates and anchoring them to the proteasome while they are prepared for degradation probably differ due to lack of the ubiquitin tag in these organisms [4, 5]. In either case, substrates are threaded through a nar-

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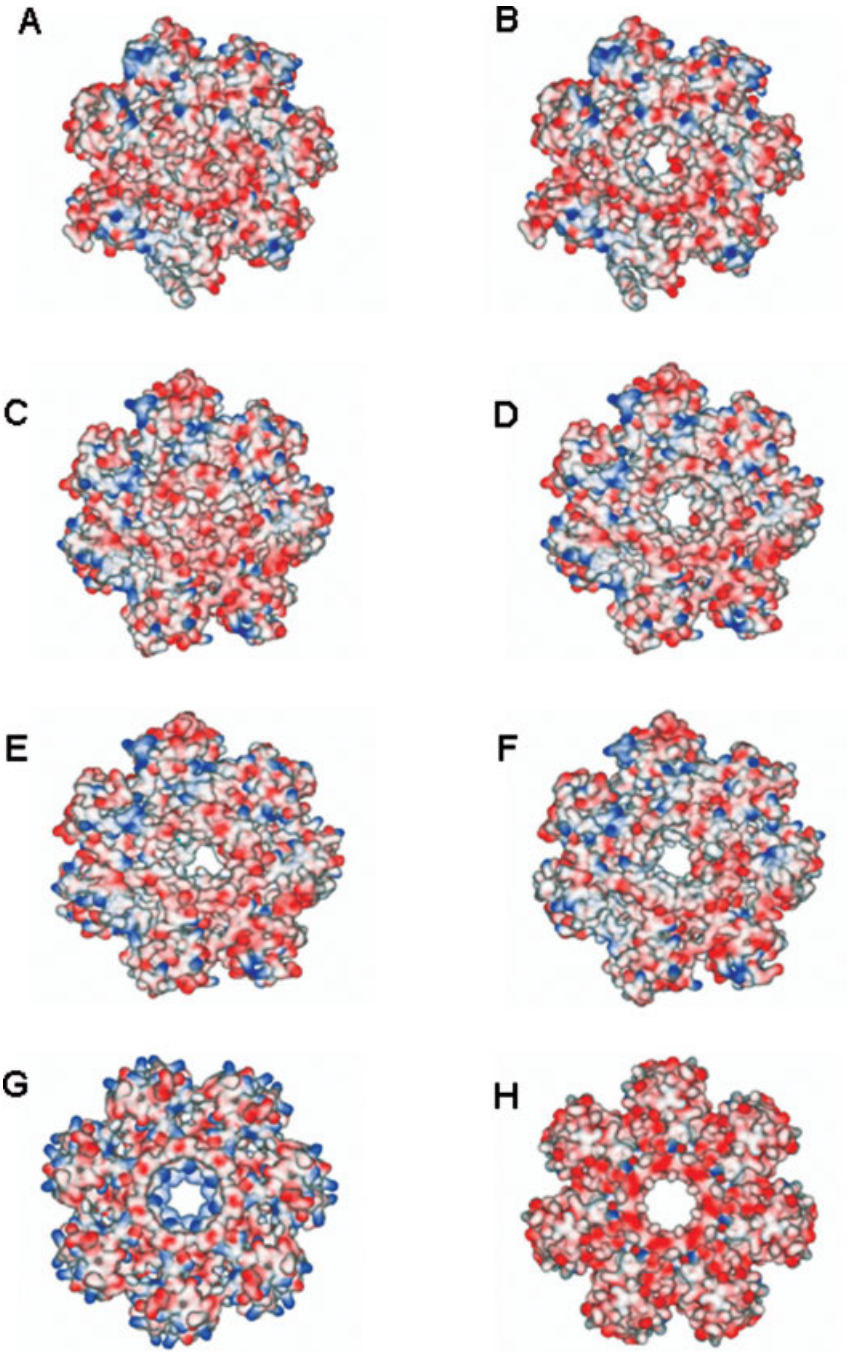
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row channel leading into the isolated internal chamber where the proteolytic active sites are located. A gated porthole at the entrance to this channel may play a defined role in controlling both the nature of preferred substrates and the rate at which they enter the proteasome lumen where they are irreversibly hydrolyzed (Figure 6.1). This chapter will focus on this gate and mechanisms of maintaining opened or closed states. Other chapters in the book series will cover in depth the myriad steps leading up to, or those following, this process.

The 26S proteasome is composed of two sub-complexes: the 20S core particle of the proteasome (CP) where proteolysis takes place and a 19S regulatory particle (RP) that prepares substrates for entry into the CP. A detailed description of proteasome structure and associated activities can be found in other chapters in this volume, as well as in many detailed reviews [6–15]. Pertinent to understanding regulation of substrate entry, the 20S CP is a cylindrical structure composed of four stacked heptameric rings engendering a sequestered proteolytic chamber. Each of the two outer rings is composed of seven α subunits, and each of the two identical inner rings is formed from seven β subunits. The β rings contain the proteolytic active sites, while the outer α rings define the channel leading into the internal pro-

Fig. 6.1. A porthole into the proteasome. Top view presentations showing the surface structure of the α ring of free 20S CPs from various preparations: (A) mammalian, (B) mammalian in theoretical open state, (C) yeast (*S. cerevisiae*), (D) yeast in theoretical fully open state, (E) the α3ΔN mutant from yeast, (F) yeast in open conformation imposed by attachment of the PA26S activator, (G) archaea (*T. acidophilum*), (H) bacteria (*Rhodococcus erythropolis*). Structures depicted in A, C, E, F, G, and H are the actual 2D determinations extracted from the crystal structure deposited in the Protein Data Bank (PDB) and visualized as a surface view with the Viewerlite program. Acidic residues are colored in red, alkali in blue, and hydrophobic in white. Note the dominance of acidic residues in the pore region of the closed state in yeast and mammalian 20S CPs. These structures indicate that the gross surface structure of eukaryotic proteasomes is remarkably similar, with slight structural divergence apparent in the archaeal and bacterial versions. The N-terminus upstream of threonine 13 in the archaeal complex (the gray region shown in Figure 6.2) is disordered and thus does not show up in the electron density map, giving the appearance of an open conformation (G). Structures shown in B and D are models in which the electron density of the corresponding tail residues in each α subunit was deleted to mimic an open conformation. Complete removal of these tail segments unveils a pore in the open state. The actual crystal structure determination of the α3ΔN 20S CP indicates that α3 is a pivotal subunit in controlling channel gating. Deletion of the α3 tail alone (E) causes disordering in neighboring subunits (up to the red arrow in Figure 6.2); however, some obstruction remains when compared with the theoretical fully open state (D). Deletion of the two opposing tails from the α3 and α7 subunits generates a fully activated complex, apparently due to removal of this residual obstruction [22]. Interestingly, attachment of PA26 to the α-ring surface rearranges the tail regions of each subunit, coupled with significant disordering (up to the blue arrow in Figure 6.2), thus imposing an open conformation (F). By deleting the electron density of the PA26 chains and focusing only on the structure of the 20S subunits, we depict in panel F the actual situation that occurs upon PA26 binding to 20S from yeast. Additional details can be found in the original publications [16–19, 55, 59].

teolytic chamber [16–19]. Overall, the 20S CP forms a $\alpha_7\beta_7\beta_7\alpha_7$ barrel structure [6]. The α and β rings of archaeal 20S proteasomes are made up of seven copies each of a single α or β subunit, giving the structure as a whole a sevenfold symmetry along the central axis. In contrast, there are seven distinct subunits in each ring of the 20S CP from eukaryotes. When visualized from outside, these subunits are labeled counterclockwise α1 through α7 and β1 through β7, respectively. Although the seven α or β subunits within the 20S CP of each species are structurally almost superimposable, their sequence identities are usually in the 20–40% range (see Ref. [7] and references therein). These differences are probably of significance as they are well maintained; sequences of orthologous subunits from different species are more than 55% identical [20, 21].

The purified 20S CP in its so-called latent form can slowly hydrolyze short or unstructured polypeptides as well as some proteins with hydrophobic or misfolded patches [22–25]. There is increasing evidence that degradation of unstructured non-ubiquitinated proteins by the 20S CP may play biological roles *in vivo* as well [22, 26–31]. To degrade ubiquitinated substrates, attachment of the ATPase-containing 19S RP to the surface of the α ring of the 20S CP is required [25, 32]. Attachment of 19S RP enhances the basal peptidase activity of proteasomes as well [33, 34]. In archaea and select prokaryotes, ATPase rings such as the proteasome-activating nucleotidase (PAN) complex or the AAA ATPase ring complex (ARC) serve as rudimentary regulatory particles that enhance proteolysis by 20S proteasomes but probably do not affect peptidase rates [35–38]. One manner by which such ATPase-containing regulatory particles activate proteolysis is by unfolding substrates and translocating them into the proteolytic chamber [36, 39–45]. However, other regulatory particles can also influence the proteolytic activity of the proteasome in an ATP-independent manner. For example, a number of non-ATPase activators – such as PA28, PA26, and 11S Reg complexes – attach to the 20S CP and enhance peptidase, but not proteolysis, rates [46–53]. Other conditions such as exposure to low ionic strength, sodium dodecyl sulfate (SDS), or small hydrophobic peptides can also activate hydrolysis rates and give rise to an activated 20S CP [18, 22, 24, 34, 54, 55]. These results (together with other observations) suggest that the latent 20S CP is found in a self-imposed repressed state and that it is possible to activate the intrinsic peptidase activity in an ATP-independent manner. Structural analysis revealed the nature of this auto-inhibition by identifying a gated channel leading substrates into the proteolytic chamber [17–19]. Alleviation of inhibition by opening the substrate channel for traffic is a basic feature of proteasomes, a key property necessary to understand their function.

6.2
The Importance of Channel Gating

In order to protect cells from mistaken degradation of random proteins, entry into the proteasome lumen must be strictly regulated. Indeed, access to the channel in the latent 20S CP of eukaryotes is restricted by the N-termini of the seven α sub-

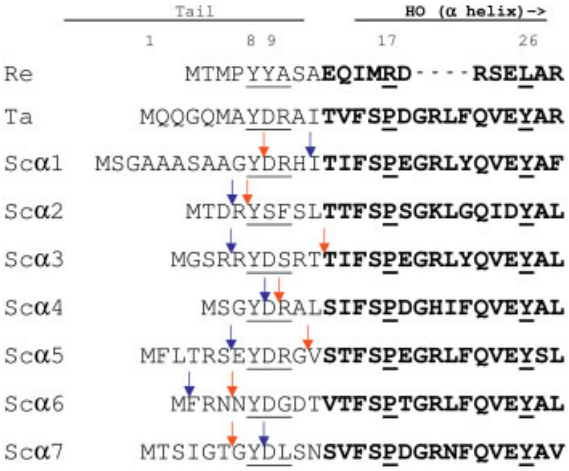

Fig. 6.2. Sequence alignment of α subunit N-termini. N-termini of the seven different α subunits from S. cerevisiae compared to archaeal and bacterial homologues. The seven α subunits (α1–α7) of yeast (Sc) are shown along with the single α subunit found in bacteria (Re) and archaea (Ta). In normal text, to the left of the bold region lie the tail sequences, which differ from one α subunit to the other. Residue numbers for the yeast and bacteria α subunits are assigned based on the alignment to archaea. Residues in the N-terminus to the arrow are disordered in α3ΔN mutant (red arrow) and upon attachment of PA26S (blue arrow). The conserved residues forming the so-called canonical cluster (YDR-P-Y) documented to play a role in stabilizing the closed or open conformations are underlined. The N-terminal sequence up to the first α helical structure of each CP α subunit (as determined by the crystal structure of the yeast CP [17, 18]) is shown in light gray. This region is homologous to the disordered segments in the N-terminal regions of the α subunit from *T. acidophilum* [16, 57]. The YD(R) motif in each tail is underlined. Note that in all subunits from yeast a tyrosine residue is present in the same location. In six of the seven subunits, an aspartate follows, and in three, an arginine completes the YDR motif. The remainder of the tail region is divergent between subunits.

units [17–19]. Each tail assumes a unique conformation while pointing inwards to the center of the ring [7, 9, 10]. Figures 6.1A and 6.1C describe the surface of the latent 20S CP as it may appear to an approaching substrate; the interlaced residues in latent proteasomes form a sealed surface obstructing passage of proteins and even short peptides. This property is a common feature of eukaryotic proteasomes and is well conserved between yeast and mammalian complexes. The blocking residues at the entrance to the proteolytic channel probably account for the repressed proteolytic activity of the latent 20S CP of eukaryotes [18, 56].

The behavior of archaeal proteasomes is somewhat different from that of the proteasomes of eukaryotes. In contrast to the sealed chamber in the eukaryotic complex, crystal structures of 20S proteasomes obtained from archaeal organisms distinctly find the proteasome in an open state. Disorder in the conformation of the first 12 amino acids of each α subunit creates a pore in the center of the α ring contiguous with the channel leading into the lumen (Figure 6.1G). This open

state accounts for enhanced peptidase rates measured for this complex [16, 36, 57]. Despite the appearance of an open pore in the archaeal 20S CP (Figure 6.1), dynamic conformations of the α-subunit tails may partially restrict passage of intact proteins through the pore, necessitating mechanisms for activation of protease activity. For instance, by locking these residues in a stable, open conformation, regulatory complexes found in these organisms (such as PAN) are able to accelerate proteolysis of full-sized proteins [36, 43, 55, 57, 58]. Certain conditions might promote switching of the N-termini from an unstructured into a structured, open conformation without requiring a regulatory complex [57]. Apparently, a similar situation occurs for 20S proteasomes present in bacteria [59]. The N-termini encompassing the first eight amino acids of the subunits in the α ring of the *Rhodococcus* proteasome are disordered (Figure 6.2), creating the appearance of an open pore (Figure 6.1H). The ARC regulatory complex found in these organisms probably stimulates proteolytic activity by anchoring these tails into a static, open conformation, similarly to the role of PAN in archaeal organisms.

Eukaryotic 20S CPs can also be found in an open conformation. Repeated freeze-thawing of purified 20S CPs; mild chemical treatments such as exposure to low ionic strength, to low levels of sodium dodecyl sulfate (SDS), or to short hydrophobic peptides; and attachment of regulatory complexes and even mutations in residues near the central pore all activate hydrolysis rates [18, 22, 24, 34, 54, 55]. Presumably, all these treatments lead to disordering in pore residues involved in gating the channel, thus opening up a porthole into the 20S CP (Figure 6.1B,D). Rearrangement of channel-blocking residues results in facilitated substrate access into the proteolytic chamber and activation of proteasomes. Activated 20S CPs can easily hydrolyze unfolded or hydrophobic proteins, in some instances more rapidly even than intact 26S holoenzymes [22, 23]. It should be noted that free 20S CPs can spontaneously switch between the latent "closed" and activated "open" conformations [60]; however, under physiological conditions it appears that the majority of free 20S CPs from eukaryotes are found in a closed and latent state.

Similar to the situation described above for archaeal proteasomes, a distinction can be made between the appearance of an open channel due to disordering in residues lining the pore region and one in which the blocking tails are secured in an open structure. Structural determination of a 20S CP–PA26 complex depicts the α subunit N-termini pointing away from the center of the ring, opening up an unobstructed porthole into the channel [55] (see also Figure 6.1). The conformation differences between the closed and open states of N-termini could explain how regulatory particles activate proteolytic activity by rearranging the blocking residues to facilitate substrate entry [43, 54, 55].

Regulatory complexes such as the 19S RP participate in channel gating and facilitate substrate entry. This activates proteolysis and allows the resulting proteasome holoenzyme to fulfill its role in regulated protein degradation [7]. Indeed, under standard growth conditions, the majority of proteasomes in yeast cells are found as 26S holoenzymes. That said, 20S CPs are abundant in certain cases and are found regularly in mammalian tissue. Given that 20S core particles are slower than 26S holoenzymes at hydrolyzing most test substrates and are unable to pro-

teolyze polyubiquitinated proteins, it is unclear what the biological function of the 20S CP is, or whether it is an important player in cellular protein breakdown. Abating bulk protein degradation upon proteasome disassembly may be a requirement for survival under certain stress conditions. For instance, prolonged starvation, oxidative stress, and severe heat-induced damage result in dissociation of proteasome holoenzymes and elevated levels of 20S CPs [22, 28, 29, 61]. There is some evidence that the catalytically repressed 20S CP can serve as a reservoir of proteasome components for reassembly when resumption of proteolysis is needed. Nevertheless, free 20S CPs may play limited roles in degradation of unstructured or non-ubiquitinated proteins under both normal growth and stress conditions [22, 26, 28–31].

6.3 A Porthole into the Proteasome

6.3.1 The Closed State

The first 12 residues in the archaeal complex, right up to the first α helix in the protein (Figure 6.2), are naturally disordered, giving the impression of an open channel (Figure 6.1G). Similar disordering is found in the equivalent sections of α subunits in the bacterial 20S proteasome purified from *Rhodococcus* (Figure 6.1H). In comparison, the corresponding N-termini of the seven α subunits in eukaryotic complexes point towards the center of the ring, sealing the entrance to the proteolytic channel (Figure 6.1A,C). In eukaryotes, the paralogous subunits within the α ring show structural and sequence similarities over the bulk of the protein, yet diverge at their amino-terminal region in both sequence and relative length (Figure 6.2). Precisely at the center of the ring, each tail accepts a unique conformation, stabilizing a well-defined and non-symmetric closed configuration [7] (see also Figure 6.3). The tail of α3 is somewhat distinct from the others in that it points directly across the surface of the α ring towards the center, maintaining close contact to every other α subunit. The importance of these tail regions is highlighted by their extreme conservation across eukaryotes; while each tail is highly conserved in different species, the corresponding regions are divergent from one subunit to another [7]. These properties suggest that the N-termini play a critical structural role that has been maintained in core particles in all eukaryotes, and it is precisely their differences that are integral to their function.

Low-energy bonds formed between specific tail residues are critical for stabilizing the open or closed conformations. For example, in latent 20S CPs, aspartate at position 9 in the N-terminus of α3 contacts both tyrosine 8 and arginine 10 in neighboring α4 [18]. A salt bridge is formed between the carboxylate group of aspartate 9 in α3 and the guanidinium group of arginine 10 in α4, simultaneous with a hydrogen bond linking aspartate 9 of α3 with tyrosine 8 of α4 [18]. Similar bonds probably link the analogous residues in mammalian 20S CPs [19]. Embedded

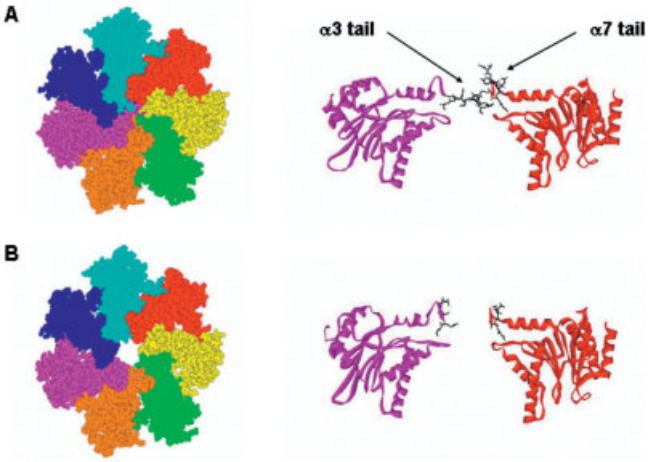

Fig. 6.3. A gate-and-latch system determines open and closed states of the substrate channel. Yeast 20S CP in a latent closed conformation (A) and in an open state imposed by the PA26S activator (B). At left a top view of the seven-member α ring of the 20S CP is shown. All α subunits are color coded, starting with α1 (light blue) on top and running counterclockwise to α7 (red). On the right, a side view focusing only on two opposing subunits – α3 (pink) and α7 (red) – highlights the conformational switch that occurs in the N-termini (black) between the closed state (A) to an open state (B) upon binding of PA26. Note that the N-terminal tails of α3 and α7 adopt different conformations in the closed state and point inwards to block access through the channel (A). In contrast, in the open state induced by PA26S, the tails adopt a new ordered conformation, pointing away from the channel region. A portion of the N-terminus (up to the blue arrow in Figure 6.2) is disordered and invisible in the crystal structure.

within the N-terminal segments of most α subunits is a short consensus sequence: Tyr8-Asp9-Arg10 or "the YDR motif" (Figure 6.2). Conservation of tyrosine at position 8 is absolute among subunits in yeast (and is invariable at this location in most subunits of other organisms as well); aspartate at position 9 is present in the tail of all yeast subunits except for α2, while conservation of arginine as residue number 10 is less strict. The direct contacts formed between these residues in adjacent subunits may explain their correlated evolutionary conservation. Interactions involving YDR residues could be critical for maintaining distinct open and closed conformations of 20S proteasomes.

Finding YDR residues in all subunits that form the α ring makes it somewhat puzzling that crystal structure determination did not pick out similar contacts between other neighboring subunits in the pore region. This raises the possibility that the interaction between α3 and α4 plays a unique and central role in maintaining the closed conformation of the proteasome. Support for the pivotal role of α3 in gating the 20S CP channel was provided upon truncation of the tail region of α3. Truncation of the N-terminus of the α3 subunit in yeast (the α3ΔN mutant) resulted in a purified 20S CP that was found in the open pore conformation (Figures

6.1 and 6.3). Furthermore, removal of the N-terminus of α3 caused disordering in neighboring subunits concomitant with stimulation of 20S CP peptidase activity. These results indicate that the tail of α3 is important for stabilizing neighboring tails in the closed conformation. Moreover, an aspartate-to-alanine substitution in the YDR motif of α3 (the α3 D9A mutant) appears to increase peptidase activity of purified 20S CP *in vitro*, on par with the activation observed upon deletion of the entire tail region in α3ΔN [18]. Both mutations associated with α3 point to a functional significance of the YDR motif in stabilization of the closed state of the gate. Interestingly, the YDR sequence is found intact in the α subunits from various archaea, even though the tail regions are not anchored in the latent state of 20S proteasomes from these organisms [16, 57]. This observation points to a wider role for α-subunit tail interactions in defining proteasome conformation.

6.3.2 The Open State

In order for substrates to enter the proteolytic chamber, and most likely for products to exit as well, the blocking N-terminal residues of the α subunits in the closed state must be rearranged. Rearrangement obviously necessitates breaking of the interactions that anchor the tails in the closed conformation, while forming competing interactions to stabilize them in an open conformation. For example, removal of the first nine residues at the N-terminus of the α3 subunit in yeast breaks stabilizing interactions with neighboring subunits, causing significant disordering in neighboring tails (Figure 6.2) and leading to an open pore roughly 13 Å across at the center of the α-ring surface (Figure 6.1F). Disordering alone is insufficient to allow unobstructed entry through the pore, as no enhancement of protein degradation rates has been observed so far with proteasomes purified from this mutant [18, 22]. Each α subunit plays a unique role in gating. Deletion of the equivalent N-terminal tail of α7 does not significantly increase the peptidase activity compared to wild type, pointing to a significant role for α3 [22]. Because of its peripheral location at the α-ring surface, truncation of α7 alone may not result in sufficient loss of order in neighboring tails to generate an opening wide enough for entry of small peptides. However, deletion of tails of two opposing α subunits (the α3α7ΔN strain; [22]) may act synergistically to relieve hindrance of entry of proteins into the proteolytic chamber. Thus, gating of the proteolytic channel emerges as a pivotal property in regulating proteolysis rates.

In fact, activated proteasomes may require more than just a disordered state of channel gating residues. Blocking residues may need to be removed (as in the truncation studies described in the preceding paragraph) or anchored into a stable, open conformation to relieve obstruction of the channel. Studies on archaeal proteasomes point to interactions involving YDR residues as influencing channel opening rather than stabilizing the closed state. While the N-terminal tails (12 amino acids) of each subunit of the α ring are disordered, they nevertheless occupy the pore region and impose a partial barrier to passage of protein substrates [16, 57]. The lack of defined electron density reflects that under the experimental con-

ditions mutual interactions were not strong enough to anchor these tails into a single stable conformation, resulting in multiple possible conformations of these segments between individual molecules in the sample. Evidence that the YDR motif may play a role in stabilizing the open state was provided by a combined mutagenesis and biochemical study of archaeal proteasomes. Purified archaeal 20S proteasomes slowly hydrolyze GFP tagged with a short C-terminal extension, GFP-ssrA. The rate is accelerated upon addition of the archaeal proteasome activator PAN. However, PAN was unable to activate proteolysis of GFP-ssrA by 20S proteasomes from the archaeon *T. acidophilum* that were mutated at positions tyrosine 8 or arginine 9 (of the YDR motif in the N-tail regions) of their α subunit [55]. It was suggested that these residues are required to stabilize the tails in an open conformation that is preferred upon attachment of PAN.

A stably open conformation was observed in proteasome assembly precursors as well. During proteasome biogenesis, the seven α subunits form an intermediate homomeric ring, the α_7 ring, which only then interacts with β subunits to yield the mature complex with $\alpha_7\beta_7$ composition [62]. In contrast to mature archaeal 20S CPs, structure determination of such an α_7 ring precursor from the archaeon *Archaeoglobus fulgidus* found the N-terminal segments anchored in a stable, open state [57]. In this conformation, the tail regions that contain the YDR motif adopted a helical structure motif and pointed away from the ring surface: tyrosine 8 of each subunit made a hydrogen bond with aspartate 9 of the preceding α subunit, whereas arginine 10 pointed inwards towards the central channel and did not partake in anchoring neighboring tails. The region N-terminal to tyrosine 8 of each subunit was disordered, creating the appearance of a pore roughly 13 Å in diameter contiguous with the substrate channel. In mature archaeal 20S CPs, the interactions between the N-termini of the α subunits appear to be broken, causing disordering in a greater portion of the tail regions up to residue number 12 (inclusive). These disordered tails, which are not stably anchored, partially block the pore and interfere with passage of proteins into the proteasome lumen.

Eukaryotic proteasomes can be found in an open state as well. Crystallography analysis of a PA26–20S CP complex depicted the α ring in an ordered, open, symmetric conformation [55]. Attachment of PA26 induces all seven of the α subunit N-terminal tails to adopt an ordered conformation for residues 7–12 away from the center of the ring. A cluster of four highly conserved residues, Tyr8 and Asp9 (part of the YDR motif in the tail region) together with downstream residues Pro17 and Tyr26 (in the first stable alpha helix; HO), is critical for the open state. Attachment of PA26 to the surface of the α ring repositions Pro17, which in turn induces a conformational change in tail segments to lift up and away from the center of the ring (Figure 6.2). In stark contrast to the closed state, the open state is remarkably symmetric, with all tails conforming to a similar structure held in place by similar interactions. In this open state, Asp9 forms a hydrogen bond with Tyr26 of the same subunit. Cooperativity between subunits is communicated via an additional hydrogen bond linking Asp9 of one subunit and Tyr8 of the preceding (clockwise) tail [55]. Consequently, the hydrogen bond that holds Asp9 and Tyr8 of α3 and α4, respectively, in the closed state must be broken and rearranged to allow for the open

state in which Asp9 of α3 now interacts with Tyr8 of α2. The term "proteasome gating" refers to switching between these two conformations. A principal role of regulatory particles is to promote channel opening by stabilizing one conformation over the other.

The interactions occurring between different subunits in the pore region of the eukaryotic PA26–20S CP complex are quite similar to those found in the α_7-ring precursor complex of archaea, which is also found in a stable, open conformation [57]. Asp9 in each subunit interacts with Tyr8 of the preceding (clockwise) tail in α_7 rings, leading to a symmetric, open structure. Asp9 of each α subunit also interacts with the downstream Tyr26 residue of the same subunit, repositioning the tail away from the center of the ring and up into the cavity of the docking proteasome activator complex (PA26 in this case). This conformation can be seen clearly in a side view of the 20S CP complex shown in Figure 6.3.

The mesh of internal interactions among α subunits described in the preceding paragraph and the apparent lack of stable interactions between α-subunit tail residues with the proteasome activator suggest that a stable, open conformation is an intrinsic property of proteasomes and may explain how, under some conditions, 20S core particles can spontaneously adopt the activated form without need for attachment of an activator complex [60]. Nevertheless, comparative studies show that the 20S CP from *Rhodococcus* is also found in an open conformation [59], even though the α subunits in this organism do not contain any of the signature YD or PY residues in the tail or HO regions (Figures 6.1, 6.2). Apparently, the semblance of an open state does not absolutely require interactions among these residues, suggesting additional mechanisms for stabilizing the closed or open states and switching between them. Additional studies will have to be completed for a clear understanding of gating mechanisms.

6.4 Facilitating Traffic Through the Gated Channel

6.4.1 Regulatory Complexes

As mentioned above, a number of ATP-independent activators are known to attach to the 20S CP and activate its peptidase and protease activities. These include the 11S Reg/PA28, PA26, and PA200 [46–53]. Attachment of PA28, for example, increases V_{max} for hydrolysis of certain peptides by the 20S CP by up to 100-fold, but in contrast to ATPase-containing activators (such as the 19S RP or PAN), PA28 does not promote protein degradation by the 20S CP [46, 63]. Activation of peptidase activity by non-ATPase-containing regulators can be attributed to imposing an open-channel conformation and facilitated substrate entry upon attachment of the regulatory complex. For example, activation has been documented for the PA26–20S hybrid complex, formed *in vitro* from 20S CP from *S. cerevisiae* and PA26 from *T. brucei* [55]. *S. cerevisiae* apparently lacks natural homologues of this

class of activators, but it is assumed that attachment of other symmetric activators induces a similar conformational change. For example, the symmetric PAN complex found in archaea appears to drive formation of the open conformation of archaeal proteasomes in much the same manner [55]. Attachment of PAN expedites proteolysis by wild-type archaeal 20S proteasomes, yet is unable to activate proteasomes mutant in any of the channel cluster residues (Tyr8, Asp9, Pro17 and Tyr26; see Section 6.3.2). This result suggests that each residue in the conserved YD-P-Y cluster is critical for stabilizing the open conformation in activated proteasomes. Mutations in these residues may lead to disordered tails that are unable to "lock" into an open conformation even upon PAN attachment, thus impinging on substrate entry. Activation is not achieved merely by realigning the residues that obstruct traffic through the alpha ring in the closed state, but necessitates locking the α tails into a stable, open conformation.

So far, it is unclear whether asymmetric activators, such as the 19S RP, open the channel in a manner similar to that of the symmetric examples given by PA26 and PAN. However, evidence linking the 19S RP to gating can be deduced from a substitution mutation in the ATP-binding site of a single ATPase (*RPT2*) that severely affects peptidase activity of the proteasome, probably due to hampering the ability of the RP to properly gate the channel into the CP [56, 64]. This observation indicates that even the entry of small peptides – which do not need to be unfolded – can be controlled by the RP. Furthermore, a constitutively open-channel 20S CP generated upon deletions of tail residues in the α3 and α7 subunits (α3α7ΔN mutant) exhibits activated peptidase activity, similar to that measured for 26S proteasome holoenzymes [22]. The implication is that attachment of the 19S RP realigns the α-subunit tails to facilitate passage of substrates, thus enhancing proteolysis rates. It should be emphasized, however, that in contrast to the homomeric rings found in PA26 or PAN, the 19S RP is a heterogeneous complex that contains six different ATPases. The two classes of regulators may induce different conformational changes on the sevenfold symmetry of the α ring. Furthermore, it has not yet been verified that the six ATPases indeed form a six-member ring at the base of the 19S RP. A limited subset of Rpt subunits have been found to come in direct contact with an α subunit (α2–Rpt4, α2–Rpt5, α4–Rpt4, α7–Rpt4, α1–Rpt6, α2–Rpt6, α4–Rpt2, α6–Rpt4; [65–68]). The pair Rpt2–α3 has been shown to be involved in gating the channel into the CP [18, 56, 64], though gating may be controlled by additional Rpt–α subunit interactions. As there does not appear to be simple pairing of each Rpt with a single α subunit, it is possible that the conformation adopted by α-subunit tails in the 26S proteasome holoenzyme will differ from the "symmetric" open state observed for the PA26–20S CP hybrid.

6.4.2
Substrate-facilitated Traffic

Recent studies show that some natively disordered proteins can enter the proteasome without assistance of ATP-dependent activators [26–28, 30]. The ability of latent 20S CP to catalyze cleavage of some unfolded proteins suggests that they may

directly interact with the α ring to promote gating to facilitate their own entry. This mechanism is not general: latent 20S CPs degrade most substrates slower than activated proteasomes [22]. Nevertheless, some substrates with unfolded domains or hydrophobic patches are degraded rapidly by latent 20S CP, faster even than by 26S holoenzymes [23]. Presumably, sequence motifs in the substrate interact with channel-gating residues in α subunits and aid in channel opening. For example, p21 and α-synuclein facilitate their own degradation and, when fused to stable and hard-to-degrade proteins such as GFP, promote their degradation as well indicating that gating sequences are transferable. Support for such a mechanism can be deduced from certain peptides that interact with the proteasome in a noncompetitive way to modulate the proteolytic activity of the proteasome [24, 69]. This stimulation was not observed for open-channel proteasomes (such as α3ΔN or the PA26–20S CP complex), suggesting that they specifically interact with channel-gating residues and promote channel opening. Whether these interactions involve the YDR motif or the YD-P-Y cluster in α subunits, similar to the manner by which regulatory particles activate proteasome activity, has not been elucidated. Interestingly, the pore region of the 20S CP, in the closed state, exposes only hydrophobic or negatively charged side chains. No positively charged groups are present on the surface of the α ring in eukaryotes (Figure 6.1). Thus, as a substrate approaches the surface of the α ring, it ''sees'' a predominantly negatively charged surface. This may explain how the latent 20S CP discriminates between substrates and why certain unstructured substrates with hydrophobic or positively charge stretches may interact with gating residues in the pore region and facilitate their own translocation inwards, whereas others do not [23]. For example, hydrolysis of casein – which is highly phosphorylated and carries multiple negatively charged groups – is remarkably slow by latent 20S CP, yet can be dramatically accelerated upon channel opening possibly by removal of tail residues as in Figure 6.1E [22].

6.5 Summary: Consequences for Regulated Proteolysis

It appears that proteasome-dependent proteolysis is a regulated process that can be enhanced or inhibited under certain conditions. There are reports that the proteasome itself can be a target of such regulation [22, 29, 61, 70]. Indeed, enhancement of overall *in vivo* proteolysis rates observed in the open-channel mutant indicates that the proteasome may be partially rate limiting in the overall cascade of ubiquitin-dependent protein degradation [22]. Polyubiquitinated substrates must be stable enough, even if only transiently, to allow for competition between degradation and reversal of fate. Channel gating within 26S holoenzymes may participate in the delicate balance between proteolysis and rescue.

A function of a gated channel leading into the CP is to impose inhibition during assembly of the mature CP. In the final stage of CP assembly, self-compartmentalization is achieved by the association of two $\alpha_7\beta_7$ half-CPs at the β–β interface. These half-CPs are inactive due to propeptides in the critical β

subunits that mask their active site. As these half-CPs are joined, inhibition by β-subunit N-termini is relieved by autolysis [17, 62], while inhibition by the blocking N-termini of the α subunits is imposed. Binding of the regulatory particles relieves this inhibition by opening the channel and thus activating proteolysis. There is increasing evidence that (at least in yeast) certain stress constitutions such as prolonged starvation or severe heat shock naturally promote proteasome dissociation into separate 20S CP and 19S RP subcomponents [22, 29, 61, 70]. These conditions may require repressed proteasome-dependent degradation for survival. One manner by which proteasome activity could be downregulated is by reinstating autoinhibition of the dissociated 20S CP. Indeed, the open-channel mutant that lacks the ability to enter the closed conformation exhibits low viability under conditions that promote proteasome disassembly [22].

An additional reason for a gated channel could be to regulate exit of products from the proteasome. It is possible that under normal conditions product release is slowed down by a gated channel in order to increase processivity or to decrease average peptide length. Most of these short peptides are quickly removed from the cytoplasm. Under certain conditions (such as during immune response) it might be beneficial to produce peptides with other lengths or properties. For example, upon interferon-γ induction, attachment of PA28/11S Reg plays a role in antigen processing by altering the makeup of peptides generated by the hybrid proteasome 19S RP–20S CP–11S Reg complexes [52, 53, 71, 72]. In analogy to the distantly related PA26, PA28 probably attaches to the α-ring surface and rearranges the blocking N-termini, promoting the open-channel conformation. It is possible that the open state increases the exit rate of peptides generated in the proteolytic chamber and alters their makeup to fit better antigen-presentation requirements.

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7
Ubiquity and Diversity of the Proteasome System

Keiji Tanaka, Hideki Yashiroda, and Shigeo Murata

7.1
Introduction

The proteasome is an ATP-dependent protease complex known to collaborate with ubiquitin (Ub), and its polymerization acts as a marker of regulated proteolysis in eukaryotic cells [1–3]. The covalent attachment of multiple ubiquitins on the target proteins is achieved by a cascade of enzymatic reactions catalyzed by the E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes [4]. The resulting polyubiquitin chain serves as a signal for trapping the target protein, and consequently the substrate is destroyed after proteolytic attack by the proteasome. Numerous studies have recently emphasized the biological importance of the ubiquitin–proteasome system, which is capable of catalyzing rapidly, timely, and unidirectionally a diverse array of biological processes that are responsible for cell-cycle progression, DNA repair, cell death (e.g., apoptosis), immune response, signal transduction, transcription, metabolism, protein quality control, and developmental programs. Details of the ubiquitin–proteasome pathway have been reviewed [5–11], but the field continues to expand rapidly.

It has become clear that most cellular proteins are targeted for degradation by the proteasome. The proteasome is an unusually large protein complex, consisting of two parts: the catalytic core and the regulatory particle, both of which are composed of a set of multiple distinct subunits. Thus, the proteasome acts as a highly organized apparatus designed for efficient and exhaustive hydrolysis of proteins; in fact, it can be regarded as the protein-destroying machinery in living cells. Whereas the proteasome complex has been highly conserved during evolution due to its fundamental roles in cells, it has also acquired considerable diversity in multicellular organisms (particularly vertebrates), the purpose of which is to adapt evolutionarily to emergencies in environmental status. Indeed, the acquisition of divergent protein factors is closely linked to the development of temporal and spatial regulations driven by the proteasome in species-specific fashions. In our current knowledge, the structural and functional heterogeneity of the proteasome expands the roles of proteolysis in the cell. In this review, therefore, we focus our attention on the diversity of the proteasome system, with a special reference to its physiological roles.

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7.2 Catalytic Machine

7.2.1 Standard Proteasome

The 20S proteasome is the central machine with multiple catalytic sites to hydrolyze the peptide bonds of proteins. There are two types of major isoforms in cells, which include standard (alias constitutive) proteasomes and immunoproteasomes (for details, see subsequent section). The standard proteasome has been well characterized at the molecular level. It is a large protein complex with a sedimentation coefficient of 20S and a molecular mass of about 750 kDa. Electron microscopic examination revealed resemblance in the cylindrical configurations of 20S proteasomes in various sources ranging from yeast to mammal [12]. It is a barrel-like particle formed by the axial stacking of four rings made up of two outer α rings and two inner β rings, which are each made up of seven structurally similar α and β subunits (Table 7.1), respectively, being associated in the order of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The overall architectures of the high-ordered structures of yeast (*Saccharomyces cerevisiae*) and mammalian (bovine) 20S proteasomes are indistinguishable, as demonstrated by X-ray crystallography [13, 14]. The subunits of the 20S proteasome exhibit a unique location with C2 symmetry.

The three β-type subunits of each inner ring have catalytically active threonine residues at their N-termini, all of which show N-terminal nucleophile (Ntn) hydrolase activity, indicating that the proteasome is a novel threonine protease, differing from the known protease family categorized into seryl-, thiol, carboxyl, and metalloproteases. Those β1, β2, and β5 subunits correspond to caspase-like/PGPH (peptidyl glutamyl–peptide hydrolyzing), trypsin-like, and chymotrypsin-like activities, respectively, which are capable of cleaving peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino acid residues, respectively. Two pairs of these three active sites face the interior of the cylinder and reside in a chamber formed by the centers of the abutting β rings (Figure 7.1).

X-ray crystallographic analysis of the bovine 20S proteasome raises the possibility that one additional novel Ntn hydrolase activity may be present in the β7 subunit, because the functional groups that satisfy the requirement for the Ntn-hydrolase active sites are located around the N-terminal threonine of the β7 subunit [14]. Intriguingly, the hollow around this active center is much smaller than the S1 pockets of β1, β2, or β5, indicating that this active site may have a small neutral amino acid–preferring (SNAAP) activity. However, the direction of the N-terminal main chain of β7 indicates that the new active site is not in the chamber formed by the two β rings but is close to the interface formed by the α and β rings. Whether or not the β7 subunit is indeed a catalytic site requires further studies.

It is obvious that the proteasome is present in both the nucleus and cytoplasm of eukaryotic cells [15]. Indeed, it is predominantly located in the nuclei of mammalian tumor cells but dynamically moves between these two compartments. How does the proteasome alter its subcellular localization? In this regard, it is

Table 7.1. Subunits and auxiliary factors of the proteasome.

Category	*Subclassification*	*Systematic nomenclature*	*HUGO*	*Miscellaneous nomenclature*		*Human (yeast) amino acids*	*Motif*	*Lethality*
				Human	*Yeast (budding/fission)*			
20S	α-type subunits	α1	PSMA6	Iota	SCL1, YC7	246	NLS	+
		α2	PSMA2	C3	PRE8, Y7	233	NLS	+
		α3	PSMA4	C9	PRE9, Y13	261	NLS	–
		α4	PSMA7	C6	PRE6	248	NLS	+
		α5	PSMA5	Zeta	PUP2, DOA5	241		+
		α6	PSMA1	C2	PRE5	263		+
		α7	PSMA3	C8	PRE10, YC1	254		+
	β-type subunits	β1	PSMB6	Y, delta	PRE3	34 + 205	Ntn	+
		β2	PSMB7	Z	PUP1	43 + 234	Ntn	+
		β3	PSMB3	C10	PUP3	205		+
		β4	PSMB2	C7	PRE1	201		+
		β5	PSMB5	X, MB1, epsilon	PRE2, DOA3	59 + 204	Ntn	+
		β6	PSMB1	C5	PRE7	28 + 213		+
		β7	PSMB4	N3, beta	PRE4	45 + 219		+
		β1i	PSMB9	LMP2, RING12	–	20 + 199	Ntn	
		β2i	PSMB10	MECL1, LMP10	–	39 + 234	Ntn	
		β5i	PSMB8	LMP7, RING10	–	72 + 204	Ntn	
PA700 (19S)	ATPase subunits	Rpt1	PSMC2	S7, Mss1	YTA3, CIM5	433	AAA	+
		Rpt2	PSMC1	S4, p56	YTA5/ mts2	440	AAA	+
		Rpt3	PSMC4	S6, Tbp7, P48	YTA2	418	AAA	+
		Rpt4	PSMC6	S10b, p42	SUG2, PCS1, CRL13	389	AAA	+
		Rpt5	PSMC3	S6′, Tbp1	YTA1	439	AAA	+
		Rpt6	PSMC5	S8, p45, Trip1	SUG1, CRL3, CIM3/let1	406	AAA	+
	Non-ATPase subunits	Rpn1	PSMD2	S2, p97	HRD2, NAS1/ mts4	908	PC	+
		Rpn2	PSMD1	S1, p112	SEN3	953	PC	+
		Rpn3	PSMD3	S3, p58	SUN2	534	PCI, PAM	+

Table 7.1 *(continued)*

Category	Subclassification	Systematic nomenclature	HUGO	Miscellaneous nomenclature		Human (yeast) amino acids	Motif	Lethality
				Human	Yeast (budding/fission)			
		Rpn4		–	SON1, UFD5	(531)	Zn finger	–
		Rpn5	PSMD12	p55	NAS5	456	PCI	+
		Rpn6	PSMD11	S9, p44.5	NAS4	422	PCI, PAM	+
		Rpn7	PSMD6	S10a, p44		389	PCI	+
		Rpn8	PSMD7	S12, p40, MOV34	NAS3	324	MPN	+
		Rpn9	PSMD13	S11, p40.5	NAS7/ mts1	376	PCI	–
		Rpn10	PSMD4	S5a, Mbp1	SUN1, MCB1/ pus1	377	UIM, VWA	–
		Rpn11	PSMD14	S13, Poh1	MPR1/ pad1, mts5	310	MPN	+
		Rpn12	PSMD8	S14, p31	NIN1/ mts3	257	PCI	+
		Rpn13		–	DAQ1	(156)	ARM	–
		Rpn14		FLJ11848	YGL004C	392	WD40, G-β	–
		Rpn15		DSS1, SHFM1	SEM1	70		–
PA28 (11S)			PSME1	PA28α, REGα	–	249		
			PSME2	PA28β, REGβ	–	239		
			PSME3	PA28γ, REGγ, Ki	–	254		
PA200			PSME4	PA200, TEMO	BLM3	1843	HEAT, ARM	–
PI31			PSMF1		–	271	Proline-rich	
Others			PSMD5	S5b, p50.5	–	504	ARM	
			PSMD9	p27	NAS2	223	PDZ	–
			PSMD10	p28, gankyrin	NAS6	226	ANK	–
				KIAA0368	ECM29	1870	HEAT	–

HUGO: Human Genome Organization; Ntn: N-terminal nucleophile hydrolase; AAA: ATPase associated with diverse cellular activities; PAM: PCI-associated module; PCI: proteasome, COP9, eIF3; MPN: Mpr1, Pad1 N-terminal; UIM: ubiquitin-interacting motif; VWA: von Willebrand factor type A; NLS: nuclear localization signal; PC: proteasome/cyclosome repeat; PDZ: PSD-95/DLG/ZO-1; ANK: ankyrin repeats; ARM: Armadillo repeats; +; lethal –; non-lethal.

noteworthy that the classical nuclear localization signal (NLS), which consists of the basic amino acid cluster whose consensus sequence is X-X-K-K(R)-X-K(R), where X is any residue, is present in the four α-type subunits; i.e., α1, α2, α3, and α4 (Figure 7.2), but lacking in other subunits including seven β-type subunits (Table 7.1). Ample evidence confirms that the NLSs are functionally active, because they are able to induce complete translocation of the reporter protein into the cell nucleus, when the NLS sequence is fused to the protein [16, 17]. Moreover, structural analysis reveals that these four NLSs are at the surface of the molecule, suggesting that they all participate in the nuclear localization of the 20S proteasome [14]. Presumably, the proteasome moves as a large particle, but not as individual subunits, through nuclear membranes, because only limited subunits have the NLS sequence and free subunits are not present in the cell in general.

In the budding yeast, gene disruption analysis reveals that deletion of all the 20S subunit genes, except the α3 subunit gene, is lethal, indicating that the proteasome is essential for cell proliferation (Table 7.1). The reason the α3 subunit is not essential is that the α4 subunit takes the position occupied by the α3 subunit in α3 subunit–deficient cells [18]. This also suggests the functional importance of molecular organization as the 20S proteasome, rather than the indispensable role of its individual subunits. Of course, the catalytic subunits themselves are of considerable importance in clarifying the biological role of the proteasome, because mutations of active threonine residues cause death of the cells. Taken together, the 20S proteasome plays a pivotal role as a basic machine for proteolysis in eukaryotes, and thereby the overall structures and functions of individual subunits are highly conserved across species, except a specialized case linked to the adaptive immune response, which will be described in the next section.

7.2.2 The Immunoproteasome

The budding yeast has seven β-type subunit genes, consistent with the configuration that the β ring of the 20S proteasome is made up of seven subunits. In contrast, mammals have 10 β-type subunit genes; this observation is puzzling, taking into consideration that the proteasomal β ring is organized by seven β subunits. On the other hand, judging from the α-ring organization, it is rational that both organisms have seven α-subunit genes. The enigma regarding the extra number of β subunits in mammals could be explained by the existence of three major immunomodulatory cytokine interferon-γ (γ-IFN) inducible subunits, β1i, β2i, and β5i, that are structurally related to β1, β2, and β5, respectively, which are regulated negatively in response to γ-IFN [19–21]. The reciprocal expression of three pairs of subunits with extremely high amino acid similarity indicates that γ-IFN may induce subunit replacement of β1, β2, and β5 by the structurally similar subunits β1i, β2i, and β5i, respectively. Based on these observations, we have proposed that γ-IFN-inducible proteasomes be called ''immunoproteasomes'' to emphasize their specialized functions in immune response and to distinguish them from those containing constitutively expressed subunits (see the simplified model depicted

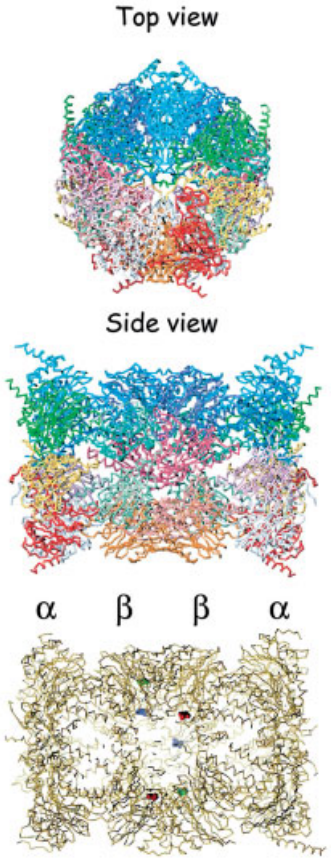

Fig. 7.1. Tertiary structure of the 20S proteasome from the bovine liver. Top panel: top view; middle and bottom panels: side view. Active threonine residues of β1, β2, and β5 appear in blue, green, and red, respectively (bottom panel). For details, see [117].

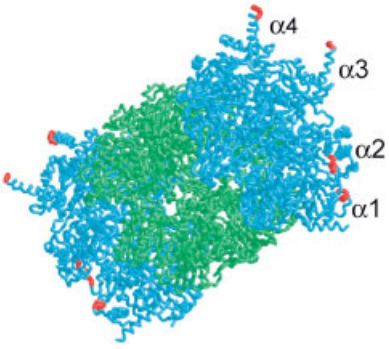

Fig. 7.2. Locations of NLSs in the bovine 20S proteasome. α subunits, β subunits, and NLSs are colored blue, green, and red, respectively.

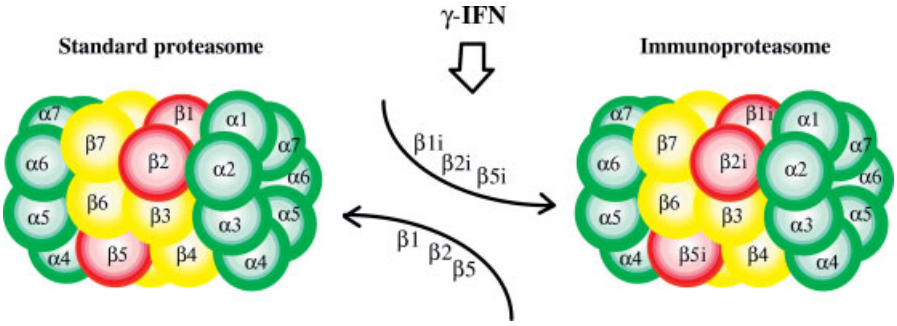

Fig. 7.3. Models of standard proteasomes (left) and immunoproteasomes (right). For details, see text.

in Figure 7.3) [19, 22]. The major histocompatibility complex (MHC) class I molecule continuously binds peptides produced by proteolysis of cytosolic proteins and displays them on the cell surface. This mechanism enables cytotoxic T lymphocytes (CTLs) to detect and destroy abnormal cells that synthesize viral or other foreign proteins [23] . Over a decade ago, the proteasome was identified as a plausible candidate-processing enzyme of intracellular antigens. To date, the roles of the immunoproteasome, which in concert contributes to the efficient production of CTL epitopes, have been highlighted in the MHC class I–restricted antigen-processing pathway [24, 25].

Of the 10 β-type subunits, three pairs of γ-IFN-regulated subunits have active threonine residues, indicating that the subunit exchanges induced by γ-IFN are likely to confer functional alterations upon the proteasome. In fact, γ-IFN alters the proteolytic specificities of the proteasomes, increasing their trypsin- and chymotrypsin-like activities for cleavage of peptide bonds on the carboxyl side of basic and hydrophobic amino acid residues of fluorogenic substrates, respectively, but decreasing their caspase-like activities for peptides containing acidic amino acid residues [21, 24, 26]. Comparison of the tertiary structures of the standard proteasome and the immunoproteasome constructed by computer-assisted modeling suggests that the caspase-like activity would be reduced and chymotryptic and tryptic activities would be enhanced in the immunoproteasome [14]. These changes of peptidase activities suggest that the immunoproteasome of γ-IFN-treated cells should generate more peptides that have hydrophobic or basic carboxyl termini and fewer peptides with acidic carboxyl termini. The peptides generated by the immunoproteasome favor settlement into the peptide-binding pocket of MHC class I molecules, because hydrophobic or basic carboxyl terminal residues normally serve as anchors for binding to MHC class I molecules. Thus, γ-IFN produces the immunoproteasome with an alteration of the proteolytic specificity that is perhaps more appropriate for the immunological processing of endogenous anti-

gens [25, 26]. It is likely that the acquisition of the immunoproteasome enabled the organism to produce MHC class I ligands more efficiently and thus combat pathogens more proficiently.

Sequence comparison of the 10 mammalian β-type subunit genes indicates that each proteasome subunit pair that undergoes exchanges upon γ-IFN stimulation emerged from the respective common ancestor by gene duplication [26]. Among the 10 β-type subunits, β1i is the most closely related to β1. The same is true with β2i and β2, and also with β5i and β5. The γ-IFN-inducible subunit genes appear to have been derived from the more ancient, constitutively expressed β1/*PSMB6*-, β2/*PSMB7*-, and β5/*PSMB5*-like genes (Table 7.1). The close evolutionary relationship of the exchangeable proteasome subunit pair is supported by the fact that yeast has the constitutively expressed β-type subunit genes β1/*PRE3*, β2/*PUP1*, and β5/*PRE2*, which resemble more closely mammalian β1/*PSMB6*, β2/*PSMB7*, and β5/*PSMB5* than their γ-IFN-inducible counterparts. For understanding the evolution of the immunoproteasome, we previously proposed a chromosomal duplication model explaining the emergence of the γ-IFN-regulated β-type subunits [26]. The basic assumption of this model is that all three sets of γ-IFN-regulated β-type subunits emerged simultaneously as a result of chromosomal duplication involving the MHC region. Many of the MHC-encoded genes including β1i/*PSMB9* and β5i/*PSMB8* appear to have emerged by an ancient chromosomal duplication that takes place as part of the genome-wide duplication, suggesting that modifications and renewal of preexisting non-immune genes were instrumental in the emergence of adaptive immunity.

7.3 Regulatory Factors

The crystal structure of 20S proteasomes reveals that the center of the α ring of the 20S proteasome is almost completely closed, preventing penetration of proteins into the inner surface of the β ring on which the proteolytically active sites are located. Thus, the 20S proteasome exists in a latent status in the cells. Accordingly, substrates gain access to the active sites only after passing through a narrow opening corresponding to the center of the α rings, and the amino termini of the α subunits form an additional physical barrier for substrates to reach the active sites. In certain cases, it is reported that unfolded proteins generated by stresses, e.g., due to oxidation, or naturally unfolded proteins without secondary structures, such as p21 and α-synuclein, are degraded directly by the 20S proteasome, but the mechanism that controls the gate opening of the closed α ring for interaction with these proteins remains a mystery [27, 28].

In most cases, however, additional protein factors that are associated with the 20S core particle are required to exert the proteolytic functions. In other words, the enzymatically active proteasome is generally capped on either and/or both ends of the central 20S proteasomal core by a regulator that can recognize target

proteins and opens the α-ring channel for entry of the substrates for their ultimate breakdown. In turn, the proteasome hardly degrades substrates, because the active sites are usually masked by their location on the inside of the β-ring cavity, preventing free interaction with the substrate proteins. Due to this catalytic mechanism, the proteasome can be referred as a self-compartmentalizing protease [2]. So far, several factors have been identified that function as activators that presumably control proteolysis catalyzed by the proteasome. Below is a brief summary of these regulatory factors.

7.3.1 PA700

The regulatory complex PA700 (also termed 19S complex or regulatory particle [RP]), associates with the 20S proteasome in an ATP-dependent manner to form the proteasome with an apparent sedimentation coefficient of 26S and a molecular mass of ~2500 kDa (Figure 7.4). The 26S proteasome is mainly responsible for ATP-dependent selective degradation of polyubiquitylated substrates [1, 6, 29, 30]. This structure is a dumbbell-shaped particle, consisting of a centrally located, cylindrical 20S proteasome that functions as a catalytic machine and two large terminal PA700 modules attached to the 20S core particle in opposite orientations. PA700 contains approximately 20 heterogeneous subunits of 25–110 kDa, which can be classified into two subgroups: a subgroup of at least six ATPases, numbered from Rpt1 to Rpt6 (i.e., RP triple ATPases 1–6), that are structurally similar and have been highly conserved during evolution, and a subgroup of over 15 heterogeneous subunits, numbered from Rpn1 to Rpn15 (i.e., RP non-ATPases 1–15), that are structurally unrelated to the members of the ATPase family [31–33]. These subunits are listed in Table 7.1.

The PA700/RP structurally consists of two sub-complexes, known as "base" and "lid" [34], which, in the 26S proteasome, correspond to the portions of PA700 proximal and distal, respectively, to the 20S proteasome (Figure 7.4). The base is made up of six ATPases (Rpt1–Rpt6) and the two large regulatory components Rpn1 and Rpn2, while the lid contains multiple non-ATPase subunits (Rpn3–Rpn15 or over). The base complex is thought to bind in an ATP-dependent manner to the outer α ring of the central 20S proteasome and is considered to be involved in opening the gate of the α ring for entry of the protein substrate. On the other hand, the lid complex is thought to be involved in the recognition of target proteins (mostly polyubiquitylated proteins), indeubiquitylation for reutilization of ubiquitin, and in interactions with various other proteins (for details, see below).

These six ATPases are most similar in their central domains of approximately 200 amino acid residues, which contain a putative ATP-binding site. They are members of a large protein family termed AAA proteins (ATPases associated with a variety of cellular activities), characterized by the conserved 200-amino-acid domain containing a consensus sequence for an ATP-binding module [35]. These six ATPases are assembled into one ring complex. One role of the ATPase is to sup-

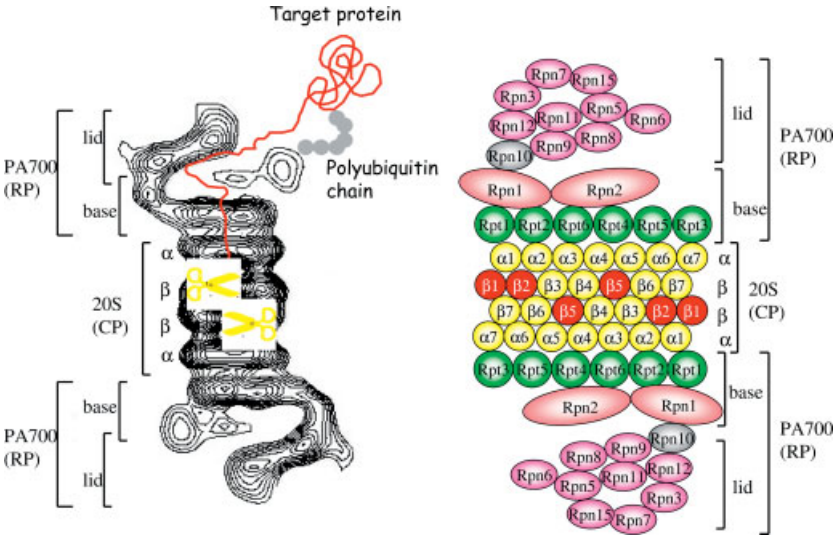

Fig. 7.4. Molecular organization of the 26S proteasome. Left panel: Averaged image of the 26S proteasome complex of rat based on electron micrographs. The α and β rings of the 20S proteasome are indicated. Photograph kindly provided by W. Baumeister. Right panel: Schematic drawing of the subunit structure. Ub: ubiquitin; CP: core particle (alias 20S proteasome); RP: 19S regulatory particle consisting of the base and lid sub-complexes; Rpn: RP non-ATPase; Rpt: RP triple ATPase.

ply energy continuously for the degradation of target proteins. In fact, the metabolic energy liberated by ATP consumption is probably utilized for assembly of the base complex with the 20S proteasome, although it may also be used for unfolding target proteins, gate opening of the 20S proteasome, and substrate translocation so that substrates can penetrate the channel of the α and β rings of the 20S proteasome [36, 37]. However, the exact reason for the presence of multiple homologous ATPases in the 26S proteasome complex remains largely unknown.

On the other hand, there are a number of Rpn subunits, but it is difficult to determine the number of bona fide stoichiometric subunits of the lid complex. As shown in Table 7.1, some subunits are not found in either mammal or yeast, but the details are largely unknown. Here we point out several mysterious aspects with a reference to subunit heterogeneity. Presumably, Rpn4 that binds to β6, Rpn13, S5b/p50.5, p27/NAS2, and p28/NAS6/gankyrin are not genuine subunits of the lid complex, which are transiently associated with the PA700, although they were initially identified by biochemical and genetic analyses as integral components of the proteasome. In addition, it is unknown whether or not Rpn14 is an actual subunit of PA700, because it was isolated by a comprehensive interaction analysis using yeast two-hybrid screening without biochemical evidence [38].

In budding yeast, all six Rpt subunits are essential, and most, if not all, Rpn subunits are also essential (Table 7.1). Nonessential Rpn subunits are of interest with respect to their roles, but their actual functions remain elusive. The 26S proteasome predominantly exists in the nucleus of the yeast [39], but the mechanism underlying the nuclear localization of PA700 has not yet been examined in detail. Indeed, the NLS motifs of PA700 subunits are largely unclear, although the sequences consisting of the basic amino acid cluster analogous to NLS are present in multiple Rpt and Rpn subunits. It has been reported that the NLS-like sequence of Rpn2 functions as the nuclear targeting signal in the budding yeast [40], but this does not seem to be the case in mammalian counterparts (our unpublished results). Indeed, when all human Rpt and Rpn subunits are fused with green fluorescence protein (GFP) and expressed in mammalian cells, their localizations showed three patterns, either in the nucleus, the cytosol, or both subcellular compartments (our unpublished results). To clarify this issue, further detailed biochemical and structural analyses of PA700 are required.

7.3.2 Rpn10

The Rpn10 subunit was identified as the first molecule capable of binding polyubiquitylated proteins *in vitro* [41]. Intriguingly, it has a unique sequence, referred to as the ubiquitin-interacting motif (UIM), that is identified as the minimal sequence bound with polyubiquitin. It is interesting to note that all subunits of the 26S proteasome conserved from yeast to mammal known so far have similar sizes, except for Rpn10. Yeast Rpn10 is approximately 30 kDa, which is 20 kDa smaller than that of other species, including human. Human Rpn10 has two UIM motifs, with cooperative roles to bind polyubiquitylated proteins [42]. In comparison, the yeast counterpart has a single UIM motif and lacks the C-terminal region containing the second UIM motif.

Importantly, Rpn10 also possesses acceptor sites for UBL domains of hHR23A/B, PLIC, and Parkin in higher eukaryotes [43]. Intriguingly, since Rad23 and Dsk2 have the ubiquitin-associated domain (UBA) that can bind polyubiquitylated proteins, beside the UBL domain, it is proposed that they may promote the targeting of substrates polyubiquitylated to the 26S proteasome [44]. Thus, there are multiple ways by which the 26S proteasome recognizes target substrates, but the pathway selected for each substrate is unknown [43, 45, 46]. Indeed, there is a genetic interaction between Rpn10 and Rad23 in yeast: the loss of both Rad23 and Rpn10 results in pleiotropic defects that are not observed in either single mutant, suggesting their functional redundancy and that Rad23 plays an overlapping role with Rpn10 [47, 48].

Surprisingly, we found that mouse Rpn10 mRNAs occur in at least five distinct forms, named Rpn10a to Rpn10e, and that they are generated from a single gene by developmentally regulated, alternative splicing [49]. Comparison of the genomic and cDNA sequences of Rpn10 revealed similar gene organizations in the medaka

fish, *Oryzias latipes*, as an example of lower vertebrates, implying that the competence for all distinct forms of Rpn10 alternative splicing is widely retained in vertebrates [50]. In contrast, no Rpn10 isoforms have so far been found in EST databases of non-vertebrate species. Interestingly, the size of Rpn10e with a single UIM motif resembles that of yeast Rpn10, suggesting that Rpn10e is an ancient form and that other species may be evolutionarily generated from Rpn10e.

The multiplicity of Rpn10 indicates that the 26S proteasome exists in multiple functionally distinct forms with distinct Rpn10 isoforms. For example, the Rpn10a form (equivalent to that originally isolated as human S5a) is ubiquitously expressed, whereas Rpn10e is expressed only in embryos, with the highest levels of expression in the brain. While the former is thought to perform proteolysis constitutively in a wide variety of cells, the latter may play a specialized role in early development. In addition, we recently found that one of the alternative products of Rpn10c is specifically associated with an apoptotic factor, suggesting that it functions as an essential subunit linking the proteasome machinery to apoptotic regulation during *Xenopus* embryogenesis (Kawahara et al., submitted).

Deletion of the Rpn10 gene in the budding and fission yeasts *Physcometrilla patens*, and *Arabidopsis* has no effect on the cell proliferation, indicating that it is nonessential. However, the deletion caused larval-pupal lethality, but did not destabilize the regulatory complex of the 26S proteasome in *Drosophila* [51]. Likewise, knockout of the mouse Rpn10 gene was embryonically lethal (our unpublished results), but the reason this defect occurs remains elusive. It is interesting that Rpn10a knock-in mice lacking the Rpn10 gene are born normally without any apparent abnormalities, suggesting that Rpn10a can rescue the lethality caused by deletion of the gene and thus is an important Rpn10 family protein (our unpublished results). To date, Rpn10 is the only subunit of the 26S proteasome that displays a variety of subspecies in higher organisms.

7.3.3 Modulator

The modulator complex was isolated as a factor that stimulates PA700-dependent proteolysis in the presence of the 20S proteasome [52]. It promotes the transformation from the 20S proteasome and PA700 into the 26S proteasome without stable association with the resulting complex [53, 54]. Intriguingly, the modulator consists of three subunits: Rpt4, Rpt5, and one additional Rpt-unrelated p27 subunit. Whereas these two ATPases are essential for cell proliferation, the deletion of p27 had no effect on cell growth in yeast [55]. It is of interest that Rpt5 is capable of binding the polyubiquitylated proteins *in vitro*, functioning as a polyubiquitin receptor [56]. However, the straightforward role of the modulator remains elusive. It is possible that the modulator is an intermediate complex before maturation into the 26S proteasome. Alternatively, p27 could act as a mediator molecule that assists in the formation of the base complex containing the ATPase ring, but there is no experimental evidence in support of this function at present.

7.3.4 PA28

PA28 (or 11S regulator/REG) was discovered as an activator protein of the latent 20S proteasome when the peptide-hydrolyzing activity was assessed [30]. PA28 is composed of subunits of 28–32 kDa, but the native molecule has a molecular weight of about 170–180 kDa, leading to the assumption that it is an oligomeric complex, perhaps hexameric or heptameric [57]. Electron microscopic investigations indicate that PA28, free of the proteasome, is a ring-shaped particle and that it associates with the 20S proteasome by forming a conical structure on both ends of the complex [58], indicating that PA28 occupies the same site on the 20S core particle as the regulator complex does in the case of the 26S proteasome. This molecular figure is confirmed by the tertiary structural analysis [59, 60].

PA28 is composed of three related family proteins, named PA28α, PA28β, and PA28γ, with approximately 50% amino acid sequence, in which PA28α and PA28β form the heteropolymeric complex and PA28γ the homopolymeric complex [61]. Immunofluorescence analysis revealed that PA28α and PA28β are located mainly in the cytoplasm and present diffusely in the nucleus, whereas PA28γ is located predominantly in the nucleus without appreciable localization in the cytoplasm [62]. Therefore, the two types of PA28 complexes containing PA28$\alpha\beta$ and PA28γ are likely to function in different subcellular compartments.

Intriguingly, PA28α and PA28β are also markedly induced by γ-IFN in various types of cells, but no obvious influence of γ-IFN was found on PA28γ. Thus, γ-IFN can alter the subunit composition of PA28 in the cells, a process similar to the replacement of immunoproteasomal subunits observed upon γ-IFN treatment. These data suggest that PA28$\alpha\beta$ could play a role in the generation by the 20S proteasome of antigenic peptides that can be presented by MHC class I molecules [63]. Thus, these newly identified γ-IFN-regulated activator genes in combination with the three pairs of γ-IFN-regulated proteasome genes perhaps act synergistically to enhance antigen presentation.

Studies in mice deficient in both PA28α and PA28β genes show that the ATP-dependent proteolytic activities were decreased in PA28$\alpha^{-/-}/\beta^{-/-}$ cells, suggesting that PA28 is involved in protein degradation [64]. Splenocytes from PA28$\alpha^{-/-}/\beta^{-/-}$ mice displayed no apparent defects in processing of ovalbumin, and PA28$\alpha^{-/-}/\beta^{-/-}$ mice also showed apparently normal immune responses against infection with influenza A virus. However, they almost completely lost the ability to process a melanoma antigen TRP2-derived peptide. Hence, PA28$\alpha^{-/-}/\beta^{-/-}$ plays an essential role in the processing of certain antigens, but it is not prerequisite for antigen presentation in general [64]. Thus, the antigen-processing pathway is clearly separated into two routes, one dependent on PA28 and the other PA28-independent (Figure 7.5).

On the other hand, the function of PA28γ remains elusive. The PA28γ-deficient mice were born without apparent abnormalities in all tissues examined, but showed postnatal growth retardation compared to PA28$\gamma^{+/-}$ and PA28$\gamma^{+/+}$ mice

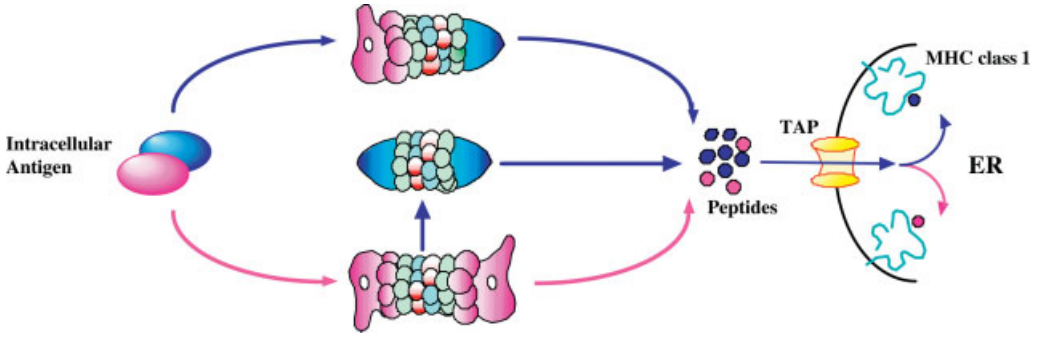

Fig. 7.5. Two distinct antigen-processing pathways mediated by the proteasome; one requires PA28, while the other is PA28-independent. Blue and red arrows represent the PA28-dependent and PA28-independent pathways, respectively. TAP: transporter associated with antigen processing; ER: endoplasmic reticulum. For details, see text.

[65], indicating that PA28γ functions as a regulator of cell proliferation and body growth in mice and suggesting that neither PA28α nor PA28β compensates for the PA28γ deficiency. In addition, PA28$\gamma^{-/-}$ mice display a slight reduction in $CD8^+$ T-cell numbers and do not effectively clear pulmonary fungal infections, indicating that that PA28$\gamma^{-/-}$ mice, like PA28$\alpha^{-/-}/\beta^{-/-}$ mice, are deficient in the processing of only specific intracellular antigens [66].

We argued earlier that the three γ-IFN-inducible subunits of the 20S proteasome most likely evolved for MHC class I–mediated antigen presentation. In this regard, it is likely that PA28α and PA28β might have coevolved with immunoproteasome subunits [26]. Because a PA28γ-like protein is found in invertebrates, the γ-IFN-inducible PA28α and PA28β subunits are probably derived from a PA28γ-like precursor [67]. This is analogous to the situation that the γ-IFN-inducible β-type subunits of the 20S proteasome emerged by gene duplication from the evolutionarily more ancient PSMB5-, PSMB6-, and PSMB7-like subunits.

7.3.5 Hybrid Proteasomes

The PA28 protein greatly stimulated multiple peptidase activities of the 20S proteasome without affecting destruction of large protein substrates, even though the proteins had already been polyubiquitylated. Thus, PA28 does not play a central role in the initial cleavage of protein substrates. It presumably has a stimulating effect on the degradation of polypeptides of intermediate size that are generated by the 26S proteasome, implying that the 26S proteasome and the PA28–proteasome complex may function sequentially or cooperatively [20, 26].

Recently, PA28 was found both in the previously described homo-PA28–proteasome complex and in the distinct proteasome complex that has one PA28 ring at one end of the 20S particle and a PA700 at the other [68]. We refer to the latter as ''hybrid proteasome'' [69], and its existence was directly demonstrated by electron microscopy [70, 71]. The formation of the hybrid proteasome proved to be ATP-dependent, like that of the 26S proteasome. The hybrid proteasome may contribute to more efficient proteolysis; perhaps intact substrate proteins are first recognized by PA700 and then fed into the cavity of the 20S proteasome, whose cleavage ability is greatly stimulated by the PA28$\alpha\beta$ complex. Indeed, it catalyzes ATP-dependent degradation of ornithine decarboxylase (ODC) without ubiquitylation but requires antizyme, an ODC inhibitory protein, as does the 26S proteasome. In contrast, the homo-PA28–proteasome complex cannot degrade ODC. Intriguingly, γ-IFN appreciably enhanced the ODC degradation through induction of the hybrid proteasome, which may also be responsible for the immunological processing of intracellular antigens (Figure 7.5). Indeed, the hybrid proteasome shows enhanced hydrolysis of small peptides and generates a pattern of peptides different from those generated by the 26S proteasome, without altering the mean product length [71]. Presumably, this change in the produced peptides accounts for the capacity of PA28 to enhance antigen presentation. Taken together, it is plausible that the two types of ATP-dependent proteases, the 26S and hybrid proteasomes, share the ATP-dependent proteolytic pathway in mammalian cells.

7.3.6 PA200

PA200 is a novel proteasome activator that stimulates 20S proteasomal hydrolysis of peptides, but not proteins, like PA28 [72]. Electron microscopy reveals that PA200 is attached to the 20S proteasome at both ends. It is a large protein of approximately 200 kDa with tandemly repeated HEAT-like motifs [73]. Homologues of PA200 are present in mammals, worms, plants, and budding yeast, but not in fruit fly and fission yeast. It is a nuclear protein, and the uniform nuclear distribution of PA200 changes to a strikingly punctate pattern in response to γ-irradiation, a behavior characteristic of many DNA-repair proteins. Indeed, mutation of the yeast *Bml3* gene-encoding mammalian PA200 ortholog results in hypersensitivity to bleomycin [74], and exposure to DNA-damaging agents induces the PA200 message [75]. Thus, it is plausible that PA200 operates in DNA repair, possibly by recruiting the proteasome to double-strand breaks. Interestingly, Blm3/PA200 was also identified as a new component of Ump1 (proteasome maturation factor)-associated precursor complexes (see Section 7.4.2). Lack of Blm3 resulted in an increased rate of precursor processing and an accelerated turnover of Ump1, suggesting that Blm3 prevents premature activation of proteasomal Cps [76]. Thus, Blm3 joins the core 20S proteasome inside the nucleus to coordinate late stages of nuclear proteasome assembly.

7.3.7 Ecm29

Ecm29 is identified as one of many proteins that are abundant in the affinity-purified proteasome, but it is absent from the proteasome, as defined previously, because elevated salt concentrations dissociate it during purification [77]. Ecm29 is a large protein of about 210 kDa with tandemly repeated HEAT-like motifs like PA200 [73]. The HEAT motif consists of two α helices and two turns; molecular modeling suggests that in the PA200 and Ecm29 repeats, the α helices may be slightly turned relative to their orientations in typical HEAT repeats. Both PA200 and Ecm29 are composed almost entirely of such repeats and therefore are likely to have α-helical solenoid structures. Based on the structural resemblance of PA200 and Ecm29, it is conceivable that they have overlapping roles in the cells.

Electron micrographs of free Ecm29 reveal a V-shaped morphology. Moreover, Ecm29 complexed with the core 20S proteasome displayed an open V-shaped morphology as well. The binding appeared to be the outer (α) ring of CP subunits. Ecm29 appears to bind the CP near the interface region, in which it contacts the RP/PA700 and CP, consistent with its function in stabilizing CP-RP association. Absence of Ecm29 leads to dissociation of the CP and RP when ATP is not provided, indicating that Ecm29 tethers the proteasome core particle to the regulatory particle. Ecm29 is conserved in various eukaryotes ranging from yeast to human.

7.3.8 PI31

PI31 was identified as a protein inhibitor of the 20S proteasome and has a molecular mass of approximately 30 kDa [78, 79]. PI31 is a proline-rich protein, particularly within its carboxyl-terminal half, where 26% of the amino acids are proline. Inhibition of the 20S proteasome by PI31 involved formation of the proteasome–PI31 complex. In addition to its direct inhibition of the 20S proteasome, PI31 inhibited the activation of the proteasome by each of two proteasome regulatory proteins, PA700 and PA28, suggesting that PI31 plays an important role in control of proteasome function, including that in ubiquitin-dependent pathways of protein degradation [79].

Previous studies reported that PI31 acts as a selective modulator of the proteasome-mediated steps in MHC class I antigen processing [20]. Consequently, overexpression of PI31 abrogates MHC class I presentation of an immunoproteasome-dependent CTL epitope and reduces the surface MHC class I levels on γ-IFN-treated mouse embryonic cells. Thus, PI31 represents a cellular regulator of proteasome formation and of proteasome-mediated antigen processing [80]. PI31 is localized at the nuclear envelope/endoplasmic reticulum membrane and selectively interferes with the maturation of immunoproteasome precursor complexes. Whereas homologues of PI31 are present in various higher organisms including mammal, *C. elegans* and budding and fission yeasts lack PI31.

7.4 Proteasome Assembly

While tremendous progress in uncovering the structure and functions of the proteasome system has been made, there is little information on the important issue of the regulatory mechanisms involved in the high-fidelity organization of the proteasome as a large multi-subunit complex. To understand this, it may be important to uncover the mechanism involved in the correct assembly of the proteasome. However, little is known about the assembly of the proteasome complex. Accumulating evidence suggests that assembly and maturation of the 20S proteasome is a precisely ordered multi-step event [20, 81]. That is, the α ring appears to assemble first, and the β subunits sequentially assemble onto the α ring, forming the 13–16S complex with an apparent size of 300 kDa that could be a pre-proteasome intermediate (alias half-proteasome), which contains one full α ring and one full β ring of unprocessed β-subunit precursors [81–84]. The processing of precursor β subunits takes place concomitantly with dimerization of half-proteasomes, forming enzymatically active mature 20S proteasomes. On the other hand, the biogenesis of 26S proteasomes remains largely elusive, especially in mammalian cells. In fact, there is no available information on how the base and lid complexes are assembled accurately. Moreover, how the 20S proteasome associates or dissociates with other multiple regulatory factors is also entirely unknown, although there is evidence that the formation of the 26S proteasome and the hybrid proteasome occurs in an energy-dependent fashion.

7.4.1 Roles of Propeptides

The three catalytic β-type proteasomal subunits β1, β2, and β5 are synthesized as proproteins (Table 7.1) and processed to their mature forms by removal of their N-terminal pro-sequences to become active assemblies, and this precursor processing occurs by an autocatalytic mechanism [85–88]. Intriguingly, precursor processing of β5 requires dimerization of the two halves of the proteasome particles (i.e., half-proteasomes) and prevents the formation of proteolytic sites until the central hydrolytic chamber is organized. Interestingly, propeptide processing itself is not required for proteasome assembly but is needed for maturation of a specific subset of active sites in yeast [86]. Unlike the propeptide of the β5 subunit, those of β1 and β2 are dispensable for cell viability and proteasome formation [89], although one study reported the importance of a propeptide of a β2 subunit whose deletion caused poor proliferation of yeast [90]. Thus, the propeptides of β subunits have unequal roles for efficient core particle maturation and a hierarchy of active-site formation [89]. In this regard, it is worth noting that another function of the propeptide is protection of the N-terminal catalytic threonine residue against $N\alpha$-acetylation [91, 92].

In addition, maturation of the catalytically inactive β-type subunits β6 and β7 appears to be exerted by active β-type subunits, forming the fully assembled 20S par-

ticle, but the role of propeptides of the non-catalytic subunits β6 and β7 has not been well documented so far.

7.4.2 Ump1

In considering the complex molecular architecture of the proteasome, a systematic pathway may be needed for the coordinated assembly of a large number of different subunits. We described above that PA200, Ecm29, and PI31 positively and negatively regulate the proteasome assembly, but there is a lack of definitive evidence in support of these actions. To date, only one molecule (termed yeast Ump1 and mammalian ortholog POMP [proteasome maturation protein], also known as proteassemblin) is known to play a crucial role in 20S proteasome assembly [93–96]. Ump1 exists in the 13–15/16S proteasome precursor complexes containing unprocessed β subunits but is not detected in the mature 20S proteasome. Upon the association of two half-proteasomes, Ump1 is rapidly degraded following the activation of proteolytic sites in the interior of the nascent proteasome, suggesting that it is a short-lived assembly chaperone. Yeast cells lacking Ump1 exhibit lack of coordination between the processing of β subunits and proteasome assembly, resulting in functionally impaired proteasomes [93]. The mammalian homologue hUmp1, POMP, or proteassemblin is a constituent of pre-proteasomes but is not a fully assembled 20S proteasome, as is Ump1 in yeast [94–96]. Moreover, it is also a constituent of the pre-immunoproteasome that contains the precursor of the γ-IFN-inducible subunit β1i [96]. Intriguingly, POMP/proteassemblin is induced by γ-IFN [94, 96], although the effect is not great, indicating that it may be involved in the immunoproteasome assembly.

A central enigma about 20S proteasome assembly is the mechanism responsible for the correct positioning of the 14 different subunits. Apart from the known functions of Ump1, i.e., the linking of β subunits to the α ring of the 20S proteasome, the mechanism of assembly of the α ring is entirely unknown. We know that the assembly starts by the formation of the α ring, which is believed to be a spontaneous process, and then the α ring provides the docking sites for the β subunits. However, we recently identified a novel heterodimeric protein factor that specifically associates with the precursor forms of 20S proteasomes and facilitates the α-ring assembly and subsequent maturation of 20S proteasomes [116]. This factor is destroyed at a late maturation stage of the assembly pathway; perhaps its proteolysis is autocatalytic, like Ump1. Interestingly, this complex has no appreciable affinity to the β subunits. Based on these findings, we propose a multi-step-ordered mechanism for mammalian proteasome assembly.

7.4.3 Immunoproteasome Assembly

As described in the preceding section, γ-IFN induces a major structural reorganization of the standard proteasome, forming the ''immunoproteasome.'' This alter-

ation of the subunit pattern is presumably due to changes in the biosynthesis of immunoproteasome subunits, because γ-IFN has no effect on the levels of preexisting standard proteasome subunits synthesized before its addition. Thus, it is unlikely that exchange of post-translationally modified subunits with subunits of preexisting proteasomes is involved in the formation of γ-IFN-induced proteasomes [97]. Accordingly, the most probable explanation for the mechanism of subunit substitution is the preferential incorporation of γ-IFN-inducible subunits and the possibly rapid degradation of the unassembled standard proteasome subunits β1, β2, and β5.

Recent studies have provided insights into the molecular mechanisms underlying the assembly of immunoproteasomes [20]. Three sets of γ-IFN-regulated catalytic β subunits (β1i, β2i, and β5i) are synthesized as proproteins and processed to the mature forms by removal of their N-terminal pro-sequences, like β1, β2, and β5. Griffin et al. [98] showed that three γ-IFN-inducible subunits can replace constitutive catalytic 20S subunits during proteasome biogenesis. β2i requires β1i for its efficient incorporation into the pre-proteasome, and the pre-proteasome containing β1i and β2i requires β5i for efficient maturation. Thus, a mechanism exists that favors the assembly of the homogenous immunoproteasomes containing all three γ-IFN-inducible subunits. However, a recent study reported that β1i incorporation does not require β2i using $\beta2i^{-/-}$ mice [90]. Indeed, there is clear evidence for the co-incorporation of β5i, rather than β5, with β1i and β2, whereas this specificity is reversed when the propeptides of β5i and β5 are switched [99]. Obviously, the β5i propeptide is responsible for the preferential incorporation, but not its catalytic activity. It is possible that β5/β5i propeptides play a critical role in preferential immunoproteasome assembly, suggesting that the differential interaction of Ump1 with β5 or β5i may play a role in the proteasome assembly [100].

7.4.4 Assembly of the 26S Proteasome

Emerging evidence indicates that mutation of certain lid subunits influences the integrity of the 26S proteasome in yeast. It was first reported that deletion of Rpn10 leads to separation of the lid–base sub-complex [31]. Thus, Rpn10 was thought to be present in the interface between the lid and base complexes and to stabilize the lid–base contacts, but it was later purified as an integral component in the lid complex [101]. Subsequently, some reports highlighted the role of certain Rpn subunits for 26S proteasome assembly [102]. Rpn9 is required for the incorporation of Rpn10 into the 26S proteasome, and it also participates in the efficient assembly and/or stability of the 26S proteasome [103]. Rpn5 plays a role in mediating correct proteasome localization and proper proteasome assembly [104]. On the other hand, Rpn6 is involved in maintaining the correct quaternary structure of the 26S proteasome, since depletion of Rpn6 affects both the structure and the peptidase activity of the 26S proteasome in the cell [105]. The loss of the temperature-sensitive mutant Rpn7-3 causes a defect in the lid complex, suggesting that Rpn7 is required for the integrity of the 26S complex by establishing a correct lid struc-

ture [106]. In evaluating these reports, it is rational to suspect that individual subunits of the 26S proteasome (if not all) require assembly of a regulatory complex containing both lid and base sub-complexes. Therefore, it is conceivable that deletion or malfunction of certain subunits causes disorganization of the complex composed of heterogeneous subunits.

Yeast two-hybrid analysis reveals a hierarchy of subunit interactions among the base and lid complexes [38, 107, 108]. Within the base, the Rpt4/5/3/6 subunits display their interaction cluster [107]. Within the lid, a structural cluster forms around Rpn5/8/9/11. Moreover, Rpn5/8/9/11 constitutes a sub-complex. However, under normal conditions, these sub-complexes are not evident, unlike half-proteasomes, suggesting that the assembly of the lid and base complexes is very rapid. Whether chaperone molecules assisting the assembly of the lid or base, or both, exist in the cell is an unresolved issue. Yeast Nob1 is a nuclear protein that forms a complex with PA700 of the 26S proteasome [109]. Nob1 serves as a chaperone-like factor to join the 20S proteasome with the 19S regulatory particle in the nucleus and facilitates the maturation of the 20S proteasome and degradation of Ump1p. Nob1 is then internalized into the 26S proteasome and degraded to complete the biogenesis of the 26S proteasome.

At present, the mechanism of 26S proteasome assembly is basically unknown, except that ATP energy is required for the association of the 20S proteasome and PA700 [110, 111]. Recently, we found a novel function for Hsp90 in the ATP-dependent assembly of the 26S proteasome [112]. Functional loss of Hsp90 using a temperature-sensitive mutant in yeast caused dissociation of the 26S proteasome. Conversely, these dissociated constituents reassembled in Hsp90-dependent fashion both *in vivo* and *in vitro*; the process required ATP hydrolysis and was suppressed by the Hsp90 inhibitor geldanamycin. We also found genetic interactions between Hsp90 and several proteasomal Rpn genes, emphasizing the importance of Hsp90 to maintain the integrity of the 26S proteasome. Thus, Hsp90 interacts with the 26S proteasome and plays a principal role in the assembly and maintenance of the 26S proteasome.

7.5 Perspectives

A recent comprehensive interactive study revealed the existence of miscellaneous molecules that could interact with the proteasome. For instance, several new proteins were identified by mild purification using affinity purification, which is coupled to high-throughput, sensitive, genome-wide proteomics analysis [77, 113]. In addition, the yeast two-hybrid analysis was introduced to define the interaction maps of multi-subunit complexes and to systematically identify new interacting proteins [38, 100, 107, 108, 114]. However, whether proteins identified by these methods are genuine subunits or transiently interacting proteins that are linked to proteasome functions await further studies.

Several factors could influence the functions of proteasomes. In this regard, many E3s and deubiquitylating enzymes (DUBs) are known to interact with the 26S proteasome. Indeed, the 26S proteasome is known to bind directly various E3 ubiquitin ligases. For example, certain E3s, such as SCF, APC, Ubr1, Ufd4, Hul5, Parkin, CHIP, and E6-AP, are reported to bind to the 26S proteasome. However, how these E3s interact with the 26S proteasome is largely unknown at present. Among these ligases, Parkin (the autosomal recessive juvenile parkinsonism–causing gene product) directly associates with Rpn10 via its UBL domain. As Hsp70 is associated with the 26S proteasome, possibly through Bag1, whose UBL motif interacts with Rpn1 and/or Rpn10, CHIP is also indirectly associated with the 26S proteasome, because CHIP binds to Hsp70 via its C-terminal EEVD sequence. The association of E3 with the 26S proteasome is functionally rational, considering the rapid destruction of substrate proteins.

In addition, a set of DUBs such as UBP6/Hsp14, UBP5, Doa4, and UCH37 are also capable of binding to the 26S proteasome. Previous studies reported that the C-terminal UBL sequence of UBP6 is responsible for the association with Rpn1. Moreover, $\beta 2$, Rpn5, and Rpn12 serve as acceptors for UBP5, Doa4, and UCH37, respectively, although the molecular basis of their interactions has not yet been defined [43, 115]. These DUBs collaborate with Rpn11 (as a genuine subunit of the lid complex) with a deubiquitylating metalloprotease activity, which allows ubiquitin peptide recycling before substrate degradation.

Recent studies have investigated the pathophysiological importance of the proteasome in the cells. For a full assessment of this issue, it is important to determine the biological significance of the diversity of the 26S proteasome system. Although we summarized our knowledge of this system in this chapter, the physiological roles of various interacting proteins are still largely unknown. Further studies should address the importance of the proteasome system in various cells and organs.

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8
Proteasome-Interacting Proteins

Jean E. O'Donoghue and Colin Gordon

8.1
Introduction

8.1.1
The Proteasome

The proteasome is the key organelle within the cell responsible for the regulated degradation of intracellular proteins. It was originally found during the search for an ATP-requiring activity that was involved with protein degradation. It has since been discovered that the proteasome itself is made up of several subunits along with various interacting regulatory subunits. In addition, in order to carry out its function as a controlled method of degradation, it interacts with a wide array of proteins. It is these interacting proteins that confer subtlety of function upon what is, at its core, a protein-degrading machine. These interacting proteins serve to control, connect and activate proteolysis. In this chapter we will first introduce the proteasome itself and the system of ubiquitination which is used to target proteins for degradation before addressing the roles of its interactors. Then we will look at the regulators of the 20S core particle (CP) and those proteins involved in the assembly and stability of the proteasome. We will address proteins involved in the ubiquitination pathway that directly interact with the proteasome, namely E2 enzymes, E3 enzymes and deubiquitinating enzymes (DUBs). In addition we will deal with those proteins that function as the ''go-betweens'' for the ubiquitination system and proteasomal degradation, and finally we will address the growing evidence for proteasomal interaction with proteins involved with transcription, translation, and DNA repair. Many of the proteins discussed in this chapter have different names in different species. In general we have used the orthologue name relevant to the species in which the work was conducted – with other orthologue names in parenthesis where possible. For a complete list of orthologue names and their appropriate species see table 8.1.

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Table 8.1. This table shows the various names assigned to the proteins discussed in this chapter. Some proteins have multiple names in one species, others have different names for different orthologues. Here *D.m.* = *Drosophila melanogaster*

	S. pombe	*S. cerevisiae*	*H. sapiens*	*Other (Species)*
Proteasome subunits	Mts1	Rpn9	S11	
	Mts2	Rpt2	S4	
	Mts3	Rpn12	S14	
	Mts4	Rpn1	S2	
	Mts8	Pre6	β1	
	Pus1	Rpn10	S5a	
	Pad1	Rpn11	POH1/S13	
UBL-UBA proteins	Rhp23	Rad23	hHR23a/b/c	
	Dph1	Dsk2	hPLIC1/2	
DUB		Doa4/Ubp4		
	Uch2		UCH37	p37A (*D.m.*)
	Ubp6	Ubp6	USP14	
Others		Blm10	PA200	
		Hul5	KIAA10	
	Cdc48	Cdc48	VCP/p97	
	Ubx3	Shp1	p47	
	Sum1	TIF34	eIF3i	
	Int6/Yin6		eIF3e/Int6	

8.1.2 Structure of the 26S Proteasome

The 26S proteasome is made up of the 20S core particle (CP) and the 19S regulatory particle (RP). The core particle contains the proteases that can degrade proteins to small peptides. It consists of 28 subunits – 14 α and 14 β proteins which form 4 stacked rings of 7 subunits each – 2 α rings and 2 β rings. The α rings sandwich the β rings to form a cylindrical structure. In this way a central channel is formed with three chambers: two antechambers on either side of a central chamber. This central chamber is lined with at least three active sites whose combined specificities can act to hydrolyse almost all peptide bonds. Access to these active sites is controlled by the α subunits which form the antechambers and can exhibit closed or open conformations. The protein to be degraded passes through this pore and the proteases degrade it to 6–9 amino acid peptide products which are released and recycled.

Access to these catalytic sites is controlled by the regulatory particle. This particle is made up of a ''base'' and a ''lid'' structure which attach to either end of the cylindrical CP (see figure 8.1). The RP functions to recognise ubiquitinated substrates and unfold proteins thus controlling access to the potent proteases contained within the 20S CP. The RP is made up of approximately 20 different protein subunits. A subset of these are the AAA ATPases which are required for the unfolding of proteins to be degraded.

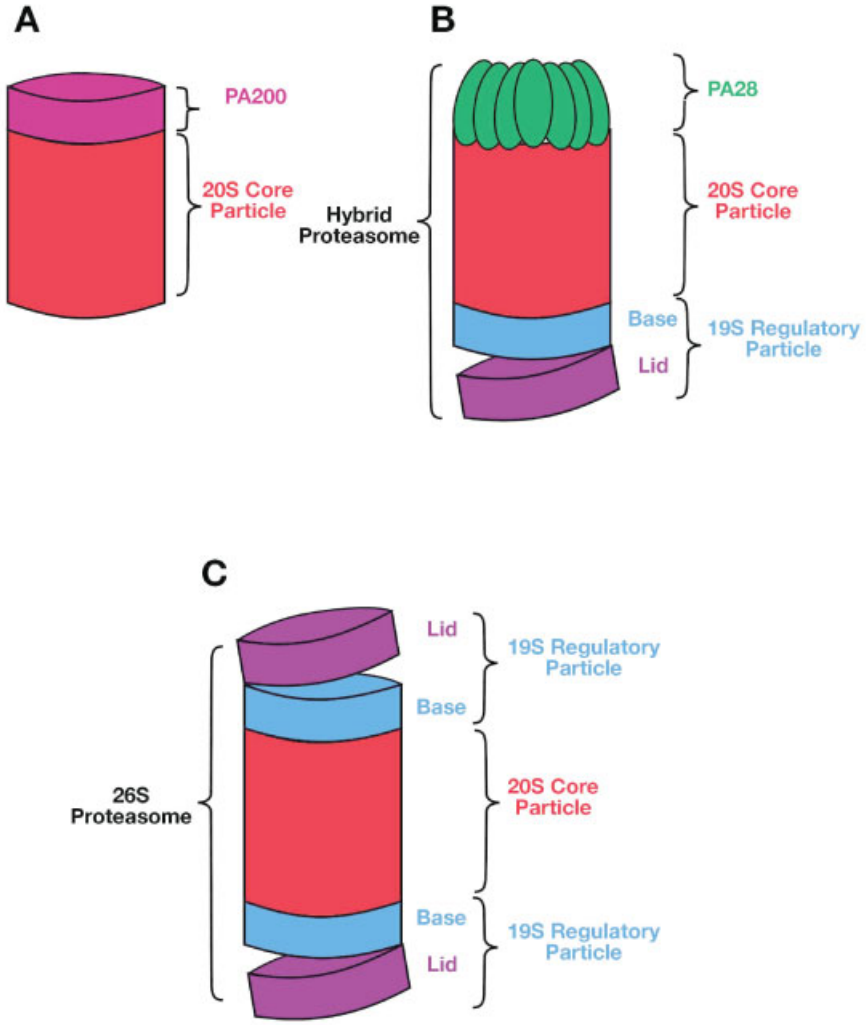

Fig. 8.1. 20S core particle with its various regulators.
A. 20S core particle with PA200. This may function in DNA repair.
B. Hybrid proteasome with one 19S particle and one PA28 particle. PA28 is a heptamericring which is interferon-inducible.
C. The classic 26S proteasome with two 19S regulatory particles for every one 20S core particle. This is the main proteasome species seen in the cell and is responsible for ubiquitin-mediated degradation.

8.1.3
Marking Proteins for Proteasomal Degradation – the Ubiquitin System

Ubiquitin is a 76 amino acid protein. It can modify proteins covalently by conjugating them through lysine linkages with ubiquitin chains forming through similar lysine linkages. This "ubiquitination" of proteins is carried out by a cascade of

enzymes – E1, E2 and E3. E1 uses ATP to activate the ubiquitin moiety, generating a high-energy thiolester intermediate in the process. The activated ubiquitin is transferred from the E1 to a cysteine residue of an E2 enzyme, thus generating another thiolester intermediate. An E3 enzyme is then required to catalyse the transfer of the ubiquitin moiety from a thiolester intermediate on the E2 to an amide linkage on the target protein or another ubiquitin moiety to create a chain. As the E3s interact directly or indirectly with targeted protein, these enzymes confer specificity on the system of ubiquitination. A protein can be monoubiquitinated, or multiubiquitinated and there is some evidence to suggest that these are not equivalent with regard to the fate of the substrate.

E4 enzymes have also been described. These enzymes target substrates that are already ubiquitinated but only have chains one or two molecules long. E4 enzymes thus serve to increase the ubiquitin chain length.

8.2 Regulators of the Holoenzyme and Chaperones Involved in Assembly of the Proteasome

8.2.1 Proteasome Assembly and Integrity

Some proteasome interactors are involved in the initial assembly of the proteasome such as the *S. cerevisiae* protein Ump1p. This was first described in 1998 as a short-lived chaperone required for the correct maturation of the 20S proteasome [1]. Ump1p was discovered in a screen for mutants defective for the degradation of test substrates. *ump1* null mutants were found to exhibit classic phenotypes of disrupted proteasome function; that is, they were hypersensitive to cadmium, canavanine and thermal stress and they showed an accumulation of ubiquitin-protein conjugates. Upon further experimentation, Ump1 was found to be a component of proteasome precursor complexes that was degraded upon the formation of the 20S proteasome and was, in fact, required to co-ordinate the proteasome's assembly and activation.

Another protein vital to the proteasome is Ecm29. This protein has been proposed to tether the 19S RP to the 20S CP to form the 26S proteasome [2].

Recently it has been proposed that Ecm29 and PA200 (see section 8.2.2) are composed almost entirely of HEAT-like repeat motifs [3] suggesting they have α-helical solenoid structures – similar to those proposed for Rpn1 and 2 [4]. The functional significance of these helical structures, however, is unclear.

8.2.2 Regulators of the Holoenzyme

While the 19S regulatory particle is the usual companion to the 20S holoenzyme, other complexes can also bind the core particle to modify its function (see figure 8.1).

The 11S REGs/PA28 proteins form a heptameric ring structure that can bind and activate the 20S proteasome. They were originally identified and characterised as molecules that could strongly activate the 20S proteasome to degrade small fluorogenic peptides [5, 6]. This heptameric structure is made up of REGα, REGβ and REGγ proteins which form homo or hetero-oligomers that bind the proteasome with differing affinities: REGα/β > REGγ > REGα > REGβ [7]. These PA28 rings can activate the proteasome without affecting the active sites of the 20S CP. This is thought to be achieved through the facilitation of entry or exit of the substrates to/from the 20S core [8]. Hybrid proteasomes, that is 20S CP attached to both 19S and PA28, have been found, and can make up a quarter of the proteasome population in mammalian cell line extracts [9]. As for the role of PA28 and its activation of the 20S CP, it is known that PA28 is inducible by interferon-γ. It has been known for some time that the proteasome has a role to play in the production of antigens for MHC class I presentation. It now seems that PA28 is a major player in this role of the proteasome. Recently hybrid proteasomes have also been shown to be induced by interferon-γ. It is worth noting that PA28 is not present in either budding or fission yeast and so it is possible that the genes that encode an interferon-γ-inducible regulator for the 20S CP evolved along with other genes responsible for adaptive immunity [10].

Another complex that can interact with the 20S proteasome has been studied in mammals and is called the PA200. This 200kD complex has been shown to exist in a monomer-dimer equilibrium and is found in the nucleus of mammalian cells [11]. There appear to be orthologues of PA200 in *C. elegans*, *Arabidopsis* and *S. cerevisiae*, but not in *S. pombe* or *Drosophila*. In mammals (mice), there are slightly differing forms of PA200. Here, while the 200kD species is abundant in testis, a 160kD form is more reactive elsewhere and a 60kD form is seen in the liver, lung and brain [11]. It is also worth noting that while the 200kD and 160kD forms are nuclear, the 60kD species is cytoplasmic. The authors suggest these different forms could arise from splicing variants – but their physiological relevance is unknown. PA200, like PA28 promotes proteasomal hydrolysis of peptides but not that of large folded proteins. As for the physiological role of PA200, or PA200-20S proteasomes, there are several pieces of evidence to support its having a role in DNA repair. The yeast orthologue of PA200, Blm10 (originally termed Blm3) was discovered in a screen for mutants sensitive to the DNA damaging agent bleomycin [12] and has also been shown to complex with Sir4p [13]. This is a chromatin component that leaves the telomeres and relocates to DSBs (Double Strand Breaks) where it binds Ku70 [14]. PA200 in mammals is most abundant in testis where double strand breaks in DNA occur during meiotic recombination and it forms intracellular foci upon γ-irradiation similar to a number of DNA repair factors [11]. In addition to its putative role in DNA repair, recent work has also shown a three- to four-fold upregulation of PA200 in four different models of muscle wasting [15]. The true physiological function of PA200, as such, remains to be determined. There has been some speculation whether Blm10 in yeast was actually a functional homologue of PA200 when evidence was found to suggest that Blm10 functions to suppress premature activity of newly-formed 20S core particles and regulate the maturation of the 20S CP [16]. Recently, however, evidence has been found that

Blm10 can associate with the mature, active proteasome, with Blm10 docking onto the end of the CP cylinder and strongly activating its peptidase activity [17]. Blm10 is usually found in a hybrid Blm10-CP-RP complex. Why these complexes were not seen in the earlier study is unclear.

8.3 Enzymes Controlling Ubiquitination and Deubiquitination

8.3.1 E2 Ubiquitin-Conjugating Enzymes

As described in section 8.1.3 the enzymes that allow for the addition of ubiquitin to its substrates are classified as the ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and substrate-recognition enzymes (E3s). We now know that E2s have a role to play at the proteasome itself.

In *S. cerevisiae*, Tongaonkar *et al.* explored the possibility that E2s may interact with the proteasome [18]. This, it could be reasoned, could lend greater efficiency to the ubiquitin-proteasome system, that is, if the machinery that constructed the ubiquitin-tagged proteins were linked to the mechanism of their degradation. Initially the intra-cellular location of Ubc4, a highly abundant E2 in *S. cerevisiae* was addressed. Orthologues of Ubc4 are seen in mammals, plants and humans and it contains a conserved catalytic domain present in all E2s.

It was shown that when the proteasome was isolated by immunoprecipitation, Ubc4 could be seen to be associated with it [18]. This was done using two different tags to pull out the proteasome, and both times Ubc4 was present in the proteasome fractions. Also, a catalytically inactive Ubc4 could still interact with the proteasome. Indeed it was seen that purified proteasomes could act as an E2 enzyme *in vitro*, that is, a test substrate could be ubiquitinated when provided with the appropriate E1 ligase and purified proteasomes [18]. Using the same strategy, Ubc1, 2 and 5 were also found to be associated with the proteasome. It appears that the E2s do not compete with each other for proteasome-binding sites, as overexpression of one Ubc does not lead to the reduction in binding of another. The authors offered two possible explanations for this. Firstly it could be possible that only a small number of proteasomes are bound to E2s at any one time – such that the increase in the number bound to one E2 is not enough to compete out the binding of another. Secondly, if the E2s each have different binding sites on the proteasome, or if they bind different E3s associated with the proteasome (see section 8.3.2), then the overexpression of one and the increase in occupation of its binding site will not necessarily impinge on another's binding.

It had been known previously that *ubc4*Δ*ubc5*Δ double mutants are susceptible to heat stress [19]. Upon examination of the association of Ubc4 with the proteasome under heat stress, Ubc4 levels in the proteasome fraction were found to increase dramatically (approx. 25-fold).

It appears therefore that E2 enzymes can, at least in part, mediate the close inter-

action between the ubiquitin-tagging system and the proteasome. In the case of Ubc4 this interaction was most important in the heat-stressed cell. Presumably the close interaction between an E2 enzyme and the proteasome would facilitate the timely removal of misfolded proteins as a result of the increase in temperature. Both the proximity of the ubiquitin-tagging system and, one can imagine, the lack of access of cellular deubiquitinating enzymes to the tagged substrate would allow for an highly efficient quality control mechanism when the cell is under stress.

8.3.2 E3 Ubiquitin Ligases

E2–E3 complexes allow for the construction of multiubiquitin chains bound to a specific substrate with the E3 conferring substrate-specificity upon this activity. Given that E2s appear to associate with the proteasome, it is not surprising that some E3s possess this property as well.

Xie *et al.* describe the proteasome-binding properties of Ubr1p and Ufd4p, the E3 components of two independent ubiquitin-related proteolytic pathways in *S. cerevisiae* [20]. This would seem to suggest that association with the proteasome has some advantage for E3 enzymes given that two separate pathways have adopted this strategy.

Ubr1p is the E3 for the N-end rule pathway whereby proteins with destabilising N-termini can be marked for degradation. Both *in vitro* and *in vivo* work proved that Ubr1 can interact with the 19S subunits Rpt1, Rpt6 and Rpn2 [20]. In addition, the *in vivo* experiments revealed that Ubr1 can also interact with Pre6 – a protein of the 20S core of the proteasome. It therefore appears that Ubr1p can bind multiple members of the 19S proteasome and potentially one of the 20S core proteases also.

The UFD (Ubiquitin Fusion Degradation) pathway provides the means to remove those proteins that have a ''non-removable'' ubiquitin moiety. By ''non-removable'' it is meant that the ubiquitin moiety shows resistance to the deubiquitinating process. This can be caused by two things, a change in the last residue of the ubiquitin moiety or, the existence, in the substrate, of a proline residue immediately C-terminal to the lysine to which the ubiquitin is attached.

Ufd4p is the E3 for this particular pathway in *S. cerevisiae*. It has been found that this E3, despite being from a different pathway and having no significant sequence similarity to Ubr1, also binds Rpt6 (but not any other proteasome subunit examined *i.e.* 9 other subunits of the 19S proteasome) both *in vitro* and *in vivo*. In the *in vivo* experiment it was shown that Ufd4 could co-immunoprecipitate Rpt6 (bearing in mind that both of these fusion proteins were overexpressed). Interestingly it was also shown that it could co-immunoprecipitate Rpn1. This meant that while Ufd4 does not interact directly with Rpn1 (GST-tagged Rpn1 did not pull down Ufd4); Ufd4, through its interaction with Rpt6 is associated with the mature proteasome *in vivo*.

In further studies on the role of Ufd4 and its association with the proteasome it was found that Ufd4 also directly interacts with Rpt4 and that the binding of both

Rpt4 and Rpt6 is dependent on the presence of the 201-residue N-terminal region of Ufd4 [21]. The N-terminal 201 amino acid residues were found to be important such that when they were deleted, Ufd4 (Ufd4$^{\Delta N}$) could no longer bind GST-Rpt4 or GST-Rpt6. This was repeated *in vivo* for the Ufd4 – Rpt6 interaction. In addition, it was shown *in vivo* that its N-terminal region was required for Ufd4 to interact with the 26S proteasome; that is, for Ufd4 to co-immunoprecipitate Pre6.

To examine the effect of this N-terminal region on the function of Ufd4, a series of experiments were performed using the *β*-galactosidase-based substrate Ub^{V76}-V-*β*gal [21]. In *ufd4*Δ cells, Ub^{V76}-V-*β*gal is long-lived and when ubiquitinated, displays only one ubiquitin moiety. When functional Ufd4 is put back in the system both of these phenotypes are rescued; that is, the half-life of Ub^{V76}-V-*β*gal decreases, and it could be multiubiquitinated. If instead an *ufd4*$^{\Delta N}$ expressing plasmid is transfected into the *ufd4*Δ cells, the degradation of Ub^{V76}-V-*β*gal remains slow while the ubiquitination effect is rescued. This implies that the loss of the 201 N-terminal residues does not affect the ability of Ufd4 to ubiquitinate its substrates but rather the speed of their degradation. This would seem to imply that the delivery of the substrate to the proteasome is important in the rate of degradation of the substrate and can be carried out by the E3 of that substrate. Therefore one would expect that if the delivery of the ubiquitinated substrate to the proteasome were impaired in some way, overexpression of a protein like Ufd4 could compensate by delivering the substrate to the proteasome. This was found to be true in *cdc48-1* and *rpn10*Δ cells [21]. In both these strains the degradation of Ub^{V76}-V-*β*gal is impaired due to the loss of the ubiquitin-binding properties of either Cdc48 or Rpn10. However when Ufd4 is overexpressed in these strains, the rate of degradation of Ub^{V76}-V-*β*gal is strongly increased while overexpression of Ufd4$^{\Delta N}$ does not change the degradation kinetics in these mutant strains.

An E3 enzyme in mammalian cells – KIAA10 – also appears to interact with the proteasome. When KIAA10 was being purified as an E3 specific to erythroid cells, contaminating proteins such as S1 (Rpn2) and S2 (Rpn1) were also co-purified [22]. Given that Ufd4 interacts with the proteasome – it was investigated if this was also true for KIAA10. This was shown to be the case *in vitro* using a GST-binding assay. Additionally it was found that GST-tagged KIAA10 could interact with the intact 19S RP by western blotting for S8/p48/Rpt6. These results were confirmed *in vivo* where KIAA10 was co-immunoprecipitated with proteasomes, using both anti-S8 and anti-S10a antibodies. As regards the region of KIAA10 that facilitates this interaction, the results are unclear. While the loss of the first 132 amino acids reduces KIAA10 – S2 binding, it does not ablate it and the *in vivo* results for KIAA10 without 132 residues of its N-terminus were inconclusive. This suggests that while there is an S2-binding site within the first 132 amino acids of KIAA10, it may not be the only point of contact between KIAA10 and the proteasome. The budding yeast orthologue of KIAA10 is Hul5, and there is also evidence that Hul5 interacts with the proteasome in *S. cerevisiae* [2].

Loss-of-function mutations in the gene encoding the Parkin protein, are implicated in causing a form of autosomal recessive juvenile parkinsonism in humans [23]. It was subsequently found that Parkin was an E3 ubiquitin ligase [24] and

that this E3 ligase contains a UBL domain which binds the 26S proteasome subunit Rpn10 in mammals [25]. There are now a growing number of Parkin substrates including α-synuclein [26] and poly-glutamine proteins [27] that suggest that Parkin is required to target potentially toxic proteins for degradation. Its location at the proteasome therefore, would appear to facilitate the efficient disposal of these potentially harmful proteins.

Other E3 ligases shown to be associated with the proteasome include SCF (Skp1/Cullin/F-box) and APC (Amphase Promoting Complex) [28]. Here, the Cdc4 subunit of SCF, tagged with a polyoma epitope, was able to interact with purified 26S proteasomes (in the presence of ATP). This was shown by the presence of Rpt1 and Rpt6 on western blots. Similarly it was shown that epitope-tagged APC could be co-immunoprecipitated with Rpt1.

8.3.3 Deubiquitinating Enzymes (DUBs)

One of the most important classes of proteins that associate with the proteasome is the deubiquitinating enzymes (DUBs). Important, because their function is integral to that of the proteasome. In order to degrade substrates efficiently the ubiquitin chain must be removed from that substrate. There are two good reasons for this to occur. Firstly, ubiquitin chains are highly thermodynamically stable and so their unfolding and degradation along with the protein to which they are attached takes a large effort. Secondly, it is more efficient for cells to recycle the ubiquitin moieties rather than to constantly translate and degrade them. In order to accomplish this, it makes sense for the deubiquitinating activity to be situated at the proteasome so that ubiquitin is released by that activity, while ensuring that the substrate gets degraded.

There are four DUBs known to interact with the proteasome; Pad1/Rpn11, Ubp6, Uch2/UCH7 and Ubp4/Doa4.

Work on DUBs revealed a ''cryptic'' deubiquitinating activity was associated with the proteasome in *S. cerevisiae* [29] and mammals [30]. The importance of removal of ubiquitin before degradation was illustrated though the *in vitro* use of ubiquitin, mutated to be irremovable from an ovomucoid moiety (Ub^{m}-OM) by bovine 26S proteasomes. When the degradation of this construct and that of removable Ub-OM was compared, it was found that the rate of degradation was reduced when ubiquitin was mutated and that the non-removable ubiquitin was degraded along with the substrate. Removal of ubiquitin was therefore important to allow efficient degradation of Ub-OM. It is worth noting that similar results were obtained using pentaubiquitin chains attached to OM. This implied the presence of a DUB that removed ubiquitin to promote efficient degradation. This DUB was also unusual in that it was resistant to Ub-aldehyde, a chemical that inhibits the majority of DUBs which are cysteine proteases. Therefore this DUB was not a cysteine protease. In addition this deubiquitinating activity actually promoted degradation rather than inhibiting it, a surprising result since one would imagine that the removal of the ubiquitin moiety/chain from a substrate would actually stabilise a substrate, as it would no longer be targeted for degradation. However if the deubi-

quitinating activity takes place at the proteasome, then the removal of ubiquitin can promote degradation, as the ubiquitin chain is highly thermodynamically stable and difficult to unfold and degrade with the substrate.

In addition to its Ub-aldehyde resistance, the proteasome-associated deubiquitinating activity was also dependent on ATP. However this dependence was only seen when the ubiquitinated substrate was incubated with 26S proteasomes. When the substrate was incubated with isolated 19S complex, deubiquitination occurred in the absence of ATP. This implied the ATP was not required for the removal of Ub but potentially for coupling the deubiquitination to downstream degradation by the ATP-dependent proteolysis of the 20S CP. The best candidate for this activity was POH1 (Rpn11 in budding yeast, Pad1 in fission yeast). POH1 is the most highly conserved 19S subunit potentially due to the presence of a catalytic domain.

In addressing the role of Rpn11 in budding yeast – the active site residues were identified and mutated [30]. This resulted in a lethal phenotype and when expression of Rpn11 was decreased there was an increase in ubiquitin conjugates. The identity of the active site residues suggested Rpn11 could be a zinc metalloprotease and when a zinc chelator was incubated with the bovine 19S proteasome – there was no deubiquitination of Ub-OM. However Rpn11 alone *in vitro* could not be shown to have deubiquitinating activity suggesting that perhaps only in the context of the 19S RP does Rpn11 have its deubiquitinating activity [30].

In *S. cerevisiae*, similar experiments were performed using Sic1-Ub as a test substrate [29]. Again it was shown that the proteasome-associated deubiquitinating activity was insensitive to Ub-aldehyde and required ATP. They also independently identified Rpn11 as the best candidate subunit and they characterised the active site of Rpn11 as a JAMM domain (Jab1/Pad1/MPN).

The JAMM domain of Rpn11 has also been examined in humans and *Drosophila* where its orthologue is S13 [31]. It was found that the *Drosophila* and human S13s are functional homologues, and also that if the JAMM domain is mutated, there is a loss of deubiquitinating activity. Another motif, similar to the cysteine box of other ubiquitin hydrolases, was also described in the JAMM domain-containing S13 and Csn5.

Ubp6 was first purified and characterised in 1997 by Park *et al.* [32] as a 58kD protein. Ubp6 proved to be sensitive to ubiquitin aldehyde and iodoacetamide suggesting it too is a cysteine protease. Its ability to hydrolyse Ub-αNH extensions and release free ubiquitin from poly-Ub-εNH protein conjugates confirmed its role as a deubiquitinating enzyme (DUB) [32].

It was initially suspected that Ubp6 could bind the proteasome given that it contained a UBL (UBiquitin Like) domain. This domain was known to be involved in binding Rad23 to the proteasome [33] and so when it was discovered in Ubp6 [34] a similar location was envisaged. It was also shown that the UBL domain was not required for Ubp6's deubiquitinating activity *in vitro*, implying that this domain has no effect on the catalytic site of the enzyme.

Association of Ubp6 with the proteasome was proven through the use of mass spectrometry to analyse affinity purified proteasomes [28]. This technique had

been used successfully in the past to identify the protein subunits of the ribosome and here it was employed to examine some of the proteins that associate most closely with the proteasome. One of these proteins was Ubp6. To confirm this finding, epitope-tagged Ubp6 was subsequently shown to co-immunoprecipitate subunits of the 19S proteasome such as Rpt1, Rpt6 and Rpn10.

A more in-depth study of this relationship between Ubp6 and the proteasome was subsequently carried out in mammalian cells (where the Ubp6 orthologue is USP14). A C-terminally modified ubiquitin derivative, ubiquitin vinyl sulphone (UbVS) was used to irreversibly label those DUBs that are cysteine proteases and in doing so, block the active site and repress the activity of these proteases [35]. This allowed an examination of activity of USP14 at the proteasome as well as confirming its association with it. [I^{125}] UbVS-labelled USP14 was detected in immunoprecipitated samples of the 26S proteasome, but not in fractions containing the 20S core particle alone. This suggested that USP14 associates only with mature 26S proteasomes, potentially via the 19S RP. In addressing some of the characteristics of USP14's deubiquitinating activity and how this is influenced by its association with the proteasome, it was found that upon inhibition of the proteasome by NLVS there was a 15-fold increase in USP14 active site labelling. Because UbVS labels the active site of cysteine proteases, an increase in labelling corresponds to an increase in activity and so this implies that when the proteasome is prevented from degrading proteins there is an increase in the deubiquitinating activity of USP14. This is not due to the increase in *de novo* synthesis of USP14 as a similar increase in activity was seen in cells where translation was inhibited by puromycin. It could be due to an increase in the recruitment of USP14 to the proteasome upon inhibition of proteolysis but for the fact that only a small amount of USP14 appears to be soluble – not enough for the associated increase in activity. It therefore seems that the activity of the USP14 present at the proteasome is enhanced upon inhibition of the proteasome. How and why this occurs is unclear. Does the inhibition of the proteasome change the physical conformation of the proteasome in the vicinity of USP14, thus allowing substrates increased access to its active site? Does this allow for the ''unclogging'' of proteasomes – that is releasing potential proteasome substrates when the CP pore is blocked? It is worth noting here that this active site labelling also picks up another proteasome-associated DUB – UCH37, but that the activity of this enzyme is not affected by proteasome inhibition. This seems to suggest that this effect is inextricably bound up with the function of USP14 at the proteasome – a function not provided by other DUBs. It is known, however that the Ubp6 null mutant in *S. cerevisiae* is viable [34] (but sensitive to canavanine) implying that the budding yeast cell can compensate for the loss of function of USP14/Ubp6.

Regarding the Ubp6 null mutant's sensitivity to canavanine, it was found that this sensitivity was rescued by the overexpression of free ubiquitin [2]. This would seem to imply that the toxicity of canavanine is due to the depletion of free pools of ubiquitin – that is not enough ubiquitin is released from substrates by Ubp6 at the proteasome to replenish free pools of ubiquitin. This was corroborated by an experiment that showed if cycloheximide was added to cells to prevent synthesis of new

ubiquitin, ubiquitin in Ubp6 null mutants was unstable over time, that is the lack of a DUB to remove ubiquitin from proteasome substrates resulted in the degradation of ubiquitin as well as their substrates [36].

Further analysis showed that Ubp6 bound the proteasome preferentially at the base of the 19S proteasome rather than the lid or 20S CP and that this binding was indeed mediated by its UBL domain [2]. Upon examination of the binding of Ubp6 to the proteasome it was found that while the UBL domain was necessary and sufficient for binding to the 19S base, binding to the lid required the presence of the catalytic site. While binding to the 19S RP stimulated the activity of Ubp6, binding to the base alone did not stimulate Ubp6 to the same extent. This suggests that although it binds the base via its UBL domain, the presence of the lid of the 19S RP is required for maximal activity. Upon closer examination it was found that the UBL domain bound Rpn1 [2]. That Ubp6's activity is important to the proteasome *in vivo* was seen when the hydrolysing activity of Ub-AMC was examined. While wild type proteasomes exhibit high levels of this activity, those from Ubp6 null cells do not, suggesting that most of this activity is attributable to the presence of Ubp6. It is also worth noting that levels of Ubp6 are similar to that of integral 19S subunits, again highlighting its importance *in vivo*.

However, while this work on Ubp6 in *S. cerevisiae* suggested that it was the main DUB at the proteasome [2], it is important to note that budding yeast do not have an orthologue of Uch2/UCH37, another proteasome-associated DUB which is found in *S. pombe* and mammals. Therefore a study of the relative importance of Ubp6 and Uch2 in *S. pombe* may be a more valid model for what occurs in mammals. A study was carried out to examine this by looking at *ubp6* and *uch2* null mutants and by using the same ubiquitin-AMC assay employed in budding yeast [37].

Neither *ubp6*Δ nor *uch2*Δ mutants were lethal, but the *ubp6*Δ mutant did exhibit synthetic lethality with *mts1* (*rpn9*), *mts2* (*rpt2*) or *mts3* (*rpn12*) temperature sensitive mutants, but not with *mts4* (*rpn1*), *mts8* (*β1*), *pad1* (*rpn11*) or *pus1* (*rpn10*) mutants. Further studies showed that this synthetic lethality was not in fact due to Ubp6's role as a DUB, but rather to its role in binding the 19S RP. Ubp6 without a UBL domain showed the synthetic lethality, while Ubp6 without a catalytic site did not. It is worth noting that the $rpn11^{D122A}$ and *ubp6*Δ are synthetically lethal in *S. cerevisiae* [38] suggesting that Ubp6 plays a different role in budding yeast as compared to fission yeast.

This difference was seen again when the deubiquitinating activity associated with the 26S proteasome was examined. Here, using the same assay that showed the importance of Ubp6 at the budding yeast proteasome [2], it was found that the main deubiquitinating activity at the fission yeast proteasome was Uch2 rather than Ubp6. It would appear therefore that with the presence of Uch2 in *S. pombe* cells, and by extension, UCH37 in mammalian cells, the importance of Ubp6 is far less than that seen in the Uch2-less *S. cerevisiae*. This has implications in the use of the budding yeast as a model for deubiquitination at the mammalian proteasome.

UCH37 (Uch2) was first recognised as an isopeptidase activity associated with the 19S of bovine proteasomes that disassembled polyubiquitin by "chewing" off

the ubiquitins one by one [39]. This activity was identified and explored further when it was described as Uch2 (Ubiquitin Carboxy-terminal Hydrolase) in fission yeast [40]. This was the *S. pombe* orthologue of mammalian UCH-L5 (mouse) / UCH37 (human). Both of these UCH DUBs had the UCH domain along with a C-terminal extension unlike other UCH proteins. The sub-cellular localisation of this protein was examined and it exhibited a perinuclear localisation during interphase and mitosis. However if the C-terminal extension was removed, Uch2 exhibited a more diffuse cell-wide location. From this evidence the authors decided to examine whether Uch2 was associated with the 26S proteasome, and if this was accomplished via the C-terminal domain. This proved to be true as Uch2 was co-immunoprecipitated with the 26S proteasome, while Uch2 lacking the C-terminal domain was not. Also upon the analysis of fractions of glycerol gradient centrifugation, Uch2 appeared to be closely associated with the proteasome and perhaps a 19S subunit [40]. Subsequent work in *Drosophila* identified the Uch2 orthologue, p37A as a subunit of the 19S RP by 2D gel electrophoresis and protein sequencing [41].

Another DUB thought to associate, albeit weakly, with the 26S proteasome is Doa4/Ubp4 [42]. Here the particular domain interacting with the proteasome is suggested to be the N-terminal 310 residues of the protein. Interestingly, *S. pombe* does not have an obvious orthologue of Doa4.

8.4 Shuttling Proteins: Rpn10/Pus1 and UBA-UBL Proteins

One of the major fields of interest regarding proteasome-interacting proteins is how the ubiquitination system and proteasome degradation system are connected or how are ubiquitinated proteins delivered to and recognised by the proteasome. One of the ways this has been studied is by looking for ubiquitin-chain interacting proteins and determining if these interact with the proteasome also.

One of the first proteins to be found to bind ubiquitin chains was, unsurprisingly, a proteasome subunit itself, the 19S RP's Rpn10 (*S. cerevisiae*) / Pus1 (*S. pombe*) / S5a (mammals). It was found that Rpn10 in *S. cerevisiae* bound ubiquitin with a preference for longer chains but that *rpn10*Δ mutants were viable [43]. It was known that when the ubiquitin-proteasome system is not functioning, for example in the case of other proteasome subunit mutants, cells are no longer viable [44]. Therefore the fact that the *rpn10*Δ mutant was viable implied that if Rpn10's role was to recognise ubiquitinated substrates, it was not the only protein to carry out that function. It was also found that while Rpn10 was a proteasome subunit, it could also exist as a slower sedimenting species indicating that a fraction of Rpn10 exists free of the proteasome [43].

Later a motif essential for multiubiquitin binding was found in Rpn10. This was a stretch of conserved hydrophobic amino acids in the C-terminal half of the protein – LAM/LALRL/V [45] – later described as a UIM (Ubiquitin Interacting Motif) domain [46]. However this motif was not required for Ub-Pro-β-gal degradation

nor did its loss affect sensitivity to canavanine. A similar result was found for the *S. pombe* orthologue, Pus1 [47]. Genetic interactions between *pus1*$^+$ and other 19S subunits, in particular *mts3*$^+$ (*RPN12* in *S. cerevisiae*), were also found. Overexpression of Pus1 could rescue the temperature sensitive mutant *mts3-1* at 32 °C, while *pus1*Δ*mts3-1* was synthetically lethal at the permissive temperature. However Pus1 could not rescue the *mts3* null mutant, which is lethal. This suggests that while Pus1 and Mts3 may interact *in vivo*, their functions were not identical. These two proteins were also shown to interact *in vitro*. However when the ubiquitin-binding motif was altered, then Pus1 could not rescue *pus1*Δ*mts3-1* or *mts3-1*, despite its still being able to bind Mts3. Interactions were also described between *pus1* and *mts4-1* (*rpn1*) and *pus1* and *pad1-1* (*rpn11*) although in this case synthetic lethality was rescued equally well by Pus1 with or without its LAMAL motif.

It was subsequently found that the DNA repair protein Rad23 (*S. cerevisiae*) could bind the proteasome through another domain – the UBL domain (Ubiquitin-Like) [33]. This domain is similar to the amino acid sequence of ubiquitin itself and had been known for sometime, as had its presence in Rad23. However its function up until then was unclear. Initially it was thought that given its similarity to ubiquitin, it targeted the protein containing it for rapid degradation [48]. However Rad23 had been found to be a highly stable protein, despite its possession of a UBL domain [49]. The significance of the UBL domain was not known therefore until it was shown that it was the means by which Rad23 could bind the proteasome [33]. There was also evidence to suggest that Rpn10 (Pus1) and Rad23 (Rhp23) played overlapping roles as the double null in budding [50] and fission [51] yeast exhibited cold sensitivity, canavanine sensitivity, slow growth and a G2/M phase delay – a more severe phenotype that either mutation alone. The increased sensitivity to the arginine analogue canavanine of *rad23*Δ*rpn10*Δ mutants suggested a defect in the ubiquitin-proteasome pathway. The same phenotype was seen in cells lacking Rpn10 and the UBL domain of Rad23. In addition, accumulation of multiubiquitinated substrates in the double mutant indicated a proteasome deficiency [50].

Other UBL-containing proteins were found to bind proteasomes in human cell lines [52]. These proteins, hPLIC1 and 2, are homologous to the *S. cerevisiae* Dsk2 and *S. pombe* Dph1. Both could immunoprecipitate elements of the proteasome although it appeared hPLIC2 associated with a subset of proteasomes bound to the cytoskeleton as well as some free proteasomes [52]. In *S. cerevisiae* a double knockout of *dsk2* and *rad23* display a G2/M cell cycle arrest [53]. This suggested that the two UBL domain-containing proteins encoded by these genes have overlapping functions.

Rad23 and Dsk2 have another domain in common – the UBA domain. The first description of a UBA (UBiquitin Associated) domain was as a theoretical motif found in several known E2s, E3s and DUBs. The function of the UBA domain was first suggested by Berolaet *et al.* when they provided evidence that Rad23 and Ddi1 in *S. cerevisiae* interacted with ubiquitin *in vitro* and *in vivo*, and that this interaction required the presence of their UBA domains [54].

The function of Rad23, Dsk2 and how these proteins overlap with Rpn10's role

in proteasome-mediated degradation was illustrated conclusively in *S. pombe*. It was shown that Rhp23 (Rad23) and Dph1 (Dsk2) could bind multiubiquitin chains (with far greater affinity than monoubiquitin) through their UBA domains and the 26S proteasome through their UBL domain *in vivo*. It was also found that the two Rhp23 UBA domains were not equivalent in their capacity to bind multiubiquitin, with the central UBA domain more potent than the C-terminal one. Given that these two proteins, Rhp23 and Dph1, were now known to bind ubiquitin chains and the proteasome, similar to Pus1 (Rpn10) – the triple deletion was constructed and found to be lethal. It should be noted here that the triple deletion in *S. cerevisiae* is viable but exhibited a severe slowed growth phenotype and a large accumulation of polyubiquitinated proteins [55]. This is probably due to the presence in *S. cerevisiae* of Ddi1, a UBL-UBA domain protein not seen in *S. pombe*. This would suggest that these three proteins perform overlapping functions that are vital to fission yeast cells. The next most severe phenotype was found in *pus1Δrhp23Δ* cells which had a severe growth defect at 25 °C and were not viable at 36 °C. It was found that, while Rhp23 could rescue this phenotype, Rhp23 without either its UBA domain or its UBL domain was unable to do so. (A similar result was obtained later in *S. cerevisiae* [56].) It could therefore be suggested that multiubiquitinated proteins can be delivered to the proteasome by these ''shuttling proteins'' – Pus1 (Rpn10), Rhp23 (Rad23) or Dph1 (Dsk2), and that the loss of this delivery system is lethal to the cell.

This model of substrate delivery has been confirmed and expanded in *S. cerevisiae* where it was found that cells lacking both Rad23 and Dsk2 were deficient in protein degradation mediated by the UFD pathway and that mutation of the UBA domain of Dsk2 resulted in cells deficient in proteolysis [57]. Also the two UBA domains of Rad23 appear to act synergistically [56]. Later Ddi1 was added to the growing list of UBA-UBL proteins that could interact with ubiquitin chains and the proteasome [55].

To elucidate the pathway further, experiments were then undertaken in *S. cerevisiae* to find where exactly on the proteasome the UBL domain of Rad23 binds. The answer appears to be Rpn1, a subunit of the base complex of the 19S RP. It was found that Rpn1 specifically recognised the UBL domain through an N-terminal region of Leucine Rich Repeat-like or LRR-like repeats with a short adjacent sequence, and that Rad23 and Dsk2 competed with each other to bind Rpn1 at the same site [58]. In *S. pombe*, it was shown that Rhp23 (Rad23) could bind Mts4 (Rpn1) with its UBL domain binding a region of Mts4 between amino acid 181 and 407 [59]. It was also shown that this domain of Mts4 could only be found in Mts4 orthologues and that another UBL-containing protein Udp7 (SPCC1442.07c) could bind this domain via its UBL domain. It therefore appeared that this region is a UBL-binding domain in the proteasome. This work also showed that Pus1 (Rpn10) could also bind Mts4 (Rpn1) but not in the same region as the UBL proteins. Pus1 bound a region of Mts4 between amino acid 408 and 582 termed the PC-repeat domain [59].

Subsequent work in human cell lines included the discovery of a hHR23 (Rad23) ''client'' protein *in vivo* – p53 [60]. Here evidence showed that hHR23 binds a poly-

ubiquitinated p53 via its UBA domain protecting it from deubiquitination *in vitro* and *in vivo*, with downregulation of hHR23 resulting in accumulation of p53.

More recently a cell-free system in *S. cerevisiae* has been used to examine the role of UBA-UBL proteins in substrate collection [61]. The biochemical evidence obtained here supports the genetic evidence in yeast for the shuttle protein hypothesis [51]. Wild type proteasomes (affinity purified) degraded the test-substrate (ubiquitinated-Sic1) efficiently, while those from *rpn10*Δ and *rad23*Δ did not. The proteasomes from the mutant strains were also deficient in deubiquitination. However if recombinant Rad23 were added to the Rad23-deficient proteasomes, efficient degradation and deubiquitination were restored. This effect was dosage dependent; at low concentrations recombinant Rad23 restored wild type degradation and deubiquitination, while at higher concentrations it inhibited these processes.

Further work has shown that the UBL domain of Rad23 can also bind Ufd2, an E4 enzyme and that mutation of the UBL domain alters this interaction and impairs the UFD proteolytic pathway [62]. This is also true for Dsk2, but not Ddi1, suggesting that Rpn1 and Ufd2 compete for the binding of Rad23 via its UBL domain. These findings raise the possibility that the UBL is not strictly a proteasome-binding domain but serves other functions in Rad23 and other UBL-carrying proteins. Also it is possible that the binding of an E4 like Ufd2 could enhance Rad23's shuttling function by placing it in the vicinity of the protein being ubiquitinated through binding the E4 via its UBL domain.

There is also evidence to suggest that Cdc48 along with its co-factors Npl4 and Ufd1 (see section 8.6) plays a role in recruiting Ufd2 to the oligoubiquitinated substrate allowing the polyubiquitination which will in turn recruit shuttle proteins like Rad23 and Dsk2 [63].

8.5 Other UBL-Containing Proteins

There are other UBL-containing proteins without UBA domains, therefore not shuttling proteins, that have been shown to interact with the proteasome. Bag1 (Bcl2-Associated athanoGene) is well conserved in eukaryotes except for the budding yeast orthologue which has no UBL domain. In mammals it had been known for some time that BAG1 could act as a cofactor of HSC70 (constitutive) and HSP70 (heat-induced) chaperone proteins [64], when it was found that its UBL domain did indeed allow it to interact with the 26S proteasome. It was also found that this association occurred in an ATP-dependent manner, and that it promoted the binding of Hsc70 and Hsp70 to the proteasome [65]. Bag1 has also been shown to stimulate the release of substrates from Hsp70 suggesting that perhaps if a protein cannot be correctly refolded by Hsp70, Bag1 functions to promote that protein's degradation.

Bag1 has also been shown to associate with an E3 ubiquitin ligase CHIP [66] (Carboxyl terminus of Hsc70-Interacting Protein) along with Hsc70 and Hsp70 and in fact that it is ubiquitinated by CHIP itself. However this ubiquitin chain

does not target Bag1 for degradation, but rather promotes the association of Bag1 with the proteasome [67, 68]. Therefore, both ubiquitinated and non-ubiquitinated Bag1 can interact with the proteasome.

Nub1 (Nedd8 Ultimate Buster) was found originally as an inhibitor of Nedd8 expression at the translation level [69] before subsequently being found to be a UBL domain-containing protein that interacts with Rpn10 in the proteasome [70]. It was also shown that Nub1 interacts with Nedd8-conjugated proteins and promotes their proteolysis by the 26 proteasome.

8.6 VCP/p97/cdc48

VCP (Valosin-Containing Protein)/p97/Cdc48 is an abundant ATPase of the AAA family of proteins. Since its initial discovery in budding yeast as a cell cycle mutant [71], it has been shown to play a role in a wide variety of cellular processes. Here we are concerned with its function as a proteasome interactor and the effect this has on the cell. In budding yeast it was found that Cdc48p is necessary for the degradation of a ubiquitin-fusion reporter protein [72] and this was shortly followed by a study in mammalian cells that described how it associates with ubiquitinated IκBα and the proteasome thereby targeting IκBα for degradation [73]. It was found that VCP interacts preferentially with polyubiquitinated IκBα, and that this interaction was necessary for the degradation of IκBα. Ubiquitinated forms of IκBα were stabilised in the absence of VCP. In addition, VCP could co-immunoprecipitate subunits of the 26S proteasome in an ATP-dependent manner.

It later became clear that Cdc48 seems to work with various adaptor proteins that aid in ''targeting'' its ATPase activity toward different cellular functions. It binds to p47 (Shp1/Ubx3) to carry out a Golgi membrane fusion role [74], and it forms a complex with Ufd1 and Npl4, which can both bind ubiquitin chains. This Cdc48-Ufd1-Npl4 complex performs a role in ubiquitin-mediated proteolysis at the endoplasmic reticulum, a process known as ERAD (Endoplasmic Reticulum Associated Degradation) [75]. Cdc48 uses its ATPase activity to physically remove ubiquitinated proteins from complexes for transport to the proteasome [76]. Specifically it has been shown that Cdc48-Ufd1-Npl4 is required to relocate ubiquitinated substrates (preferentially polyubiquitinated chains) from the ER into the cytosol for ubiquitin-mediated degradation [77].

Subsequently however, it became clear that p47/Shp1/Ubx3 can also bind ubiquitin, especially monoubiquitin, via a UBA domain and that this domain is essential for the role of Cdc48-p47 in membrane fusion [78]. It was therefore suggested that perhaps while Ufd1-Npl4 bound Cdc48 performs a role in ERAD by binding multiubiquitinated proteins and targeting them for degradation, p47-Cdc48 perhaps binds monoubiquitin to some other end. p47 (Shp1 in *S. cerevisiae* Ubx3 in *S. pombe*) contains a UBX domain (Ubiquitin regulatory X) a well-conserved domain believed to mediate Cdc48 binding [79]. It was established that there are seven family members and that all seven can bind Cdc48. In addition, *shp1* and

ubx2 null strains show defects in the degradation of a test substrate [80], although not in that of an ERAD substrate [81]. It has also been shown that *ubx3* null mutants in *S. pombe* are heat and canavanine sensitive and display synthetic lethality with *pus1* (*rpn10*) null mutants [81]. This suggests that p47/Shp1/Ubx3 function in ubiquitin-mediated proteolysis as well as membrane fusion, and that it serves an overlapping function with Pus1 (Rpn10).

Recently, new evidence has been found to support a novel model of Cdc48's role in ubiquitin-mediated proteolysis [63]. Richly and co-workers found that Cdc48 is required for the E4 enzyme Ufd2 to bind ubiquitin chains, and in mutants lacking Cdc48 or its co-factors, Rad23 (or Dsk2) is no longer able to bind Ufd2. This suggests that not only does Cdc48 facilitate Ufd2 binding to oligoubiquitinated substrates but it is also involved in recruiting Rad23 to substrates for degradation. It had been found previously, in a screen for ERAD-defective mutants, that Rad23 and Dsk2 were important in this process [82]. This implies a model whereby a substrate has a chain of one or two ubiquitin molecules added by its E1, E2 and E3 enzymes which in turn allows the binding of Cdc48 via its co-factors Npl4 and Ufd2. This recruits the E4 (such as Ufd2) to increase the length of the multiubiquitin chain which in turn allows the binding of UBL-UBA shuttle proteins (such as Rad23) which transfer the ubiquitinated substrate to the proteasome [63].

8.7 Proteasome Interactions with Transcription, Translation and DNA Repair

We have seen that the proteasome associates closely with various components of the ubiquitin system in order to make its recognition of proteins ready for degradation both efficient and accurate. But what of the role it plays in other cellular processes? Does the proteasome interact with proteins in order to connect itself to those processes requiring efficient degradation? Here we look at the proteasome's role in transcription and translation through the interactors that connect these vital cellular processes.

There is some evidence to suggest that the proteasome has a part to play in transcription, in particular that the proteasome can interact with RNA polymerase II. It was shown that some subunits of the 19S regulatory particle could be recruited to the Gal1-10 promoter upon induction of transcription [83]. These subunits included Rpt1-6 which are found in the base of the 19S RP. No other subunits from the lid or the 20S proteasome were found to associate with activated promoters and so the authors termed the subset of 19S subunits that were present at active promoters, the APIS complex – AAA Proteins Independent of the 20S proteasome. However there is no evidence to confirm the existence of such a complex in the cell. This reflected earlier work by the same group, where evidence was presented that the 19S complex is required for efficient elongation of RNA polymerase II [84]. Recent work by the same authors suggests that the 26S proteasome is associated physically with regions of induced genes that correlate with a build-up of RNA polymerase II [85]. These regions include the 3′ end of genes, sites of UV damage

and other locations that could represent pauses in elongation. The authors suggest therefore, that the 26S proteasome may be involved in some aspect of transcription termination. However the ubiquitin-proteasome system has been shown to be involved with transcription in more ways than one. These include regulation of chromatin structure and the controlled degradation of transcription activators. (See review [86]). Therefore the relative importance of the observed proteasome–RNA polymerase II interactions are unknown.

The proteasome also has a role to play at the level of translation. eIF3 is a translation initiation factor made up of many subunits. One of these, Sum1 (eIF3i) was found to change its intracellular localisation in *S. pombe* upon stresses such as osmotic and heat shock [87]. Following heat shock, Sum1 relocated to the 26S proteasome at the nuclear rim; this localisation of Sum1 was also shown to be dependent upon Cut8, the protein believed to localise the proteasome to the nuclear periphery. In temperature sensitive mutants of proteasomal subunits such as *mts4-1*, *mts2-1* or *pad1-1*, Sum1 no longer goes to the nuclear periphery upon heat shock. This implies that the relocation observed depends on a fully-functioning proteasome. In addition when Sum1 is over-expressed in *mts4-1* cells, they exhibit an elongated phenotype consistent with cell cycle arrest. In fact Mts4 and Sum1 physically interact *in vivo* [87].

In a similar vein, the mammalian eIF3 subunit eIF3e or Int6 was found to interact with Rpt4 in the yeast two hybrid system and *in vivo* by co-immunoprecipitation [88]. Int6 also co-immunoprecipitated the 20S proteasome subunit HC3 under conditions that allowed for an intact 26S proteasome suggesting that Int6 associates with the mature 26S proteasome.

This relationship between eIF3e/Int6 and the 26S proteasome was confirmed in fission yeast [89]. Here it was noticed that mutants of Yin6, the *S. pombe* orthologue of eIF3e/Int6, exhibit a similar phenotype to proteasome subunit mutants. They are sensitive to canavanine, they accumulate ubiquitinated proteins and they can increase the severity of proteasome subunit mutants. This showed that not only does Yin6 associate with the proteasome, but that it has an important role to play in the degradation of ubiquitinated protein, given that its loss had an effect on proteasome function. In addition *yin6* null mutants exhibited a cell cycle defect in that they had abnormally long mitosis and inefficient chromosome segregation.

Closer examination of the *yin6* null mutant uncovered a particular effect upon the 19S lid subunit Rpn5. It was found that while the *yin6* null mutant exacerbated proteasome subunit mutants, it did not affect *rpn5* mutants, suggesting their presence in the same pathway. The localisation of Rpn5 was also affected in the *yin6* null mutant in that it localised to the cytoplasm rather than at the nuclear periphery while Yin6 location was unaffected in the *rpn5* mutant. The overexpression of Rpn5 also partially rescued the *yin6* null phenotype but not vice versa. This seems to suggest that Yin6 functions upstream of Rpn5, and also that it may be required to target Rpn5 to the 19S lid. It is worth noting that Rpn5 lacks a nuclear localisation signal and so it is likely that it enters the nucleus as part of a complex. Yin6 has a nuclear export signal and a nuclear localisation signal. In fact, the whole proteasome appears misassembled in *yin6* null cells and the authors report they have

found some evidence that this is the case in *rpn5* null cells too. Whether the proteasome needs all its subunits to be correctly assembled or Rpn5 plays a particularly important role in proteasome assembly is unclear.

It is also worth noting that a *ras1* null mutant exacerbates the phenotypes of the *yin6* null mutant, while overexpression of Ras1 can rescue these phenotypes. This suggests a pathway whereby Ras1 can affect the proteasome via the interaction of Yin6 and Rpn5.

One of the more interesting aspects of this interaction between Yin6/Int6/eIF3e and the proteasome is the possibility of the crosstalk between the translation apparatus and proteolytic degradation apparatus via this interaction. Conceivably, translation and degradation need to be linked as a proofreading method of degrading incorrectly folded newly-translated proteins. This interaction could also be required as a means of co-ordinating the levels of certain critical proteins by the translation machinery receiving information from the degradation machinery and/or vice versa.

This relationship could also include crosstalk with the COP9 signalosome as there is evidence that these three complexes – eIF3, COP9 and the lid component of the 26S proteasome have similar structures and may have evolved in a similar fashion [90].

While we have discussed a shuttling role for Rad23 with regard to the ubiquitin-proteasome system (see section 8.4), it should be noted that Rad23 also has a vital role to play in DNA repair where it interacts with the nucleotide excision-repair (NER) factor Rad4 forming a dimer that can bind damaged DNA. What impact therefore does Rad23's interaction with the proteasome make on its role in DNA repair? It has been found that when Rad23 lacks a functional UBL domain *S. cerevisiae* cells are more susceptible to UV damage, and that this domain is required for optimal levels of NER [33]. Also Rad23 possesses a Rad4 binding domain that binds and stabilises Rad4 and in itself can rescue NER in *rad23*Δ cells. This action of Rad23 appears to occur entirely separately from its UBL-proteasome mediated function in NER [91]. There are also data to suggest that the Rad23-proteasome role in NER is independent of the 20S proteasome [92], but the mechanism of this role in NER is unknown.

8.8 Concluding Remarks

The proteasome is a simple machine – it degrades proteins to peptides. We have shown here, however that that the role the proteasome plays in the cell is far from simple. The first layer of control of degradation is via the 19S regulatory particle which restricts access to the protease activity of the 20S core particle. The ubiquitin signal introduces another level of regulation upon degradation. In this review we have discussed how the proteins which interact with the proteasome affect its function and direct its activity such that it becomes a finely controlled tool regulating most cell processes. For a summary of these interactions see Figure 8.2.

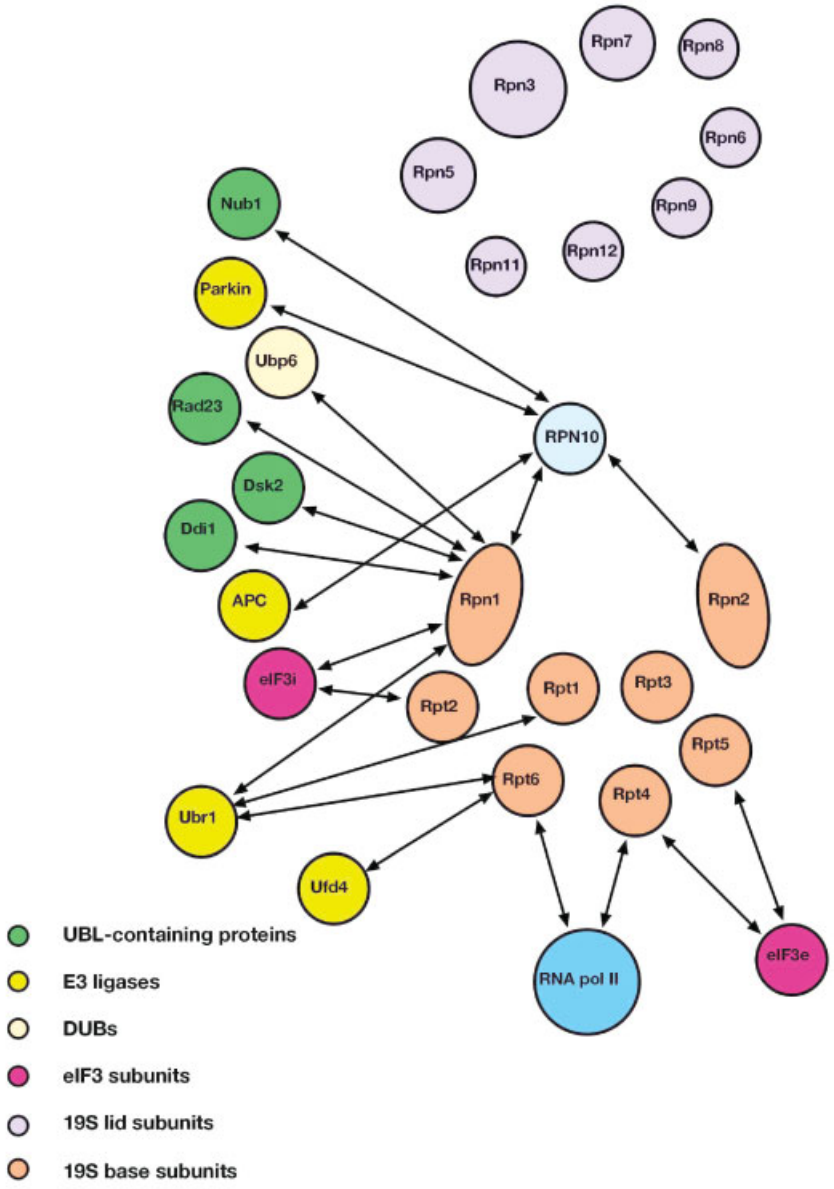

Fig. 8.2. Proteasome-interacting proteins that have been shown to interact with specific subunits of the 19S regulatory particle. Note: There is evidence that a pool of Rpn10 exists free in the cell as well as being a 19S subunit. It also functions as a shuttling protein in a similar fashion to UBA-UBL proteins. Therefore the interactions between it and subunits of the 19S RP are also shown here.

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9
Structural Studies of Large, Self-compartmentalizing Proteases

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9.1
Self-compartmentalization: An Effective Way to Control Proteolysis

Within the metabolic pathways of a cell, proteolysis plays a key role at different levels. The basic or ''housekeeping'' function is the degradation of proteins that are nonfunctional or misfolded due to mutations or as a result of stresses such as heat or oxidation. Such proteins are prone to aggregation and therefore should be removed. Regulatory proteins such as transcription factors or components of signal transduction chains need to be degraded at specific times of their life span. In the immune system, the activity of proteases ensures the availability of immunocompetent peptides that are produced via degradation of foreign proteins.

Intracellular proteolysis, however, is a hazard, and the destruction of proteins not destined for degradation must be prevented. An effective strategy for this purpose is to confine proteolysis to secluded compartments, where access is limited to proteins exhibiting degradation signals. Such a compartment can be a membrane-delimited organelle – such as the lysosome – or the proteolytic chamber of a self-compartmentalizing protease, a structural design that has evolved in prokaryotic cells, which are devoid of membrane-bound compartments [1]. This principle of self- or auto-compartmentalization has been implemented successfully in several unrelated proteases, the proteolytic subunits of which self-assemble into barrel-shaped complexes. The active sites of these protease complexes are sequestered physically in internal chambers and thus are accessible only for unfolded polypeptides. Accessory proteins – either transiently or continuously associated with the protease – recognize their target proteins, unfold them in an energy-dependent manner, and finally aid in translocating them into the interior. The translocation occurs through narrow orifices, which are likely to prevent immediate discharge and enforce a retention period, eventually leading to a minimum product size.

This concept has been realized successfully in all kingdoms of life, and the ATP-dependent proteolytic systems typically are linear assemblies, where the accessory proteins flank the protease unit. Examples for such adaptor-protease complexes are the 26S proteasome in eukaryotes; ClpX, ClpA, and ClpY associated with the

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Clp proteases ClpP or ClpQ in bacteria [2]; and the proteasome-activating nucleotidase PAN, which prepares proteins for degradation by the 20S proteasome in archaea.

In the successive degradation of the resulting, relatively small, products into amino acids, large complexes are also involved. Despite the relatively small size of their substrates, some of them, such as the tricorn protease in archaea and the eukaryotic tripeptidyl peptidase II, have masses of several megadaltons [3, 4]. In addition, their molecular architecture differs considerably from the linear barrel-shaped assemblies, and the route along which substrates enter and exit is less obvious.

In this review, we describe the structural and functional organization of key proteolytic complexes that are found in eukarya, eubacteria, and archaea, with the main emphasis on the giant proteases that have thus far been visualized in their fully assembled and fully functional oligomeric form only via electron microscopy (Figure 9.1).

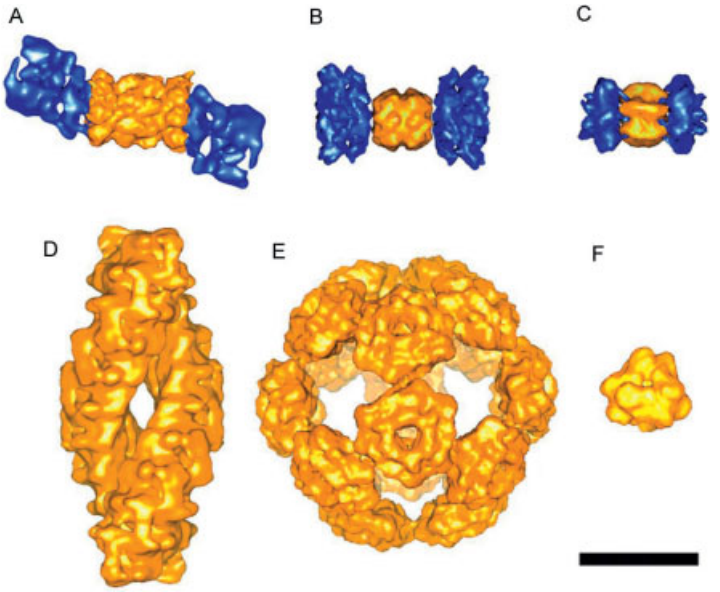


Fig. 9.1. Surface representations of large cytosolic proteolytic complexes drawn to scale. (A) 26S proteasome (20S: yellow; 19S caps: blue); (B) ClpAP complex (ClpP: yellow; ClpA: blue); (C) HslUV (HslV: yellow; HslU: blue); (D) tripeptidyl peptidase II; (E) tricorn protease capsid; (F) tetrahedral aminopeptidase. The structures of the 19S caps, tripeptidyl peptidase II, and tricorn were obtained from electron microscopy; for all other structures, the respective crystal structure was low-pass filtered to a resolution of 1.5 nm.

9.2
ATP-dependent Proteases: The Initial Steps in the Proteolytic Pathway

9.2.1
The Proteasome

The proteasome has frequently been described as ''the paradigm'' for a self-compartmentalizing protease. It is an ancestral particle that is ubiquitous and essential in eukarya and ubiquitous but not essential in archaea [5]. In eubacteria, where the proteolytic systems are redundant and the proteases Lon, Clp, HslV, and FtsH coexist, proteasomes are rare and not essential; genuine proteasomes have hitherto been found only in actinomycetes [6].

9.2.1.1 The 20S Proteasome

Architecture The quaternary structure of 20S proteasomes is the same in all kingdoms: the barrel-shaped 700-kDa complex consists of 28 subunits (termed α and β) that are arranged into four seven-membered rings. The four rings enclose three large cavities that are separated by narrow constrictions. One α ring and one β ring jointly form the outer (ante-) chambers, whereas two β rings enclose the central, proteolytic chamber. Crystal structures of 20S proteasomes from eukaryotes, archaea, and bacteria illustrate that α and β subunits share the same basic fold. This fold, which is typical for the superfamily of Ntn (N-terminal nucleophile) hydrolases [7, 8], consists of a pair of five-stranded β sheets flanked on both sides by α helices [9–13].

The 20S proteasome of the archaeon *Thermoplasma acidophilum*, like most prokaryotic proteasomes, exhibits $\alpha_7\beta_7\beta_7\alpha_7$-stoichiometry. The 20S proteasomes of the actinomycete *Rhodococcus erythropolis* contain two different α and β subunits. In eukaryotes, α- and β-type subunits have each diverged into seven distinct subunits; thus, eukaryotic proteasomes have a $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ stoichiometry and show pseudo-sevenfold symmetry.

The β subunits, which enclose the proteolytic chamber, are N-terminal hydrolases; the N-terminal threonine acts as both the catalytic nucleophile and the primary proton acceptor [9, 14]. In contrast to archaea, where all 14 β subunits are active, there are only six proteolytically active sites in a fully assembled eukaryotic proteasome, since four out of seven β subunits lack the N-terminal threonine residue. However, this discrepancy in the number of active sites in eukaryotic and prokaryotic proteasomes is not reflected in the size of the degradation products. Their average length is 7–8 residues and appears to be independent of number, specificity, and spatial arrangement of the active sites [15–17].

Assembly Unassembled proteolytically active β subunits carry propeptides, i.e., N-terminal extensions of variable lengths, which require post-translational removal for the formation of the active sites. Removal of these propeptides occurs autocata-

lytically and is delayed until the 20S complex is fully assembled, thereby ensuring that the active sites reside in a secluded, proteolytic chamber. The α subunits from *T. acidophilum* form seven-membered rings in the absence of β subunits [18], whereas the α subunits of the *Rhodococcus* proteasome do not form rings on their own, probably due to the size of their contact region, which is considerably smaller than corresponding α-subunit contact regions in *Thermoplasma*, yeast, and mammalian 20S proteasomes [11]. Instead, in the presence of β subunits, they assemble into αβ heterodimers and subsequently into half-proteasomes, which dimerize and eventually are activated by propeptide cleavage.

While the assembly of prokaryotic proteasomes proceeds independent of cofactors, assembly of eukaryotic proteasomes requires extrinsic maturation factors and must be carefully orchestrated to ensure the correct positioning of each of the 14 different subunits [19]. Some of the eukaryotic α subunits also assemble into ring structures that serve as a scaffold for subsequent beta-subunit assembly [20, 21].

Substrate access The three inner compartments of the *Thermoplasma* 20S proteasome are accessible only through the narrow entry ports at both ends of the particle. This was corroborated by electron microscopic studies of *Thermoplasma* 20S proteasomes where Nanogold-labeled insulin was used as substrate [22]. In the crystal structure of the *Thermoplasma* 20S proteasome, the entrance ports are 1.3 nm wide and are constricted by an annulus built from turn-forming segments of the seven α subunits. Thus, they appear to be open and accessible for unfolded proteins [9]. In contrast, entrance of substrate into yeast 20S proteasomes appeared to be blocked by a plug formed by the interdigitating N-terminal tails of the α subunits [23]. These N-terminal tails were disordered in *Thermoplasma* 20S proteasomes and thus not visible in the crystal structure. Deletion of the nine N-terminal residues of α3 of the yeast 20S proteasome led to an opening similar to that seen in *Thermoplasma* and to a much-enhanced peptidase activity compared to eukaryotic, wild-type 20S proteasomes, which have a low basal activity [23]. In fact, a similar activation also occurs in *Thermoplasma* proteasomes when the N-termini of the α subunits are deleted [24].

In vivo, eukaryotic proteasomes associate with regulatory complexes such as 19S caps, PA28, or PA26, which are known to activate the 20S complex. All of these adaptors interact with the terminal α rings of the proteasome and are likely to function by mechanisms that open the gate for substrate uptake (for reviews, see Refs. [25–29]). The same mechanism might be valid for archaeal proteasomes, which assemble at least transiently with hexameric ATPase complexes [30–33].

9.2.1.2 The PA28 Activator

The PA28 activator of the eukaryotic 20S proteasome is restricted to organisms with an adaptive immune system [34]. It is ATP-independent and stimulates the hydrolysis of small peptides, albeit not of denatured or ubiquitinylated proteins [35, 36]. PA28 induces dual substrate cleavages by the 20S proteasome and thus can enhance the generation of antigenic peptides [37, 38]. The expression of PA28

and that of the immunoproteasome subunits β1i, β2i, and β5i is induced by γ-interferon [39].

PA28 is a predominantly cytosolic complex of 200 kDa consisting of two related 28-kDa subunits, PA28α and PA28β, which assemble into a heteroheptamer [40, 41]. The PA28α heptamer, which is able to stimulate 20S proteasomes similarly to native PA28α/β, is composed of a bundle of α helices forming a cone-shaped structure traversed by a central channel [42, 43]. PA28 complexes can bind to both ends of 20S proteasomes, as has been shown by electron microscopy [44, 45].

PA26, a PA28-related protein in *Trypanosoma brucei* that stimulates the peptidolytic activity of *Trypanosoma*, rat, and yeast 20S proteasomes [46, 47], has been crystallized in complex with the yeast 20S proteasome. The structure reveals that the C-terminal regions of the PA26 subunits insert into pockets formed by the α subunits of the proteasome. This in turn induces conformational changes in the 20S α subunits, resulting in the proposed gate opening: the α tails are straightened out and pushed away from the entrance gates (Figure 9.2) [28]. Interestingly, the residues that stabilize the open conformation in eukaryotic proteasomes (i.e., Y8, D9, P17, and Y26) are also conserved and are important for proteolysis in archaeal proteasomes [33].

As shown by electron microscopy, opening of the axial channel also occurs when PA200, a large, 200-kDa nuclear protein with a dome-like structure, binds to the alpha-ring of 20S proteasomes [48].

9.2.1.3 The 19S Cap Complex

The major player in intracellular proteolysis in eukaryotes is the 26S proteasome, a 20S proteasome flanked by one or two 19S regulatory complexes that associate with the 20S core in an ATP-dependent manner [49–52]. The 26S proteasome links the ubiquitin system for targeting substrates for degradation with the machinery executing their degradation. The 19S regulatory complexes recognize ubiquitinylated proteins and prepare them for degradation via the 20S complex. These preparatory steps involve the binding of ubiquitinylated substrates, their deubiquitinylation, unfolding, and subsequent translocation into the 20S complex. The 19S complex is composed of two sub-complexes, the lid and the base, which are located distally and proximally in relation to the 20S core, respectively (Figure 9.3). 19S caps of *Drosophila melanogaster* have a mass of approximately 890 kDa and consist of 18 subunits, which were identified by two-dimensional gel electrophoresis [53]. Recognition and binding of ubiquitin-tagged substrates appear to be mediated by the eight subunits of the lid complex, since the 26S holoenzyme but not the 20S-base complex is capable of degrading ubiquitinylated proteins [54, 55]. 19S particles from mammals, *Drosophila*, and fission yeast, but not budding yeast, contain a deubiquitinylating subunit [53, 56, 57], which, using gold-labeled ubiquitin aldehyde [53], was mapped to the lid–base interface close to the proposed location of Rpn10, where multi-ubiquitinylated chains have been proposed to bind *in vitro* [58].

The base of the 19S cap contains the two largest subunits of the 26S proteasome S1/Rpn2 and S2/Rpn1 as well as six paralogous AAA ATPases. Attachment of the

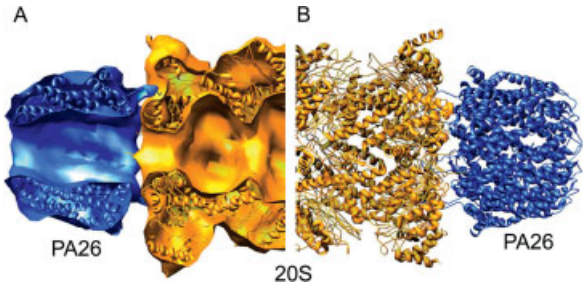


Fig. 9.2. The PA26–20S–PA26 complex. Structure of the hybrid complex between *T. brucei* PA26 (blue) and yeast 20S (yellow). (A) Cut-open view of the crystal structure low-pass filtered to a resolution of 1.5 nm combined with a ribbon representation showing the orifice of the proteasome. (B) Crystal structure of the PA26–20S–PA26 complex (PDB entry 1FNT [28]).

base complex only is sufficient to activate the 20S proteasome; thus, the ATPases must be involved in the gating of the α-ring channel and in controlling access to the proteolytic core. However, the symmetry mismatch between the 19S base and the 20S α ring suggests a gate-opening mechanism differing from the one found in PA26–20S complexes. Since both the base complex and its evolutionary ancestor PAN exhibit chaperone activity *in vitro* [32, 59], the ATP-dependent unfolding of substrates can be attributed to the ATPases acting in a "reverse chaperone" or unfoldase mode [31, 60].

The six paralogous ATPases of the base contain one copy of the AAA module [61–63] and an N-terminal coiled-coil region, which might mediate the binding of substrate proteins [64] and promote interactions between individual ATPases [65], hence playing a role in the assembly of the heterohexameric ring. All 19S ATPases are essential, as was shown by deletion analysis in fission and budding yeast [66–68]. Site-directed mutations in the Walker A motif of individual yeast ATPase sub-

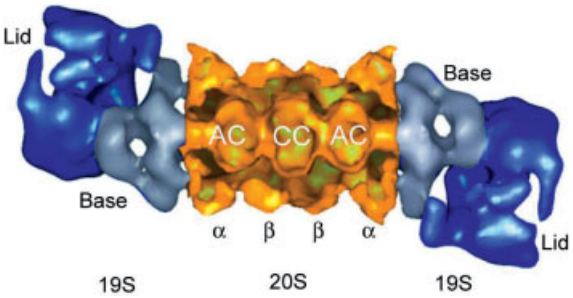


Fig. 9.3. The 26S proteasome. Composite image of the 3D structure of the 19S caps (lid: blue; base: gray) from *Drosophila melanogaster* [78] with a cut-away view of the crystal structure of the activated yeast proteasome (PDB entry: 1FNT [28]) low-passed filtered to 1.5 nm. AC: antechamber; CC: catalytic chamber.

units resulted in different phenotypes, indicating that the six ATPases of the base complex are not functionally redundant [69–71].

When observed in the electron microscope, the fully assembled 26S proteasome appears as an elongated dumbbell-shaped particle. 26S proteasomes have been isolated from different organisms; all preparations show a mixture of 20S particles associated with either one or two 19S units, resulting in total lengths of 30 nm and 45 nm, respectively [72–74]. Immunoprecipitation experiments suggest that 19S–20S–PA28 heterocomplexes also occur *in vivo* [75]; however, these appear to be very labile and have thus far been visualized only in the electron microscope after *in vitro* reconstitution [76, 77]. 2D averages of 26S proteasomes feature the characteristic "dragon head," where the 19S complexes in the double-capped particles face in opposite directions, reflecting the C2-symmetry of the eukaryotic 20S particle. In general, structural studies with 26S proteasomes are hampered by their low stability and tendency to dissociate into various sub-complexes. It is notoriously difficult to obtain a sufficiently homogenous and stable 26S preparation. Therefore, the only 3D structure of fully assembled 26S complexes available so far has been obtained from negatively stained 26S particles adsorbed on carbon film. Here, the 19S caps appear not to be in a fixed position with respect to the core but rather undergo an up-and-down "wagging-type" movement with a maximum amplitude of 2°, the functional relevance of which is not yet clear [78]. An even larger variety of states are observed when 26S proteasomes are frozen in a thin self-supporting layer of vitreous ice, where nearly mirror, symmetric, double-capped complexes also occur. This might hint at a possible rotary movement between the α rings of the 20S complex and the ATPase heterohexamer of the 19S base complex (Kapelari et al., unpublished results, [49]). In contrast, a recent immunoelectron microscopic study employing monoclonal antibodies against subunits α4 and α6 of the 20S complex outer rings suggests that the 19S cap complexes are attached to the 20S core complex in a defined orientation [79].

The 19S complex is an intricate, spongy structure from which it is not obvious what path a substrate protein will take before it is translocated to the 20S core (Figure 9.3) [78]. The observed flexible linkage between the caps and the core complicates structural analysis of the 26S complex, and a detailed analysis of a large set of ice-embedded 26S particles will be required to monitor the proposed rotational freedom of the 19S caps in the holocomplex and to confirm the proposed gate opening by the base complex.

9.2.1.4 Archaeal and Bacterial AAA ATPases Activating the 20S Proteasome

While eukaryotic 20S proteasomes can be isolated in association with a regulatory complex, interactions of activators with the 20S complex in bacteria and archaea seem to be rather transient. Some archaea contain the AAA ATPase proteasome-activating nuclease (PAN), which was first discovered in *Methanococcus jannaschii* as a homologue to the ATPases of the eukaryotic 19S base complex [80]. When mixed with 20S proteasomes from *Thermoplasma*, *Methanococcus* PAN stimulates the degradation of substrate proteins [81], and 20S–PAN complexes have been vi-

sualized whereby PAN apparently associates with the ends of the 20S proteasome cylinder [82]. Heterologously expressed PAN assembles into a 650-kDa complex, probably representing a dodecamer [81]. It recognizes ssrA-tagged green fluorescent protein as a substrate and mediates its energy-dependent unfolding and subsequent translocation into the 20S proteasome for degradation [24, 32, 83]. No homologues of PAN exist in *T. acidophilum* and its close relatives [84, 85]. Thus, in these organisms other complexes must substitute for the missing PAN function. In *T. acidophilum* the role of PAN is likely to be fulfilled by VAT, an archaeal AAA ATPase for which chaperone-like activity was demonstrated [86, 87]. Its eukaryotic homologues p97 and Cdc48 have been implicated in the degradation of substrate proteins via the ubiquitin proteasome pathway, thus making a role for VAT in protein degradation plausible [88, 89]. However, the putative substrate-binding domain of VAT and p97 is not a coiled coil, as characteristic for the proteasomal AAA ATPases, but rather a two-domain structure consisting of a double ψ barrel and a six-stranded β-clam fold [90, 91]. Both VAT and p97 form homohexameric 500-kDa toroids that have been characterized by electron microscopy [91–95]. The detailed intersubunit contacts have been revealed recently by crystal structures of the p97 complex [96–98].

A candidate for interaction with the bacterial proteasome is the ARC complex, a more distant member of the AAA family found in most bacteria possessing genuine 20S proteasomes [2, 99, 100]. ARC from *Rhodococcus erythropolis* is composed of two hexameric rings and, like the proteasomal ATPases, it contains an N-terminal coiled-coil domain [101, 102]. However, a functional interaction between the ARC complex and the *Rhodococcus* proteasome has not been demonstrated yet.

All of the hexameric complexes described above contain a central channel, which might serve to translocate the unfolded substrate into the 20S complex. However, the route taken by substrates during binding, unfolding, and translocation has not been visualized for the eukaryotic 26S proteasome, where the molecular understanding of the steps involved in transferring ubiquitinylated subunits from the lid to the base and eventually into the 20S core is substantially incomplete, nor has it been visualized for the corresponding 20S-activator complexes in bacteria and archaea. Although a pore-threading mechanism is conceivable, substrate unfolding via PAN occurs at the surface of the AAA ATPase ring [83]. Furthermore, in one of the recently solved crystal structures of the VAT homologue p97, a zinc ion occludes the central pore of the hexamer, which might prevent threading of the substrate [96, 97].

9.2.2 The Clp Proteases

Clp proteases are proteolytic complexes found in bacteria as well as in mitochondria and chloroplasts of eukaryotic cells [103]. Like the 26S proteasome, they function in ridding the cell of abnormal proteins as well as in regulatory circuits. Aside from Clp proteases, two other protease families are found in bacteria, Lon and

FtsH, in which both ATPase activity and proteolytic activity are joined in a single polypeptide chain.

As in the 26S proteasome, ATPase activity and proteolytic activity reside in different sub-complexes within ClpAP: ClpP is the proteolytic component, and ClpA is the ATPase [103, 104]. A second ATPase, ClpX, also assembles with ClpP to form ClpXP complexes [105, 106]. ClpA and ClpX recognize different signals for degradation and fulfill different regulatory functions within the cell [107].

The 21.5-kDa subunits of the serine protease ClpP assemble into two heptameric rings, isologously bonded, that enclose the proteolytic chamber lined with 14 active sites. The chamber is large enough to accommodate a protein of about 51 kDa [104, 108]; however, as in the 20S proteasome, the entry port is very narrow (10 Å) and thus not wide enough to admit most folded proteins [109].

The ATPase complexes ClpA and ClpX belong to the family of AAA^+ ATPases. ClpA consists of three structural domains: an N-terminal domain, representing a helical pseudo-dimer, followed by two AAA^+ modules [110]. In contrast, the N-domain of ClpX is shorter, is unrelated to that of ClpA, and is followed by a single AAA^+ module only. As was revealed by its solution structure, the ClpX N-domain contains a zinc-binding domain that forms a stable dimer [111, 112]. Both ClpA and ClpX are homohexameric in the presence of ATP or non-hydrolyzable ATP analogues [113, 114] and both have been crystallized [110, 115], but co-crystals with ClpP are not available as yet. The fully functional assembly has thus far been visualized only by electron microscopy, where heterocomplexes of the ClpAXP type also have been observed [116, 117].

In the assembled ClpAP and ClpXP complexes, the ATPase rings are positioned over the entry ports to the proteolytic chamber, analogous to the base complexes in the 26S proteasome. Substrates interact with ClpA and ClpX on the surface distal to ClpP, where the N-domains are likely to provide additional interaction sites [117–119]. Before being translocated through the narrow entrance site of ClpP, target proteins must be unfolded. Both ClpA and ClpX can act as chaperones and unfold proteins even in the absence of proteolytic activity [120–124].

In the fully assembled proteasome and Clp complexes, the protease and the ATPases form linear assemblies, an arrangement reflecting the sequence of steps necessary for substrate degradation. Time-resolved electron microscopic studies of ClpAP and ClpXP incubated with their respective model substrates RepA (ClpA) and λO (ClpX) allowed the visualization of the substrate pathway through a proteolytic machine "par excellence" [125–127]. The interaction of ClpAP and ClpXP with protein substrates involves several steps. Firstly, substrate binds to specific sites on the distal surface of the ATPase. Subsequently, the substrate is unfolded and fed into the digestion chamber of ClpP. Translocation – at least in ClpAP – appears to be a stepwise process whereby portions of substrate accumulate at the inner surface of the ATPase [125, 126] (Figure 9.4).

As is the case for the 26S proteasome, there is a symmetry mismatch between the (sixfold) ATPase unit and the (sevenfold) proteolytic unit in the ClpAP and ClpXP complexes [113]. For the ClpAP system, small rotational increments of

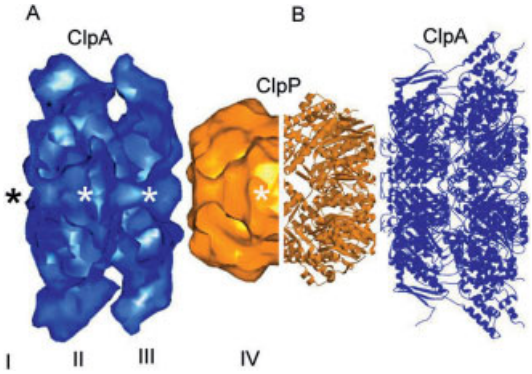


Fig. 9.4. The ClpAP complex. (A) Composite model of ClpAP as cut-away view. Crystal structures of ClpA (blue, PDB entry 1KSF [110]) and ClpP (yellow, PDB entry 1TYF [109]) have been low-pass filtered to 1.5 nm. The numbers describe the route of a protein substrate through ClpAP: (I) Substrates bind to specific sites on the distal side of ClpA. (II) Substrates are unfolded and translocated into the proteolytic chamber (IV). (III) Translocation in ClpAP appears to be a stepwise process during which portions of substrate accumulate at the inner surface of ClpA [125, 126]. (B) Composite crystal structure of ClpAP.

8.6° are sufficient to bring six- and sevenfold rings into (pseudo-)equivalent positions, a movement which might occur during the processive translocation of substrate into ClpP [30, 113]. Presumably, no symmetry mismatch occurs in HslUV (also called ClpYQ). Here, unlike the heptameric ClpP, the protease HslV (ClpQ) is a dimer of hexamers [128], whereas the ATPase HslU (ClpY) is dimorphic and assembles *in vitro* into hexameric and heptameric rings [129]. However, in the crystal structure of HslUV, both the ATPase and the protease complexes are hexameric [130–132]. The relative orientation of the ATPase HslU to the protease HslV has been a controversial issue: whereas in the crystal structure of HslUV obtained by Bochtler et al. [131] the "intermediate" (I) domains of HslU are pointing towards HslV, in the crystal structure of Sousa et al. [130] they extend outwards from the complex and the hexameric ATP-binding rings of HslU bind intimately to the HslV protease, an arrangement that is also consistent with the appearance of the HslUV complex in the electron microscope [129, 133].

While in proteasome and in Clp complexes the protease unit is flanked by one or two ATPase complexes, recent electron microscopic studies of CodWX, an N-terminal serine protease complex from *Bacillus subtilis*, reveal a strikingly different picture: the protease CodW, consisting of two stacked hexameric rings, binds to either one or both ends of a "spool-like" CodX-ATPase double ring, and even more elongated particles are found, in which an additional CodX double ring is bound to CodW [134, 135]. The physiological relevance of these linear assemblies with alternating ATPase and protease units is currently unclear.

9.3
Beyond the Proteasome: ATP-independent Processing of Oligopeptides Released by the Proteasome

The length of the fragments generated by the proteasome, Clp, and HslV varies from eight to 15 amino acids [15–17], but peptides of this length cannot be recycled by the cell and require further degradation down to the level of single amino acids. This task is performed by a number of ATP-independent proteases. In eukaryotes, proteasome degradation products are cleaved further by prolyl oligopeptidase (POP), thimet oligopeptidase (TOP), and tripeptidyl peptidase II. In archaea, this downstream processing is performed by the tricorn protease and its cofactors F1, F2, and F3 or by the tetrahedral aminopeptidase TET, which is found in archaea not containing any tricorn homologues (see Ref. [136] for a review).

9.3.1
Tripeptidyl Peptidase II

Among the proteases involved in downstream processing of oligopeptides released by the 26S proteasome, the tripeptidyl peptidase II (TPP II) complex has attracted attention owing to its extraordinary size, its versatility, and its apparent potential to substitute for some of the proteasome's functions.

TPP II was discovered in 1983 in the extralysosomal fraction of rat liver during the search for peptidases specific for proteins phosphorylated by cAMP-dependent protein [137]. It is a serine peptidase of the subtilisin type, which has broad substrate specificity, except that proline is not accepted in the P1 or P1′ positions [138]. The basic activity of TPP II is the removal of tripeptides from the free N-terminus of oligopeptides [137], but in addition to this exopeptidase activity, a much lower endopeptidase activity of the trypsin type was demonstrated [139]. Aside from soluble TPP II, a membrane-bound TPP II form exists in brain and in liver, which cleaves and inactivates the neuropeptide cholecystokinin as well as a number of different neuropeptides *in vitro* [140, 141].

Mammalian TPP II has a molecular subunit mass of 138 kDa, whereas the insect, worm, plant, and fungal forms carry an additional insert in the C-terminal region and have a molecular mass of 150 kDa [136, 142, 143]. The cDNA for TPP II does not contain any obvious signal peptide or membrane-spanning domain; thus, the membrane-bound TPP II is believed to bind to the membrane via a glycosyl phosphatidyl inositol anchor [141]. Since a number of variants of the TPP II–encoding mRNA exist (i.e., mRNA with long and short untranslated 3′ ends [144, 145]), one of them might encode this membrane-bound form. Pyrolysin, a membrane-bound serine endopeptidase from the archaeon *Pyrococcus furiosus* is the closest structural, albeit not functional, homologue [146].

Thus far, TPP II has been found only in eukaryotes and has been purified to apparent homogeneity from a variety of sources [4, 139, 142, 147, 148]. Like the pro-

teasome, it occurs in a variety of tissue types, and its broad substrate specificity indicates its participation in general intracellular protein turnover.

In addition to its proposed housekeeping function, cytosolic TPP II participates in the trimming of antigenic peptides to be presented by the MHC class I complex. Whereas the proteasome releases some antigenic peptides directly in their final form, others are produced as precursor peptides, possessing the correct C-terminus of the final antigenic peptide, but with an extended N-terminus that requires additional trimming [149]. Although this additional trimming activity occurs in the endoplasmic reticulum, where the interferon gamma–inducible aminopeptidase ERAP1 is involved [150–153], a screen using antigenic peptide precursors released from the proteasome revealed two cytoplasmic peptidases that are involved in this process: puromycin-sensitive aminopeptidase (PSA, a widely expressed monomeric cytosolic amino peptidase of 100 kDa) and TPP II [154]. Since TPP II never cleaved within the antigenic peptide sequence in this screen, it is speculated that it might play a role in protecting antigenic peptides from their complete hydrolysis in the cytosol [155].

Some studies suggest that TPP II can in part compensate for the loss of proteasomal function. Thus, cells treated with a high dose of proteasome inhibitor normally undergo apoptosis, but EL-4 cells adapted to this treatment responded with an increased TPP II activity [156]. When TPP II was overexpressed in EL-4 cells, they resisted otherwise-lethal proteasome inhibitor concentrations and did not accumulate polyubiquitinylated proteins [157]. Such an upregulation of TPP II activity also occurred in apoptosis-resistant cells derived from large *in vivo* tumors exhibiting decreased proteasome activity [158]. The same was observed in Burkitt's lymphoma cells, which are likewise apoptosis-resistant and did not accumulate polyubiquitinylated proteins in response to normally lethal doses of proteasome inhibitors. When TPP II activity was inhibited, this apoptosis resistance was abolished [159].

Whether TPP II is *de facto* able to substitute for the proteasome is still discussed. Cells with low proteasome activity apparently are still able to process ubiquitinylated proteins; however it is not clear whether this is due to residual proteasomal activity or to increased TPP II activity. Princiotta et al. [160] found that EL-4 cells adapted to NLVS (4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-leucinal-vinyl sulfone) still require proteasome function for the degradation of polyubiquitinylated proteins as well as antigen processing, and they concluded that the adaptation is not due to an induced alternative protease. They suggest that a shared substrate pool between proteasomes and TPP II might explain the observed survival of NLVS-treated EL-4 cells in which TPP II was overexpressed [157].

Many functional data are controversial, and our understanding of the structure is incomplete. As was shown by electron microscopy, the 138-kDa subunits of mammalian TPP II occur as discrete double-bow structures (ca. 50×20 nm), as single-bow structures, and as dissociation products of lower mass [161]. However, the predominant species is a double-bow oligomer composed of two single bows twisted together to a short double helix. Dissociation experiments to produce homogeneous breakdown products led to an accumulation of 8×9-nm structures, which

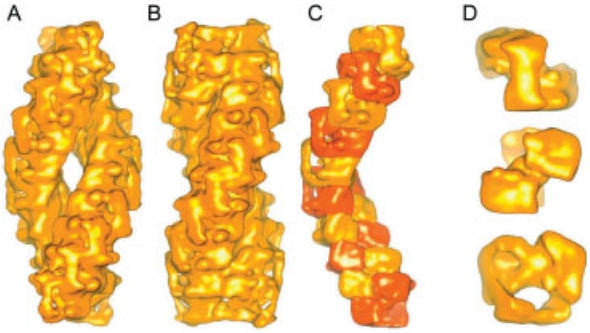


Fig. 9.5. Surface representations of TPP II as obtained by single-particle electron microscopy and 3D reconstruction. (A, B) Two perpendicular views of the TPP II complex. (C) Single TPP II strand with alternating coloring of the dimers in order to stress their interdigitation. (D) Different views of a computationally excised dimer.

in the presence of polyethylene glycol assembled into linear paracrystalline arrays. It is likely that these 8 × 9-nm structures represent the dimeric or tetrameric state of the enzyme with intersubunit contacts different from those in the bow structures [162].

TPP II complexes isolated from *Drosophila melanogaster* embryos are spindle-shaped 28 × 60-nm particles consisting of two segmented and twisted strands [4]. In the 3D reconstruction of the *Drosophila* TPP II complex at 2.2-nm resolution, each of the two strands is composed of a linear assembly of 10 interdigitated dimers (Figure 9.5) [163]. Intact TPP II complexes isolated from mammals and *Drosophila* are of defined length [4, 139], while TPP II particles heterologously expressed in *E. coli* often possess extensions beyond the spindle poles or occur as single strands of variable lengths, presumably as a consequence of the comparatively high TPP II concentration in those preparations. Treatment of such extended particles and single strands with destabilizing agents led to trimming of extensions and disassembly of single strands and demonstrated that the spindles observed in native preparations are the thermodynamically favored conformation. This stabilization of the spindles probably results from a double-clamp structure at the spindle poles in which the terminal dimer of one strand "locks" the two terminal dimers of its neighboring strand and vice versa [163].

TPP II exhibits the highest activity only when assembled into strands. Dissociation of the complex (e.g., upon dialysis) results in loss of activity. The minimal, active unit has been described as a dimer [143, 164]. Residue G252, which is conserved in all homologues, is apparently involved in complex formation since in mammalian TPP II the mutation G252R led to impaired complex formation and loss of activity. This effect is possibly a consequence of the location of G252 within the catalytic domain of TPP II (Asp-44, His-264, Ser-449) and its proximity to the catalytic His-264 [165, 166]. In wild-type TPP II, association and dissociation appear to be reversible provided the protein concentration is sufficiently high [164] and the equilibrium between both states is considered as a means of regulating

the enzyme's activity; however, the underlying mechanism of this regulation remains unknown at present. Some clues might be obtained by investigating conditions where TPP II activity is upregulated. Is the proposed equilibrium between associated and dissociated forms shifted to the fully assembled complex, resulting in higher TPP II activity, or is the increase in activity always accompanied by an increase in TPP II protein and mRNA, as was observed for septic muscles [167]?

Up to now, no substrate-localization studies on the TPP II complex have been performed. Thus, the questions of whether the active sites are buried in a channel, how many peptides are processed simultaneously, and where they enter and leave the TPP II complex remain unsolved. From electron microscopic studies of TPP II constructs containing a bulky tag at their N-terminus, we can conclude that the N-terminal domains including the catalytic residues are located at the inner backbone of the strands. Indeed, the linear stacking of the dimers into strands leads to the formation of a channel or an arcade with lateral openings through which substrate flow might occur [163].

9.3.2 Tricorn Protease

In the course of searching for regulatory components of the proteasome from *T. acidophilum*, a new, high-molecular-weight protease was found [168]. The purified protein eluted with a molecular mass of 720 kDa from size-exclusion columns; when subjected to SDS-polyacrylamide gel electrophoresis, only a single polypeptide chain of 121 kDa was observed. At the C-terminal end (residues 878–1036), this polypeptide showed significant homology to the *E. coli* tail-specific protease (Tsp) and to the mammalian interphotoreceptor retinol–binding protein (IRBP). Subsequent biochemical experiments indicated a preference for trypsin-like substrates, although one chymotrypsin-like substrate (alanyl-alanyl-phenylalanyl-7-amino-4-methylcoumarin, H-AAF-AMC) was also cleaved. This homohexameric protease was named tricorn due to its triangular shape in electron micrographs. Simultaneously, a much larger assembly of tricorn, approximately 50 nm in diameter and exhibiting icosahedral symmetry, was observed. These capsid-like structures were found in *Thermoplasma* cells as well as in the void volume of Superose 6-fractionated *Thermoplasma* lysate; recombinantly overexpressed His6-tagged tricorn failed to produce such capsid structures. Three-dimensional reconstructions of ice-embedded capsids led to a significantly improved representation of the tricorn hexamers within the tricorn capsids, with a nominal resolution of 1.3 nm [3, 169]. Such a tricorn capsid is assembled from 20 homohexameric toroids, resulting in a combined molecular mass of 14.6 MDa (Figure 9.5B). This form of supramolecular organization has been proposed to make substrate channeling more efficient by positioning the tricorn protease into close spatial relationship with potentially interacting aminopeptidases. Hitherto, a physiological function of these icosahedral complexes in the *Thermoplasma* cell could not be demonstrated experimentally; however, Tamura and colleagues characterized three aminopeptidases, F1, F2, and F3, the tricorn-interacting factors, that enable tricorn to accept a

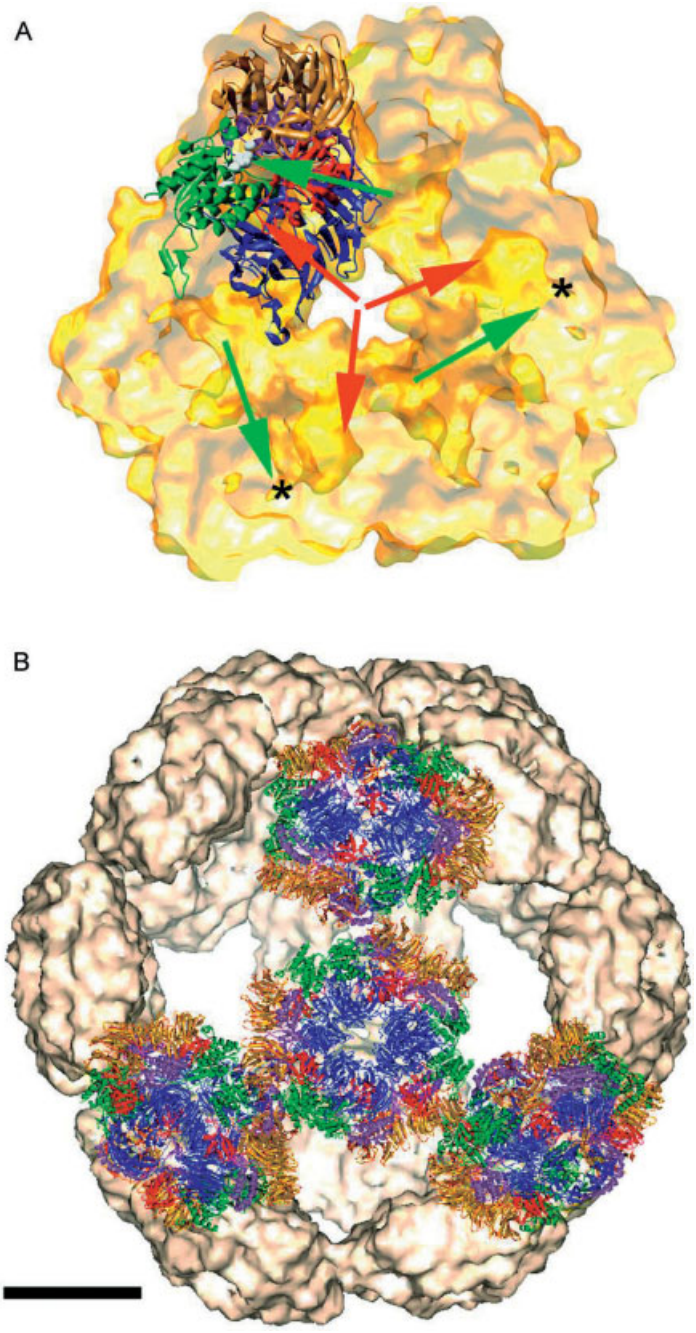
broader substrate range when mixed together [170, 171]. Like the eukaryotic, functionally homologous TPP II, the tricorn protease seems to act downstream of the proteasome, as polypeptides that are degraded by the proteasome to 6–12 mers are further degraded to di-, tri-, and tetrapeptides by tricorn.

These oligopeptides have to be processed further in order to recycle the amino acids within the cell, a task accomplished by the aminopeptidases F1, F2, and F3. F1 is a proline iminopeptidase (PIP) with 14% sequence identity to the catalytic domain of prolyl oligopeptidase (POP) [172]. A number of homologues of the F1 peptidase are known in bacteria and eukaryotes, all of them belonging to the superfamily of α/β hydrolases [136]. As judged by gel filtration studies, F1 migrates as a monomeric enzyme with a molecular mass of 33.5 kDa. The active site residues S105, H271, and D244 were identified by sequence alignments and verified by mutational studies. While functioning as a peptidase, releasing proline residues from short oligomers, F1 also enhances the cleavage activity of tricorn and, moreover, generates novel peptidase activities when assayed together with the tricorn peptidase [170].

The aminopeptidases F2 and F3 are closely related to one another, with an overall sequence identity of 56.3%, but are unrelated to F1. They harbor zinc finger motifs in the N-terminal half of their respective polypeptide sequences, and homologues are known in yeast and bacteria (e.g., PepN [173]). The enzymatic activities of F2 and F3 are inhibited completely by removing the coordinated zinc by metal chelators. Both enzymes migrate as monomers with a molecular mass of 89 kDa in gel filtration studies. They have overlapping substrate spectra, but each of them hydrolyzes specific substrates as well. All three interacting factors F1, F2, and F3 are efficient in cleaving only very short peptides of two to four residues; while F2 is mainly responsible for the release of basic residues, F3 releases acidic residues.

In vitro incubation studies revealed a sequential manner of peptide degradation in *T. acidophilum*: peptides released by the proteasome are further cleaved by the tricorn protease to di- and tetrapeptides, which eventually are degraded to free amino acids by either the tricorn-interacting factors alone or in their interaction with tricorn. Changing the incubation order of tricorn, F1, F2, and F3 with substrates released from the proteasome failed to produce free amino acids in an efficient way [171].

Formerly, the occurrence of tricorn protease appeared to be restricted to thermophilic archaeal genera such as *Thermoplasma* and *Sulfolobus*, but meanwhile, orthologous tricorn genes have also been found in several bacterial genomes [136]. For instance, the structural and functional characteristics of the tricorn-like enzyme from *Streptomyces coelicolor*, which was studied in some detail by Tamura and coworkers, are very similar to the respective complex from *T. acidophilum*. Interestingly, the genome of *S. coelicolor* harbors two genes with significant similarity to tricorn, one with a calculated molecular mass of 115 kDa and another of 125 kDa [174]. In *Streptomyces* cells, however, tricorn was expressed as a homohexamer selectively composed of the 115-kDa polypeptide type and not as a hetero-oligomer from both types. It is presently unclear whether the second tricorn gene is expressed in *S. coelicolor* at all or only under special environmental conditions. Judg-


A
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B

ing from the scattered distribution of tricorn genes across species, it seems plausible that different protein-degradation pathways exist in different species whereby functional homologues could replace tricorn's peptidase function (see Section 9.3.3).

In order to reveal the atomic structure of the tricorn protease and, by that means, to obtain more detailed functional and mechanistic insights, several groups have performed crystallization experiments. Two different crystal forms (C2, $P2_1$) of the *T. acidophilum* tricorn protease were reported [175, 176]. In both cases, the crystal lattice was built from hexameric toroids of 720 kDa. The D3-symmetric tricorn particle is assembled from two staggered and interdigitating trimeric rings. Due to the different contact areas within a tricorn hexamer, its assemblage can be described as a trimer of dimers. The 2.0-Å structure solved by Brandstetter et al. [176] revealed the subunit structure (Figure 9.6A). Thus, a single subunit is divided into five subdomains: a six-bladed β-propeller (residues M39–D310) followed by a seven-bladed β-propeller (A326–K675), and a PDZ-like domain (R761–D855) integrated between two mixed α-β domains (S681–G752 and R856–N1061). Both β6- and β7-propellers are open, Velcro-like structures, enabling a certain structural plasticity, possibly even a widening of the domain during substrate uptake [177, 178]. Such open, Velcro-like structures have so far been observed in POP, dipeptidyl peptidase IV (DPP IV/CD26), and alpha-L-arabinanase [172, 179, 180].

Co-crystallization experiments with specific tricorn inhibitors identified the residues S965 (nucleophile), H746 (proton donor), and D966 together with G918 (oxyanion hole) as the peptidase active site [176]. This active-site arrangement suggests that hydrolysis follows the classical mode of action of trypsin-like serine proteases (catalytic triad). After covalently binding the substrate C1 position to the active-site serine, a negatively charged tetrahedral transition state is formed. The other residues of the peptide are kept in place by hydrogen bonding, usually along a β strand or extended loop. During this step, the peptide bond is cleaved, one peptide product is attached to the enzyme in the acyl-enzyme intermediate, and the other peptide product diffuses away rapidly. In a second step of the reaction, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the second peptide product and to restore the active-site serine.

The dissociation of the product (di- or tripeptide) from the active-site residues generates the space necessary for the unprimed product to move forward for further processing [181]. A prominent cluster of basic residues (R131, R132) delineates the binding site of the substrate carboxy terminus. These basic residues together with the primed site topology clearly identify tricorn as a carboxypeptidase.

Fig. 9.6. Tricorn protease. (A) Cut-away representation of a tricorn hexamer with an overlay of one chain modeled as ribbons (PDB entry 1K32 [176]). The active-site residues are depicted as white spheres in the ribbon display and as asterisks on the cut-away representation. Blue: β7-propeller domain; yellow: β6-propeller domain; red: PDZ domain; purple and green: mixed alpha-beta domains. The main substrate pathway through the β7-propeller domain is marked by red arrows, whereas the alternative pathway through the inner cavities is highlighted by green arrows (see Section 9.3.2). (B) Reconstructed, three-dimensional density map of the icosahedral capsid at 1.3-nm resolution with an insert of four tricorn hexamers represented as ribbons.

The geometric dimensions explain tricorn's preferential di- and tripeptidase activity. In contrast to PDZ domains in other structures, the tricorn PDZ domain is not involved in substrate recognition; instead, it mainly serves to scaffold the subdomains [182].

Neighboring subunits have an effect on substrate recognition, especially the mobile side chain of residue D936, which is provided by a symmetry-related subunit and serves as a substrate-specificity switch accommodating both hydrophobic and basic P1 residues (preceding the cleavable bond). The P1 residue is held in place by main-chain interactions with the oxyanion hole (G918, D966). P2–P4 residues are bound through unsaturated main-chain hydrogen bonds at the strand I994-P996.

Based on the domain topology, the β7-propellers were suggested to serve as substrate filters for the active site in analogy to POP, while the β6-propellers might release the hydrolyzed peptides from the active site. A double cysteine mutant located at the entrance to the β7 channel (R414C and A643C) resulted in significant decreases in activity towards fluorogenic substrate and insulin B-chain, of 20% and 40%, respectively, compared to the wild-type activity. Co-crystallization experiments with long peptides with a C-terminal, inhibitory chloromethyl ketone group revealed a trapped peptide density stretching from the active site towards the β7 tunnel. Therefore, it has been suggested that the β7 domains might be suitable as major channels for substrate access to the active site, although the entrance of the β7 pores is obstructed by basic side chains, which form a lid (R369, R414, R645 and K646). An alternative but longer substrate pathway also seems plausible (Figure 9.6A). Following this scenario, the substrate peptide would reach the active site directly through funnel-like cavities emerging from the central channel of the tricorn hexamer. Convincing evidence for the role of the β6-propeller domain was obtained with the L184C mutant, which restricts the channel through the β6 domain when alkylated, thus leading to a reduced enzyme activity due to product accumulation [181]. Accordingly, F1 might bind in close proximity to the exit pore of the β6 domain, thus being well positioned for further degrading the released di- and tripeptides to free amino acids. This configuration would also be consistent with an arrangement in the whole icosahedral capsid, where only three F1 molecules could bind to a tricorn hexamer β6 domain, while the other three β6 domains would serve as anchoring points for neighboring tricorn hexamers [183] (Figure 9.6B).

Earlier biochemical studies indicated the presence of certain elements of the modular tricorn protease in humans, including their cofactor proteins [184]; however, other functional tricorn homologues might be difficult to detect in eukaryotes. Tricorn is not built of a single domain, but of five folding domains. Therefore, tricorn homologues might assemble noncovalently from different gene products.

9.3.3
Tetrahedral Aminopeptidase

Recently, a role analogous to that of tricorn in *Thermoplasma* has been ascribed to tetrahedral aminopeptidase (TET) from the halophilic archaeon *Haloarcula maris-*

mortui [185], a 500-kDa complex that degrades peptides of different length in organisms where tricorn is not present. TET is an aminopeptidase with broad substrate specificity that has a preference for neutral and basic residues and can progressively degrade peptides of up to 30–35 amino acids in length. This property assigns TET a role in processing peptides released by the proteasome (6–12 mers) or Lon (3–24 mers) [15]. Its 42-kDa subunits assemble into a dodecamer with a tetrahedral shape (edge length: 15 nm). In the 3D reconstruction obtained from negatively stained TET particles, four 2.1-nm-wide central channels emanate from the middle of each facet and converge into a central cavity. Additionally, a smaller channel of 1.7 nm emanates from the apices. Their asymmetry is discussed as reflecting the route of substrates through the complex, and it is suggested that the wider channels serve as entry ports for the peptide chain and the narrower channels as exit sites for the released peptides [185]. In the recently determined crystal structure of the TET homologue FrvX from *Pyrococcus horikoshii* (Figure 9.7), the smaller channel is nearly completely blocked by a phenylalanine residue [186]. Based on the crystal structure of TET from *Pyrococcus horikoshii*, a unique mechanism of substrate attraction and orientation is discussed. Here, the interior of the four central openings leading to the central, proteolytic chamber is negatively charged, forcing the peptide substrates to enter the proteolytic chamber with their N-termini. While substrate access is proposed to occur through the 1.8-nm-wide central openings, the proposed exit channels are not located at the vertices of the tetrahedron but are arranged in close proximity to the central openings at the facets [187] (Figure 9.7C).

9.4 Conclusions

Large, self-compartmentalizing proteases occur in all three kingdoms of life and all along the degradation pathway. The proteolytic complexes participating in the first steps of proteolysis associate linearly with hexameric ATPase complexes; their task is the recognition, unfolding, and subsequent translocation of target proteins into the respective proteolytic compartments. In general, such assemblies are labile, and thus their crystallization is challenging. Consequently, the functional assembly of most large, multicomponent proteolytic complexes has so far been visualized only by electron microscopy. Substrates en route through ATPase–protease complexes have hitherto only been observed with ClpAP/ClpXP. While the sequence of events is likely to be exemplary for the whole group of ATP-dependent protease complexes, the final objective must be the mapping of substrate-interaction sites, e.g., within the 19S subunit of the 26S proteasome, as well as the study of the mechanisms involved in substrate translocation. Also, the role of giant oligomeric superstructures formed by tricorn and TPP II is still rather enigmatic. While the proteolytic activity of tricorn capsids appears not to be different from the activity of hexamers, TPP II is fully active only when assembled into strands. For tricorn, a possible pathway for the substrates has been proposed, whereas for TPP II, no

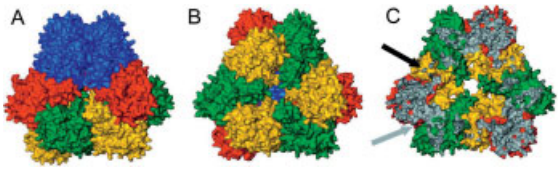

Fig. 9.7. Tetrahedral aminopeptidase. (A) Surface representation of the TET homologue FrvX from *Pyrococcus horikoshii* as a side view (PDB entry 1XFO, color-coding as in Ref. [186]). (B) Top view down the crystallographic 3-fold axis at one facet with the wide channel at the center. (C) Cut-open view down the crystallographic 3-fold axis along one edge. The black and gray arrows describe the location of the wider and the narrower channels, respectively. Around the large opening in the facet, three small openings are visible that might represent exit channels as discussed in Ref. [187].

data of that kind are available as yet. Thus, further structural studies including hybrid electron microscopy and X-ray crystallography approaches will be required to obtain insight into the modes of operation of these large proteolytic machines.

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10
What the Archaeal PAN–Proteasome Complex and Bacterial ATP-dependent Proteases Can Teach Us About the 26S Proteasome

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10.1
Introduction

Much of what we have learned about biochemical pathways, gene transcription, and protein synthesis emerged initially from studies in bacteria that provided the basis for the subsequent elucidation of these processes in eukaryotic cells. Studies in prokaryotes have also provided fundamental insights into the physiological significance and mechanisms of protein degradation, although these major contributions have often been overlooked in discussions of the ubiquitin–proteasome pathway. Surprisingly, the importance of intracellular protein breakdown was not appreciated by microbiologists for a long time. In fact, until the mid-1970s, it was generally taught that in bacteria, in contrast to mammalian cells, proteins were stable after synthesis (Goldberg and Dice 1974). This conclusion was based upon classic, but over-interpreted, studies by Monod and coworkers, who showed that rates of protein breakdown (compared to rates of synthesis) are very low in *Escherichia coli* during exponential growth (Hogness et al. 1955). However, in the 1970s, our understanding of the importance of protein degradation in *E. coli* changed dramatically with the discovery that these cells rapidly degrade misfolded or incomplete proteins (Goldberg 2003; Goldberg and Dice 1974); that the overall degradation of normal proteins is regulated and increases rapidly in cells lacking amino acids or a carbon source; and that in bacteria, as in the mammalian cytosol, proteins are degraded by a process requiring ATP (Goldberg and St John 1976).

Because bacteria lack lysosomes, this discovery implied that the energy requirement for intracellular proteolysis was a universal feature of protein breakdown and was not related to the functioning of lysosomes, which were then believed to be the exclusive site of protein breakdown (Ciechanover 2005; Goldberg 2005). Our subsequent discovery of the existence of the soluble (non-lysosomal) ATP-dependent proteolytic system in reticulocytes (Etlinger and Goldberg 1977) was followed by establishment of similar cell-free systems in bacteria (Murakami et al. 1979) and subsequently in mitochondria, in which turnover of proteins uses enzyme systems quite similar to those in eubacteria (Desautels and Goldberg 1982a, 1982b). In analyzing this process in bacteria, we discovered that it depended on a new kind

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of enzyme, large ATP-dependent proteolytic complexes that degrade proteins and ATP in linked processes (Chung and Goldberg 1981; Gottesman 1996). As discussed below, bacteria and archaea were later found to contain several such proteolytic complexes, which function in the degradation of different types of proteins (Gottesman 1996). We had initially chosen to work in *E. coli* because of the opportunity to use genetic approaches, and, in fact, mutants lacking these ATP-dependent proteases (e.g., *lon*$^-$ strains) are defective in breakdown of misfolded and certain regulatory proteins (Gottesman 2003). Unexpectedly, with the advent of recombinant DNA, these protease-deficient strains have also proven particularly useful for expression of cloned proteins, many of which are rapidly degraded in bacteria (Baneyx and Mujacic 2004; Goldberg 2003).

Ironically, the discovery of the first ATP-dependent protease (lon/La) came at the same time as the classic discovery of the role of ubiquitin in protein breakdown in the reticulocyte system by Hershko, Ciechanover, and Rose (Ciechanover 2005; Glickman and Ciechanover 2002). This modification was proposed to explain the ATP requirement for intracellular proteolysis. Thus, two very different explanations for this requirement emerged in eukaryotes and prokaryotes, and they were initially assumed to constitute a fundamental distinction between these organisms. However, with time, it became clear that after proteins are ubiquitinated, ATP is still necessary for their breakdown (Tanaka et al. 1983), and by the late 1980s, the 26S proteasome, an ATP-dependent proteolytic complex that degrades ubiquitinated proteins, was identified. As discussed here, many of its special properties are similar to those of bacterial and archaeal ATP-dependent proteases. In fact, the isolation of the 26S proteasome by Rechsteiner's and our lab (Hough et al. 1987; Waxman et al. 1987) utilized stabilizing conditions (e.g., glycerol) and biochemical assays originally developed in studies of the bacterial ATP-dependent proteases.

As discussed below, studies of these ATP-dependent protease complexes from archaea and bacteria have proven very valuable in illuminating the structure and enzymatic mechanisms of the 26S proteasome (Voges et al. 1999). Certainly, the 20S proteasome from archaea and its regulatory ATPase complex, PAN, have been most informative in this regard. Apparently, this ancestral system evolved before protein breakdown became linked in eukaryotes to ubiquitination to enhance the selectivity and regulation of this process. Because the key properties of the archaeal complex have been conserved, its study offers many unique advantages for elucidation of the 26S proteasome function.

Eubacteria can utilize any of a number of ATP-dependent proteases (e.g., Lon, ClpAP, ClpXP, HslUV, FtsH) to eliminate short-lived regulatory or unwanted abnormal proteins. In contrast, the cytosol and nucleus of eukaryotic cells contain only one ATP-dependent proteolytic complex, the 26S proteasome, which is much larger and has a much more complex structure than these prokaryotic enzymes. In addition, although several prokaryotic ATP-dependent proteases have an architectural organization similar to that of the eukaryotic 26S proteasomes, for all of them, except HslUV, the peptidase component does not share homology with the eukaryotic 20S proteasome.

Archaea and eukarya have a common ancestor that is not shared by eubacteria. Therefore, although archaea resemble eubacteria in most cytological features, many archaeal proteins and biochemical pathways are more closely related to those of eukarya (Doolittle and Brown 1994). An excellent illustration is the presence in archaea of 20S proteasomes, which are not found in eubacteria with the exception of actinomycetes, such as *Rhodococcus erythropolis* and *Mycobacteria*. Although archaea lack ubiquitin and the lid components of the 19S regulatory complex, their 20S proteasomes function in ATP-dependent degradation. Indeed, in place of the large 19S regulatory particle and its many distinct subunits, archaeal proteasomes depend on hexameric ATPase complexes of the AAA^+ family. Some archaeal species, including *Methanococcus jannaschii* and *Archaeoglobus fulgidus*, contain one AAA ATPase, the proteasome-activating nucleotidase (PAN), that exhibits high sequence similarity to all six ATPases of the 19S. In the presence of ATP, PAN was shown to stimulate protein degradation by 20S proteasomes from *Thermoplasma acidophilum*, the best-characterized prokaryotic proteasomes (Maupin-Furlow et al. 2004; Zwickl et al. 2000). PAN appears to be the ancestor of the 19S base, before protein degradation became linked to ubiquitin conjugation and the evolution of the 19S lid from the signalosome particle. Because of the simplicity of archaeal PAN and 20S proteasomes, their homologous subunit organization, and their ease of expression in *E. coli*, their structural and biochemical properties have been extensively studied. The discovery of the PAN complex has provided a powerful experimental system for investigating the role of ATP and the biochemical mechanisms involved in the process of substrate translocation into 20S particles. In this chapter, we describe the archaeal PAN–20S complex and review how this complex and related eubacterial ATP-dependent proteases have helped us in understanding the biochemistry of energy requirement in protein breakdown.

10.2 Archaeal 20S Proteasomes

The 20S proteasome was first identified in an archaebacterium (*T. acidophilum*) by Dahlmann et al. (1989). Since then, 20S proteasomes have been found in many other archaeal species (for reviews, see Zwickl 2002 and Zwickl et al. 2000). Dahlmann et al. (1989) pointed out that archaeal 20S particles have a cylindrical shape similar to that of eukaryotic particles, but a much simpler subunit composition and a more limited spectrum of proteolytic activities. Indeed, most archaeal 20S particles contain only two different subunits, the α- and the β-subunits, although the genome of some species such as *Haloferax volcanii* and *Pyrococcus furiosus* contain two types of α- and β-subunits. Whether these different types of subunits exist within the same 20S particle, as has been shown for *R. erythropolis* particles, or in different class of proteasomes remains to be established. As in eukaryotic 20S particles, archaeal proteasomes are composed of four stacked rings, two inner rings composed of seven β-subunits and two outer rings composed of seven α-subunits.

The outer α-rings mediate interaction with proteasome activators (in eukaryotes, 19S ATPases or 11S) and control substrate entry and/or exit.

The α-subunit from *T. acidophilum* is homologous to the seven α-subunits from *Saccharomyces cerevisiae* (27–39% similarity as shown by multiple sequence alignment in Figure 10.1). A cluster of four conserved residues located at the amino-terminal extremities (Tyr8, Asp9, Pro17, and Tyr26, based on the numbering of *T. acidophilum* sequence) stabilizes a conformation with an open entry pore (Forster et al. 2003, 2005).

The β-subunits are responsible for the proteolytic activity. The β-subunit from *T. acidophilum* and three of the β-subunits from eukaryotic 20S (β1, β2, and β5) are produced as precursors, and are processed to an active form by removal of a prosequence (Voges et al. 1999). This processing leads to a primary sequence starting with a threonine residue. The primary sequence of the processed β-subunit from *T. acidophilum* has 23–25% similarity to those of *S. cerevisiae*, as shown by multiple sequence alignment with two absolutely conserved motifs (GXXXD and GSG) (Figure 10.2). Most conserved residues are located in the N-terminal region and certain ones are of particular importance for catalysis as Thr1, Glu17, and Lys33 (based on the numbering of *T. acidophilum* sequence).

Proteasomes from *T. acidophilum* were initially reported to exhibit only chymotrypsin-like activity (cleave after hydrophobic residues using standard model fluorogenic peptide substrates) and no substantial trypsin-like cleavages (after basic residues) or caspase-like activity (after acidic residues) as found in eukaryotic 20S particles (Dahlmann et al. 1989). Similar observations were made on proteasomes from other archaea such as *H. volcanii* (Wilson et al. 1999). However, subsequent studies indicated that *T. acidophilum* particles have a clear capacity to hydrolyze standard basic and acidic peptide substrates, although much more slowly than the standard substrate of the chymotrypsin-like activity (Akopian et al. 1997). Furthermore, when the peptides produced during polypeptide degradation by *T. acidophilum* 20S were examined, chymotryptic cleavages were neither the exclusive nor the predominant type, suggesting that proteasome active sites have a broader specificity than can be assayed by studies with several fluorogenic or chromogenic peptides (Akopian et al. 1997; Wenzel et al. 1994). In fact, these particles were recently shown to rapidly cleave after glutamine residues, which the three specialized active sites of eukaryotic proteasomes cannot do (Venkatraman et al. 2004). Also, the 20S proteasomes from *Methanosarcina thermophila* and *M. jannaschii* exhibited both chymotryptic- and caspase-like activity against model substrates (Maupin-Furlow et al. 1998; Maupin-Furlow and Ferry 1995).

Certainly the clearest difference between archaeal and eukaryotic proteasomes lies in the mechanism for recognition of the substrate. In eukaryotes, the main pathway to select proteins for degradation by 26S proteasomes is by covalent attachment of multiple ubiquitin moieties through a complex enzymatic cascade involving at least three types of enzymes (E1, ubiquitin activating; E2, ubiquitin conjugating; E3, ubiquitin ligase) and ATP hydrolysis (Pickart and Eddins 2004). A ubiquitin conjugation machinery has never been found in any bacterium, nor has any other general mechanism to target proteins for degradation been identified in

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T20S α      ----MQQGQMAYDRAITVFSPDGRLFQVEYAREAVKKG-STALGMKFANGVLLISDKKVR 55
Y20S α1     MSGAAAASAAGYDRHITIFSPEGRLYQVEYAFKATNQTNINSLAVRGKDCTVVISQKKVP 60
Y20S α2     -------MTDRYSFSLTTFSPSGKLGQIDYALTAVKQG-VTSLGIKATNGVVIATEKKSS 52
Y20S α3     ------MGSRRYDSRTTIFSPEGRLYQVEYALESISHA-GTAIGIMASDGIVLAAERKVT 53
Y20S α4     --------MSGYDRALSIFSPDGHIFQVEYALEAVKRG-TCAVGVKGKNCVVLGCERRST 51
Y20S α5     ----MFLTRSEYDRGVSTFSPEGRLFQVEYSLEAIKLG-STAIGIATKEGVVLGVEKRAT 55
Y20S α6     ------MFRNNYDGDTVTFSPTGRLFQVEYALEAIKQG-SVTVGLRSNTHAVLVALKRNA 53
Y20S α7     ----MTSIGTGYDLSNSVFSPDGRNFQVEYAVKAVENG-TTSIGIKCNDGVVFAVEKLIT 55

T20S α      SRLIE-QNSIEKIQLIDDYVAAVTSGLVADARVLVDFAR-ISAQQEKVTYGSLVNIENLV 113
Y20S α1     DKLLD-PTTVSYIFCISRTIGMVVNGPIPDARNAALRAK-AEAAEFRYKYGYDMPCDVLA 118
Y20S α2     SPLAM-SETLSKVSLLTPDIGAVYSGMGPDYRVLVDKSRKVAHTSYKRIYGEYPPTKLLV 111
Y20S α3     STLLEQDTSTEKLYKLNDKIAVAVAGLTADAEILINTAR-IHAQNYLKTYNEDIPVEILV 112
Y20S α4     LKLQDTRITPSKVSKIDSHVVLSFSGLNADSRILIEKAR-VEAQSHRLTLEDPVTVEYLT 110
Y20S α5     SPLLE-SDSIEKIVEIDRHIGCAMSGLTADARSMIEHAR-TAAVTHNLYYDEDINVESLT 113
Y20S α6     DELS---SYQKKIIKCDEHMGLSLAGLAPDARVLSNYLR-QQCNYSSLVFNRKLAVERAG 109
Y20S α7     SKLLV-PQKNVKIQVVDRHIGCVYSGLIPDGRHLVNRGR-EEAASFKKLYKTPIPIPAFA 113

T20S α      KRVADQMQQYTQ-YGG-----VRPYGVSLIFAGIDQIG--PRLFDCDPAGTINEYKATAI 165
Y20S α1     KRMANLSQIYTQRAY------MRPLGVILTFVSVDEEL-GPSIYKTDPAGYYVGYKATAT 171
Y20S α2     SEVAKIMQEATQSGG------VRPFGVSLLIAGHDEFN-GFSLYQVDPSGSYFPWKATAI 164
Y20S α3     RRLSDIKQGYTQHGG------LRPFGVSFIYAGYDDRY-GYQLYTSNPSGNYTGWKAISV 165
Y20S α4     RYVAGVQQRYTQ-SGG-----VRPFGVSTLIAGFDPRDDEPKLYQTEPSGIYSSWSAQTI 164
Y20S α5     QSVCDLALRFGEGASGEERLMSRPFGVALLIAGHDADD-GYQLFHAEPSGTFYRYNAKAI 172
Y20S α6     HLLCDKAQKNTQSYGG------RPYGVGLLIIGYDKS--GAHLLEFQPSGNVTELYGTAI 161
Y20S α7     DRLGQYVQAHTLYNS------VRPFGVSTIFGGVDKNG--AHLYMLEPSGSYWGYKGAAT 165

T20S α      GSGKDAVVSFLEREYKENLP---EKEAVTLGIKALKSSLEEGEE---------------- 206
Y20S α1     GPKQQEITTNLENHFKKSKIDHINEESWEKVVEFAITHMIDALGTEFSK----------- 220
Y20S α2     GKGSVAAKTFLEKRWNDELE---LEDAIHIALLTLKESVEGEFNGDTIELAIIGDE---- 217
Y20S α3     GANTSAAQTLLQMDYKDDMK---VDDAIELALKTLSKTTDSSALTYDRLEFATIRKGAND 222
Y20S α4     GRNSKTVREFLEKNYDRKEPPATVEECVKLTVRSLLEVVQTGAKNIEITVVKPDSD---- 220
Y20S α5     GSGSEGAQAELLNEWHSSLS---LKEAELLVLKILKQVMEEKLDE--------------- 214
Y20S α6     GARSQGAKTYLERTLDTFIK---IDGNPDELIKAGVEAISQSLRDESLT----------- 207
Y20S α7     GKGRQSAKAELEKLVDHHPEGLSAREAVKQAAKIIYLAHEDNKEKDFELEISWCSLSETN 225

T20S α      ---------LKAPEIASITVGNKYRIYDQEEVKKFL------------------------ 233
Y20S α1     ----------NDLEVGVATKDKFFTLSAENIEERLVAIAEQD------------------ 252
Y20S α2     ------NPDLLGYTGIPTDKGPRFRKLTSQEINDRLEAL---------------------- 250
Y20S α3     --GEVYQKIFKPQEIKDILVKTGITKKDEDEEADEDMK----------------------- 258
Y20S α4     ------IVALSSEEINQYVTQIEQEKQEQQEQDKKKKSNH--------------------- 254
Y20S α5     ----------NNAQLTCITKQDGFKIYDNEKTAELIKEL---------------------- 243
Y20S α6     ---------VDNLSIAIVGKDTPFTIYDGEAVAKYI------------------------ 234
Y20S α7     GLHKFVKGDLLQEAIDFAQKEINGDDDEDEDDSDNVMSSDDENAPVATNANATTDQEGDI 285

T20S α      ---
Y20S α1     ---
Y20S α2     ---
Y20S α3     ---
Y20S α4     ---
Y20S α5     ---
Y20S α6     ---
Y20S α7     HLE 288
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Fig. 10.1. Sequence alignment of 20S proteasome α-subunits. The sequence of the *T. acidophilum* 20S proteasome α-subunit was aligned with those of the seven α-subunits of the *Saccharomyces cerevisiae* 20S proteasome with ClustalW. Identical residues in all sequences are shown in gray.

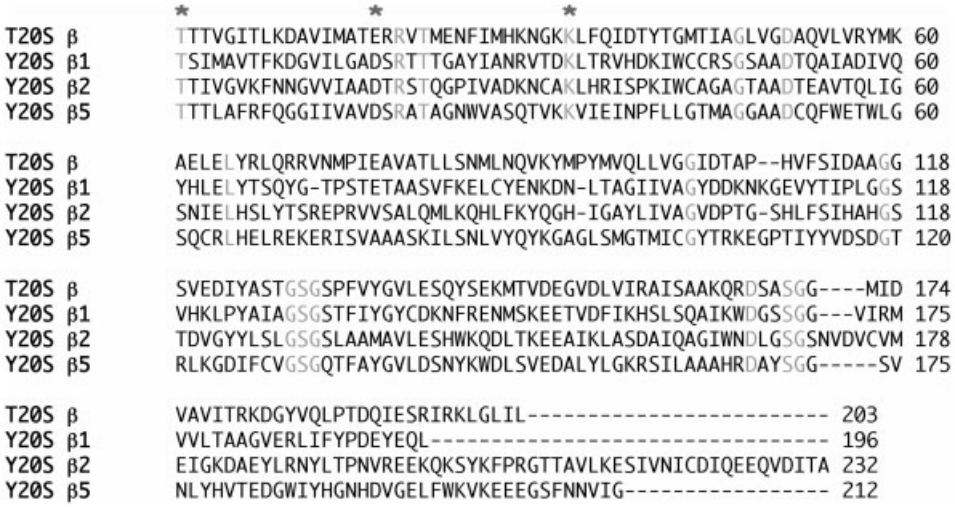

Fig. 10.2. Sequence alignment of 20S proteasome β-subunits. The sequence of the *T. acidophilum* 20S proteasome β-subunit was aligned with those of the processed β1, β2, and β5 subunits of the *S. cerevisiae* 20S proteasome with ClustalW. Identical residues in all sequences are shown in gray. The asterisks at the top of the *T. acidophilum* 20S sequence indicate the residues (Thr1, Glu17, and Lys33) that are of particular importance in catalyzing peptide bond cleavage.

these organisms. Wenzel and Baumeister (1993, 1995) showed that purified archaeal 20S particles by themselves in the absence of any ATPase component can degrade certain unfolded proteins such as phenylhydrazine-treated hemoglobin, oxidant-damaged α-lactalbumin, and reduced α-lactalbumin. Likewise, the loosely folded casein, oxidized alkaline phosphatase, and reduced insulin-like growth factor (IGF-1) are rapidly degraded by these particles (Akopian et al. 1997; Kisselev et al. 1998), although low levels of SDS can activate these archaeal particles further by facilitating entry of protein substrates, as they do in eukaryotic 20S.

The simpler organization of archaeal proteasomes has made possible major advances in our knowledge of the biochemical and structural properties of proteasomes. A key step was the cloning and efficient co-production of its α- and β-subunits in *Escherichia coli* by Zwickl et al. (1992). The first crystal structure of a 20S particle was solved by Huber and Baumeister's laboratories in 1995 using the *T. acidophilum* 20S (Lowe et al. 1995). The X-ray analysis revealed that α- and β-subunits both had a novel fold that was later defined as a characteristic feature of the N-terminal nucleophile (Ntn) hydrolase protein family. Each subunit is made of two central antiparallel β-sheets flanked by two α-helixes on one side and three α-helixes on the other side. The four stacked rings have an elongated cylindrical shape and appear tightly packed, so that peptide and protein substrates can enter the particle only through a central channel in the α-ring (Figure 10.3). The central channel has three large cavities separated by narrow constrictions. The two outer

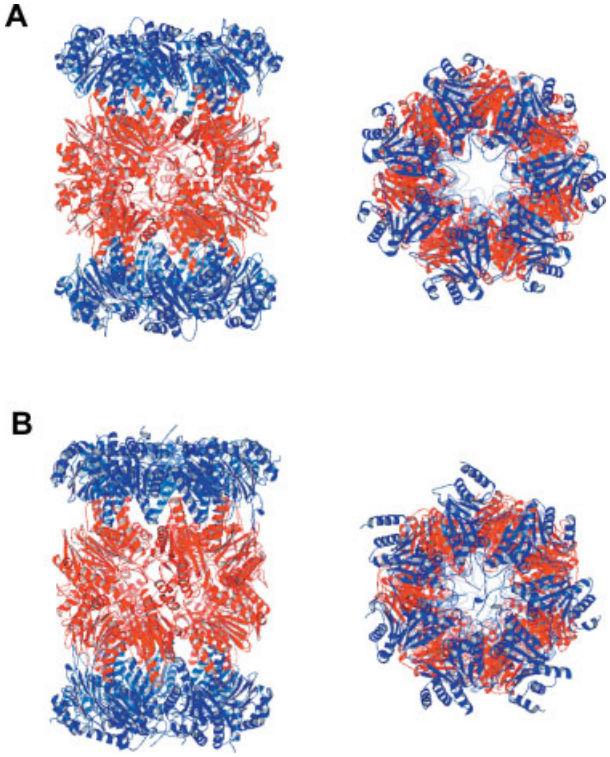

Fig. 10.3. Crystal structures of archaeal and yeast 20S proteasomes. Side (left panels) and top (right panels) views of *T. acidophilum* (panel A) and *S. cerevisiae* (panel B) 20S proteasomes. α- and β-subunits are represented in blue and red, respectively.

antechambers are located at the interface between the α- and β-rings, while the central cavity is formed by the β-rings that contain the active sites.

The catalytic mechanism of 20S proteasomes had long been unclear, and solving the tridimensional structure of archaeal 20S proteasomes, especially in the presence of a competitive inhibitor, elucidated its novel mechanism. These studies and site-directed mutagenesis (Seemuller et al. 1995) identified the amino-terminal threonine of the β-subunits as responsible for the nucleophilic attack and as the primary proton acceptor in peptide bond cleavage. These findings led to the classification of 20S proteasomes in the new superfamily of Ntn hydrolases. The ease of production of α- and β-subunits in *E. coli* has also made possible studies that clarified the processing event that generates an N-terminal threonine on the β-subunits. Seemuller et al. (1996) showed that the processing is autocatalytic and mediated by the amino-terminal threonine, another common feature of Ntn hydrolases.

Studies of the products generated during the degradation of protein substrates showed that *T. acidophilum* 20S, like eukaryotic particles, digests proteins in a processive manner to oligopeptides whose sizes range between 3 and 24 residues (with a mean size of 6–10 residues) without the release of degradation intermediates (Akopian et al. 1997; Kisselev et al. 1998; Wenzel et al. 1994). Because product size is very similar to that in eukaryotic proteasomes, this distribution of peptide size cannot be due to the number, specificity, or tridimensional location of the active sites. Therefore, the size of the products generated is probably determined by a kinetic competition between further cleavages and the ability of a product to diffuse out of the catalytic chamber (Kisselev et al. 1998; Kohler et al. 2001). These fundamental findings about the particle's structure, catalytic mechanism, and processivity were all rapidly extended to eukaryotic proteasomes (Fenteany et al. 1995; Groll et al. 1997; Kisselev et al. 1999; Nussbaum et al. 1998), where many of these questions had proven harder to resolve.

10.3
PAN the Archaeal Homologue of the 19S Complex

Although studies in bacterial and animal cells had clearly established that ATP was required for intracellular proteolysis (for reviews, see Ciechanover 2005, Goldberg 2005, and Goldberg and St John 1976), it was initially assumed that archaeal proteasomes degrade proteins independently of ATP hydrolysis. Also, several groups had failed to demonstrate proteasome-regulatory ATPases in archaea. The X-ray diffraction of the 20S had clearly demonstrated that a narrow opening controlled access to the proteolytic chamber (Lowe et al. 1995). Using nanogold-labeled insulin, Wenzel and Baumeister (1995) nicely demonstrated that protein substrates reached the central chamber by passing the narrow entry pore in the α-rings, through which only unfolded proteins could enter. In the crystalline structure, this entry pore at each end of *T. acidophilum* 20S particles appeared initially to be open (Lowe et al. 1995), whereas the equivalent entry channels in the eukaryotic particles are sealed (Groll et al. 1997; Unno et al. 2002) and necessitate an ATP-dependent mechanism for gate opening and translocation (Groll et al. 2000; Kohler et al. 2001). Because *T. acidophilum* 20S particles were able to degrade certain unfolded proteins (Wenzel and Baumeister 1993, 1995) and because no clear gate was evident in the X-ray structure (Lowe et al. 1995), it was generally assumed that protein unfolding was the only prerequisite for degradation by archaeal 20S. In eukaryotes, it has long been clear that ATP was necessary for the hydrolysis of ubiquitinated proteins and for the degradation of certain proteins that could not be conjugated to ubiquitin, because of a lack of free amino groups (Tanaka et al. 1983). These observations eventually led to the isolation by Rechteiner's (Hough et al. 1986, 1987) and Goldberg's (Waxman et al. 1987) laboratories of the large ATP-dependent complex, now known as the 26S proteasomes (for review, see Ciechanover 2005 and Goldberg 2005). At that time, this structure was believed to be distinct from the 600-kDa multicatalytic particle we later named the proteasome

(Arrigo et al. 1988). Subsequent studies using antibodies demonstrated an essential role for the 20S particle in degradation of ubiquitin conjugates (Matthews et al. 1989) and its association with other components to form the ATP-dependent 26S proteasome (Eytan et al. 1989). Further characterization of the 19S regulatory complex, especially by Rechsteiner, De Martino, Tanaka, and coworkers, led to the cloning and identification of the key ATPase subunits in eukaryotes.

The 19S ATPases, PAN, and the ATP-hydrolyzing protease complexes in bacteria and mitochondria (ClpA, ClpX, HslU, FtsH, and Lon) are all members of the AAA^+ (ATPases associated with various cellular activities) ATPase superfamily (for review, see Ogura and Wilkinson 2001). These ATPases are found in all living organisms and in all cell compartments, where they participate in a variety of essential cellular processes such as mitosis, protein folding and translocation, DNA replication and repair, membrane fusion, and proteolysis. AAA^+ ATPases are characterized by the presence of one or two conserved ATP-binding domains (200–250 residues), called AAA motifs, consisting of a Walker A and a Walker B motif (Confalonieri and Duguet 1995). The 19S-associated ATPases and PAN belong to a subfamily of AAA^+ ATPases that contains an additional motif called the second region of homology (SRH) (Lupas and Martin 2002). Despite the large variety of cellular processes in which AAA^+ ATPases participate, they have some common features. A recurrent structural feature of most AAA^+ ATPases is their assembly in oligomeric (generally hexameric), ring-shaped structures with a central pore. In addition, most appear to be involved in protein folding or unfolding, assembly or disassembly of protein complexes through nucleotide-dependent conformational changes.

Several groups had unsuccessfully attempted to demonstrate the ATP dependence of proteolysis by archaeal proteasomes. In 1996, the complete sequencing of the methanogenic archaeon *M. jannaschii* revealed the presence of two genes (named *S4* and *S8*) that are highly homologous to the genes encoding for the 19S ATPases (Bult et al. 1996). To test whether it regulates proteolysis, the *S4* gene was expressed in *E. coli*, and the 50-kDa product (PAN) was purified and characterized by Zwickl et al. (1999). The primary sequence of PAN contains only one AAA domain (residues 200–342) that includes hallmarks of this ATPase family: one P-loop motif (which includes the Walker A and B motifs) and an SRH motif at its C-terminus (Figure 10.4). PAN shares 41–45% similarity with human and yeast 19S ATPases (Zwickl et al. 1999). As seen by multiple sequence alignment with yeast Rpt1–6 19S subunits, a number of conserved residues in the PAN and 19S ATPases are found in the P-loop and SRH motifs (Figure 10.5). Both PAN and 19S ATPases possess a predicted coiled-coil motif at their N-termini (Zwickl et al. 1999).

When purified to homogeneity, PAN was shown to exist in solution as a homooligomeric complex of 650 kDa that has a hexameric ring structure (P. Zwickl, unpublished data; Wilson et al. 2000) and exhibits Mg^{2+}-dependent ATPase activity (Zwickl et al. 1999). In its initial characterization, PAN with ATP present was shown to promote selectively the breakdown of proteins lacking tertiary structure, including casein and oxidized RNAse A (Zwickl et al. 1999). Subsequently, PAN

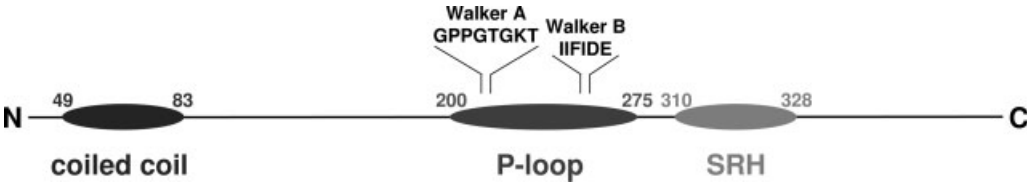

Fig. 10.4. Schematic representation of the primary sequence of PAN. PAN exhibits typical features of an AAA ATPase, i.e., a P-loop domain with Walker A and Walker B motifs and a second region of homology (SRH) at its C-terminal. The N-terminal part of PAN was predicted to contain a coiled-coil region (Zwickl et al. 1999).

was also shown to promote degradation of globular proteins such as green fluorescent protein (GFP) in a reaction requiring ATP hydrolysis (Benaroudj and Goldberg 2000). In fact, PAN by itself could catalyze ATP-dependent unfolding of stable globular proteins. As yet, no similar ATP-dependent unfolding process has been demonstrated with pure 19S particles or 26S complexes.

Although the association of an ATPase chaperone–like complex with a proteolytic particle appears to be a common feature of several systems for intracellular protein degradation (the 26S proteasome and the bacterial ClpAP, ClpXP, and HslUV complexes), an association between PAN and the 20S particle was difficult to observe by standard biochemical approaches. In fact, this failure to demonstrate such a complex led some investigators to suggest alternative mechanisms to explain the stimulation of protein degradation by PAN acting on the substrate, without formation of a PAN–20S complex (Forster et al. 2003). Alternatively, the complex between PAN and 20S proteasomes could be hard to detect because it is short-lived (e.g., compared to the complex between the 19S and eukaryotic 20S). In recent studies, the association of PAN with archaeal 20S proteasomes was demonstrated by Smith et al. (submitted for publication). Using immunoprecipitation, surface plasmon resonance, and electron microscopy, complex formation between PAN and the 20S was observed in the presence of ATP and non-hydrolyzable analogue (AMPPNP or ATPγS) but not in the presence of ADP or in the absence of any nucleotide. Thus, the association of PAN with the 20S is favored upon ATP binding and is reduced by ATP hydrolysis.

Electron microscopy of the PAN–20S complex demonstrated that PAN could associate with 20S at either one or both ends (Figure 10.6B,C). PAN appears as a two-ring structure, with a large inner ring and a smaller outer ring, and resembles a ''top hat'' capping the 20S cylinder. The position of the outer ring resembles the coiled coil containing the intermediate domain of HslU that protrudes outward from the HslUV complex (Bochtler et al. 2000; Sousa et al. 2000). Interestingly, the PAN–20S complex structure is remarkably similar to a 26S proteasome lacking its lid components and the non-ATPase subunits of the base (Figure 10.6D). In fact, the PAN–20S complex images can be exactly superimposed on the densities in the 26S complex and strongly suggest that an unidentified density in the 19S corresponds to the coiled-coil domain of the ATPase. These studies demonstrate

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PAN     MVFEEFISTELKKEKK------------AFTEEFKEEKEINDNSN---LKNDL--LKEELQEKAR--------IAE 59
Rpt1    MPPKEDWEKYKAPLEDDDKKPDDDKIVPLTEGDIQVLKSYGAAPYAAKLKQTENDLKDIEARIKEKAGVKESDTGL 76
Rpt2    -MGQGVSSGQDKKKKK------------GSNQKPKYEPPVQSKFGRKKRKGGP--ATAEKLPNIYPSTRCKLKLLR 61
Rpt3    MEELGIVTPVEKAVEE------------KPAVKS-YASLLAQLNG--TVNNNS--ALSNVNSDIY------FKLKK 59
Rpt4    MS--EEQDPLLAGLG-------------ETSGDNHTQQSHEQQP--EQPQETEEHHEEEPSRVDPEQ---EAHNKA 59
Rpt5    MATLEELDAQTLPGDD------------ELDQEILNLSTQELQTRAKLLDNEIRIFRSELQRLSHEN---NVMLEK 64
Rpt6    ---------MTAAVT----------------SSNIVLETHESGI----KPYFEQKIQETELKIRSKT--------- 47

PAN     LESRILKLELEKK---------ELERE--NLQLMKENEILRRELDRMRVPP---LIVGTVVDKVGERKVVVKSSTG 116
Rpt1    APSHLWDIMGDRQRLGEEHPLQVARCTKIIKGNGESDETTTDNNNSGNSNSNSNQQSTDADEDDEDAKYVINLKQI 152
Rpt2    MERIKDHLLLEEE---------FVSNSEILKPFEKKQEEEKKQLEEIRGNP---LSIGTLEEIIDDDHAIVTSPTM 128
Rpt3    LEKEYELLTLQED---------YIKDE--QRHLKRELKRAQEEVKRIQSVP---LVIGQFLEPIDQNTGIVSSTTG 118
Rpt4    LNQFKRKLLEHRR-----YDDQLKQRRQNIRDLEKLYDKTENDIKALQSIG---QLIGEVMKELSEEKYIVKASSG 127
Rpt5    IKDNKEKIKNNRQ---------LPYLVANVVEVMDMNEIEDKENSESTTQG---GNVNLDNTAVG-KAAVVKTSSR 128
Rpt6    --ENVRRLEAQRN--------ALNDKVRFIKDELRLLQEPGS-------------YVGEVIKIVSDKKVLVKVQPE 102

PAN     PSFLVNVSHFVNPDDLAPGKRVCLNQQTLTVVDVLPENKDYRAKAMEVDERPNVRYEDIGGLEKQMQEIREVVELP 189
Rpt1    AKFVVGLGERVSPTDIEEGMRVGVDRSKYNIELPLPPRIDPSVTMMTVEEKPDVTYSDVGGCKDQIEKLREVVELP 228
Rpt2    PDYYVSILSFVDKELLEPGCSVLLHHKTMSIVGVLQDDADPMVSVMKMDKSPTESYSDIGGLESQIQEIKESVELP 201
Rpt3    MSYVVRILSTLDRELLKPSMSVALHRHSNALVDILPPDSDSSISVMGENEKPDVTYADVGGLDMQKQEIREAVELP 191
Rpt4    PRYIVGVRNSVDRSKLKKGVRVTLDITTLTIMRILPRETDPLVYNMTSFEQGEITFDGIGGLTEQIRELREVIELP 200
Rpt5    QTVFLPMVGLVDPDKLKPNDLVGVNKDSYLILDTLPSEFDSRVKAMEVDEKPTETYSDVGGLDKQIEELVEAIVLP 200
Rpt6    GKYIVDVAKDINVKDLKASQRVCLRSDSYMLHKVLENKADPLVSLMMVEKVPDSTYDMVGGLTKQIKEIKEVIELP 167

PAN     LKHPELFEKVGIEPPKGILLYGPPGTGKTLLAKAVATETNATFIRVVGSELVKKFIGEGASLVKDIFKLAKEKAPS 265
Rpt1    LLSPERFATLGIDPPKGILLYGPPGTGKTLCARAVANRTDATFIRVIGSELVQKYVGEGARMVRELFEMARTKKAC 304
Rpt2    LTHPELYEEMGIKPPKGVILYGAPGTGKTLLAKAVANQTSATFLRIVGSELIQKYLGDGPRLCRQIFKVAGENAPS 277
Rpt3    LVQADLYEQIGIDPPRGVLLYGPPGTGKTMLVKAVANSTKAAFIRVNGSEFVHKYLGEGPRMVRDVFRLARENAPS 267
Rpt4    LKNPEIFQRVGIKPPKGVLLYGPPGTGKTLLAKAVAATIGANFIFSPASGIVDKYIGESARIIREMFAYAKEHEPC 276
Rpt5    MKRADKFKDMGIRAPKGALMYGPPGTGKTLLARACAAQTNATFLKLAAPQLVQMYIGEGAKLVRDAFALAKEKAPT 276
Rpt6    VKHPELFESLGIAQPKGVILYGPPGTGKTLLARAVAHHTDCKFIRVSGAELVQKYIGEGSRMVRELFVMAREHAPS 243
                                Walker A
PAN     IIFIDEIDAIAAKRTDALTGGDREVQRTLMQLLAEMDGFDARGDVKIIGATNRPDILDPAILRPGRFDRIIEVPAP 341
Rpt1    IIFFDEIDAVGGARFDDGAGGDNEVQRTMLELITQLDGFDPRGNIKVMFATNRPNTLDPALLRPGRIDRKVEFSLP 380
Rpt2    IVFIDEIDAIGTKRYDSNSGGEREIQRTMLELLNQLDGFDDRGDVKVIMATNKIETLDPALIRPGRIDRKILFENP 353
Rpt3    IIFIDEVDSIATKRFDAQTGSDREVQRILIELLTQMDGFDQSTNVKVIMATNRADTLDPALLRPGRLDRKIEFPSL 343
Rpt4    IIFMDEVDAIGGRRFSEGTSADREIQRTLMELLTQMDGFDNLGQTKIIMATNRPDTLDPALLRPGRLDRKVEIPLP 352
Rpt5    IIFIDELDAIGTKRFDSEKSGDREVQRTMLELLNQLDGFSSDDRVKVLAATNRVDVLDPALLRSGRLDRKIEFPLP 352
Rpt6    IIFMDEIDSIGSTRVEGSGGGDSEVQRTMLELLNQLDGFETSKNIKIIMATNRLDILDPALLRPGRIDRKIEFPPP 319
        Walker B                                                    SRH
PAN     -DEKGRLEILKIHTRKMNLAEDVNLEEIAKMTEGCVGAELKAICTEAGMNAIRELRDYVTMDDFRKAVEKIMEKKK 416
Rpt1    -DLEGRANIFRIHSKSMSVERGIRWELISRLCPNSTGAELRSVCTEAGMFAIRARRKVATEKDFLKAVDKVISGYK 455
Rpt2    -DLSTKKKILGIHTSKMNLSEDVNLETLVTTKDDLSGADIQAMCTEAGLLALRERRMQVTAEDFKQAKERVMKNKV 428
Rpt3    RDRRERRLIFGTIASKMSLAPEADLDSLIIRNDSLSGAVIAAIMQEAGLRAVRKNRYVILQSDLEEAYATQVKTDN 419
Rpt4    -NEAGRLEIFKIHTAKVKKTGEFDFEAAVKMSDGFNGADIRNCATEAGFFAIRDDRDHINPDDLMKAVRKVAE-VK 427
Rpt5    -SEDSRAQILQIHSRKMTTDDDINWQELARSTDEFNGAQLKAVTVEAGMIALRNGQSSVKHEDFVEGISEVQARK- 427
Rpt6    -SVAARAEILRIHSRKMNLTRGINLRKVAEKMNGCSGADVKGVCTEAGMYALRERRIHVTQEDFELAVGKVMN--K 394

PAN     VKVKEPAHLDVLYR 430
Rpt1    KFSSTSRYMQYN-- 467
Rpt2    EENLEGLYL----- 437
Rpt3    TVDKFDFYK----- 428
Rpt4    KLEGTIEYQKL--- 437
Rpt5    -SKSVSFYA----- 434
Rpt6    NQETAISVAKLFK- 405
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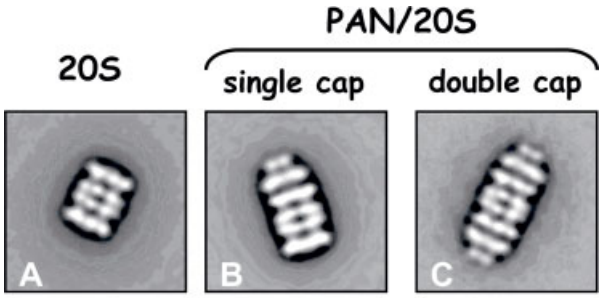
Fig. 10.5. Sequence alignment of proteasomal ATPases. The sequence of *M. jannaschii* PAN was aligned with those of the six Rpt ATPase subunits of the *S. cerevisiae* 19S proteasome with ClustalW. Identical residues in all sequences are in gray. Walker A and B motifs, as well as the SRH, are underlined.



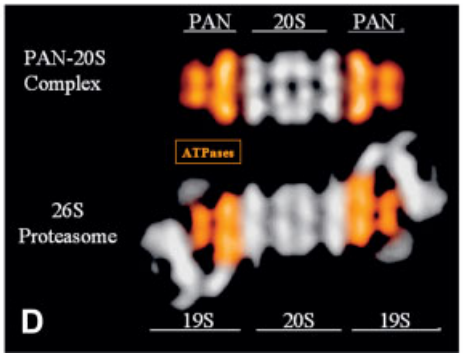


Fig. 10.6. Electron microscopy analysis of the archaeal PAN–20S and proteasomes complex. Electron micrographs of the negatively stained archaeal 20S proteasome (A), the singly capped archaeal PAN–20S complex (B), and the doubly capped archaeal PAN–20S–PAN complex (C). Negatively stained particles of the doubly capped PAN–20S complex are compared to the mammalian 26S proteasome in (D).

that PAN physically associates with 20S proteasomes for ATP-dependent protein degradation to occur.

Degradation of tri- or tetrapeptides by archaeal 20S proteasomes is not enhanced in the presence of PAN and ATP, probably because such small peptides can readily diffuse into the particle. In fact, this inability to stimulate degradation of small peptides distinguishes PAN from the 19S complex, which stimulates ATP-dependent degradation of small peptides (Kohler et al. 2001). However, Smith et al. (submitted) recently showed that PAN and ATP do stimulate degradation of peptides of seven residues or longer. Thus, a gated pore exists in the archaeal particles that allows entry of small peptides, but longer peptides (over seven residues), like proteins (Benaroudj et al. 2003), are excluded in the absence of PAN and ATP.

Thus, because PAN in the presence of ATP interacts with 20S particles and stimulates proteasomal degradation of oligopeptides and of globular and unfolded proteins, it exhibits features similar to those of the 19S complex in protein degradation. These many functional similarities between PAN and the 19S complex further confirm that PAN was the evolutionary precursor of the eukaryotic 19S com-

plex. Moreover, because of its greater simplicity, the PAN–20S complex has proven to be tremendously useful in studies of the detailed mechanisms of protein degradation by 26S proteasomes.

10.4 VAT, a Potential Regulator of Proteasome Function

PAN is present in most archaeal species (Zwickl 2002), except *T. acidophilum* (Ruepp et al. 2000) and *T. volcanium* (Kawashima et al. 2000). In these organisms, ATP hydrolysis is probably still necessary for proteasomal degradation, which is likely supported by another AAA ATPase. One candidate is the protein VAT (VCP-like ATPase from *Thermoplasma acidophilum*), which is closely related to CDC48 of *Saccharomyces cerevisiae* and p97 (VCP) of vertebrates. These AAA family members are distantly related to the eukaryotic 19S ATPases and contain two AAA motifs. In eukaryotes, these proteins were first shown to participate in ER and Golgi membrane fusion (Latterich et al. 1995; Rabouille et al. 1995). More recent studies have demonstrated that mammalian p97/VCP and CDC48 are required for the ER-associated degradation (ERAD) of misfolded and ubiquitinated membrane proteins. Indeed, CDC48 interacts directly with the ubiquitin chains on the substrate and somehow facilitates hydrolysis of ubiquitinated protein by the 26S proteasomes (Dai and Li 2001; Rabinovich et al. 2002; Ye et al. 2001). This complex has also been proposed to be important in the degradation of subunits of cytosolic complexes, such as IκB (Dai et al. 1998; Dai and Li 2001). It is very likely that an ATP-dependent chaperone-like activity of p97/CDC48 is involved in the removal of the ubiquitinated protein from the ER and its subsequent association with 26S proteasomes (Jarosch et al. 2002; Ye et al. 2001). In archaea, where there is no ubiquitin pathway or ER or Golgi apparatus, the physiological function of VAT is still unknown. VAT may function with archaeal proteasomes in the breakdown of misfolded proteins. Purified VAT protein has an Mg^{2+}-dependent ATPase activity and assembles into a hexameric ring-shaped structure. Chaperone and potential unfoldase activities were also demonstrated for VAT (Golbik et al. 1999), but it remains to be established whether the VAT complex can activate protein degradation by archaeal 20S particles, in a fashion similar to PAN.

10.5 The Use of PAN to Understand the Energy Requirement for Proteolysis

Since the early 1970s, it has been clear that protein degradation in prokaryotes, as well as in eukaryotes, requires metabolic energy (Ciechanover 2005; Goldberg 2005; Goldberg and St John 1976). Although much has been learned about the requirement for ATP, the detailed mechanisms of the ATP-dependent proteolytic complexes are still unclear. In particular, elucidating the mechanisms whereby the 19S regulatory particle unfolds substrates and facilitates their entry into the 20S

proteolytic core particle has long been a great challenge. Investigating these processes in the 26S proteasome is difficult because of the requirement for ubiquitination of substrates and the instability and complexity of the 19S particle, which contains at least 17 different subunits, including six nonidentical ATPases. To elucidate multiple roles of ATP in proteasome function in recent years, we have studied the regulatory complex PAN because many of its structural and enzymatic properties resemble those of the 19S ATPases. Moreover, because of its simple structural organization, lack of requirement for substrate ubiquitination, and ease of expression in *E. coli*, PAN offers many advantages for dissecting and clarifying the mechanism by which ATPases promote protein degradation by 20S proteasomes.

10.5.1 ATP Hydrolysis by PAN Allows Substrate Unfolding and Degradation

Because ATP hydrolysis by PAN did not enhance degradation of tri- or tetrapeptides by archaeal 20S, we initially hypothesized that the major role of PAN's ATPase activity in protein breakdown was to unfold globular substrates, a key step in facilitating their entry into the central proteolytic channel in the 20S (step 3, Figure 10.8). This unfoldase activity was verified by using as a substrate GFPssrA, a variant of GFP whose C-terminus has been fused to the 11-residue peptide ssrA. By itself, GFP is not a substrate and does not bind to PAN. In eubacteria, this unfolded C-terminal sequence is incorporated via a specific tRNA into nascent chains when ribosomes are stalled, and its presence targets the proteins for degradation by several ATP-dependant proteases (Gottesman et al. 1998; Herman et al. 1998; Keiler et al. 1996). GFP is a particularly stable protein, even at high temperatures ($Tm > 65\ ^{\circ}C$) (Bokman and Ward 1981), whose unfolding can be easily monitored by a loss of its fluorescence by using the method introduced by the Horwich laboratory (Weber-Ban et al. 1999). As seen in Figure 10.7, PAN by itself catalyzed the unfolding of GFPssrA by a mechanism that required ATP hydrolysis (Benaroudj and Goldberg 2000; Benaroudj et al. 2003). This PAN-catalyzed unfolding of GFPssrA was critical in allowing its degradation by archaeal 20S proteasomes because 20S particles, in the absence of PAN, cannot degrade GFPssrA (Benaroudj and Goldberg 2000).

These findings provided the first experimental evidence that a proteasomal-associated ATPase has an unfoldase activity (Benaroudj et al. 2001). In fact, thus far no such unfoldase activity for purified eukaryotic 19S ATPases or 26S particles has been demonstrated, although such an activity seems very likely (Murakami et al. 2000), especially because GFP fusion proteins used for studying protein degradation in eukaryotes can be rapidly degraded *in vivo*. The 26S and the base of the 19S particles from yeast and mammals exhibit several activities characteristic of molecular chaperones, such as the ability to reduce protein aggregation and to promote the refolding of denatured proteins (Braun et al. 1999; Strickland et al. 2000). Also, 19S particles have been shown to be able to remodel certain substrates, e.g.,

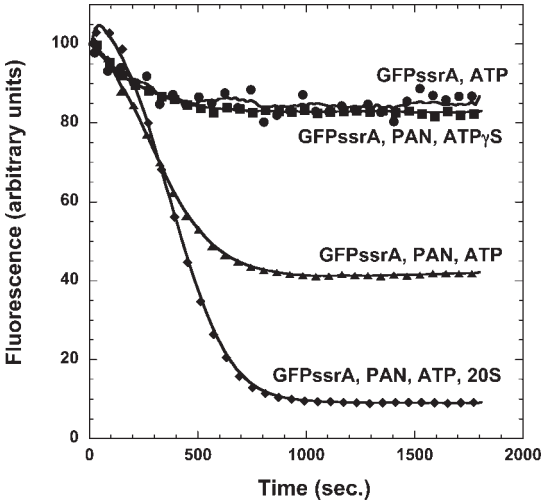


Fig. 10.7. PAN promotes ATP-dependent unfolding and proteasomal degradation of GFPssrA. The time course of fluorescence change of 500 nM of GFPssrA was followed at 45 °C (excitation at 400 nm and emission at 510 nm) in 50 mM tris (pH 7.5), 1 mM DTT, and 10 mM $MgCl_2$ in the presence of 2 mM of ATP (●); or 2 mM of ATP and 250 nM of PAN (▲); or 2 mM of ATPγS and 250 nM of PAN (■); or 2 mM of ATP, 250 nM of PAN, and 53.5 nM of archaeal 20S proteasomes (◆). The loss of GFPssrA fluorescence observed upon addition of PAN and ATP or of PAN, ATP, and 20S indicates GFPssrA unfolding and degradation, respectively.

they have the capacity to catalyze the reactivation of misfolded RNAse A and to expose otherwise buried chymotryptic sites in a folded substrate, the polyubiquitinated DHFR (Liu et al. 2002). However, ATP binding or hydrolysis by the 19S ATPases does not seem to be necessary for this remodeling activity. Moreover, a relationship between the chaperone-like activities of the 19S and protein breakdown by the 26S proteasomes remains to be established, although these activities seem very likely based upon the findings with PAN.

10.5.2
ATP Hydrolysis by PAN Serves Additional Functions in Protein Degradation

Our early findings indicated that ATP hydrolysis by PAN enhanced the degradation of various substrates that were loosely folded, such as casein (Zwickl et al. 1999). These findings suggested that ATP consumption by PAN facilitated additional steps in protein degradation aside from protein unfolding. Like the several AAA ATPases that promote protein degradation in *E. coli*, PAN's ATPase activity is stimulated two- to fivefold by protein substrates (step 1, Figure 10.8). Surprisingly, ATP hydrolysis by PAN is stimulated similarly by the globular GFPssrA, by the loosely folded casein, and even by the 11-residue ssrA recognition peptide. There-

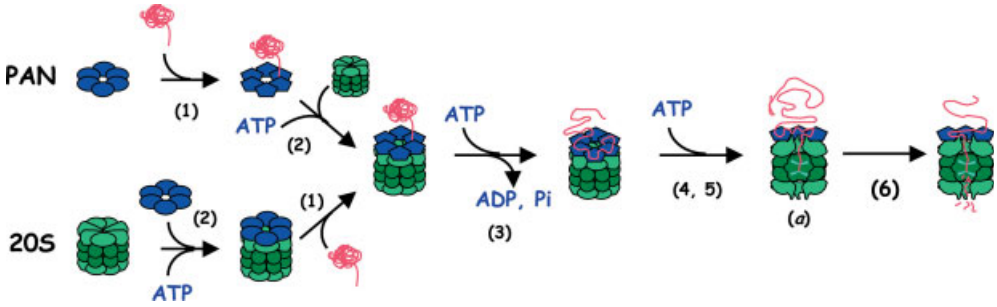


Fig. 10.8. Schematic representation of the different steps of archaeal PAN–20S-mediated protein degradation. PAN is shown as a blue hexameric ring, and the 20S proteasome is in green. Formation of the PAN–20S complex occurs upon ATP binding (step 2), and this association causes opening of the entry channel in the 20S (step 4). Gate opening by PAN requires ATP binding but not hydrolysis. Protein substrates (represented in pink) bind to PAN or to the PAN–20S complex (step 1) and trigger activation of ATP hydrolysis. The circular and pentagonal shapes of PAN subunits represent the substrate-free and substrate-bound forms of PAN, respectively. Protein substrates are unfolded in an ATP hydrolysis–dependent manner on the surface of the PAN ATPase ring (step 3). After protein unfolding, unfolded substrates are translocated into the internal chamber of 20S proteasomes (step 5). Particle *a* is a cross-section of the PAN–20S complex with an open gate that translocates an unfolded protein. 20S active sites are represented as cyan triangles inside the 20S internal chamber. Unfolded substrates studied thus far appear to be translocated by PAN into the 20S particle by a mechanism that requires only ATP binding, not ATP hydrolysis. Inside the 20S particles, proteins are processively degraded to small peptides by the multiple 20S active sites (step 6). The gate that precludes entry of protein substrates inside 20S particles also controls exit of peptide products out of the 20S particles.

fore, ATP hydrolysis is activated by substrate binding and not by the unfolding process (Benaroudj et al. 2003). Furthermore, prior denaturation of the GFPssrA did not accelerate its degradation by proteasomes, nor did it eliminate the requirement for PAN and ATP.

By measuring simultaneously the rate of ATP consumption and the rate of protein degradation, it was possible to determine the amount of ATP hydrolyzed during breakdown of different proteins. During the degradation of one molecule of the globular substrate GFPssrA, the PAN–20S complex consumed the same amount of ATP molecules (300–400 molecules) as during degradation of one molecule of denatured GFPssrA or the natively unfolded protein casein. Interestingly, this amount of ATP hydrolyzed during proteolysis corresponds to approximately one-third of the amount of ATP consumed during synthesis of these proteins. No such analysis has been carried out for the 26S proteasome, although presumably the amount of ATP utilized in degradation of proteins in eukaryotes is even higher due to the additional requirement of ATP for ubiquitination. It is also noteworthy that substrate unfolding by the ATPases does not appear to be the rate-limiting step in protein degradation.

10.5.3
PAN and ATP Regulate Gate Opening

By using a deletion variant of the archaeal 20S proteasome that lacks the N-terminal extremities of the α-subunits, we found that these residues, which correspond to those comprising the gated entry channel in the yeast proteasome (Groll et al. 2000; Kohler et al. 2001), also limit the entry of protein substrates in archaeal particles. The deletion of these residues facilitates the degradation of acid-denatured GFP and casein and eliminates the requirement for PAN and ATP for their degradation. These findings indicate that one role of PAN and ATP is to promote opening of the 20S gate (Benaroudj et al. 2003) (step 4, Figure 10.8). Recently, we found that this gateless 20S variant cleaved a variety of peptides, whose lengths range between 7 and 18 residues, at a much higher rate than did wild-type 20S (Smith et al., submitted). Therefore, the 20S particles from *T. acidophilum* possess a functional gate that excludes proteins and even peptides as small as heptamers. This discovery of a gate in the α-ring of the archaeal proteasomes was surprising because X-ray crystallography of this particle failed to indicate a specific density in this region (Lowe et al. 1995). In fact, the absence of a regulated gating mechanism for archaeal 20S proteasomes has been assumed to represent a major difference between archaeal and eukaryotic proteasomes (Groll et al. 2003; Groll and Huber 2003).

It is now clear that PAN and ATP regulate this gated entry channel into the archaeal 20S particles from *T. acidophilum*. PAN together with ATP or non-hydrolyzable ATP analogues was found to support gate opening. Thus, ATP binding by PAN, which also favors formation of the 20S–PAN complex, stimulates peptide entry through the 20S gate (Smith et al., submitted). In fact, a variety of observations strongly suggest that the association of PAN with the 20S triggers gate opening. One possible model is the non-homologous proteasome activator complex PA26 (11S) from *Trypanosoma brucei*, which (without ATP present) opens the pore into the yeast 20S proteasome by stabilizing an ordered conformation of the N-terminal extremities of α-subunits (Forster et al. 2003, 2005). Certain conserved residues in the gate of yeast 20S α-subunits appeared to be important in the stabilization of this open, ordered conformation. Because the corresponding residues in the archaeal 20S particles are also important in precluding substrate entry, it is attractive to hypothesize that PAN's association with the 20S upon ATP binding also stabilizes an open, ordered conformation of α-subunit N-terminal extremities through these residues. In support of this conclusion, we have recently shown that three conserved C-terminal residues in PAN are essential for both the ATP-dependent association with the 20S and gate opening (Smith, Chung, and Goldberg, in preparation). In fact, a peptide corresponding to the C-terminal residues by itself activates gate opening in a manner that requires the residues found in the motif that is conserved in most 19S ATPases. Most importantly, the peptide sequence that activates gate entry into the *T. acidophilum* 20S can do so in the 20S particle from rabbit muscle. These findings indicate a highly conserved mechanism for gate opening and for the role of ATP in this process. These detailed mechanisms, however, ap-

pear to differ from those controlling gate opening in the ATP-independent PA28 activators, which bear no sequence homology to PAN or the AAA family ATPases, although both activating mechanisms seem to involve C-terminal residues and binding sites on the 20S particle (Smith, Chung, and Goldberg, in preparation; Forster et al. 2005).

10.5.4
PAN and ATP Are Required for Translocation of Unfolded Substrates

A major challenge in studying the ATP requirement for protein translocation into the 20S is to dissociate this process from the process of substrate unfolding and gate opening. To study the role of ATP hydrolysis by PAN during substrate translocation, we tested whether the gateless 20S variant required ATP hydrolysis by PAN to degrade GFPssrA once it has been already unfolded by PAN. Interestingly, we found that although GFPssrA is unfolded by PAN, its degradation by 20S still requires ATP and PAN even when the 20S gate is open (Benaroudj et al. 2003). By using non-hydrolyzable analogues, we found that ATP binding is sufficient for translocation of certain unfolded substrates (Smith et al., submitted). Thus, after opening of the 20S gate, PAN in its ATP-bound form can allow translocation of unfolded proteins (casein, denatured ovalbumin, or denatured GFP) into the 20S proteolytic cavity (step 5, Figure 10.8). Thus, once a globular protein has been unfolded on the surface of PAN, it is not simply released into the medium to diffuse to nearby 20S particles. It remains possible that, with longer polypeptides or unfolded proteins with a tendency to refold, ATP hydrolysis-dependent unfolding may increase the rate of translocation and degradation. In any case, it is clear that while ATP hydrolysis is absolutely essential for the unfolding process, translocation of the bound, unfolded or loosely folded polypeptide can proceed by passive diffusion facilitated by PAN in its ATP-bound form.

10.6
Direction of Substrate Translocation

To reach the active sites within the 20S particle, substrates have to penetrate the narrow axial pore formed by the α-ring, presumably after traversing the pore in the ATPase ring of PAN or the 19S complex. These models raise the obvious questions of whether the degradation of a polypeptide chain starts from one specific end or the other and whether a substrate assumes a preferred or exclusive orientation when entering the 20S proteasome. To determine whether a polypeptide chain enters the 20S particle by its N- or C-terminus or by an internal loop, we attached bulky moieties to protein substrates that prevented their translocation through the pore in the PAN ATPase (Navon and Goldberg 2001; Navon et al., in preparation). GFPssrA was shown to be translocated exclusively in a C-to-N orientation. However, different substrates were found to be translocated in different fashions. While some proteins (maltose binding protein and GFPssrA) are transported into the pro-

teasome by their C-terminus, others (casein) are exclusively translocated from their N-terminus, and some (calmodulin) from both directions (or by an internal loop). By contrast, the isolated 20S showed no such directional preference. Thus, the orientation of entry seems to be a property of the substrate's termini and its interaction with the ATPase. Accordingly, it has been observed that different substrates appear to enter eukaryotic 26S by different extremities (Prakash et al. 2004; Zhang and Coffino 2004; Navon et al., in preparation), and some even seem to enter by an internal loop (Liu et al. 2003). However, these observations were made with crude cell lysates or with pure 26S proteasomes and substrates that do not require ubiquitin conjugation, such as casein (Navon et al., in preparation), $p21^{cip1}$, and α-synuclein (Liu et al. 2003). The influence of ubiquitination on the unfolding process and the directionality of substrate translocation remain to be ascertained.

It is widely assumed that protein degradation by proteasomes is a highly processive process *in vivo*, as it is with isolated 20S particles (Akopian et al. 1997). Through studies of the degradation of a multi-domain polypeptide in cell extracts, Matouschek and colleagues (Lee et al. 2001; Prakash et al. 2004) concluded that proteasomal degradation requires not only a "degradation signal" on the polypeptide (e.g., ubiquitination) but also an unstructured region that is necessary for unfolding by the 19S complex. Once this sequence has been translocated into the proteasomes by the ATPase ring, the whole polypeptide chain is pulled into the 20S particle and degraded. Interestingly, if the polypeptide chain contains independent globular domains, it is degraded vectorially, starting from the domain that is closest to the part that first enters the 20S particle. However, if it contains a particularly stable globular domain that obstructs the pore entrance, the fragment containing the globular domain is released from the proteasomes. This finding can explain why certain substrates, such as the p105 precursor of NFκB, are degraded only partially, releasing an active p50 protein. The use of pure archaeal PAN–20S complex has allowed more rigorous investigations of ATP-dependent translocation of multi-domain proteins by proteasomal ATPases. Using protein fusions containing the GFP domain and an easily translocatable and degradable domain (e.g., the first 70 amino acids of casein or calmodulin) with the PAN–20S complex or mammalian 26S, Navon et al. (in preparation) recently found that the translocation process stops at the globular domain, which is then released from the proteasomes. Thus, simple translocation of casein or calmodulin cannot lead to the unfolding or translocation of the upstream globular GFP, unlike attachment of the tight-binding ssrA peptide, which induced efficient unfolding, translocation, and degradation of GFP by the PAN–20S complex (Benaroudj and Goldberg 2000; Navon et al., in preparation).

Remarkably, attachment of the ssrA peptide to the C-terminus of this multi-domain GFP–calmodulin fusion (generating GFP–calmodulin–ssrA) allowed translocation and degradation of the GFP by the PAN–20S complex (Navon et al., in preparation). Therefore, the nature of the terminal sequence and whether it binds tightly to the ATPase ring appears to be critical in determining whether a polypeptide is unfolded and whether a multi-domain protein is translocated processively. Surprisingly, this same ssrA sequence on the C-termini can also cause

unfolding, complete translocation, and degradation of even distant domains. Other sequences such as casein and calmodulin, while readily degraded, cannot facilitate the unfolding process, and recent studies indicate that they bind to PAN much less tightly. Presumably, in eukaryotes the polyubiquitin chain functions like ssrA to promote tight binding and processive degradation.

10.7 Degradation of Polyglutamine-containing Proteins

Certain proteins in mammals (e.g., huntingtin or ataxin) contain long sequences of glutamine residues (polyQ) that can reach 20–30 residues in humans. When mutational events increase the length of these repeats to more than 35 residues, neurodegenerative disorders, such as Huntington's disease or the several spinocerebral ataxias (SCA 1–7), result (Zoghbi and Orr 2000). One of the characteristic features of these diseases is the presence of large protein inclusions in the neurons of specific regions of the brain. In addition to the aggregated polyQ proteins, these inclusions contain ubiquitin, components of 26S proteasomes, and the PA28$\alpha\beta$ activator complex. Therefore, a failure of the ubiquitin–proteasome pathway to degrade polyQ-containing proteins has been proposed to explain the accumulation of aggregated polyQ-containing proteins (Verhoef et al. 2002). In addition, several studies have suggested that the presence of these aggregated polyQ proteins in cells impairs the ability of the ubiquitin–proteasome pathway to degrade other proteins (Bence et al. 2001; Michalik and Van Broeckhoven 2004).

To test more directly the ability of pure 20S and 26S proteasomes to degrade polyQ-containing proteins, Venkatraman et al. (2004) used short peptides containing 10–30 Gln residues and found, surprisingly, that eukaryotic proteasomes could not cut in polyQ stretches in soluble peptides. Accordingly, when long glutamine repeats were fused with myoglobin, the open-gated yeast or activated 20S mammalian proteasomes hydrolyzed peptide bonds within the polypeptide chain but spared the polyQ repeat. By contrast, the less specialized active sites of the proteasome from *T. acidophilum* could rapidly degrade polyQ sequences in peptides and proteins. Therefore, even though eukaryotic and archaeal proteasomes have similar architecture, structure, and threonine-based catalytic mechanisms, they differ in their active site specificities. Presumably, the evolution of the three more specialized active sites in the eukaryotic particles provided some advantage in proteolytic rates, but because these more specialized active sites cannot bind glutamine repeats, this binding may have contributed to the occasional appearance of these relatively rare, late-onset neurodegenerative diseases.

These findings also enabled us to propose a new mechanism that may contribute to the pathogenesis of polyQ diseases and the remarkable association of these diseases with polyQ sequences longer than 30 residues. Since polyQ fragments are not degraded by mammalian proteasomes, they must be released from the proteasomes during the breakdown of polyQ-containing proteins and normally are degraded by cytosolic peptidases. However, the peptides that exit the proteasome nor-

mally range up to 25 residues, and presumably the longer the peptide, the slower the exit (Kisselev et al. 1999; Kohler et al. 2001). A failure of long polyQ fragments (>30 Gln) to diffuse out of 20S particles may lead to an inhibition of proteasome function and promote further accumulation of aggregated proteins and the inclusion formation observed in these neurodegenerative diseases.

10.8 Eubacterial ATP-dependent Proteases

Although eubacteria (aside from actinomycetes) lack 20S proteasomes and ubiquitin, they contain five different types of ATP-dependent proteases that have provided useful insights about intracellular proteolysis and the functioning of 20S proteasomes: ClpAP, ClpXP, HslUV, Lon, and the membrane-bound protease FtsH (Gottesman 2003) (see Table 10.1). Closely related proteases are also present in mitochondria and chloroplasts of eukaryotes. In ClpAP/XP and in HslUV, the ATPase and peptidase activities are located in separate subunits that form distinct sub-complexes; thus, they share with the PAN–20S and 26S complexes certain architectural and functional features. In Lon and FtsH, the ATPase and peptidase activities are located in different domains of a single polypeptide chain. Like proteasomes, the proteolytic components of these enzymes form a distinct compartment and use ATP hydrolysis to support processive protein breakdown. The ATPase components of all these ATP-dependent proteases belong to the AAA ATPase family and share identical motifs, although each ClpA subunit contains two ATP-binding domains, whereas the others (ClpX, HslU, Lon, FtsH), like the 19S and PAN ATPases, contain only one. HslUV and ClpAP/XP are the best characterized among the eubacterial proteolytic complexes, and the following section summarizes our current knowledge of their biochemical properties.

10.8.1 HslUV (ClpYQ)

In eubacteria, HslV (also called ClpQ) is a two-ring peptidase complex that, unlike ClpP, is a member of the proteasome family. HslV subunits are 18% identical to the *T. acidophilum* 20S β-subunits and share a similar fold (Bochtler et al. 1997). As in the 20S proteasome β-subunits, the N-terminal threonine of HslV acts as the nucleophile in peptide bond cleavage. HslV subunits self-associate in a dimer of two hexameric rings to form a barrel-shaped dodecamer. Thus, HslV closely resembles in structure and function the β-ring of the 20S archaeal proteasome, although it contains six rather than seven subunits and lacks the α-rings (Rohrwild et al. 1997). These complexes associate with the ATPase component HslU (also called ClpY), which is an AAA ATPase homologous to ClpA, ClpX and PAN. ATP hydrolysis by HslU is normally coupled to peptide bond cleavage in the degradation of small peptides and proteins (Rohrwild et al. 1996; Yoo et al. 1996). This feature distinguishes HslV from other eubacterial peptidases because pro-

Table 10.1. Features of ATP-dependent proteases present in eubacteria. The main ATP-dependent proteases from eubacteria are divided into two oligomeric classes, depending on whether the peptidase and ATPase activities are located on different (hetero-oligomers) or the same (homo-oligomers) polypeptide chains. Both ClpP and Lon are Ser proteases (Amerik et al. 1991; Maurizi et al. 1990), but they differ by the nature of their active-site residues. ClpP has a typical serine protease catalytic triad (Ser^{97}, His^{122}, Asp^{171}) (Maurizi et al. 1990; Wang et al. 1997), but Lon has a Ser^{679}, Lys^{722} dyad in its active site (Botos et al. 2004b). HslV, like the β-subunits of 20S proteasomes, has a single residue (Thr)-based proteolytic activity (Bochtler et al. 1997). FtsH is a metalloprotease whose active-site components include two histidine (His^{417} and His^{421}) residues and one glutamate (Glu^{479}) residue as ligands for a zinc atom (Ito and Akiyama 2005; Saikawa et al. 2002). The active-site motif of FtsH (His^{417}-Glu-Ala-Gly-His^{421}) is indicated. The oligomeric structure of ClpP has been determined by electron microscopy (Flanagan et al. 1995) and by X-ray diffraction (Wang et al. 1997). Those of HslU and HslV are based on analysis by X-ray diffraction (Bochtler et al. 1997, 2000; Sousa et al. 2000; Wang et al. 2001a). Hexameric ring structures have been observed for ClpA and ClpX by electron microscopy (Beuron et al. 1998; Grimaud et al. 1998) and modeled from the crystal structure of their monomers (Guo et al. 2002; Kim and Kim 2003). The oligomeric status of Lon and FtsH proteases is based on analogy of the crystal structures of their AAA domains and other AAA ATPases (Botos et al. 2004a; Krzywda et al. 2002).

	Family	*Peptidase*	*Active site*	*Oligomeric state of the peptidase complex*	*ATPase*	*Number of AAA domains*	*Oligomeric state of the ATPase complex*
Hetero-oligomers	ClpAP	ClpP	Ser protease: Ser^{97}, His^{122}, Asp^{171} triad	Tetradecamer (2 heptameric rings)	ClpA	2	Hexamer
	ClpXP	ClpP	Ser protease: Ser^{97}, His^{122}, Asp^{171} triad	Tetradecamer (2 heptameric rings)	ClpX	1	Hexamer
	HslUV	HslV	Threonine protease	Dodecamer (2 hexameric rings)	HslU	1	Hexamer
Homo-oligomers	Lon	C-terminal region	Ser protease: Ser^{679}, Lys^{722} dyad	Hexamer	Central region	1	Hexamer
	FtsH	C-terminal region	Zn^{2+} metalloprotease $H^{417}EAGH^{421}E^{479}$	Hexamer	Central region	1	Hexamer

tease Lon requires ATP binding but not hydrolysis for peptidase activity (Goldberg and Waxman 1985), and ClpP does not require the presence of ATP to degrade small peptides (Thompson and Maurizi 1994; Woo et al. 1989). However, it was possible to eliminate this requirement for ATP hydrolysis for protein and peptide breakdown under certain experimental conditions such as in the presence of KCl (Huang and Goldberg 1997; Yoo et al. 1998).

X-ray diffraction studies have established that the association of HslU with HslV induces conformational changes in the peptidase active site and increases the pore size of HslV, indicating that HslU increases peptidase activity of HslV by allosteric activation and probably also by promoting peptide entry and/or products release (Huang and Goldberg 1997; Sousa et al. 2000, 2002; Wang et al. 2001a; Yoo et al. 1998). Facilitating peptide entry thus appears to be a common property among HslU, the 19S ATPases, and PAN.

Another role of the ATPase activity of HslU in protein degradation by HslV is to unfold and translocate the protein substrate. HslU must unfold globular substrates because the HslUV complex has been shown to degrade stable folded proteins (Burton et al. 2005; Kwon et al. 2004; Park et al. 2005). However, unlike for ClpX/A and the PAN complex, an unfoldase activity has not yet been directly demonstrated for HslU because of the lack of a substrate, such as GFP, whose folding status can be easily monitored and that can be recognized by HslU.

10.8.2 ClpAP and ClpXP

The most thoroughly characterized ATP-dependent proteolytic complexes from a physiological and mechanistic perspective are ClpAP and ClpXP from *E. coli*. Although often viewed as models of the proteasomes, the peptidase component of these enzymes, ClpP, is unrelated to 20S proteasome β-subunits and to HslV in both amino acid sequence and proteolytic mechanism. ClpP is a serine protease with a canonical catalytic triad instead of the N-terminal threonine active-site residue characteristic of the proteasome family (Maurizi et al. 1990). ClpP is a hollow cylindrical particle composed of a heptameric ring particle, within which are found its 14 active sites (Wang et al. 1997). Alone, ClpP is unable to degrade polypeptides longer than six residues, presumably because they cannot enter the peptidase complex. Upon binding to ClpA and ClpX, which are hexameric ring ATPase complexes of the AAA family, ClpP can degrade longer peptides and proteins in a ATP-dependent processive manner (Hwang et al. 1988; Thompson et al. 1994; Woo et al. 1989).

Much has been learned during the past 10–15 years about the ATP dependence of protein degradation by the ClpAP and ClpXP complexes. Upon nucleotide binding, the ClpA and ClpX ATPases bind polypeptide substrates through a recognition motif that can be located at the ends or middle of the polypeptide (Hoskins et al. 2002; Sauer et al. 2004). Then, ClpA and ClpX catalyze the unfolding of the substrate by a process that requires ATP hydrolysis (Hoskins et al. 2000; Kim et al. 2000; Singh et al. 2000; Weber-Ban et al. 1999). It has been suggested that unfolding is initiated from the recognition signal by sequential unraveling of the polypeptide chain (Lee et al. 2001), as is also suggested for the 19S ATPase (Prakash et al. 2004). After unfolding, substrate release from ClpA also requires hydrolysis of ATP, which suggests that the movement of the unfolded substrate from ClpA to the ClpP chamber also requires metabolic energy (Hoskins et al. 2000). Chemical inactivation of ClpP's peptidase sites has enabled investigators to capture unfolded

substrates inside the catalytic chamber. Because release of the trapped unfolded substrate occurred only upon ATP hydrolysis by the ClpX, it seems likely that the ATPase allows opening of a central pore in ClpP (Kim et al. 2000). As we have found for the PAN–proteasome complex (Benaroudj et al. 2003), a large amount of ATP is consumed during degradation of a polypeptide by ClpXP (Burton et al. 2001). Once the unfolded substrate has been translocated into the ClpP catalytic chamber, it is degraded processively into small peptides (Thompson et al. 1994). If the peptides are small enough, they probably exit the ClpP chamber by passive diffusion, but longer peptides may require the ATPase to exit the chamber (Kim et al. 2000).

10.9 How AAA ATPases Use ATP to Catalyze Proteolysis

It is now clear that PAN and the bacterial AAA family of ATPases (Lon, HslU, ClpA, and ClpX) utilize ATP in multiple steps during protein degradation. As hypothesized for AAA ATPases, ATP-driven changes in the conformation of the ATPases must underlie protein unfolding, gate opening, and substrate translocation. A variety of experimental evidence indicates that PAN undergoes conformational changes upon ATP binding and/or hydrolysis. For example, since ATPγS or AMPPNP stabilize the association of PAN with 20S particles (as well as ClpA with ClpP and HslU with HslV), ATP binding must induce a conformation of PAN (or ClpA or HslU) that has a higher affinity for the 20S. This association-prone conformation is not evident in the absence of any nucleotide, or in the presence of ADP, because the PAN–20S complex is not demonstrable under those conditions (Smith et al., in preparation). Also, assays of the protease sensitivity of PAN indicate that its conformation in the ATP-bound state is different from that in its ADP-bound state (Navon et al., in preparation), as has also been demonstrated upon nucleotide binding to ClpA/X (Singh et al. 2001). Unfortunately, efforts using X-ray crystallography to resolve the structure of PAN or the 19S or to define the conformational changes upon ATP binding have thus far not been successful. However, much has been learned about the effects of ATP binding and hydrolysis on the structure of HslU (Wang et al. 2001a, 2001b). ATP binding induces a movement of its C-terminal, α-helical domain that narrows the nucleotide-binding cleft, which further narrows upon ATP hydrolysis. As a consequence, the diameter of the central pore of the HslU hexameric ring decreases, and it has been proposed that these conformational changes provide a mechanical force to thread the substrate through the pore, and perhaps to promote unfolding. Most likely, the transitions induced by ATP binding and hydrolysis are similar for all AAA ATPases and underlie their ability to unfold or remodel their substrates. Depending on the intrinsic stability of the substrate, these ATPases presumably need multiple iterative cycles of ATP-driven mechanical force for the unfolding and threading to reach completion, as indicated by the large ATP consumption during proteolysis by PAN–20S and ClpAP (Benaroudj et al. 2003; Burton et al. 2001).

Another important role of the AAA ATPases in protein degradation by certain ATP-dependent proteases is to stimulate the activity of the associated peptidase complex. In the eukaryotic 26S proteasomes, the Rpt2 ATPase controls the rate of peptide hydrolysis by regulating gate opening of 20S particles and thus limiting substrate entry and/or exit (Groll et al. 2000; Kohler et al. 2001; Rubin et al. 1998). The association of the archaeal PAN ATPase with the 20S particles causes gate opening in a similar fashion (Benaroudj et al. 2003; Smith et al., submitted).

In addition to promoting substrate entry, association of the peptidase complex with the ATPase can lead to conformational changes that enhance peptidase activity, as shown by X-ray analysis of HslUV from *Haemophilus influenza* and *E. coli* (Sousa et al. 2000; Wang et al. 2001a, 2001b). In the *H. influenza* complex, the C-terminal extremities of HslU subunits move in between two HslV protomers (Sousa et al. 2000), while two apical helixes of HslV protrude close to the nucleotide-binding cleft in HslU. As a consequence, the threonine active sites are altered, causing an allosteric activation of peptidase activity in the presence of HslU and ATP (Kwon et al. 2003; Sousa et al. 2002). In the *E. coli* complex, binding of HslU in the presence of ATP causes both HslU and HslV rings to twist round their mutual sixfold axis, thereby enlarging HslV's central pore and closing partially that of HslU (Wang et al. 2001a). In addition, upon ATP hydrolysis, a tyrosine residue in a conserved motif (GYVG) at the HslU central pore moves from inside HslU toward HslV. These findings led to the proposal that threading through the HslU ring is initiated from one end of the polypeptide chain, as has been shown for PAN (Navon and Goldberg 2001; Navon et al., in preparation), and that in the ATP-bound state, this tyrosine residue interacts with hydrophobic residues on the folded polypeptide, and upon ATP hydrolysis, the movement of the tyrosine residue toward HslV and constriction of the central pore in the HslU can promote unfolding and translocation into HslV.

It remains to be established whether this elegant model is valid and whether PAN and the 19S ATPases work in the same manner in stimulating proteasomal degradation. The many conserved motifs among AAA ATPases suggest strongly that they function through similar mechanisms.

However, the recent finding that translocation of unfolded polypeptides through PAN or the 19S ATPases into the 20S (Smith et al., in preparation) can occur in the absence of ATP hydrolysis indicates that this hypothesized "power stroke" is not essential for the degradation of most proteins. In addition, ATP-induced activation of the peptidase as shown for HslU sites in the 20S proteasome seems unlikely because PAN plus ATPγS stimulate markedly the hydrolysis of peptides excluded by the 20S gate, but they do not enhance degradation of tetrapeptides, which freely enter this particle even through the closed gate (Smith et al., in preparation)

10.10 Conclusions

A full understanding of the molecular mechanisms for protein degradation by the proteasome will require detailed structural information about the ATP- and ADP-

bound forms of the PAN–20S and 19S complexes. Most likely, X-ray crystallography will first be achieved with PAN, whose many advances for study have been summarized here. Already, however, a great deal has been learned concerning the multiple steps in this process and about the multiple roles of ATP through studies of the PAN–20S complex, as well as the bacterial ATP-dependent proteases. Our present understanding of this process is illustrated by the reaction scheme in Figure 10.8:

1. Nucleotide binding to PAN promotes the association between the ATPase ring and the 20S complex (step 2).
2. Complex formation triggers gate opening in the α-ring (seen in step 4).
3. The binding of the protein substrate induces a conformational change in PAN that activates ATP hydrolysis (step 1).
4. Repeated cycles of ATP hydrolysis catalyze unfolding of globular proteins (step 3).
5. The unfolded polypeptide can diffuse through the ATPase ring (in its ATP-bound form) and the open gates in the α-ring (step 5).
6. The polypeptide in the central chamber of the 20S particle is processively degraded to small peptides (step 6).

These steps appear to be well established for the PAN–20S complex and clearly evolved before the linkage of ubiquitination to proteolysis in eukaryotes. Many detailed questions about this scheme and its general applicability to the 26S complex remain uncertain, and one outstanding issue will be resolved only through studies of the 26S proteasome, i.e., how these steps are integrated with the binding and disassembly of the polyubiquitin chain.

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11
Biochemical Functions of Ubiquitin and Ubiquitin-like Protein Conjugation

Mark Hochstrasser

Abstract

Protein modification by ubiquitin and ubiquitin-like proteins (Ubls) plays a pervasive role in eukaryotic cell regulation. One aim of this chapter is to survey the ubiquitin and Ubl conjugation systems in order to highlight key mechanistic and functional features. Another is to discuss some of the gaps in our understanding of both the evolutionary origins of these conjugation systems and the changes Ubl attachment can impart on a conjugated protein. The ubiquitin and Ubl systems use related enzymes to activate and attach ubiquitin and Ubls to proteins (and, in at least one case, to phospholipids). Most ubiquitin and Ubl attachments are dynamic, with efficient reversal of the modifications by a battery of deconjugating enzymes. The versatility of these systems is reflected in the enormous array of biological processes they control. It is likely that ubiquitin and Ubl attachments function fundamentally as a means of regulating macromolecular interactions. Best known is the ability of polyubiquitinated protein to bind with high affinity to polyubiquitin receptor sites on the proteasome, causing the rapid degradation of the tagged protein. Specific examples of physiological deployment of ubiquitin and Ubl attachment will be used to illustrate distinct mechanisms of regulation by these highly conserved protein modifiers.

11.1
Introduction

The biological functions of many proteins are altered by their covalent attachment to polypeptide modifiers [1–5]. Among these types of modification, probably the best known is ubiquitination. Ubiquitin can target proteins for degradation by the 26S proteasome, but additional effects of protein ubiquitination are now well documented. Ubiquitin is joined reversibly to proteins by amide linkage between the carboxy terminus of ubiquitin and primary amino groups of the acceptor proteins [3, 5]. The primary amine is usually a lysine ε-amino group (the bond with ubiqui-

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tin is then called an isopeptide bond) but can also be the N-terminal N^{α} amino group [6].

11.1.1 The Ubiquitin Conjugation Pathway

The C-terminus of ubiquitin must be activated before it can form a covalent bond with another protein [3, 5] (see Figure 11.1, which depicts a more general Ubl cycle). Initially, the ubiquitin is adenylated by the ubiquitin-activating enzyme E1. A high-energy mixed anhydride bond links the ubiquitin–AMP, which remains bound to the E1. This bond is then attacked by a sulfhydryl group of a cysteine in the E1 enzyme, yielding a high energy E1–ubiquitin thioester intermediate. The activated ubiquitin is subsequently passed to one of a large number of distinct

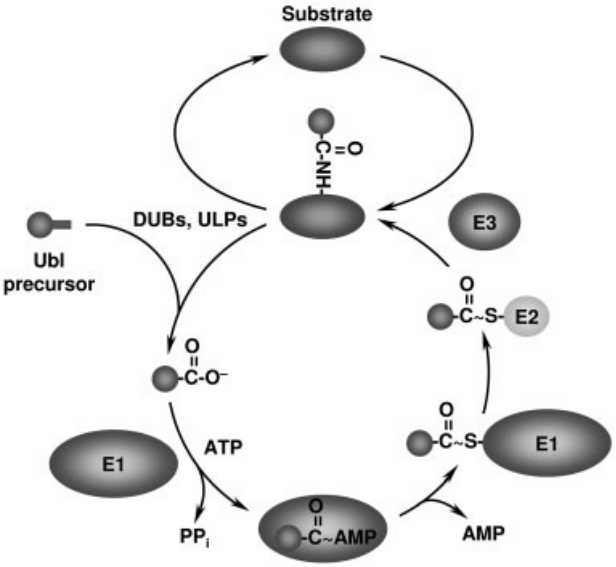

Fig. 11.1. A general ubiquitin-like protein (Ubl) conjugation cycle. After precursor processing, the C-terminal carboxyl group of the Ubl is activated by an E1 Ubl-activating enzyme, which catalyzes formation of a Ubl–AMP intermediate from ATP and the Ubl (the high-energy bond between the AMP phosphate and the C-terminal carboxyl group is indicated by "~"). This Ubl adenylate remains noncovalently bound to the E1 but is then attacked by an active-site cysteine of the E1, leading to formation of a thioester bond between the E1 and Ubl and release of AMP. An E2 Ubl-conjugating enzyme receives the Ubl from the E1, creating an E2–Ubl thioester intermediate. An E3 Ubl–protein ligase then stimulates transfer of the Ubl to a substrate amino group. Additional Ubl molecules can be added either to other lysine side chains on the substrate or to the Ubl itself, the latter leading to polymeric Ubl chains. Ubl chain formation is well documented for ubiquitin; SUMO can form chains *in vivo*, but their functional significance is uncertain. Ubl modifications are usually dynamic and can be removed by deubiquitinating enzymes (DUBs) or Ubl-specific proteases (ULPs). Most Ubls are also synthesized in precursor forms, and the C-terminal extensions are removed by DUBs or ULPs as well.

ubiquitin-conjugating (E2) enzymes by transthiolation to a conserved cysteine side chain of the E2. The E2 proteins catalyze substrate ubiquitination in conjunction with a ubiquitin–protein ligase (E3). For one structural class of E3 proteins (the "HECT domain" E3s), the ubiquitin is first transferred to a conserved cysteine of the E3 before the final transfer to a substrate amine. For most other ubiquitination reactions, a primary role for the E3 appears to be as an adaptor that positions the substrate in close proximity to the reactive E2–ubiquitin thioester bond. The majority of such E3s are characterized by a RING domain, which coordinates a pair of zinc ions and participates in E2 binding [7].

Additional roles for E3s in the catalytic cycle, such as allosteric activation of the E2, remain a distinct possibility [8, 9]. For instance, all E2s have an asparagine residue upstream of the active site cysteine. This asparagine is implicated in the formation of an oxyanion hole that stabilizes the tetrahedral intermediate formed by nucleophilic attack of a substrate amino group on the activated carbonyl of ubiquitin [9]. However, the side chain of the asparagine is fully hydrogen-bonded and oriented away from the active cysteine in the atomic structures determined for isolated E2 enzymes. E3 binding to the E2 and/or ubiquitin thioester formation on the E2 may trigger local structural changes that allow rotation of the E2 asparagine side chain to a position where it can help generate a functional oxyanion hole [10].

11.1.2 Ubiquitin Polymers

In many cases, particularly for proteolytic substrates, more than one ubiquitin is attached to the substrate protein. These ubiquitin molecules can be attached to different substrate amino groups, or they can be attached to each other to form a polyubiquitin chain that is linked to a single substrate site [11, 12]. The ubiquitin molecules in these polymers are linked through the lysine side chain of one ubiquitin with the C-terminal carboxyl of the next ubiquitin. Ubiquitin has several different lysines that contribute to such linkages. For instance, the polyubiquitinated proteins recognized by the proteasome usually have ubiquitin Lys48-linked chains, and the chain must include at least four ubiquitins for tight binding to the proteasome [13]. Ubiquitin chain formation is also essential for certain types of DNA repair and signal transduction pathways, but these chains have Lys63 linkages and do not target the proteins to the proteasome. Ubiquitin polymers of distinct topology are generally thought to have intrinsically different binding affinities for particular target proteins [14]. However, some proteins, such as the S5a proteasome subunit, can bind different types of ubiquitin chains with comparable affinity [15]. Monoubiquitination has distinct signaling functions, as will be discussed below.

11.1.3 Ubiquitin Attachment Dynamics

Ubiquitinated proteins are in a dynamic state, subject to either further rounds of ubiquitin addition or ubiquitin removal by deubiquitinating enzymes (DUBs)

(analogous enzymes act on Ubls; see Figure 11.1). The DUBs comprise one of the largest classes of ubiquitin-system enzymes, but their individual functions are just now beginning to come into view [16]. Many DUBs have negative roles in ubiquitin-dependent signaling. For example, removal of a polyubiquitin chain from a proteolytic substrate prior to its binding to the proteasome will prevent degradation of the substrate. Several DUBs have been demonstrated to have substrate-specific deubiquitinating activity. An illustrative example is the herpesvirus-associated ubiquitin-specific protease (HAUSP). HAUSP can specifically deubiquitinate the p53 tumor suppressor protein; this limits p53 degradation, thereby enhancing p53 pro-apoptotic and growth inhibitory functions [17]. Other DUBs can have positive functions in ubiquitin-dependent processes. The best-known examples of this are the enzymes that recover ubiquitin from proteasome-bound polyubiquitinated substrates [16, 18]. Failure to remove the polyubiquitin from the tagged proteins severely impedes their degradation, presumably because it is difficult to unfold and degrade the highly structured ubiquitin molecules [19]. Ubiquitin (and most Ubls) is synthesized in C-terminally extended precursor forms, which are also processed by DUBs to expose a terminal Gly–Gly dipeptide that is necessary for ubiquitin activation and conjugation.

The fates of ubiquitinated proteins vary greatly. Ubiquitin-induced functional changes depend on whether a single ubiquitin or a polyubiquitin chain is attached (and, if a chain, with what topology), where and when in the cell the modification occurs, and exactly what protein receives the modification. Similar considerations apply to the Ubls and their targets, although Ubl chain formation is not widely observed. Dynamic modification of proteins by ubiquitin and Ubls allows reversible switches between different functional states, as is true for other transient covalent protein modifications such as phosphorylation. A major focus of the remainder of this chapter will be on the general properties of the resulting ubiquitin and Ubl conjugates and the functional consequences of these modifications.

11.2
Ubls: A Typical Modification Cycle by an Atypical Set of Modifiers

The existence of potential ubiquitin-related protein modifiers first became apparent in the late 1980s with the discovery that an interferon-stimulated gene product of 15 kDa, or ISG15, shares significant sequence similarity with ubiquitin and is recognized by anti-ubiquitin antibodies [20]. In 1992, ISG15 was shown to modify other proteins by what seemed likely to be a similar post-translational enzymatic mechanism [21]. Like ubiquitin, ISG15 is synthesized in precursor form, and its C-terminal tail is processed off to expose a diglycine motif that is essential for ISG15–protein conjugation [22]. The mature protein is composed of two domains, both with substantial sequence and structural similarity to ubiquitin (Table 11.1) [23]. Although ISG15 is the prototypical Ubl, it remains one of the least understood. Only very recently have the ISG15 E1 and E2 enzymes been identified [24–26]. These enzymes, like the Ubl itself, are strongly induced by type I interferons,

Table 11.1. Known or suspected Ubls.

Modifiera	*Identity with Ub (%)*	*E1a*	*E2a*	*Comments*	*Reference*
Ubiquitin (Ub)	100	Uba1	Many	Viral form more diverged (75% identity)	123
ISG15	32/37b	Ube1L	UbcH8	First Ubl identified	21
Rub1/NEDD8	55	Uba3-Ula1	Ubc12	Substrates: cullins, p53	110
Smt3/SUMO1–4	18	Uba2-Aos1	Ubc9	Vertebrates have 4 distinct *SUMO* genes	111
Atg12	NSc	Atg7	Atg10	~20% identical to ATG8	112
Atg8	NS	Atg7	Atg3	3 known human isoforms; has the Ub fold	33
Urm1	NS	Uba4	–	Related to MoaD, ThiS	44
UFM1	NS	Uba5	Ufc1	Has the Ub fold	115
FUBI/MNSFβ	38	–	–	Derived from ribosomal precursor	113
FAT10	32/40b	–	–	Substrates unknown	116
Ubl-1	40	–	–	Nematode ribosomal precursor	114
Hub1	22	–	–	Might bind only noncovalently to targets	117–119
BUBL1, 2	variable (up to 80%)	–	–	Ciliate putative autoprocessed proteins	32
SF3a120	30	–	–	Ubl at C-terminus; no data for conjugation	120
Oligo(A) synthetase	42	–	–	Ubl at C-terminus; no data for conjugation	121

[a] Yeast names are listed for E1s and E2s except for the ISG15 and UFM1 systems, which are not found in *S. cerevisiae*; for Ubl names, the yeast names are given (listed first if a vertebrate ortholog is known and goes by a different name) if present in yeast;
[b] Two Ub-related domains;
[c] Not statistically significant.

and they are presumed to be important in antiviral responses; to date, however, the only genetic evidence supporting this idea is the finding that mice lacking a protease with ISG15-deconjugating activity, UBP43, have a more vigorous innate immune response to viral infections [27]. Current data also suggest that ISG15 functions in signal transduction, particularly the Jak–STAT pathway, but its exact role is unclear. Multiple ISG15–protein conjugates are likely, but only a small number of substrates have been reported so far [28].

Since the discovery of ISG15, at least 10 additional ubiquitin-related proteins have been identified that can covalently modify other macromolecules or are strongly suspected of having this ability (Table 11.1). The widespread occurrence of Ubls underscores the potential regulatory importance of protein attachment to other proteins (or to lipids). Ubiquitin can modify hundreds if not thousands of different proteins [29, 30]. Some Ubls, such as small ubiquitin-related modifier (SUMO), appear to rival ubiquitin in the number and diversity of substrates targeted, while others are likely to have a very limited number of substrates. Atg12 (autophagy protein 12), for example, is thought to have but a single target (Atg5), and Atg8 is specifically attached to phosphatidylethanolamine, a phospholipid.

Most Ubl modification pathways utilize highly similar enzymatic mechanisms involving E1-like, E2-like, and often E3-like enzymes as well as specific Ubl-deconjugating enzymes (Figure 11.1). Several unusual ubiquitin and Ubl conjugation mechanisms have been proposed as well. These noncanonical mechanisms, which at this point still have little supporting experimental evidence, range from ubiquitin hydrolases effectively working in reverse [31] to a set of unusual ciliate self-splicing polyproteins [32]. The ciliate polyproteins consist of a series of Ubl domains flanked by self-splicing bacterial intein-like (BIL) domains. The BIL domains are postulated to excise the flanking Ubl segments and attach them to substrates during autocatalytic *in cis* processing reactions. Such atypical mechanisms most likely account for only a very small percentage of Ubl conjugation reactions. The major pathways of protein conjugation appear to have evolved by repeated rounds of duplication and diversification of enzymes and protein modifiers derived from ancient enzyme–cofactor biosynthetic pathways (see below).

It should be noted that there are also many ubiquitin-related proteins in which a ubiquitin-like domain (UBL) is built into a larger polypeptide, but the UBL is neither excised nor attached to other proteins. Such UBLs may impart properties on a protein similar to those conferred by a transferable Ubl, but the UBL is locked into a single target. The UBL-containing proteins include several proteins that also have ubiquitin-binding domains. These multi-domain proteins are thought to help transfer polyubiquitinated proteins from E2 and E3 complexes to the proteasome and will be discussed later in the chapter.

11.2.1 Some Unusual Ubl Conjugation Features

Among the eight Ubl conjugation pathways for which E1 enzymes have been identified (the first eight entries in Table 11.1), there are several with unusual properties that deserve additional comment. First, the Atg8 and Atg12 Ubls share a single E1-like enzyme but have different E2-like proteins [33]. This remains the only known example in which a single E1, Atg7, can activate two different Ubls. The ability of Atg7 to transfer Atg8 and Atg12 to distinct E2s suggests that these E2s bind to the E1–Ubl complex in ways that are productive for transfer only of the E2's cognate Ubl. The structural basis of this discrimination remains to be determined. Second, there is now also an example of different E1 enzymes specific for

different Ubls sharing the same E2 enzyme to effect cognate substrate modification. UBEL1, the E1 for ISG15, and UBA1, the E1 for ubiquitin, can both use the same E2, UbcH8 [25, 26]. How Ubl substrate targeting specificity is maintained is an important question, although it might be explained largely by mass action. ISG15 and UBEL1 are only strongly expressed when cells are induced with interferon. Potentially, activated ISG15 would be present at sufficiently high levels under these conditions to ensure that UbcH8 picks up a sizeable amount of it and, together with ISG15-specific E3s, which might also be interferon-inducible, will be able to transfer it to the appropriate targets. No such E3 has yet been described, but it would be predicted to be able to recognize ISG15-charged, but not ubiquitin-charged, UbcH8. For the SUMO pathway, recent studies reveal direct contacts between an E3 and the SUMO protein [10, 34]. Similarly, ISG15-specific E3s might be able to use direct ISG15 contacts to identify the cognate ISG15–E2 thioester even if the same E2 is sometimes linked to ubiquitin.

In the remainder of the review, several areas will be emphasized. First, I will expand on earlier speculations about the possible evolutionary origins of the ubiquitin and Ubl modification systems [1]. Such an evolutionary perspective is useful when potential mechanistic variations in Ubl activation and conjugation are considered. It also suggests an explanation for the otherwise mysterious existence of the widespread E1–E2 couple in Ubl conjugation. Second, I will take examples from both the ubiquitin and Ubl literature to highlight common and divergent themes regarding the biochemical functions of ubiquitin and Ubl ligation. In other words, the emphasis will be on what happens after the modifier is attached to its target rather than focusing on what determines how a target is chosen for modification in the first place. The latter topic is extensively reviewed elsewhere in these volumes. Finally, I will attempt to generalize these examples to give a broader account of the biochemical and physiological consequences of ubiquitin and Ubl conjugation.

11.3 Origins of the Ubiquitin System

For many years, the evolutionary antecedents to the ubiquitin system were completely mysterious. Ubiquitin itself was regarded as perhaps the most highly conserved of all eukaryotic proteins, yet no eubacterial or archaeal proteins shared any obvious primary sequence similarity to it [35]. An early hint to ubiquitin's origins came from the cloning and sequencing of the gene encoding the E1 ubiquitin-activating enzyme; the protein displays weak but significant similarity to ChlN/MoeB, an *E. coli* protein required for the biosynthesis of the molybdenum cofactor (Moco) [36]. At the time, the biochemical function of MoeB was unknown, and thus this similarity was not informative by itself. During the late 1990s, however, the protein sequences and catalytic mechanisms of the enzymes used to synthesize Moco (and thiamin [vitamin B1]) began to be deciphered, and intriguing similarities to ubiquitin activation were noted [37–39].

11.3.1

Sulfurtransferases and Ubl Activation Enzymes

Biosynthesis of both Moco and thiamin requires insertion of sulfur atoms into their precursor forms. In each case, the sulfur is donated by a small sulfur carrier protein termed MoaD and ThisS, respectively. The donor sulfur derives from a thiocarboxylate group generated at the C-termini of these proteins (see Figure 11.2). MoaD and ThiS are related and, like ubiquitin, end with a pair of glycines. Most interestingly, conversion of the C-terminal glycine carboxylate to a thiocarboxylate

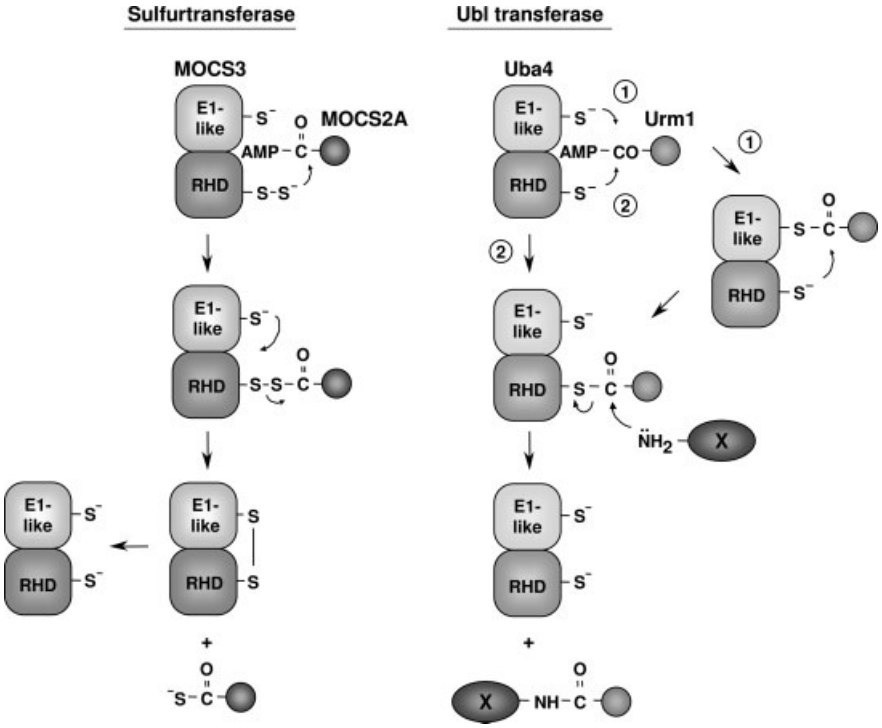

Fig. 11.2. Potential parallels between the MOCS2A–MOCS3 (MoaD–MoeB) and Urm1–Uba4 pathways. In MOCS2A activation, the C-terminally adenylated protein is thought to be attacked by a persulfide on the C-terminal RHD in MOCS3 (for *E. coli* MoeB, a persulfide on a separate, unidentified protein can be postulated). Release of the MOCS2A thiocarboxylate from its persulfide linkage to the MOCS3 RHD requires a second thiol group, which is proposed to be the E1-like domain cysteine, but other reducing factors may serve as well, depending on the species. For Uba4, it is not yet known whether a thioester between Urm1 and the conserved cysteine in the E1-like domain is formed (as depicted in pathway 1). Pathway 2 supposes that the adenylated Urm1 is directly attacked by a thiol from the RHD (a persulfide is also conceivable). No experimental evidence for the postulated RHD-linked Urm1 intermediate is currently available. Nucleophilic attack by substrate (X) transfers Urm1 from Uba4 to the substrate.

in these proteins is preceded by C-terminal adenylation by an E1-related enzyme: MoeB for MoaD and ThiF for ThiS [38–40]. Both MoaD and ThiS were later shown to share the ubiquitin fold despite the lack of obvious overall sequence similarity to ubiquitin [41, 42]. Therefore, ubiquitin, MoaD, and ThiS are all structurally related proteins whose C-termini are activated through adenylation by homologous E1-related enzymes [5, 43].

Further evidence for an evolutionary link between these sulfur transfer systems and ubiquitin activation came from a bioinformatics analysis of proteins that might be related to MoaD or ThiS in the predicted *S. cerevisiae* proteome [44]. This eukaryote lacks any Moco-containing enzymes and uses a different mechanism for thiamin synthesis; therefore, the aim of the sequence searches was to identify potential new Ubls that might have been missed in scans with ubiquitin. One previously uncharacterized protein was uncovered and named ubiquitin-related modifier-1 (Urm1), although no sequence similarity to ubiquitin could be detected. By yeast two-hybrid interaction screening with Urm1, Furukawa et al. [44] then identified a novel E1-related protein, which they named Uba4. Uba4 is more closely related to ThiF and MoeB than it is to the E1 ubiquitin-activating enzyme. Nevertheless, Uba4 appears to form a thioester intermediate with Urm1 and to stimulate covalent addition of Urm1 to cellular proteins. (Subsequent analysis revealed that a major target of Urm1 conjugation is Ahp1, a thiol-specific antioxidant protein [45].) The conclusion from these findings is that Urm1 and Uba4 function as a Ubl-protein conjugation system despite bearing much closer sequence relatedness to biosynthetic sulfur transfer factors than to ubiquitin and ubiquitin-activating enzyme. As such, the Urm1–Uba4 system might represent a kind of "missing link" in the evolution of the ubiquitin system from these sulfur transfer pathways [1, 44].

11.3.2 The E1–E2 Couple

Of course, the ubiquitin system involves a number of additional enzymes beyond the E1-activating enzyme. E2, E3, and ubiquitin- and Ubl-deconjugating enzymes are also central components, although E3s and Ubl-cleaving enzymes might not be part of all Ubl conjugation systems. On the other hand, an E1–E2 couple may be obligatory in all conjugation pathways that utilize an E1-like enzyme (Figure 11.2). For the eight Ubl conjugation systems in which an E1 has been identified (Table 11.1), all but one – the Urm1 system – is known to require a separate E2 protein. The transfer of Ubls from a cysteine side chain of an E1 to a cysteine on an E2 is not chemically necessary insofar as the Ubl C-terminus is already activated when it is bound to the E1, and the transfer to E2 also yields an enzyme–Ubl thioester bond. Enhanced regulatory flexibility or substrate specificity might help to explain the existence of E2s. Multiple E2 isozymes characterize the ubiquitin pathway, and in conjunction with different E3s, this enzyme diversity might increase the range or specificity of substrate modification. An E2 might also be needed to generate polymerized forms of ubiquitin or of certain Ubls [46, 47]. However, these

explanations do not account for the evolutionary appearance of E2s in the first place, and no protein obviously related in sequence to any E2s has been identified in the biosynthetic sulfur donor pathways.

A possible exception to the E2 requirement for E1-catalyzed Ubl-protein conjugation is Urm1. Because Urm1 ligation appears to be poised, evolutionarily speaking, between the MoaD/ThiS activation and Ubl-protein ligation mechanisms, this exception is potentially instructive. A unique and conserved feature of Uba4 (the Urm1 E1 enzyme) compared to other E1-like proteins is the presence of a rhodanese homology domain (RHD) in the protein (Figure 11.2). Rhodanese and a number of RHD proteins are sulfurtransferases that form a persulfide (–S–S–H) on their active-site cysteine. Many MoeB family proteins, such as human MOCS3, have a similar domain organization, with an E1-like domain followed by an RHD. Based on these and other similarities, we previously proposed that thiocarboxylate formation in MoaD (MOCS2A in humans) catalyzed by MoeB/MOCS3 is closely related mechanistically to Uba4-catalyzed Urm1 activation and transfer [1] (see Figure 11.2). The Uba4 RHD in this scenario functions as a kind of built-in E2.

In the part of this speculative model pertaining to the sulfurtransferase, it was suggested that a persulfide is generated at the RHD active site of MoeB and that this attacks the activated MoaD to form an acyl disulfide intermediate (Figure 11.2, left side). In a second step, reductive cleavage of the MoaD acyl disulfide by the conserved E1-domain cysteine releases the MoaD thiocarboxylate. Recent experiments on human MOCS2A thiocarboxylate formation support many features of this model [48, 49]. We and others [1, 37, 39] had also initially suggested that the cysteine in the E1-like domain forms a thioester with MoaD. This appears not to occur, and the importance of this cysteine for Moco synthesis varies between species. The model in Figure 11.2 incorporates this revision. Interestingly, an acyl disulfide intermediate linking *E. coli* ThiF and ThiS has also been isolated, but this occurs on the conserved E1 domain cysteine [50]; such a covalent complex is not universally observed [51].

What are the implications for the Uba4 mechanism of Urm1 activation? As noted, the RHD of Uba4 was proposed to function as a built-in E2, using its active-site cysteine to attack a thioester intermediate on the E1-like domain [1] (Figure 11.2). We have found that the conserved RHD cysteine of Uba4 is required for its physiological function and for Urm1-protein conjugation *in vivo* (I. Velichutina, M. Hochstrasser, unpublished data). However, it remains to be shown that Urm1 is transferred onto the RHD or even that it forms a thioester at the E1 site, as has been assumed (Figure 11.2, right side). More generally, the E1-to-E2 transthiolation of Ubls may reflect the derivation of these protein-conjugation systems from sulfurtransferases that mobilize sulfur from a protein-linked persulfide through reductive cleavage by a second enzyme thiol group. Urm1 conjugation may retain features of the more ancient sulfurtransferases, while a process of ''molecular takeover'' might have occurred for other Ubl ligation pathways such that the RHD was replaced by a distinct E2 species. It is noteworthy that the E2s for the Atg8 and Atg12 pathways, while very weakly related to each other, are not detectably similar

in sequence to the identified E2s for the remaining Ubl pathways, suggesting the possibility of convergent evolution of E2s from at least two separate lineages.

11.4 Ubiquitin-binding Domains and Ubiquitin Receptors in the Proteasome Pathway

Most early work on ubiquitin focused on its role in proteolysis [52–54]. Once it became clear that the 26S proteasome was responsible for the degradation of polyubiquitinated proteins, an obvious question was how the polyubiquitin chain facilitated degradation of the substrate protein. Several functions for such chains were proposed, but the most patent was that they enhanced the association between substrate and proteasome by directly binding to the proteasome. Elegant biochemical experiments eventually demonstrated that direct binding between the Lys48-linked polyubiquitin chain and the proteasome could fully account for the observed affinity of model polyubiquitinated proteins for the protease complex [13].

11.4.1 A Proteasome "Ubiquitin Receptor"

Beginning with the assumption that a polyubiquitinated protein could directly bind to a single subunit of the proteasome, Rechsteiner and colleagues used far-Western analysis to search for a ubiquitin receptor within the proteasome [55]. The subunits of purified human 26S proteasomes, which consist of one or two 19S regulatory complexes bound to a 20S proteasome core, were resolved by denaturing gel electrophoresis, blotted onto a membrane, and incubated with a radiolabeled polyubiquitinated protein. Remarkably, a single subunit of the 19S regulatory complex called S5a (Rpn10 in yeast) bound tightly to the radiolabeled substrate despite having been denatured initially in SDS and separated from the other subunits of the complex. Binding in solution was later shown as well. Two related ~30-residue hydrophobic segments in S5a are responsible for the binding to polyubiquitin [56]. A subsequent bioinformatics analysis recognized a more general ~20-residue core related to the S5a ubiquitin-binding element, and this element was christened the ubiquitin interaction motif (UIM) [57].

11.4.2 A Plethora of Ubiquitin-binding Domains

Since the description of the UIM, a substantial number of distinct ubiquitin-binding modules have been discovered by a combination of bioinformatic, biochemical, and structural studies [58]. Ubiquitin-binding domains include the UBA, CUE, UEV, NZF, DAUP/ZnF-UBP/PAZ, and GAT domains [59–61]. These domains range between ~35 and ~145 amino acids in length and vary considerably in structure. Nevertheless, several generalizations unite them. First, all of the struc-

turally characterized ubiquitin-binding proteins contact the same general region on the ubiquitin molecule. The interface centers on a hydrophobic surface on the β-sheet of ubiquitin, which includes Ile44 and is sometimes called the Ile44 face. Hydrophobic interactions dominate the binding. Second, the binding to a single ubiquitin is generally very weak, with apparent dissociation constants ranging from ~20 μM to nearly 1 mM. Despite this unimpressive affinity, mutational studies have made clear the physiological relevance of these weak binding interactions in many cases [58, 59].

Amplification of the ubiquitin signal by linking multiple ubiquitin moieties into a chain can have dramatic effects on the affinity or avidity of interaction with target proteins. Again, the first and probably clearest example comes from studies on the proteasome and S5a/Rpn10. In the original far-Western analyses that identified S5a as a ubiquitin-binding subunit, binding of S5a to ubiquitin chains that had at least four ubiquitin units was clearly far tighter than to shorter chains [55]. Later, quantitative studies of Lys48–ubiquitin chain binding to full 26S proteasomes revealed a similar discontinuity in binding affinity [13]. Using competitor ubiquitin chains of various lengths, Thrower et al. [13] measured the inhibition of degradation of a model substrate. They found that tetrameric chains displayed very strong inhibition ($K_i \sim 170$ nM), i.e., high affinity, whereas inhibition was extremely weak with trimeric chains ($K_i \sim 1.9$ μM) and undetectable with dimeric chains ($K_i > 15$ μM). Such nonlinear effects imply that a unique binding signal is created by formation of a tetrameric chain rather than independent binding of multiple monoubiquitin moieties [62].

11.4.3 Ubiquitin-Conjugate Adaptor Proteins

Although S5a/Rpn10 was the first identified ubiquitin-binding subunit in the proteasome, it is not the only one, and for the degradation of many proteasome substrates, S5a/Rpn10 is completely dispensable. In *S. cerevisiae* for instance, loss of Rpn10 leads to only very minor phenotypic abnormalities [63]. Examination of individual substrates *in vivo* also suggests that only a subset are affected by loss of Rpn10 [63, 64]. Another major way by which polyubiquitinated proteins bind to the proteasome is through "adaptor proteins" that are thought to shuttle on and off the proteasome, ferrying their cargo from ubiquitin–ligase complexes or other intermediaries to sites on the 19S proteasome regulatory complex [64–67].

Although the details of the apparent substrate handoffs to and from these adaptors are still unclear, some common features of the adaptor proteins are emerging. One commonality is that the adaptors bear separate modules for proteasome and polyubiquitin binding. Three structurally related adaptors, Rad23, Dsk2, and Ddi1, were first characterized in yeast. They have an N-terminal ubiquitin-like domain (UBL) and one or two UBA elements, which, as noted earlier, are ubiquitin-binding domains. The UBL of these adaptors binds directly to either of two specific subunits in the proteasome 19S regulatory complex [68, 69]. These subunits, Rpn1 and Rnp2, share a series of leucine-rich repeats (LRRs). The LRRs, at least for

Rpn1, directly bind to the UBL of Rad23, and presumably the same holds for the other adaptors [68].

Only a subset of polyubiquitinated proteasome substrates requires any of these UBL–UBA adaptor proteins [64, 70]. Genetic studies suggest that Rpn10 and Rad23 have overlapping functions in substrate targeting, but degradation of some ubiquitinated proteins requires neither [64, 71]. Such substrates might be targeted by unidentified adaptors, or they might be recognized by an integral proteasome subunit [122]. One question raised by these data is how adaptor proteins with apparently generic proteasome- and polyubiquitin-binding modules can discriminate between different polyubiquitinated substrates (or even whether there is any functional difference between targeting such substrates directly to the proteasome or to a proteasome-binding adaptor protein). Moreover, while the bipartite nature of these adaptors suggests how they can bind simultaneously to both the polyubiquitinated substrate and the proteasome, it does not indicate how substrate transfer, e.g., between the adaptor and the proteasome, takes place.

A provocative recent study suggests some unexpected features of polyubiquitinated substrate transfers and also addresses the question of substrate specificity. This work indicates that the UBL of Rad23 binds not only to the proteasomal Rpn1 subunit but also to a second protein, Ufd2, which participates in polyubiquitination of a limited set of proteins [66]. Rpn1 and Ufd2 compete for Rad23 binding. One interpretation of these findings is that binding of Rad23 to a Ufd2-containing ubiquitin–ligase complex engaged in substrate polyubiquitination displaces the substrate-linked polyubiquitin chain from Ufd2. This in turn could facilitate binding of the polyubiquitin chain to the UBA domains of Rad23. The net effect will be the transfer of the polyubiquitinated substrate from Ufd2 to Rad23. Subsequently, the Rad23 UBL must somehow release Ufd2 and bind the proteasome, initiating the final transfer of substrate. Whether additional factors are required for these transfer reactions remains to be determined.

11.5
Ubiquitin-binding Domains and Membrane Protein Trafficking

Targeting proteins to the proteasome is not the only function of ubiquitin. An intricate array of dynamic ubiquitinated protein–protein target interactions has been described in a completely different arena, namely, membrane protein trafficking [58]. As with polyubiquitinated protein trafficking to the proteasome, sequential interactions of ubiquitinated substrate proteins with multiple ubiquitin-binding factors is a central feature of membrane protein sorting. However, in membrane protein trafficking, monoubiquitin is the predominant signal. As noted earlier, the binding of monoubiquitin to a ubiquitin-binding domain is generally weak; therefore, association is usually very transient unless additional substrate-binding sites are combined with the ubiquitin-binding motif. In principle, this allows considerable flexibility and sensitivity in the regulation of endocytosis and other membrane protein trafficking events.

Initial evidence for the importance of membrane protein ubiquitination for trafficking came from yeast [72–74]. These early studies demonstrated that cell surface receptors are ubiquitinated at the plasma membrane and that this ubiquitination correlates with their endocytosis and eventual degradation in the vacuole (the yeast equivalent of the lysosome). Subsequent research from many laboratories working in a variety of organisms has revealed that monoubiquitin attachment to receptors at the cell surface is a commonly employed endocytic signal [75]. Moreover, monoubiquitination of transmembrane proteins also helps to sort them once they have entered the endosomal membrane system.

An intensively investigated ubiquitin-dependent trafficking pathway in higher eukaryotes is signal transduction by receptor tyrosine kinases, the best studied of which is the epidermal growth factor (EGF) receptor [58, 76]. Beginning at the cell surface, ubiquitin modification functions at several stages in the endocytosis and intracellular sorting of EGF receptors to the lysosome, where the receptors are ultimately degraded. Thus, as with yeast plasma membrane proteins, ubiquitination is a means of downregulating or attenuating the surface expression of EGF receptors.

Once in endosomal vesicles, EGF receptors either can be sorted to a recycling compartment and thence back to the plasma membrane, or they can continue on toward late endosomes [76]. Late endosomes mature by a processing of invagination and vesiculation to form multivesicular bodies (MVBs). Receptors either stay in the limiting membrane of the MVB, which allows them to make their way back to the plasma membrane, or sort into the internal vesicles. MVBs eventually fuse with lysosomes, and the internalized vesicles and their cargo receptors are destroyed by lysosomal lipases and proteases.

Surprisingly, the E3 that ubiquitinates the EGF receptor at the cell surface continues to colocalize with the receptor along the endocytic pathway all the way to the internal vesicles of MVBs. This sustained colocalization may be crucial for maintaining the EGF receptor in its ubiquitin-modified state and for its sorting to the lysosome [77]. In yeast, there is no requirement for continued E3 association with the ubiquitinated receptor during trafficking [78]. It could be that in mammalian cells, the endocytosed receptors are more susceptible to deubiquitination by DUBs and therefore require repeated rounds of ubiquitin re-addition. This sustained requirement for ubiquitin on receptor proteins reflects ubiquitin-dependent endosomal sorting steps that occur within both yeast and mammalian cells. When either endocytosed proteins or biosynthetic membrane cargo proteins moving from the Golgi to the vacuole or lysosome are ubiquitinated, they are sorted into the invaginating regions of the late endosome. This sorting requires the sequential action of at least four highly conserved protein complexes that act at the endosome surface [79].

The first of these complexes is a heteromultimer formed by Hrs and STAM. Both the Hrs and STAM subunits contain ubiquitin-binding UIMs, which are necessary for sorting of cargo into internal MVB vesicles in yeast and might directly bind ubiquitinated membrane cargo proteins [80]. In mammalian cells, the Hrs–STAM complex is required for EGF receptor sorting in the MVB and degradation

in the lysosome. Based on these and other data, this ubiquitin-binding complex has been proposed to be the sorting receptor for ubiquitinated membrane proteins at the endosome [80, 81].

Following initial recognition of ubiquitinated cargo by Hrs–STAM, the cargo is passed on to the ESCRT-I complex, which also includes a ubiquitin-binding subunit, Tsg101. ESCRT-I is recruited to the endosome through direct interactions between Tsg101 and both a tetrapeptide motif in Hrs and, apparently, the monoubiquitinated membrane protein cargo [82, 83]. Therefore, as was true for trafficking of certain polyubiquitinated proteins to the proteasome, ubiquitinated membrane proteins destined for the lysosome also need to be exchanged between a series of ubiquitin-binding factors. In both cases, these escort or adaptor proteins might shield the substrate from DUBs that could otherwise prematurely remove the ubiquitin signal. By interrogating the ubiquitin–substrate conjugate multiple times along the pathway to degradation, these factors might also enhance substrate selectivity.

Finally, two other complexes important for protein sorting at the late endosome membrane, ESCRT-II and ESCRT-III, help to drive the ubiquitinated cargo into invaginating membrane domains. Prior to scission of an invaginating region, a specific DUB is recruited to the ESCRT-III complex to recover the ubiquitin from the targeted receptors [84, 85]. This prevents degradation of ubiquitin and the depletion of cellular ubiquitin pools.

For simplicity, only ubiquitination of the endocytic or biosynthetic membrane protein cargo itself was noted in the preceding discussion. However, this is not the only point at which ubiquitination is important in membrane protein trafficking. Multiple endocytotic factors and membrane protein-sorting factors are also monoubiquitinated, sometimes in response to the same ligands that trigger receptor ubiquitination, e.g., EGF binding to EGF receptor [58, 75]. Many of these factors also contain ubiquitin-binding domains. These observations have led to the proposal that these soluble factors form dynamic protein networks in which ubiquitination of one factor allows intramolecular or intermolecular binding to other trafficking factors. This might contribute to or regulate the assembly of the ubiquitin-binding factors at plasma membrane sites for endocytosis or at endosomal sites for MVB sorting.

11.5.1 The MVB Pathway and RNA Virus Budding

A source of considerable excitement in the area of ubiquitin-dependent trafficking has been the discovery that enveloped RNA viruses such as HIV-1 and Ebola commandeer the MVB machinery to bud from the surface of the cell [86]. Budding is the way by which such viruses detach from the membrane of infected host cells. Outward budding of the plasma membrane and inward vesiculation of the late endosome membrane are topologically equivalent, and thus it is not entirely surprising that the same vesiculation machinery might be used in both cases. A key issue is how these viruses recruit MVB components to sites of virus particle assembly on

the plasma membrane. Expression of the HIV-1 Gag protein is sufficient for membrane budding and release, and a specific region in Gag called the late or L domain is necessary for these events. Interestingly, the L domain in HIV-1 (and other retroviruses) includes a tetrapeptide similar to the sequence in Hrs mentioned earlier; as with Hrs, this sequence provides a docking site for the Tsg101 subunit of the ESCRT-1 complex. In this case, however, recruitment of ESCRT-I serves to hijack the MVB machinery to viral budding sites. In this way, the L domain of Gag binds to the MVB machinery, bringing the ESCRT complexes to Gag-associated plasma membrane sites and thereby stimulating the budding and release of virus particles.

What is unclear in all this is the exact role of ubiquitin [86]. Ubiquitin is somehow necessary for enveloped RNA virus budding based on ubiquitin depletion and mutagenesis experiments. Many retroviruses incorporate ubiquitin into their virions, and their Gag proteins are ubiquitinated in infected cells. However, Gag ubiquitination does not appear to be necessary for efficient viral budding and release, at least for the HIV-1 and MuLV retroviruses [87]. Components of the MVB machinery such as Hrs are also modified by ubiquitin, so it could be that this is where ubiquitin ligation is most important for viral egress from the cell.

11.6 Sumoylation and SUMO-binding Motifs

Besides ubiquitin, SUMO has been the most extensively studied ubiquitin-related protein modifier, and it is already clear that it can modify many proteins, possibly hundreds, *in vivo*. Recent results from studies on SUMO ligation and sumoylated protein interactions underscore and extend many of the ideas about ubiquitin–protein interactions, which were summarized in the preceding two sections.

RanGAP1 was the first SUMO-modified protein to be described, and it has been a useful prototype for understanding how conjugation to SUMO can modify a protein's function [88]. RanGAP1, the Ran GTPase-activating protein, is modified at a specific lysine that conforms to what has turned out to be a widely, but not universally, utilized sumoylation consensus sequence (ΨKxD/E, where Ψ is a large aliphatic residue and x is any residue). Protein sumoylation is also different from ubiquitination in that site-specific modification can occur with just the cognate E1 and E2 but no E3. This can be explained by the observation that the consensus sumoylation site provides a set of specific side-chain interactions with the E2 active-site pocket [89].

Attachment of SUMO to RanGAP1 dramatically changes the subcellular localization of the enzyme, resulting in its concentration on the cytoplasmic fibrils of the nuclear pore complex (NPC) where it binds to RanBP2/Nup358. Binding of SUMO–RanGAP1 to RanBP2 is not competed off by an excess of either free RanGAP1 or SUMO, suggesting either that essential RanBP2-binding determinants are present in both the RanGAP1 and SUMO portions of the SUMO–RanGAP1 conjugate or that formation of the conjugate somehow alters the conformation of

RanGAP1 or SUMO to generate a RanBP2-binding site [90]. Recent work on noncovalent SUMO interactions with RanBP2 and other proteins points toward the first of these two models and has helped to identify the first SUMO-binding motif (SBM).

11.6.1 A SUMO-binding Motif

Unlike the description of ubiquitin-binding motifs, which has become something of a cottage industry, there are relatively few studies thus far that address noncovalent binding of SUMO to other proteins. The first published foray into this area came from a two-hybrid screen for proteins that interacted with the p53-related protein p73α [91]. Both SUMO and a series of known SUMO-interacting proteins were found, and SUMO was shown to be conjugated to p73α. Comparison of the SUMO-interacting proteins suggested a short common motif that was potentially important for SUMO binding, and this was supported by subsequent mutagenesis experiments. The consensus derived from this small group of proteins was hhXSXS/Taaa, where *h* is a hydrophobic amino acid, *a* is an acidic amino acid, and *X* is any residue.

Building on these findings, Song et al. [34] carried out quantitative binding and NMR chemical shift perturbation studies to map the interface between SUMO and peptides bearing an SBM. With as few as nine residues, these peptides are substantially smaller than the domains that typically bind ubiquitin (20–145 residues); despite this small size and the fact that the peptides and SUMO form 1:1 complexes, the dissociation constants are between 5 μM and 10 μM, which is much tighter than the binding seen with all the ubiquitin-binding motifs discussed earlier. The refined SBM consensus sequence derived by Song et al. emphasized a central group of 3–4 hydrophobic residues. A region of RanBP2 known to bind sumoylated RanGAP1 has a sequence that conforms to this consensus. Mutagenesis experiments supported the significance of these hydrophobic residues but also indicated a contribution from flanking acidic residues to SUMO binding. Viewed together, the data from Minty et al. [91] and Song et al. [34] suggest that the SBM is composed primarily of a hydrophobic amino acid cluster flanked on one or both sides by acidic residues.

Interestingly, a two-hybrid screen for proteins that interacted with yeast SUMO, Smt3, pointed to the presence of a potential Smt3-binding sequence related to the above SBM sequences, namely, a cluster of 3–4 hydrophobic residues flanked by acidic amino acids [92]. This finding suggests that the mechanism of noncovalent SUMO interaction with target proteins is conserved from yeast to mammals. There will almost certainly be additional SUMO-binding domains distinct from the SBM, but it is notable that unrelated screens as different as the yeast and mammalian two-hybrid studies could yield such similar consensus sequences.

A very recent report on the crystal structure for a complex containing a SUMO1-RanGAP1 conjugate, a segment of RanBP2, and the SUMO E2 revealed the structure of the RanBP2 SBM and its mode of binding to SUMO [10]. The SBM peptide

from RanBP2 forms a β strand that sits in a hydrophobic surface depression on SUMO and extends the SUMO β sheet; the ends of the SBM segment are acidic residues that interact with basic residues on the SUMO surface. The SBM–SUMO interface determined from this structure is therefore consistent with the mutagenesis and binding data mentioned above.

Importantly, both the crystallographic analysis of SUMO–RanGAP1 and an earlier NMR study of the same conjugate [93] revealed minimal direct interaction between SUMO and RanGAP1, except around the isopeptide linkage, and no obvious structural changes from the unligated proteins. From the NMR analysis, the loop of RanGAP1 linked to SUMO and the SUMO tail were both highly dynamic. Therefore, the mechanism of SUMO–RanGAP1 binding to RanBP2 does not appear to be through a conformational switch in the conjugate but rather by cooperative and simultaneous interaction of RanBP2 to a bipartite binding site created by the physical linkage of SUMO to RanGAP1. This type of binding is likely to typify many other SUMO–protein conjugate interactions.

11.6.2 A SUMO-induced Conformational Change

In contrast to the structural independence of the SUMO and substrate moieties in the SUMO–RanGAP1 conjugate, an elegant series of mechanistic studies on the function of SUMO conjugation to the DNA repair enzyme thymine-DNA glycosylase (TDG) suggests that in this case, SUMO conjugation to the substrate induces a substantial and functionally important conformation change in the protein [94]. TDG initiates base excision repair of a mismatched thymine or uracil nucleotide by removing the base, leaving an abasic site in the DNA. *In vitro*, TDG dissociation from the abasic site is strongly rate limiting; tight binding to the potentially harmful abasic site likely shields the site until downstream enzymes can complete the repair process. Nonetheless, a mechanism for enzyme turnover is required, and unexpectedly, site-specific SUMO ligation to TDG turns out to greatly accelerate its release from the DNA [95].

Even more surprisingly, the stimulation of TDG–DNA dissociation by sumoylation of the TDG C-terminal domain appears to operate through a sumoylation-induced conformational change in the TDG N-terminal domain, which causes the enzyme to loosen its grip on the abasic site [94]. Based on a protease sensitivity assay, the N-terminal domain of unsumoylated TDG triggers a conformational change in the enzyme when it binds to a G–U DNA mismatch substrate, enhancing its affinity but preventing catalytic turnover. If, instead, a sumoylated version of TDG is used in the reaction, TDG conformation no longer changes upon incubation with this DNA substrate, suggesting that in its sumoylated form, TDG does not assume the high-affinity DNA-binding state. N-terminally deleted TDG and sumoylated versions of both full-length and N-terminally truncated TDG show identical kinetic behavior when base excision is assayed (in all cases, turnover is still very slow: $k_{cat} \sim 0.05\ min^{-1}$). These data suggest that sumoylation allows enzymatic turnover by somehow facilitating an N-terminal domain-dependent confor-

mational switch back to a state with low DNA-binding affinity. In the cell, one would assume, TDG sumoylation should occur only after the base excision step. This way, DNA damage recognition and base excision can occur efficiently (non-sumoylated, tight binding state), but substrate release and handoff to the downstream DNA endonuclease will then also be possible (sumoylated, weak binding state). What controls the timing of sumoylation and desumoylation relative to DNA binding and base excision is an interesting question that remains to be examined.

11.6.3 Interactions Between Different Sumoylated Proteins

While SUMO ligation to TDG is likely to be critical to its normal *in vivo* function, a noncovalent interaction between the two also seems to be important [96]. A mutation in TDG that blocks noncovalent SUMO binding also blocks its covalent attachment. In addition, TDG associates with the promyelocytic leukemia (PML) protein, and the same mutation that blocks TDG binding to SUMO blocks *in vivo* colocalization with PML; this association is not impaired by simple elimination of the TDG sumoylation site (sumoylated TDG does bind slightly better to PML *in vitro*). In other words, noncovalent association of SUMO with TDG is important for TDG interaction with PML. Interestingly, PML is itself a sumoylated protein and also has an SBM, so an obvious model is that the SUMO portion of sumoylated PML binds to TDG, and the SUMO on TDG binds to the PML SBM. Indeed, mutations that eliminate PML sumoylation inhibit TDG binding as well.

PML functions as a scaffold for the assembly of so-called PML nuclear bodies, subnuclear structures that have been implicated in transcriptional regulation and DNA repair. PML must be sumoylated to associate with these bodies and to concentrate a number of other sumoylated proteins there as well [97]. By combining sumoylation sites and SUMO-binding motifs into the same polypeptide, networks of protein interactions, typified by the TDG-PML association, can be created (also see Ref. [92]). This is reminiscent of what seems to occur with ubiquitin modification of and association with components of the endocytic machinery. As we saw earlier, a number of endocytic factors are ubiquitinated and also carry ubiquitin-binding domains. This is thought to contribute to the activation and interaction of such factors and/or to the exchange of ubiquitinated cargos between them.

Besides being relevant to the question of how SUMO–protein conjugates interact specifically and noncovalently with their protein partners, these data might also be significant when considering SUMO ligation specificity. As noted earlier, site-specific protein sumoylation is frequently observed *in vitro* with only the E1 and E2 enzymes, at least when they are at high concentration. We saw that one element of this specificity comes from E2–substrate contacts at consensus sumoylation sites. However, direct but noncovalent SUMO–substrate binding could provide an important additional binding determinant, particularly in combination with a consensus sumoylation site. This notion is supported by the finding that TDG is not sumoylated if its noncovalent SUMO-binding site is mutated [96].

11.7
General Biochemical Functions of Protein–Protein Conjugation

The examples chosen in the previous sections illustrate various ideas about the biochemical functions of protein modification by ubiquitin and Ubls. The simplest generalization to come out of all these examples is that attachment of ubiquitin or a Ubl to a protein (or other macromolecule) creates a distinct physiological state by altering the protein's interactions with other macromolecules (Figure 11.3). This is an almost trivial assertion, but more specific versions of the statement will be for-

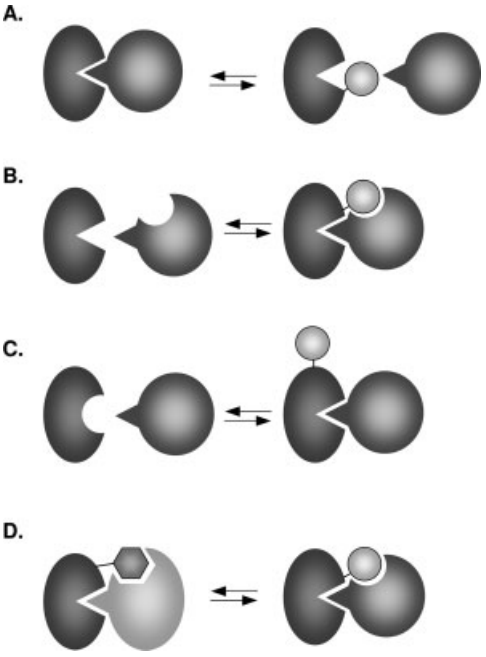

Fig. 11.3. Possible molecular functions for ubiquitin or Ubl-protein modification. (A) Ubl conjugation directly blocks an interaction between two proteins. A potential example of this is the sumoylation of the vaccinia A40R protein, which prevents association and aggregation between A40R monomers. (B) Ubl conjugation facilitates protein binding by providing a (additional) binding site. The best-documented case for this type of regulation is the sumoylation of RanGAP1, which leads to the binding of the conjugate to the nuclear pore protein RanBP2. (C) Ubl-conjugation causes a conformational change that enhances binding (or could have the reverse effect). For instance, it appears that SUMO attachment to thymine-DNA glycosylase (TDG) triggers a conformational change in TDG that lowers its affinity for DNA. (D) Modification by one Ubl helps to recruit a factor that is different from the protein that would be recruited were the substrate modified by another type of Ubl. Alternatively, one Ubl (red hexagon) might simply block conjugation of the substrate to another Ubl (or to another molecule), thereby preventing the substrate's interaction with another protein (green). The modification of IκB by SUMO on the same sites used by ubiquitin has been proposed to reflect such a mechanism. (Note: Not all possible variations on these basic mechanisms are shown.)

mulated below that may help give a sense of which mechanisms of altering macromolecular interaction by ubiquitin or Ubl attachment are more common and why this might be so.

11.7.1 Negative Regulation by Ubl Conjugation

Because ubiquitin and Ubls are bulky modifiers, one obvious way they could function is by steric occlusion: the attached Ubl simply blocks the ability of its substrate to bind to another protein (or another part of the same protein) (Figure 11.3A). There are still relatively few well-established examples of this inhibitory mode of action. One possible reason is that for such a mechanism to operate effectively in many cases, a very large fraction of the protein would need to be modified by the Ubl. However, for many proteins, only a very small fraction is observed in the conjugate form. This can sometimes be attributed to artifactual deconjugation during protein isolation, but even when such deconjugation is largely prevented, often only a few percent of a particular protein is modified, e.g., with SUMO or ISG15.

Nevertheless, if the small fraction of modified protein were localized to some functionally unique cellular site, or if a transient modification were sufficient to put the protein in a new state, such an inhibitory mechanism could still operate. An example that combines both of these mechanisms is the transient ubiquitination of histone H2B at chromosomal sites of induced transcription [98]. (The exact biochemical consequences of this histone ubiquitination are not yet known, so this might not be an example of a modification that inhibits interaction.) Local ubiquitination is brought about by the recruitment of a histone H2B-specific ubiquitin ligase complex. Such transient histone H2B ubiquitination triggers histone H3 methylation, and this new histone state, which is necessary but not sufficient for gene activation, no longer requires that ubiquitin remain on histone H2B. Indeed, deubiquitination of the histone is needed for completion of the switch to the transcriptionally active state. Another example of such molecular memory is the sumoylation of TDG discussed earlier: SUMO attachment is needed to weaken the interaction with DNA after base scission, but once TDG has released from the DNA, the SUMO is no longer necessary or desirable [94]. In this case, however, SUMO attachment seems to inhibit macromolecular interaction (DNA binding) indirectly by inducing a change in enzyme conformation.

If a large percentage of a protein were Ubl-modified, the notion of negative regulation of protein interaction and function would be more straightforward. A recent example of a protein that is nearly quantitatively modified by SUMO is the vaccinia virus A40R early protein [99]. A40R sumoylation is required for its localization to ER viral replication sites. Mutation of the A40R sumoylation site causes the protein to self-associate and aggregate into long rods. Thus, SUMO attachment to A40R appears to block its interaction with another protein (another copy of A40R). A second potential example of quantitative sumoylation is the plasma membrane K2P1 potassium leak channel [100]. The apparent SUMO modification is proposed to block channel opening and thereby leakage of K^+ ions from the cell.

11.7.2
Positive Regulation by Ubl Conjugation

When the Ubl modifier enhances an interaction with another macromolecule, it usually does so by participating directly in the formation of part or all of the binding interface with the target molecule (Figure 11.3B). In principle, such an interaction can also be modulated if an allosteric change in a target binding site were induced by the attached Ubl (Figure 11.3C). Many examples of Ubl regulation fall into the former category. Here it is easy to see that even if only a small fraction of a particular protein were modified, its new activity could suffice to effect a change in physiological state. Many of the examples in the preceding sections reflect this kind of mechanism. As discussed, noncovalent ubiquitin–protein or Ubl–protein interactions tend to be weak. Binding can be greatly enhanced either by polymerization of the ubiquitin signal (no clear example of obligatory Ubl chain formation is known) or by combining the weak binding from ubiquitin or Ubl with additional weak binding sites (possibly created by additional ubiquitin or Ubl modifications). The combination of multiple weak interactions to give highly specific protein–protein binding is a well-established idea in the signal transduction field. An example of such multivalent binding was discussed earlier for SUMO–RanGAP1 binding to the nuclear pore complex.

11.7.3
Cross-regulation by Ubls

Interestingly, alternative Ubl or ubiquitin modifications sometimes occur on the same substrate, and these can direct the protein to different targets. The clearest illustration of this is proliferating cell nuclear antigen (PCNA), which can be monoubiquitinated, polyubiquitinated, or sumoylated, and each of these forms results in the recruitment of distinct downstream effector proteins. PCNA functions as a DNA polymerase processivity factor in various modes of DNA replication and DNA repair. It forms a homotrimer that encircles the DNA double helix and associates with multiple DNA polymerases. Ubiquitin or a Lys63-linked polyubiquitin chain is attached to a single PCNA lysine, and SUMO is primarily attached to this same site, with a small amount at a second lysine [101]. When the DNA replication machinery encounters a DNA lesion and cannot replicate past it, the type of post-translational modification of PCNA determines which of several distinct mechanisms will be engaged to correct or bypass the lesion.

A model that accounts for these different outcomes was recently outlined [102]. The ubiquitin E2 Rad6 and the E3 Rad18 are responsible for monoubiquitination of PCNA, and this ubiquitin can be extended by a heterodimeric E2, Ubc13-Mms2, and the E3 Rad5 into a Lys63-linked polyubiquitin chain [101, 103]. During normal replication, the replicative DNA polymerase is associated with PCNA at the primer–template junction. Monoubiquitination of PCNA is proposed to prevent the polymerase from accessing the junction when DNA damage is encountered, allowing a translesion synthesis (TLS) polymerase to enter the complex [102, 104].

These TLS polymerases bind directly to PCNA and promote either error-free or mutagenic replication through the lesion, depending on the TLS polymerase and the type of DNA damage. If a Lys63–ubiquitin chain has formed on PCNA, it is postulated to cause complete dissociation of the replicative DNA polymerase complex, allowing a template-switching mechanism with error-free copying of the other replicated DNA strand.

The consequences of SUMO ligation to PCNA have been less clear, but PCNA sumoylation, which occurs during a normal S phase without induced DNA damage [101], prevents recombinational bypass of lesions [102]. Such recombination during normal DNA replication can be deleterious because of the risk of chromosome rearrangements. Very recent results strongly support this function for SUMO–PCNA, and argue that the sumoylated form specifically recruits the Srs2 DNA helicase to the replication fork [105, 106]. Srs2 disassembles the nucleoprotein filaments that are necessary intermediates for DNA strand invasion and homologous recombination [107, 108]. The exact protein–protein interactions affected by the different ubiquitin and SUMO modifications have not been worked out in full, but it appears that both positive and negative regulation of such interactions occurs (Figure 11.3). Because the same site of PCNA is used for both ubiquitin and SUMO attachment, there also appears to be some antagonism between these two modifications [105] (Figure 11.3D). Similar competition between ubiquitin and SUMO for the same substrate lysine has been seen with IκB, an inhibitor of the NF-κB signaling pathway [109]. For IκB, Lys48 polyubiquitination leads to IκB degradation and NF-κB activation, but sumoylation of the same IκB lysines prevents this.

11.8 Conclusions

As should be evident from the above survey, ubiquitin and Ubl modification of proteins represents a highly versatile means of regulating protein function. Nature has made widespread use of such conjugation through the elaboration of multiple variants of the same basic enzymatic mechanism. On the order of a dozen or so Ubls have been documented to date, and for eight of these, at least one enzyme in the pathway for substrate conjugation has been identified. Ubiquitin itself can attach to proteins in the form of polymers of different topology, and these topological variants impart differences in function as well. The fundamental E1–E2 couple, which probably arose very early in the evolution of the ubiquitin system from more ancient sulfur transfer pathways, has been supplemented with an array of specificity factors (E3s) in some of the pathways, especially the ubiquitin pathway. Deconjugating enzymes have turned ubiquitin and many of the Ubls into dynamic modifiers whose attachments are tightly regulated both spatially and temporally. The basic biochemical consequence of protein modification by ubiquitin or Ubls is usually a change in the target's association with other proteins. This change can occur by both direct and indirect mechanisms and can either stimulate or inhibit partic-

ular protein–protein interactions. Given the intricacy of the ubiquitin–Ubl system, research into its functions and mechanisms should continue to tax and reward investigators for years to come.

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Cell Biology of the Ubiquitin-Proteasome System

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Preface

There is an incredible amount of current global research activity devoted to understanding the chemistry of life. The genomic revolution means that we now have the basic genetic information in order to understand in full the molecular basis of the life process. However, we are still in the early stages of trying to understand the specific mechanisms and pathways that regulate cellular activities. Occasionally discoveries are made that radically change the way in which we view cellular activities. One of the best examples would be the finding that reversible phosphorylation of proteins is a key regulatory mechanism with a plethora of downstream consequences. Now the seminal discovery of another post-translational modification, protein ubiquitylation, is leading to a radical revision of our understanding of cell physiology. It is becoming ever more clear that protein ubiquitylation is as important as protein phosphorylation in regulating cellular activities. One consequence of protein ubiquitylation is protein degradation by the 26S proteasome. However, we are just beginning to understand the full physiological consequences of covalent modification of proteins, not only by ubiquitin, but also by ubiquitin-related proteins.

Because the Ubiquitin Proteasome System (UPS) is a relatively young field of study, there is ample room to speculate on possible future developments. Today a handful of diseases, particularly neurodegenerative ones, are known to be caused by malfunction of the UPS. With perhaps as many as 1000 human genes encoding components of ubiquitin and ubiquitin-related modification pathways, it is almost certain that many more diseases will be found to arise from genetic errors in the UPS or by pathogen subversion of the system. This opens several avenues for the development of new therapies. Already the proteasome inhibitor Velcade is producing clinical success in the fight against multiple myeloma. Other therapies based on the inhibition or activation of specific ubiquitin ligases, the substrate recognition components of the UPS, are likely to be forthcoming. At the fundamental research level there are a number of possible discoveries especially given the surprising range of biochemical reactions involving ubiquitin and its cousins. Who would have guessed that the small highly conserved protein would be involved in endocytosis or that its relative Atg8 would form covalent bonds to a phospholipid during autophagy? We suspect that few students of ubiquitin will be surprised if it or a

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ubiquitin-like protein is one day found to be covalently attached to a nucleic acid for some biological purpose.

We are regularly informed by the ubiquitin community that the initiation of this series of books on the UPS is extremely timely. Even though the field is young, it has now reached the point at which the biomedical scientific community at large needs reference works in which contributing authors indicate the fundamental roles of the ubiquitin proteasome system in all cellular processes. We have attempted to draw together contributions from experts in the field to illustrate the comprehensive manner in which the ubiquitin proteasome system regulates cell physiology. There is no doubt then when the full implications of protein modification by ubiquitin and ubiquitin-like molecules are fully understood we will have gained fundamental new insights into the life process. We will also have come to understand those pathological processes resulting from UPS malfunction. The medical implications should have considerable impact on the pharmaceutical industry and should open new avenues for therapeutic intervention in human and animal diseases. The extensive physiological ramifications of the ubiquitin proteasome system warrant a series of books of which this is the third one.

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1
Ubiquitin: A New Player in the Peroxisome Field

Astrid Kragt, Rob Benne, and Ben Distel

1.1
Introduction

Peroxisomes are single-membrane-bound organelles found in almost all eukaryotic cells. The name "peroxisome" reflects its role in hydrogen peroxide metabolism, since it was found in the 1960s [1] that this organelle contains a variety of hydrogen peroxide-producing oxidases and catalase. As we know now, peroxisomes are not just hydrogen peroxide-detoxification organelles. They also play essential roles in cellular metabolism, hosting a set of enzymes that varies depending on species, tissue, developmental state and/or nutritional status of the cells. A metabolic pathway common to all peroxisomes is the β-oxidation of fatty acids. In yeasts the entire breakdown of fatty acids takes place inside peroxisomes whereas in mammalian cells a second β-oxidation system is present in mitochondria. Furthermore, peroxisomes in mammalian cells harbour processes such as the detoxification of oxygen radicals and glyoxylate, and the synthesis of cholesterol, dolichol, etherphospholipids and bile acids. The α-oxidation of 3-methyl-branched fatty acids and the breakdown of polyamines, purines and some amino acids such as L-lysine also occur inside peroxisomes (reviewed in Refs [2, 3]). In yeasts and other fungi, peroxisomes can be involved in such diverse processes as methanol utilization and penicillin biosynthesis [4, 5]. Other examples of specialization that can be displayed by peroxisomes are provided by trypanosomatids and plants. In addition to more universal peroxisomal proteins, peroxisomes in trypanosomes contain a unique set of glycolytic enzymes that catalyze the conversion of glucose into 3-phosphoglycerate, hence the term "glycosome" (reviewed in Ref. [6]). In plants (and in many other organisms, but not in mammals), peroxisomes house the "glyoxylate cycle", a reaction sequence that converts two-carbon compounds into four-carbon units, allowing the organism to subsist on C2 compounds. For this reason plant peroxisomes have been called "glyoxysomes" (reviewed in Ref. [7]).

The importance of functional peroxisomes for cellular metabolism has been emphasized by the discovery of severe human genetic disorders that are caused by deficiencies in peroxisomal functions (reviewed in Ref. [8]). In the most severe forms of these disorders, the peroxisome biogenesis disorders (PBDs), peroxisomes fail to

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be formed normally and matrix enzymes are mislocalized to the cytosol, where most of them are rapidly degraded. Although studies of PBDs have greatly contributed to the current knowledge of peroxisomal functions, it was mainly the use of yeast genetics that resulted in the unravelling of the details of peroxisome biogenesis.

At present, 32 genes (*PEX* genes) have been identified that encode proteins (peroxins) required for the biogenesis of peroxisomes [9, 10]. One of the first *PEX* genes characterized was *PEX4* (also known as *PAS2*) [11], which codes for a protein (Pex4p) belonging to the E2 family of ubiquitin-conjugating enzymes that has been identified as Ubc10p [11]; (for a review on ubiquitination see Ref. [12]). In the yeast species *Pichia pastoris*, it was demonstrated that Pex4p conjugates with ubiquitin [13], while its conserved active site cysteine is essential for the function of the protein in peroxisome biogenesis [11, 13]. Following the identification in 1993 of Pex4p as a genuine ubiquitin-conjugating enzyme, it was suspected for a long time that ubiquitination played a role in peroxisome biogenesis. However, the substrate(s) of Pex4p remain largely unknown, and only since 2001 have a number of ubiquitinated peroxins been identified [14–17].

In this chapter we will first review the putative roles of the peroxins that, most likely, function directly in peroxisomal matrix protein import. Next, we will discuss the identification and characterization of the ubiquitinated peroxins, with emphasis on Pex5p, which has a central role in the import of proteins into peroxisomes. Finally, we will present a hypothetical model in which we summarize our ideas as to how Pex5p is ubiquitinated, what other peroxins may be involved and how ubiquitination may regulate Pex5p function.

1.2 Matrix Protein Import into Peroxisomes is Mediated by Cycling Receptors

Peroxisomal matrix proteins are nuclear encoded, synthesized on cytosolic polyribosomes and posttranslationally imported into peroxisomes (reviewed in Ref. [18]). The targeting of matrix proteins to peroxisomes requires one of two distinct peroxisomal targeting signals: type I (PTS1) or type II (PTS2). Most matrix proteins contain a PTS1, a tripeptide with the sequence serine-lysine-leucine or a derivative thereof, which is present at the extreme C-terminus of these proteins [19, 20]. Only a few matrix proteins contain a PTS2, which is located in the N-terminal region and has the consensus sequence (R/K)-(L/I/V)-X5-(H/Q)-(L/A/F) [21, 22]. The receptors for PTS1 and PTS2 proteins are encoded by the *PEX5* and *PEX7* genes, respectively [23, 24]. Pex5p interacts with PTS1-containing cargo proteins via six conserved tetratricopeptide repeat (TPR) motifs in its C-terminal half [25, 26]. In contrast, the N-terminal half of Pex5p is poorly conserved, with the exception of multiple pentapeptide motifs (WxxxF/Y) that are thought to function in membrane association [27–30]. In *Saccharomyces cerevisiae*, Pex7p requires either of two auxiliary proteins, Pex18p or Pex21p, for correct import of PTS2 proteins [31]. These Pex7p-assisting proteins bind the receptor, but not thiolase, the PTS2 cargo. It has

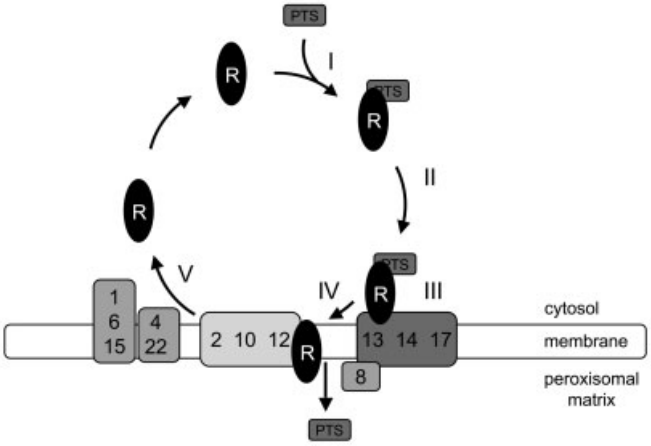

Fig. 1.1. Model for peroxisomal matrix protein import and receptor cycling. The following steps in the receptor cycle have been proposed: (I) binding of the receptor to matrix proteins in the cytosol. (II) Transport of the receptor–cargo complex to the peroxisomal membrane. (III) Docking of the receptor–cargo complex on the membrane. (IV) Dissociation of the receptor–cargo complex and translocation of cargo into the peroxisomal matrix. (V) Recycling of the receptor to the cytoplasm. R represents the (PTS1 or PTS2) receptor, and the numbers refer to specific peroxins. See text for details.

been shown that Pex18p also contains a WxxxF/Y motif and can functionally replace the Pex5p N-terminus. This suggests that Pex18p may facilitate membrane association of the PTS2 receptor, in analogy to the role of the Pex5p N-terminus in PTS1 import [32, 33].

Both Pex5p and Pex7p are predominantly cytosolic, partly membrane-associated proteins that cycle between cytosol and peroxisome (reviewed in Ref. [18]; Figure 1.1). The receptors bind cargo proteins in the cytosol, subsequently dock on the peroxisomal membrane and facilitate the dissociation and translocation of the cargo across the membrane in a hitherto unknown fashion. Recent evidence seems to extend the route followed by the receptors, suggesting that they enter, at least partly, the peroxisomal matrix, then release their cargo and subsequently recycle back to the cytoplasm to initiate another round of import [34–36]. In the peroxisomal membrane, a diverse group of twelve peroxins is present that plays an important role in matrix protein import and receptor cycling, as judged from the fact that deletion of any of the corresponding genes results in mislocalization of matrix proteins to the cytosol [18, 37]. Two large membrane protein complexes have been identified: (1) the docking complex formed by Pex13p, Pex14p and Pex17p, and (2) the RING complex consisting of the RING finger-containing integral membrane proteins Pex2p, Pex10p and Pex12p. The docking complex facilitates docking of the cargo-bound receptor, whereas the RING complex may mediate cargo translocation into the peroxisomal matrix or, as suggested recently, may facilitate export of the receptor from the matrix to the cytosolic face of the membrane [38–40]. In the latter model the docking complex has a dual function: it binds cargo-loaded recep-

tors and subsequently translocates them to the trans-side of the membrane. An important role in the organization and coordination of the import process has been ascribed to the intraperoxisomal peroxin Pex8p, which is able to assemble the docking and the RING complexes into a larger import complex, suggestively called the Importomer [38, 41].

The other peroxins on the membrane are the E2 enzyme Pex4p, which is anchored to the peroxisomal membrane by the integral membrane protein Pex22p [42], and the two interacting AAA (ATPases associated with various cellular activities) proteins Pex1p and Pex6p. Pex1p and Pex6p belong to the family of type II AAA proteins that are characterized by the presence of two ATPase domains, D1 and D2 [43, 44]. Each of these domains consists of a Walker A and Walker B motif, which bind and hydrolyze ATP, respectively. The basic activity of the AAA ATPases is thought to be protein unfolding or disassembly of protein complexes, an activity that may be employed in a broad range of cellular processes [45]. Pex1p and Pex6p form a complex that associates with the peroxisomal membrane via the interaction between Pex6p and the integral membrane protein Pex15p in *S. cerevisiae* (or Pex26p in mammals) [46–50].

So far, evidence for direct physical interaction between Pex1p, Pex6p, Pex15p, Pex4p, Pex22p and the docking and RING complexes is lacking. However, Pex4p was shown to be in close proximity to Pex10p, providing a link between the Pex4p/Pex22p complex and the RING finger complex [51]. Based on genetic studies, it has been suggested that Pex1p, Pex6p, Pex15p, Pex4p and Pex22p act at the final stages of peroxisomal matrix protein import, after receptor docking and translocation of cargo across the peroxisomal membrane, and most likely play a role in Pex5p recycling from the peroxisomal compartment to the cytosol [39, 41, 52]. For Pex4p, this is in line with the two following observations. First, in the absence of (functional) Pex4p, the amount of Pex5p associated with peroxisomes increases and PTS1 import is reduced in *S. cerevisiae* and *P. pastoris* [11, 52]. Second, overproduction of Pex5p partially suppresses the PTS1 protein import defect in *Hansenula polymorpha pex4*Δ cells [53]. The observation that recycling of Pex5p from the peroxisomal compartment to the cytosol requires ATP hydrolysis [54], supports the notion that Pex1p and Pex6p, the only peroxins that exhibit ATPase activity, play a role in Pex5p recycling as well. This has recently been substantiated by the demonstration in *S. cerevisiae* that these peroxins indeed mediate the ATP-dependent dislocation of Pex5p from the peroxisomal membrane to the cytosol [55]. The (possible) role played by the AAA proteins in this process will be discussed in more detail in Section 1.5.

1.3 Pex5p is Monoubiquitinated in Wild-type Cells, but Polyubiquitinated in Late-acting *pex* Mutants

The effect of ubiquitination on a protein substrate depends on the length of the appended ubiquitin chain. Monoubiquitination, that is the attachment of a single

ubiquitin molecule to a given lysine residue, is a nonproteolytic, reversible modification that controls cellular processes such as endocytic trafficking, DNA repair, virus budding and transcription [56, 57]. In contrast, polyubiquitin chains of at least four molecules linked through Lys 48 serve as a signal to target proteins for degradation by the proteasome [58]. Interestingly, *S. cerevisiae* Pex5p can either be monoubiquitinated or polyubiquitinated. Pex5p monoubiquitination seems to occur only in wild-type cells grown on fatty acids, conditions in which active peroxisomes are essential for survival, while polyubiquitination is found mainly in certain *pex* mutants or in cells cultivated in glucose (which represses peroxisome biogenesis) or grown under adverse conditions (see below).

Before discussing the implications of these findings, we will first summarize the experimental evidence that resulted in the identification of Pex5p as a substrate for ubiquitination. Pulse-chase experiments in oleate-grown yeast cells, in which peroxisome formation is induced, demonstrated that Pex5p is a stable, posttranslationally modified protein [16]. Immunoprecipitation analysis of cells overexpressing myc-tagged ubiquitin revealed that Pex5p is monoubiquitinated at two different lysine residues. In mutant strains defective in vacuolar or proteasomal degradation the level of monoubiquitinated Pex5p remains unaltered, ruling out that the monoubiquitinated Pex5p species represent a breakdown intermediate of either system. The subcellular site of Pex5p ubiquitination proved to be the peroxisomal membrane, since monoubiquitinated Pex5p localized almost entirely to the peroxisome-enriched pellet fraction in subcellular fractionation experiments. In addition, in *pex3*Δ cells that lack peroxisomal membranes, ubiquitination of Pex5p was blocked.

To address the question at which step of the import cycle Pex5p is ubiquitinated, a series of *pex* deletion strains was constructed in which components of the docking complex (Pex14p), the RING complex (Pex2p, Pex10p, Pex12p), or the intralumenal membrane-associated Pex8p were deleted one at a time. In all these strains, ubiquitination of Pex5p was strongly reduced. Importantly, deletion of peroxins not involved in the Pex5p receptor cycle had no effect on Pex5p ubiquitination. These results imply that Pex5p monoubiquitination requires a functional Importomer and, most likely, takes place late in the receptor cycle, after docking and import of PTS1 proteins. Given these observations, Kragt et al. [16] also investigated the role of the late-acting peroxins Pex1p, Pex6p, Pex15p, Pex4p and Pex22p in the ubiquitination process. Surprisingly, single deletion of each of these peroxins did not inhibit ubiquitination of Pex5p *per se*, but instead changed the pattern of ubiquitination. Two groups of mutants could be distinguished. In the first group, consisting of *pex4*Δ and *pex22*Δ, two ubiquitinated Pex5p species were found. The second group, comprising *pex1*Δ, *pex6*Δ and *pex15*Δ displayed three, and occasionally four, ubiquitinated Pex5p species, of which the smallest co-migrated on an SDS-gel with the largest of the first group. Together, the data from the deletion mutants corroborated the results reported by two other groups, who found similar patterns of Pex5p ubiquitination [15, 17]. In order to determine whether in these deletion mutants Pex5p was multiple monoubiquitinated or polyubiquitinated, mutant ubiquitin of which lysine 48 was replaced by arginine (Ub-K48R) was used [17]. Ub-K48R

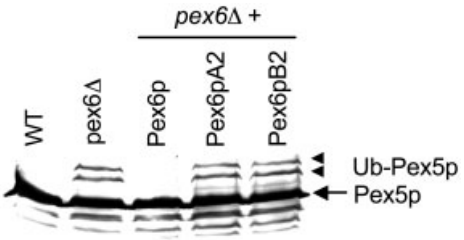

Fig. 1.2. Pex6p ATP-binding and -hydrolysis mutant cells accumulate (poly)ubiquitinated forms of Pex5p. TCA lysates of oleate-induced *pex6*Δ cells expressing wild-type Pex6p, or Pex6p point mutants were analyzed by anti-Pex5p immunoblotting. Lysates of untransformed wild-type and *pex6*Δ cells were analyzed as controls. Pex6pA2 and Pex6pB2 contain an inactivating point mutation in the second ATP-binding or -hydrolysis domain, respectively.

can still be conjugated to protein substrates, but cannot function as an acceptor for ubiquitin-chain elongation via lysine 48, the site normally used for polyubiquitination [59]. Overexpression of Ub-K48R in *pex1*Δ and *pex4*Δ cells resulted in a significant reduction of all but the smallest ubiquitinated Pex5p species, indicating that in the *pex* deletion strains these larger ubiquitinated Pex5p species represent polyubiquitinated forms. However, the ubiquitin chains that are added in these late-acting *pex* mutants are rather short, ranging from two in the *pex4*Δ and *pex22*Δ to maximally four molecules in the group comprised by *pex1*Δ, *pex6*Δ and *pex15*Δ.

The accumulation of polyubiquitinated forms of Pex5p in late-acting *pex* mutants may be caused either by the complete absence of a particular peroxin or by a deficiency in its activity. This was tested for the AAA ATPase Pex6p (Figure 1.2). Total cell lysates of *pex6*Δ cells expressing Pex6pA2 and Pex6pB2, which are mutated in the second ATP-binding and -hydrolysis domain, respectively, were analyzed for Pex5p ubiquitination. Figure 1.2, lane 2 shows the characteristic pattern of (poly)ubiquitinated forms of Pex5p that accumulate in *pex6*Δ cells (but not in wild-type cells, lane 1). A virtually identical pattern was found in *pex6*Δ cells expressing either Pex6pA2 or Pex6pB2. Similar results have been reported by Kiel et al. [15] for *pex1* deletion cells expressing Pex1pK744E, which harbours a mutation in the second ATP-binding domain, and for *pex4*Δ cells expressing a catalytically inactive variant of Pex4p (Pex4p-C115S). Thus, the formation of polyubiquitinated forms of Pex5p in *pex1*, *pex4* and *pex6* mutants is a direct consequence of the lack of ATPase activity of Pex1p or Pex6p, or ubiquitin-conjugating activity of Pex4p.

A rather puzzling observation was that in the strain deleted for the presumed ubiquitin-conjugating enzyme Pex4p, ubiquitination of Pex5p is not inhibited. However, it could be envisaged that in the absence of Pex4p another E2 enzyme might function as ubiquitin donor. To address this issue, several groups constructed double deletions of each of the non-essential, ubiquitin-specific *UBC* genes and *PEX4*, and analyzed the ubiquitination state of Pex5p in the mutant cells [15–17]. The experiments revealed that polyubiquitination of Pex5p in the *pex4* de-

letion strain depends on Ubc4p. Also in the *pex1* and *pex6* deletion strains, Pex5p polyubiquitination is mediated by Ubc4p.

Several lines of evidence suggest that Pex5p polyubiquitination in late-acting *pex* mutants also occurs at the peroxisomal membrane. First, polyubiquitinated forms of Pex5p are found exclusively in the organellar pellet in *pex1* and *pex4* deletion cells [15, 17]. Second, Pex5p polyubiquitination is blocked in cells in which the *pex1*, *pex4* or *pex6* null mutation was combined with a deletion in the gene encoding Pex3p, a protein required for the formation of peroxisomal membranes [15]. Finally, it was demonstrated that Pex5p polyubiquitination requires the function of a specific set of membrane-associated peroxins, which all act prior to receptor recycling [15, 17]. When the *pex1* or *pex4* null allele was combined with deletions in *PEX* genes required for receptor docking (*PEX13*, *PEX14*), or translocation (*PEX2*, *PEX8*, *PEX10*), Pex5p polyubiquitination was no longer observed. Together, these data suggest that the polyubiquitinated Pex5p species have actually followed most of the translocation route at the peroxisomal membrane, and get stuck at a stage where Pex5p is normally recycled to the cytosol. As will be discussed later (see Section 1.6) the reasons for the membrane accumulation of Pex5p may vary depending on the peroxin that is mutated.

Although both mono- and polyubiquitination of Pex5p take place at the peroxisomal membrane and seem to occur at a similar stage in the Pex5p receptor cycle, there is compelling evidence that Ubc4p only plays a role in Pex5p polyubiquitination. First and foremost, Kragt et al. [16] showed that deletion of *UBC4* does not affect the level of monoubiquitination of Pex5p in wild-type cells. Since Ubc1p, Ubc4p and Ubc5p are redundant E2 enzymes, a *ubc4*/*ubc1* double deletion strain was constructed, which also showed the wild-type pattern of Pex5p ubiquitination. In addition, several groups tested *ubc4* mutant strains for growth on oleate, which is a measure of the functionality of peroxisomal matrix protein import [15–17]. These experiments revealed no significant difference between wild-type, *ubc4* and *ubc4*/*ubc1* cells, indicating that Ubc4p and, thus, Ubc4p-dependent polyubiquitination of Pex5p, is not essential for the formation of functional peroxisomes. Slightly different results were reported by Platta et al. [17] for a *ubc4*/*ubc5* double mutant, which showed a small growth defect on oleate and a minor deficiency in PTS1 matrix protein import. However, since *ubc4*/*ubc5* double mutants are temperature-sensitive and grow very slowly on most culture media [60], it is very likely that the observed effects are consequences of the poor growth phenotype of *ubc4*/*ubc5* mutants in general and are not related to a specific role of either Ubc4p or Ubc5p in peroxisome biogenesis.

Although our pulse-chase experiments indicate that in wild-type cells Pex5p is a very stable protein and we never observed Pex5p polyubiquitination [16], Kiel and coworkers obtained indirect evidence that under certain conditions, a small fraction of Pex5p may be degraded by the proteasome [15]. These authors carried out a careful analysis of the steady-state levels of Pex5p in glucose-grown wild-type and *pex* mutant cells and found increased levels of Pex5p in *pex* mutants blocked in the early stages of PTS1 protein import. These observations suggest that in glucose-grown wild-type cells, Pex5p concentration is modulated, possibly by proteasomal

degradation. Pex5p degradation in glucose-grown cells, conditions in which protein import into peroxisomes and peroxisome biogenesis are repressed [61], may occur via a quality-control mechanism (see model below) that disposes of non-functional Pex5p, that is docked Pex5p without cargo and/or Pex5p stuck in the import pathway.

Additional evidence for proteasomal degradation of Pex5p comes from experiments with temperature-sensitive mutants blocked in proteasome function [15, 17], using either the *cim5–1* mutant carrying a mutation in the *CIM5* gene encoding a regulatory subunit of the 26S proteasome, or the *cim3–1* mutant, which carries a mutant allele of the gene encoding the proteasomal ATPase Rpt6p [62]. In both mutants, polyubiquitinated forms of Pex5p accumulated upon a shift to the non-permissive temperature, which appeared to be Pex10p-dependent, indicating that ubiquitination does indeed occur at the peroxisomal membrane [15, 17]. In the *cim3–1* mutant, Pex5p polyubiquitination was Ubc4p-dependent [15]. These data should be interpreted with caution, however. First, to elicit the phenotype, the mutant cells were incubated in either oleic acid or glucose medium at 37 °C, the non-permissive temperature, at which the mutants arrest the cell cycle [62] and are unable to grow. Neither paper mentions how long the mutant cells were incubated at the high temperature before samples were taken for analysis. It is possible, therefore, that the observations reported were made in non-dividing cells, in which proteolytic pathways have been initiated that only operate under these adverse conditions. Second, it is a generally accepted notion that heat stress, that is elevating the temperature from 30 °C to 37 °C, leads to the accumulation of damaged and aberrantly folded proteins that must be disposed of by the cell. The polyubiquitinated Pex5p species in heat-stressed mutant cells may represent misfolded Pex5p that is targeted for degradation. In line with this suggestion, we have found polyubiquitinated Pex5p species in heat-stressed wild-type cells (unpublished observations). However, we have never observed Pex5p polyubiquitination in proteasomal mutants that display their phenotype at a normal growth temperature ([16] and our unpublished results).

Taken together, the data suggest that Pex5p is a stable monoubiquitinated protein in wild-type cells that is modified at a late step of the receptor cycle. Although it is currently unclear which E2 enzyme is involved in Pex5p monoubiquitination in wild-type cells, Pex4p is the most likely candidate: Pex4p is associated with peroxisomes through its interaction with the peroxisomal membrane protein Pex22p and cells lacking Pex4p are deficient in PTS1 import into peroxisomes [11, 52, 53]. The fact that PTS2 import is also affected in *S. cerevisiae* and *P. pastoris pex4*Δ cells may even suggest that ubiquitination plays a role in both pathways. In line with this suggestion, Pex18p, a peroxin involved in the import of PTS2-containing proteins, was found to be ubiquitinated (but see below).

We would like to propose that Pex5p monoubiquitination plays a role in recycling the receptor from the peroxisome. In mutants blocked at a stage where Pex5p is normally recycled to the cytosol, that is *pex1*, *pex6*, *pex15*, *pex4*, *pex22*, the protein is polyubiquitinated in a Ubc4p-dependent manner, and most likely destined for degradation by the proteasome. Polyubiquitination and degradation

may also occur in wild-type cells when Pex5p function is not required, that is in glucose-grown cells to remove excess useless Pex5p, or under poor physiological conditions that may induce Pex5p misfolding (i.e. very low growth rates, heat-stressed cells). Such a mechanism may be required to retain a functional PTS1 import machinery.

1.4 Ubiquitination of Pex18p

The second putative substrate for Pex4p-mediated ubiquitination is Pex18p, the Pex7p auxiliary protein [31]. In wild-type *S. cerevisiae* cells, FLAG epitope-tagged Pex18p is modified by either one or two ubiquitin molecule(s) [14]. Since Pex18p is constitutively degraded in wild-type cells, but not in a *doa4* deletion mutant in which ubiquitin homeostasis is impaired, ubiquitination of Pex18p appears to function in turnover. Furthermore, Pex18p degradation depends on Ubc4p/Ubc5p, but does occur in a mutant lacking the Pep4p vacuolar protease, indicative of degradation by the proteasome but not the vacuole. The level of Pex18p increases in the absence of either a functional docking complex, the E2 enzyme Pex4p, or the AAA protein Pex1p, while *PEX18* mRNA levels or Pex18p synthesis rates are unaffected. On the basis of these results, the authors suggested that the rapid turnover of Pex18p is associated with its role in peroxisome biogenesis. Although there is no other published experimental evidence for a role of Pex4p in Pex18p ubiquitination, Lazarow [63] claimed to have preliminary data that Pex4p conjugates the second, but not the first ubiquitin onto Pex18p. In such a scenario, it could be envisaged that Ubc4p/Ubc5p are involved in conjugation of the first ubiquitin, providing an explanation for the co-dependence of Pex18p ubiquitination on both Ubc4p/Ubc5p and Pex4p [14]. Whether this is indeed the case and how the E2 enzymes act together to regulate Pex18p ubiquitination remains to be determined.

At first sight, the above results indicate that Pex18p and Pex5p ubiquitination in wild-type cells have different functions. However, Pex18p is functionally similar to the N-terminus of Pex5p, and the PTS1 and PTS2 import pathways use the same set of membrane-associated peroxins, making it unlikely that different mechanisms are employed in the two pathways.

1.5 Role for the RING Finger and AAA Peroxins in Pex5p Ubiquitination and Recycling

Recent biochemical and genetic data suggest that many of the membrane-associated peroxins function in ubiquitination and recycling of Pex5p [15–17, 52, 55]. As discussed above there is strong evidence that both mono- and polyubiquitination of Pex5p take place at the peroxisomal membrane. This implies that the E3 ligase(s) involved in this process is (are) either recruited to or present at the perox-

isomal membrane. There are three membrane-localized peroxins, Pex2p, Pex10p and Pex12p, that qualify as potential Pex5p-E3 ligases based on the following criteria. First, all three proteins seem to be required for Pex5p ubiquitination, either directly or indirectly [15–17]. Second, they all contain a RING finger domain, which is the hallmark of one of the two types of E3 ligase that have been identified [64–67]. In particular, the Pex10p RING finger domain has a high similarity to the RING finger domain of the human E3 ligase c-Cbl [68]. Third, Pex10p has been suggested to interact with Pex4p (the putative E2 enzyme, see above) [51]. Fourth, Pex10p and Pex12p physically interact with Pex5p [39, 69, 70]. Finally, the three proteins form a heteromeric membrane-bound complex [38]. Together, these observations make it tempting to speculate that the RING finger peroxins function as a multisubunit E3 ligase, although direct experimental evidence (e.g. from ligase activity assays) is lacking that the complex, or any of the individual peroxins, actually has E3 ligase activity.

The AAA proteins Pex1p and Pex6p are essential in peroxisomal matrix protein import. We and others have shown that ATP hydrolysis is crucial for proper functioning of both proteins, and that blocking their ATPase activity results in the accumulation of polyubiquitinated Pex5p [15, 46, 52, 54, 71] (and see Figure 1.2). In an elegant series of *in vitro* export experiments, Platta et al. [55] have recently shown that Pex1p and Pex6p are indeed essential for the release of Pex5p from the peroxisomal membrane, but the molecular mechanism of Pex5p recycling is still obscure and questions as to the (possible) involvement of Pex5p monoubiquitination remain unanswered. However, lessons can be learned from another AAA ATPase, Cdc48p, the closest type II AAA-relative of Pex1p and Pex6p [72, 73].

In the next couple of paragraphs, we will briefly review the proposed roles of Cdc48p in different cellular processes and point out the possible structural and functional similarities to Pex1p/Pex6p. This information will be used to construct a model for the role of Pex1p and Pex6p in Pex5p recycling. Cdc48p (in mammals also known as p97 or VCP (valocin-containing protein)) can function in different cellular processes depending on the cofactors it associates with [74]. When Cdc48p is complexed with the adaptor Shp1p (suppressor of high-copy phosphoprotein phosphatase 1; the mammalian homologue is p47), it is involved in membrane fusion. Combined with the heterodimeric cofactor Ufd1p/Npl4p, it mediates the retrotranslocation of misfolded proteins from the ER (also known as ERAD, ER-associated protein degradation), activation of the ER-bound transcription factor Spt23p and spindle disassembly. Of particular interest in this context is the proposed mechanism of action of Cdc48p in retrotranslocation. Ye and coworkers [75] recently identified a novel ER membrane protein with a predicted type I orientation (N_{lumen}–$C_{cytosol}$), which recruits the soluble Cdc48p ring-shaped hexameric complex and its associated cofactors to the ER membrane. On the ER membrane, the Cdc48p complex recognizes and binds the emerging retrotranslocation substrate, concomitant with the attachment of polyubiquitin chains to the substrate catalyzed by an ER-associated E3 ligase [76]. Next, the Cdc48p complex pulls the substrate out of the ER, moving it through the central pore, reminiscent of the mechanisms by which ring-shaped hexameric helicases move along single-

stranded nucleic acids and hexameric ATPase rings move polypeptides into the proteolytic chambers of the eukaryotic proteasome or the bacterial ClpP protein [77]. ATP hydrolysis has been suggested as the driving force for the movement of the ubiquitinated substrate into the cytosol. Recently, Jentsch and colleagues were able to dissect this process into smaller steps. Based on their results they propose a similar but slightly different model for the action of the Cdc48p complex [78], in which the Cdc48p complex first recognizes and binds mono- or diubiquitinated substrates and induces the dissociation of the substrate from its interacting partner protein. Subsequently, Cdc48p recruits the cofactor Ufd2p, which extends the ubiquitin chain on the substrate by a few ubiquitin moieties. Finally, the ubiquitinated protein is handed over to a second set of cofactors (Rad23p, Dsk2p) that escort it to the proteasome for degradation. Compelling evidence in favour of such a mechanism is provided by recent structural analysis of Cdc48p and Ufd1p, whose N-termini are similar in that they both adopt a so-called double-psi β barrel fold [79]. Importantly, this fold was identified as a ubiquitin-binding domain with two binding sites for mono- and polyubiquitin, respectively. This confirms and extends earlier work in which it was demonstrated that Cdc48p can bind ubiquitin directly with its N-domain, and that this interaction is more efficient in the presence of Ufd1p [80, 81].

The following observations, summarized in Table 1.1, suggest that the mechanism of action of Cdc48p in ERAD is similar to that of Pex1p and/or Pex6p in Pex5p recycling. First, there is a resemblance in membrane association of the three AAA proteins. Pex1p and Pex6p associate to the peroxisomal membrane via the interaction between Pex6p and the integral membrane protein Pex15p, whereas Cdc48p is recruited to the membrane through its interaction with the membrane anchoring protein VIMP [46, 75]. Although there is little similarity in primary sequence between Pex15p and VIMP they have a similar domain structure, consisting of a single transmembrane domain, a short lumenal segment and a larger cytosolic domain [47, 75]. Second, the N-terminal domain of Pex1p contains the double-psi barrel fold [82], while the N-terminus of Pex6p, although lacking the double-psi β barrel motif, appears to have other structural features in common with Cdc48p [83]. Indeed, the N-terminal domains of Cdc48p and Pex6p are required for association with a membrane anchoring protein, that is with VIMP and Pex15p, respectively [46, 75]. Third, both Cdc48p- and Pex1p/Pex6p-dependent

Table 1.1. Functional and structural similarities between Cdc48p and Pex1p/Pex6p.

Type II AAA proteins
Membrane-associated via proteins with similar domain structure
(Predicted) similar structural motifs in N-terminus
Involved in routing of ubiquitinated proteins (not formerly proven for Pex1p/Pex6p, but see Section 1.6)

pathways involve ubiquitinated proteins. The interaction between Cdc48p and ubiquitin is well documented (see above), but a direct interaction of Pex1p and Pex6p with ubiquitin has not yet been shown. However, efficient interaction of Pex1p/Pex6p with ubiquitin may depend on cofactors that have eluded detection so far.

1.6 Pex5p Monoubiquitination: A Role in Receptor Recycling

Based on data described so far and similarities between certain key peroxins and proteins involved in other cellular ubiquitination events (such as Pex1p/Pex6p and Cdc48p), we propose a hypothetical model for Pex5p functioning (Figure 1.3). The essence of the model is that in wild-type cells, Pex5p monoubiquitination functions as a signal for recycling. At a late stage of peroxisomal matrix protein import, that is after release of its cargo, Pex5p appears to be localized in or at

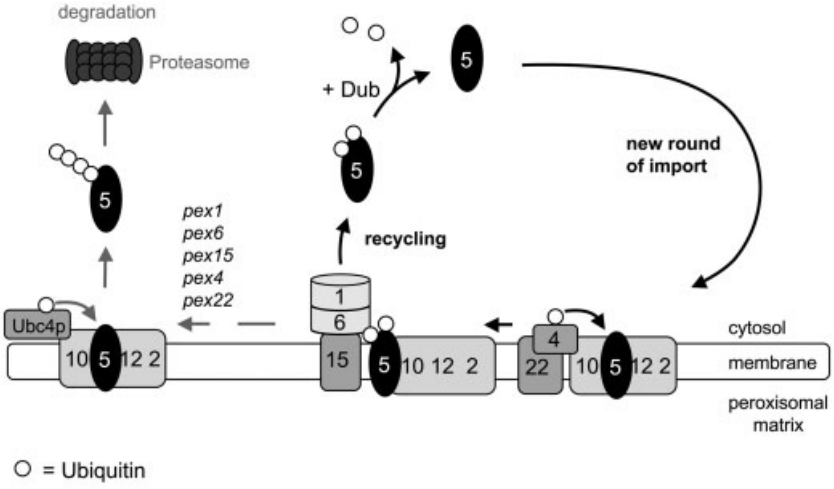

Fig. 1.3. Hypothetical model for Pex5p ubiquitination and ubiquitin-dependent recycling of Pex5p. After release of its cargo, Pex5p is present at the membrane tightly associated with the RING complex, consisting of Pex2p, Pex10p and Pex12p. Subsequently, Pex5p is monoubiquitinated at two different lysine residues by the E2 enzyme Pex4p, the RING complex supplying the E3 ligase activity. Next, monoubiquitinated Pex5p is recognized and bound by the AAA ATPases Pex1p and Pex6p, dissociated from the RING complex and recycled to the cytosol. Deubiquitination of Pex5p by one of the cytosolic deubiquitinating enzymes (Dubs) prepares Pex5p for a new round of matrix protein import. For clarity, Pex5p binding to PTS1 cargo and docking of the Pex5p–cargo complex has been omitted in the model (but see Figure 1.1). In the absence of functional Pex1p, Pex6p, Pex15p, Pex4p or Pex22p, or under poor physiological conditions, Pex5p gets stuck at the import site. This triggers Ubc4p-dependent polyubiquitination of Pex5p, possibly involving the same E3 ligase complex, resulting in targeted degradation by the proteosome. See text for further details.

the peroxisomal membrane, tightly associated with one or more other peroxins. Indeed, peroxisome-associated Pex5p behaves like a transmembrane protein [84]. Nevertheless, membrane-associated Pex5p is accessible for externally added proteases, suggesting that the protein does not completely enter the peroxisomal matrix during the import cycle, but remains associated with the membrane. At this stage, Pex5p is most likely bound to the RING finger complex. First, a RING finger complex function is required at a late stage of peroxisomal protein import (i.e. after the docking step) and, second, two of the RING finger complex subunits, Pex12p and Pex10p, directly interact with Pex5p [39, 69, 70]. We envisage that the interaction between Pex5p and the RING proteins prevents complete translocation of the receptor to the *trans*-side of the membrane. This notion is supported by the observation that in Pex10p- and Pex12p-deficient human fibroblasts, Pex5p is found inside peroxisomes [39, 40]. In the next step, the RING finger complex may recruit the E2 enzyme Pex4p, possibly mediated by the RING finger domain of Pex10p, to facilitate Pex5p monoubiquitination. In this scenario, Pex10p functions as an E3 ligase. Monoubiquitinated Pex5p is then recognized and bound by the AAA Pex1p/Pex6p complex, during which ATP is bound and hydrolyzed, inducing conformational changes that result in dissociation of Pex5p from the RING protein complex and its release into the cytosol. The released, monoubiquitinated Pex5p is subsequently deubiquitinated by one of the cytosolic deubiquitinating enzymes to prepare it for a new round of import.

When Pex5p recycling cannot occur, owing to a missing or defective component of the recycling machinery (i.e. Pex1p, Pex6p, Pex15p, Pex4p or Pex22p), or in strains cultivated under adverse physiological conditions, Pex5p gets stuck at the membrane and obstructs the PTS1 protein import pathway. Such a situation seems to trigger polyubiquitination of Pex5p in a Ubc4p-dependent manner, presumably also involving the RING finger complex as E3 ligase. The observation that membrane-associated polyubiquitinated Pex5p isolated from *pex1*Δ or *pex1*Δ/*pex6*Δ cells can still be released from the membrane by the AAA complex (Pex1p/Pex6p) *in vitro*, suggests that this form of Pex5p is both mono- and polyubiquitinated. This is in line with the proposed model in which Pex4p-dependent monoubiquitination of Pex5p precedes the recognition and dislocation by the AAA complex, and with the observation that the ubiquitinated Pex5p species in the *pex1*Δ, *pex6*Δ and *pex15*Δ mutants are larger than those found in *pex4*Δ and *pex22*Δ cells, and contain up to four ubiquitin moieties. Along the same lines, we hypothesize that the ubiquitinated Pex5p that accumulates in membranes of *pex4*Δ and *pex22*Δ cells cannot be dislocated by the AAA complex, either *in vivo* or *in vitro*, because it lacks monoubiquitin. Not withstanding these differences, in all the above mutants Pex5p is polyubiquitinated in an attempt to eliminate the import block by targeting Pex5p for degradation by the proteasome. Whether such an attempt succeeds appears to depend on the organism. In *S. cerevisiae*, Ubc4p-dependent ubiquitination of Pex5p does not lead to degradation [15–17]. This could be explained by the inefficiency of the Ubc4p-dependent machinery in *S. cerevisiae*, which adds relatively short ubiquitin chains to Pex5p in the *pex* mutants, whereas efficient degradation by the proteasome requires a chain length of at least four molecules.

Alternatively, membrane-localized polyubiquitinated Pex5p may not be easily accessible for the proteasome. In *Hansenula polymorpha*, on the other hand, there is strong evidence that the chain length of polyubiquitinated Pex5p is sufficient for degradation by the proteasome, since addition of a proteasome inhibitor to cells lacking Pex4p leads to a substantial increase of Pex5p levels [85]. Also *P. pastoris pex4*, *pex22*, *pex1* and *pex6* mutants, human *pex1* and *pex6* cell lines and *Arabidopsis thaliana pex6* cells, harbour severely reduced amounts of Pex5p, although it has not yet been determined whether this is the result of proteasomal degradation [40, 42, 52, 86]. Ubc4p-dependent polyubiquitination of Pex5p in *H. polymorpha* and *S. cerevisiae* appears to occur at equivalent, conserved lysine residues, *Hp* Pex5p lysine 21 [85] and *Sc* Pex5p lysine 18 (our unpublished results), respectively. This suggests that we are dealing with the same type of ubiquitination, in spite of the different outcome with respect to ubiquitin chain length and Pex5p stability. Mutation of the conserved Pex5p lysine does not affect the growth of cells on media that require functional peroxisomes in both yeasts ([85] and our unpublished observation), indicating that Ubc4p-dependent Pex5p polyubiquitination is not required for normal functioning of the receptor. We have found that a K-to-R mutation of residue 18 of *Sc* Pex5p did not affect monoubiquitination in wild-type cells (our unpublished data). Together, these results support the idea that Pex5p mono- and polyubiquitination target different lysines and, thus, may have different functions.

In conclusion, we would like to argue that the ability of the cell to switch between mono- and polyubiquitination of Pex5p might serve as a control mechanism. In this scenario, Pex5p monoubiquitination is required for receptor release from the membrane thereby maintaining functional cycling. However, once the Pex5p cycle is blocked at the membrane, the obstructing receptor must be removed from the translocation site. This is mediated by a switch from mono- to polyubiquitination, which targets Pex5p for proteasomal degradation if sufficient ubiquitin molecules are added. By a similar mechanism, yeast Cdc48p regulates the function of the ER-bound transcription factor Spt23p [87, 88]. Monoubiquitination of Spt23p activates the protein and moves it from the ER membrane to the nucleus. In contrast, Spt23p polyubiquitination inactivates the protein via proteasomal degradation.

1.7 Conclusions/Future Prospects

Thirteen years after the discovery that *PEX4*, one of the 32 genes essential for peroxisome formation, encodes a ubiquitin-conjugating enzyme, the first (putative) substrates of this E2 enzyme have been identified. As outlined in this chapter, the PTS1 receptor Pex5p is the most intensively studied potential substrate, and two different types of Pex5p ubiquitination have been found: mono- and polyubiquitination. Ironically, the best-characterized ubiquitination event, Pex5p polyubiquitination, is not mediated by Pex4p, but by the E2 enzyme Ubc4p. It is important to realize, however, that Pex5p polyubiquitination probably plays only a minor role in wild-type cells and is not essential for Pex5p functioning. Monoubiquitination, on

the other hand, is thought to be essential for receptor cycling and peroxisome biogenesis in wild-type cells, but many aspects of the mechanism remain unclear. For example, definitive evidence that Pex4p is the E2 enzyme is still missing. A similar situation exists for the E3 ligase(s), for which the RING finger proteins Pex2p, Pex10p and Pex12p are the most likely candidates, without a clear demonstration of E3 ligase activity for any of these proteins. *In vitro* ubiquitination experiments using purified proteins will be required to address these important issues. A crucial experiment will be the identification of the target lysine(s) of Pex5p monoubiquitination. Mutation of the residues involved and *in vivo* analysis of the mutant phenotype should provide further insight as to how monoubiquitination regulates Pex5p function.

Another important question is how Pex1p and Pex6p work together in Pex5p recycling. Pex1p and Pex6p have been shown to interact [89], which requires the first ATPase domain of both proteins and the second ATP-binding domain of Pex1p [71]. Whether Pex1p and Pex6p, like many other AAA ATPases, operate as a ring-shaped hexameric complex remains to be elucidated. For Pex6p, there are indications that the second ATPase and ATP-binding domains play a role in Pex5p recycling, since Birschmann et al. [46] showed that a mutation in either of these domains results in a larger fraction of organelle-bound Pex5p, while our own experiments indicate that such mutations result in the accumulation of polyubiquitinated Pex5p (Figure 1.2). Platta et al. [55] have convincingly demonstrated the importance of the second ATPase domain of Pex1p for Pex5p recycling, since mutations in this domain impaired Pex5p release from the membrane fraction in their *in vitro* export assay. Using cell fractionation and affinity chromatography, these authors also showed that Pex1p, Pex6p and Pex15p associate with Pex5p in a membrane-bound complex. However, with a two-hybrid-based experimental approach, several groups were unable to detect an interaction between Pex5p and the AAA-proteins. One possible reason for the latter result could be that the N-terminal domains of Pex1p and/or Pex6p might only interact with Pex5p via the attached ubiquitin, a mechanism that would be similar to that described for Cdc48p and its substrates. It is likely that soon after its recycling from the peroxisomal membrane monoubiquitinated Pex5p is deubiquitinated by one of the cytosolic deubiquitinating enzymes to prepare Pex5p for another round of import. Since the ubiquitin-specific protease Ubp3p has been reported to preferentially cleave ubiquitin from a conjugated protein rather than from polyubiquitin chains, Ubp3p might be a likely candidate for Pex5p deubiquitination [90].

Clearly, as indicated in the title of this chapter, ubiquitin is a new player in the peroxisome biogenesis field and many more new discoveries on its role in this process can be expected in the future.

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2
The Ubiquitin Proteasome System and Muscle Development

Johnny Kim and Thorsten Hoppe

2.1
Introduction

Muscle protein turnover has been a field of intense research for many years, which has led to the discovery of several factors involved in this process. Degradation of skeletal muscle proteins can occur through at least four different protein degradation mechanisms: through the lysosome [1], by calpain proteases [2], through the caspase or apoptotic protease system [3], [4] but most prominently through the ubiquitin proteasome system (UPS) [5]. The versatility of protein ubiquitination as a regulatory mechanism is underlined by the number of processes in which the UPS is involved in muscle tissue. Selective protein destruction is required to (1) ensure the development of muscle, (2) regulate the maintenance and remodelling of the sarcomere, the major component of the myofibrillar apparatus and (3) to mediate the destruction of the sarcomeric structure.

The UPS is intricately involved in dictating the delicate balance between intracellular signalling pathways that regulate muscle protein synthesis and breakdown. In this chapter we review the involvement of the UPS in muscle development and the implications of ubiquitin ligases that maintain and organize muscle filament structures. We then discuss recently identified UPS-associated factors that influence the homeostasis of muscle during physiological and pathophysiological conditions.

2.2
Muscle Histology

Muscle is categorized on the basis of its main functional property: the ability to contract. Three types of muscle tissue can be distinguished histologically: smooth muscle, skeletal muscle and cardiac muscle. The last two exhibit cross striations at the light microscope level and are thus both referred to as striated muscle. Owing to the evolutionarily conserved abundance of striated muscle among different species, this class has been the most extensively analyzed, and implications of the

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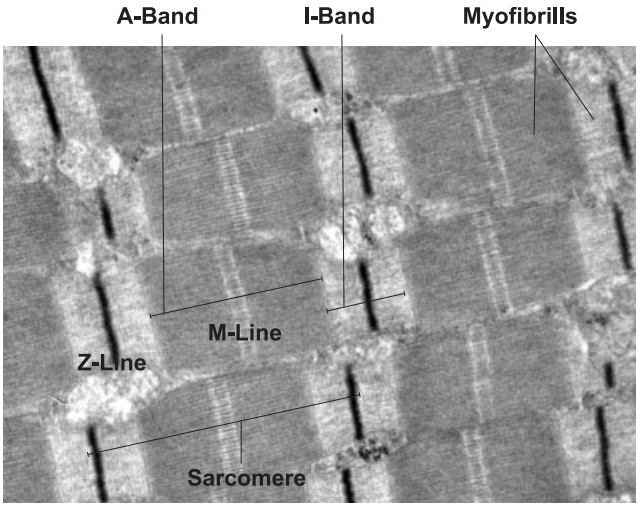

Fig. 2.1. Mouse skeletal muscle viewed by transmission electron microscopy. See text for details Magnification 12 000×. (Courtesy of Dr. Michaela Schweizer)

UPS in muscle physiology have been predominantly identified in this type. Thus, much of the information in this chapter focuses on striated muscle, in particular on skeletal muscle.

Skeletal muscle is arranged in parallel fibres, which show striations due to the arrangement of actin and myosin filaments within them. The fundamental repeat unit within muscle that is responsible for contraction is the sarcomere, which consists of a bundle of myosin-containing thick filaments flanked and interdigitated with bundles of actin-containing thin filaments (Figure 2.1). The striated appearance of muscle results from the alternation of thick-filament-containing A-Band and thin-filament-containing I-Band regions. The centre of each A-Band comprises a specialized region (M-line) which is thought to provide a link between the thick and the elastic filament systems. The centre of the I-Band consists of a specialized region called the Z-line (or Z-disc) and extends as a partition across the fibril. One sarcomeric unit is considered to be from Z-line to Z-line. In live animals, it is ultimately the sliding interaction of microscopic filaments that enables the muscle to contract.

Unlike smooth muscle, each muscle fibre remains the same width throughout its length. In contrast to cardiac muscle cells, which are mono- or binucleate, skeletal muscle fibres are multinucleate and the nuclei are located at the periphery of the fibres. Each individual muscle fibre is enveloped by a thin layer of connective tissue called the endomysium. Bundles of muscle fibres (fasiculi) are enveloped by a thicker layer of connective tissue called the perimysium, and the entire muscle is enveloped by the epimysium [6].

2.3 UPS and Developing Muscle

Most vertebrate skeletal muscles derive from a population of proliferative precursor cells called myoblasts, which themselves arise from the somitic mesoderm. During embryonic development, separate processes trigger myoblasts to exit proliferation allowing specification of mesodermal precursor cells to the myogenic lineage. Terminally differentiated myocytes finally fuse into mature, syncytial multinucleated myotubes [7, 8].

Commitment of muscle precursors to specify into fully developed myocytes depends on the muscle-specific transcriptional activator MyoD which belongs to the protein family of MRFs (muscle regulatory factors) [9]. MyoD was identified in a subtractive hybridization screen [10] and shortly after its discovery three other members of the MyoD family were identified: Myf5, Myogenin and MRF4 [11]. Astonishingly, ectopic expression of MyoD results in converting mouse 10T1/2 fibroblasts, and also a variety of other cell types, into myogenic cells capable of terminal muscle differentiation [10, 12–14]. Understandably, MyoD activity is precisely regulated at both the gene expression and the protein level in the process of myogenic differentiation, and disruption of this regulation at any time point can lead to developmental defects and disease. Although much is known about transcriptional regulation and protein activation of MyoD, recent data have identified the UPS to be the main pathway in regulating the stability and therefore the activity of MyoD protein throughout myogenesis.

2.3.1 Ubiquitin-dependent Degradation of MyoD

The first indications of ubiquitin-dependent regulation of MyoD derived from the observation that MyoD protein levels fluctuate dramatically along the course of the cell cycle in synchronized proliferating myoblasts [15]. Furthermore, in myoblast primary cell cultures from rat, Gardrat et al. reported an increase of unidentified ubiquitin conjugates during fusion of myotubes, which is followed by the upregulation of proteasomal subunits. In addition, proteasomal inhibitors like MG132 and PSI, antisense DNA targeted to three proteasomal subunits (iota, RC3α and RC7β) and blocking of E3 ubiquitin ligase activity with Leu–Ala dipeptides are able to prevent fusion of myocytes into myotubes [16].

Indeed, in the groups of Bengal and Ciechanover, MyoD was identified as a target for the UPS [17, 18]. In this context, it is noteworthy that MyoD was the first identified substrate for which the N-terminal methionine residue instead of an internal lysine can serve as the ubiquitin conjugation site to mediate proteasomal degradation. If all lysine residues in MyoD are converted to arginines, the N-terminal methionine of MyoD can be ubiquitinated via the C-terminal glycine of the first attached ubiquitin molecule followed by the subsequent synthesis of a Lys48-linked polyubiquitin chain [17]. N-terminal ubiquitination should not be confused with the N-end rule degradation pathway in which a target substrate re-

quires an N-terminal degradation signal (also referred to as an N-degron) and an internal lysine residue on which the ubiquitin chain can be synthesized (for a review see Ref. [19]). However, it was shown that MyoD can alternatively be ubiquitinated on internal lysine residues when the amino-terminal methionine has been blocked, for example by epitope tagging, methylation or carbamylation [17, 20, 21].

MyoD contains nine lysine residues as putative ubiquitination sites [22], and progressive replacement of the lysine residues by arginine has led to the identification of Lys133 as the specific ubiquitination site involved in the lysine-dependent degradation pathway of MyoD. Unexpectedly, stabilization of MyoD by mutating Lys133 does not promote myogenic differentiation [20]. However, Lys133 might additionally affect binding of MyoD interactors, since it is located in the bHLH (basic helix loop helix) domain, which is known to be necessary for the dimerization with MyoD [23]. These interactors include co-transactivating E-proteins which enhance muscle-specific gene transcription and Id proteins (inhibitor of DNA Binding) that antagonize the DNA-binding properties of MyoD [24–26]. Interestingly, Id and two splice variants of the E-protein E2A, E12 and E47, not only modulate DNA binding, cellular localization and turnover rate of MyoD but are themselves targets for the UPS, underlining the complexity of MyoD degradation [21, 27–32]. These findings lead to the speculation that dissociation of MyoD complexes may have to occur prior to ubiquitination.

But what could trigger dissociation of MyoD complexes, and thus ubiquitination and subsequent degradation? In addition to activating muscle-specific genes during proliferation, MyoD expression leads to cell cycle arrest, even in the absence of terminal myogenic differentiation [33]. It has been shown that overexpression of cyclin D1 results in an inhibition of MyoD-dependent transcription and a concomitant increase of a phosphorylated form of MyoD [34, 35]. This indicates that the activity of MyoD protein could be controlled by its direct phosphorylation through a cyclin-dependent kinase. Indeed, comparative peptide mapping and site-directed mutagenesis led to the observation that MyoD is phosphorylated on Ser200 by Cdk1 and Cdk2 both *in vitro* and in proliferating myoblasts. Prevention of phosphorylation at this site not only leads to the stabilization of MyoD protein but also to an enhancement of MyoD-dependent gene transactivation and myogenic conversion of 10T1/2 fibroblasts to muscle cells [22, 36]. It is thus attractive to speculate that phosphorylation of MyoD could be the initial step in promoting its degradation.

2.3.2 Degradation of MyoD by SCF^{MAFbx}

Degradation of phosphorylated MyoD was shown to depend on the 26S proteasome and on the ubiquitin-conjugating activity of the E2 enzyme Cdc34, which is known to associate with SCF complexes [22]. SCF complexes are conserved multisubunit ubiquitin ligases consisting of the invariable components Skp1, Cul1 and a variable component, known as an F-box protein, which is the main determinant of substrate specificity [37, 38]. Together with the cell cycle-dependent decrease of

MyoD protein before S phase [39], the described requirement of MyoD phosphorylation at G2/M phase transition [40] indicated that ubiquitin-mediated degradation would probably be mediated by an SCF complex. Tintignac et al. revealed that Atrogin-1/MAFbx, a muscle-specific F-box protein, interacts with MyoD, specifically in a highly conserved core region of its bHLH domain [41]. Moreover, they were able to show that Atrogin-1/MAFbx can indeed mediate MyoD ubiquitination together with Cdc34 and a recombinant SCF complex *in vitro*. Importantly, exchange of Lys133 to arginine suppresses ubiquitination of MyoD, which indicates that Lys133 is the specific target site for SCF^{MAFbx} ligase activity [41].

Posttranslational modifications of target proteins are often prerequisite for their recognition by the F-box protein component of certain SCF ubiquitin ligase complexes [38, 42–44]. F-box proteins frequently contain WD40 repeats or leucine-rich repeats, both of which have been found to bind phosphorylated substrates to the SCF complex [38]. However, phosphorylation of MyoD does not seem to be a requirement for Atrogin-1/MAFbx interaction since the non-phosphorylatable mutant $MyoD^{S5A/S200A}$ can still interact with Atrogin-1/MAFbx. SCF^{MAFbx} derived from skeletal muscle as well as recombinant SCF^{MAFbx} can mediate ubiquitin-dependent degradation of N-terminally tagged MyoD which is resistant to N-terminal ubiquitination [17, 41]. Taken together, these findings suggest that phosphorylation of MyoD is required for its N-terminal ubiquitination rather than for SCF^{MAFbx}-mediated ubiquitination at the internal Lys133.

The differential signals for N-terminal or Lys133 directed ubiquitination of MyoD could also depend on dynamic spatial and temporal localization because MyoD contains both an NLS (Nuclear Localization Sequence) and an NES (Nuclear Export Sequence) [45]. Several studies indicate that degradation of MyoD can be mediated in both compartments, in line with the fact that the UPS is present in the cytoplasm and nuclei of all eukaryotic cells [46]. Moreover, subcellular distribution of the proteasome appears to be regulated during myogenesis since the proteasome localizes to nuclei during myotube fusion but later co-localizes with actin fibres in the cytoplasm [47]. Indeed, MyoD is still degraded in HeLa cells even when inhibiting nuclear export with LeptomycinB [48]. Some observations indicate that both the N-terminal- and the Lys133-dependent ubiquitin-conjugation pathways are equally active in the nucleus whereas the latter seems to be more active in the cytoplasm. The sum of both degradation pathways appears to be overall more efficient in the nucleus than in the cytoplasm [21]. These findings corroborate the notion that different mechanisms exist to orchestrate the turnover of MyoD in a concerted dependence of phosphorylation state and subcellular localization.

It is important to consider that simultaneous disruption of the NLS and the NES of MyoD is not sufficient to abolish cytoplasmic MyoD, indicating that other factors are involved in regulating its subcellular localization [21]. Such a function has been attributed to the aforementioned E-proteins and Id proteins, which have also been implicated in determining the degradation rate of MyoD. For example, a study by Schwartz and co-workers showed that two splice variants of the E-protein E2A, E12 and E47, modulate the cellular distribution and half-life of MyoD. E12 and E47 can

shuttle MyoD to the nucleus and co-expression of E12 or E47 leads to the stabilization of MyoD and Id1 protein levels in HeLa cells [28]. In addition, it has been demonstrated that MyoD and Id1 co-localize within the nucleus in proliferating myoblasts. However, in mature myotubes, MyoD localizes to the nucleus and Id1 exclusively to the cytosol [27]. Owing to the evident necessity for precise spatial and temporal regulation of MyoD activity, it is not surprising that E12, E47, and Id themselves have been shown to be degraded by the UPS [28, 29].

The complex regulation of MyoD is summarized in a model illustrating the different pathways involved in MyoD degradation (Figure 2.2). In the initial phase of myogenesis, MyoD is synthesized in the cytoplasm and rapidly shuttled to the nucleus where it binds to target genes in collaboration with E-proteins. Phosphorylation of MyoD might be the initial step in promoting its N-terminal ubiquitination and hence its degradation through the 26S proteasome. However, the exact ubiquitin ligase machinery that is responsible for N-terminal ubiquitination of MyoD remains to be identified. Excess amounts of MyoD could be transported back to the cytosol via its NES or, alternatively, could be conjugated with ubiquitin at Lys133 via the E3 ligase SCF^{MAFbx} complex in both the cytosol and the nucleus. In this case, phosphorylation of MyoD is not necessary. That within the nucleus the two pathways have equivalent activities is supported by the similar half-lives of N-terminal-blocked MyoD and lysine-less MyoD [21].

2.3.3 Other Muscle Regulatory Factors

Of the different MRFs identified so far, MyoD is the best-described myogenic transcription factor subject to proteasomal degradation. It is reasonable to suggest that the UPS could represent the proteolytic machinery for all the MRFs to precisely balance their transcriptional function during the myogenic process. Indeed, Myogenin and Myf5 have been indicated to be targets for the UPS. However, enzymes that mediate their ubiquitination have not yet been identified. Interestingly, Myf5 contains a D-box motif, which is thought to be a hallmark of substrate degradation mediated by the multisubunit E3 ubiquitin ligase complex, APC (anaphase-promoting complex), and disruption of this motif impairs the degradation of Myf5 at M-Phase in U2OS cells [49]. However, Myf5 is still degraded in the presence of a dominant negative inhibitor of APC ligase activity, indicating that the D-box-like motif may participate in the recognition of Myf5 by a different ubiquitin ligase [49, 50]. All of the described MRFs are highly conserved at the protein level and each of them possesses an LXXLL motif, which has been shown to be the interaction site for the F-Box protein Atrogin-1/MAFbx [41]. Thus, it is tempting to speculate that Atrogin-1/MAFbx or putative homologues could also be involved in the recognition and degradation of other MRFs besides MyoD. However, despite the high homology between MyoD and Myf5, Atrogin-1/MAFbx only interacts with MyoD but not with Myf5 in co-immunoprecipitation assays [41]. It is known that variant residues in the LXXLL core motif could influence the affinity and selectivity for Atrogin-1/MAFbx binding [51]. Alternatively, we might speculate that other

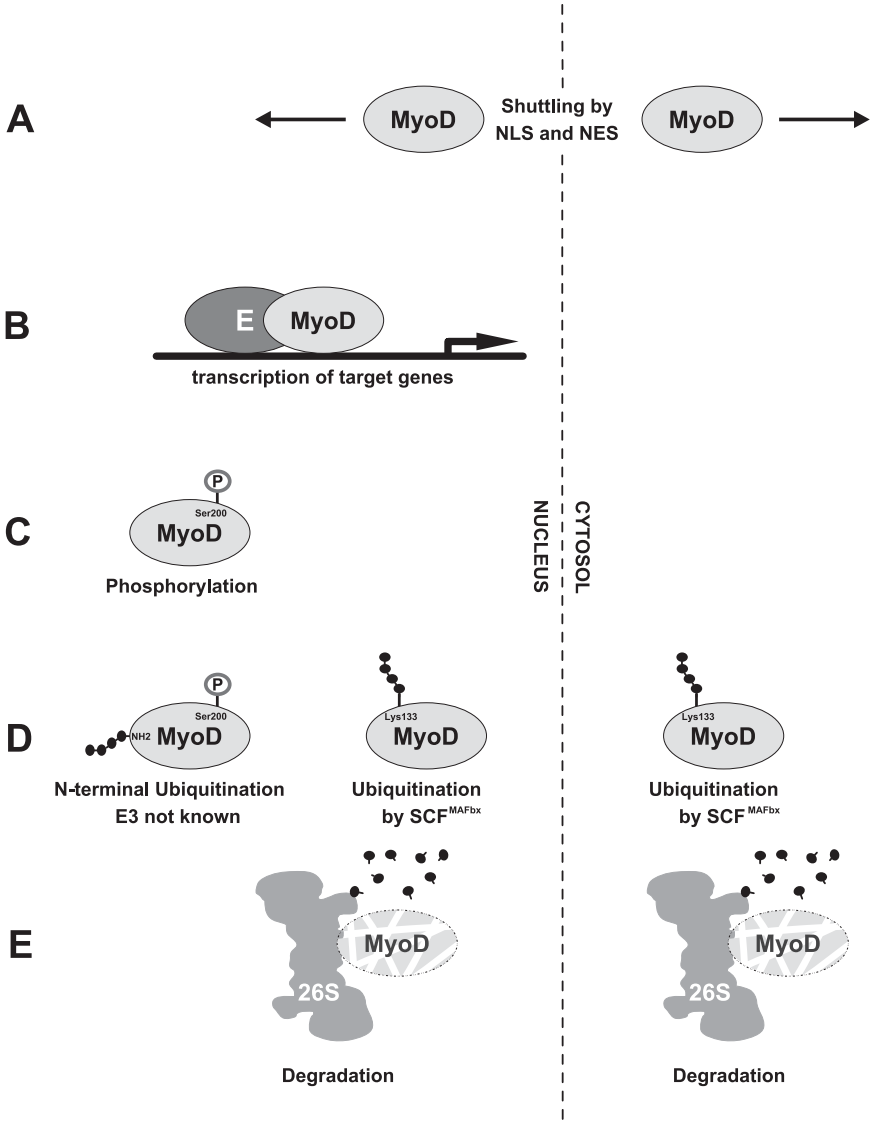

Fig. 2.2. Degradation pathways of MyoD. (A) MyoD is rapidly synthesized in the cytoplasm and can be transported to the nucleus via its intrinsic NLS and/or through the modulating activity of E-proteins and Id proteins. Excess amounts of MyoD protein are shuttled back to the cytosol via its NES. (B) Together with E-proteins, MyoD mediates the expression of target genes. (C) Phosphorylation of MyoD on Ser200 could be the initial signal for degradation. (D) In the nucleus, phosphorylated MyoD is N-terminally ubiquitinated. Alternatively, excess amounts of non-phosphorylated MyoD are ubiquitinated by the E3 ligase SCF^{MAFbx} complex on Lys133, in both the nucleus and the cytosol. (E) Both ubiquitinated forms of MyoD are degraded by the 26S proteasome with equivalent degradation rates in the nucleus. The excess MyoD ubiquitinated at Lys133 is alternatively degraded by the 26S proteasome in the cytosol.

unidentified co-activators also modulate the degradation process of the different MRFs.

2.4 UPS and Organizing Muscle

Amazingly, although the muscle cytoarchitecture must be maintained with almost crystalline order for its efficient contractile function, it is not a passive, static framework. Instead, the components are in a requisite dynamic equilibrium with constant coordinated alterations in protein synthesis, degradation, assembly and maintenance. An impressive example of this is human cardiac muscle, where the dynamic process of synthesizing and replacing contractile proteins occurs even while force production is maintained at rates of more than one hundred beats per minute [6]. It is clear that the muscle cytoskeleton is under tight regulation and again the UPS seems to be a likely candidate to achieve protein level homeostasis.

Indeed, several recently identified ligases have been reported as being implicated in regulating myofibril organization. One of these is Ozz-E3 which ubiquitinates membrane-bound β-catenin, whose turnover appears to be required for the alignment and growth of the sarcomere [52]. A similar sarcomere-assembly pathway is mediated by the E3 enzymes UFD-2 and CHN-1 in the nematode *Caenorhabditis elegans*. UFD-2 and CHN-1 dynamically regulate protein levels of the myosin-assembly chaperone UNC-45, assuring proper myosin assembly throughout the development of striated muscle [53].

2.4.1 Ozz-E3-dependent β-Catenin Regulation in the Muscle

β-catenin has two important functions in the cell: In the nucleus, β-catenin acts as a transcription factor in activating the Wnt signal transduction cascade, thereby controlling cell fate determination and cell proliferation [54]. At the plasma membrane, together with cadherins, β-catenin controls cell adhesion and tissue morphogenesis by mediating the physical anchorage of neighbouring cells [55].

In both of these compartments it has been shown that β-catenin protein levels are regulated through distinct mechanisms of ubiquitin-mediated proteasomal degradation. Cytosolic β-catenin degradation can occur through ubiquitination by the SCF-E3$^{\beta\text{-TrCP}}$ ubiquitin ligase [56, 57] or alternatively through the Ebi-E3 complex [58, 59]. In contrast, membrane-associated β-catenin is targeted for proteasomal degradation by the RING finger E3 ligase Hakai, which specifically binds to the phosphorylated intracellular domain of E-cadherin and hence promotes its ubiquitination and that of associated β-catenin [60].

In identifying Ozz-E3, Nastasi et al. [52] discovered an alternative pathway for proteasomal degradation of membrane-bound β-catenin in the developing muscle. Indeed, Ozz-E3 was shown to be a bona fide E3 ubiquitin ligase by exhibiting ubiquitination of β-catenin *in vitro* in collaboration with Cullin-5, Elongin B/C and

Rbx1, and ubiquitination of membrane-bound β-catenin *in vivo* in cultured myocytes. Additionally, Ozz-E3 activity is specifically involved in the alignment and growth of the sarcomere. Ozz-E3 is a muscle-specific protein belonging to the SOCS (suppressor of cytokine signalling) family of proteins, and its expression is induced during muscle differentiation. SOCS-box-containing proteins have been implicated in acting as a bridge between specific substrate-binding domains and SCF-like complexes, similar to F-box proteins [61]. Moreover, Ozz-E3 also contains two NHRs (neuralized homology repeats) that represent protein–protein interaction domains which were originally identified in the *Drosophila* RING-E3 ligase neuralized [52, 62–64].

Although Ozz-E3 is expressed in differentiating myoblasts, it seems that it is required for organized myofibril growth or maintenance but not for myogenesis since Ozz-E3$^{-/-}$ knockout mice do not show an obvious phenotype. However, there is severe disorganization and misalignment of sarcomeres, resulting in perturbation of the myofibrils and the striated pattern of muscle fibres [52]. Inhibition of Ozz-E3 leads to an accumulation of membrane-bound β-catenin causing myofibril abnormalities similar to those observed in Ozz-E3$^{-/-}$ myocytes. However, it cannot be excluded that this inhibition could be due to an accumulation of other substrates involved in myofibril organization. It is therefore reasonable to consider that additional proteins may be regulated by Ozz-E3 activity.

It has been postulated that the cadherin/β-catenin complex functions to restrain the Z-discs of sarcomeres to the sarcolemma and align sarcomeres within and between myofibres [52, 65]. The degradation of sarcolemmal β-catenin by the Ozz-E3 ligase during muscle cytoskeletal breakdown might lead to the disassembly of Z-line connections, thus destabilizing the terminal sarcomeres to permit assembly of new sarcomeric units. Hence, the regulation of sarcolemmal β-catenin protein levels is likely to be critical for the alignment, growth and organization of myofibrils.

2.4.2
Regulation of Myosin Assembly by CHN-1 and UFD-2

Identifying the regulation of membrane-bound β-catenin protein levels by Ozz-E3 has shed more light on the process of organizing the sarcomeric structure. However, the assembly of myosin into thick filaments during muscle development is still a largely unexplored phenomenon. Recent data suggest that the organization of myosin into sarcomeric structures is the result of a regulated multistep assembly pathway that requires additional factors. Candidates for this process are members of a protein family containing a UCS (UNC-45/CRO1/She4p) domain, which have been indicated to be necessary for proper myosin function [66, 67]. One founding member of this family is UNC-45, for which homologues have been identified in a variety of organisms, from yeast to humans. It was demonstrated that the UCS domain of UNC-45 interacts with muscle myosin and exerts chaperone activity onto the myosin head, whereas its N-terminal TPR domain (tetratricopeptide repeat) binds the general molecular chaperone Hsp90 [66]. Thus, UNC-45

functions both as a molecular chaperone and as an Hsp90 co-chaperone for myosin during muscle thick filament assembly. Consequently, mutations in *C. elegans unc-45* [68] result in paralyzed animals with severe myofibril disorganization in striated body-wall muscles [69].

Our work has revealed that protein levels of the myosin chaperone UNC-45 are subject to stringent regulation, which appears to be dependent on UFD-2 and CHN-1 ubiquitination activity [70]. UFD-2 is an orthologue of the yeast E4 enzyme UFD2 known to bind oligoubiquitinated substrates to catalyze the addition of further ubiquitin moieties in the presence of E1, E2 and E3 enzymes [71]. Thus, UFD2 defines a novel enzymatic activity that mediates multiubiquitin chain assembly, needed for subsequent proteasomal degradation, and was thus termed E4 enzyme [72]. The human CHN-1 orthologue CHIP was identified both as a co-chaperone of Hsc70 and Hsp90 and as an E3 enzyme. Thus, CHIP probably acts as a protein quality-control ubiquitin ligase, which selectively leads abnormal proteins recognized by molecular chaperones to degradation by the 26S proteasome [73, 74].

We were able to show that either UFD-2 or CHN-1 alone, in collaboration with E1 and E2, conjugates UNC-45 with one to three ubiquitin moieties [70]. Therefore, both CHN-1 and UFD-2 work independently as E3 enzymes in this pathway. However, in combination, CHN-1 and UFD-2 increase the ubiquitination of UNC-45. Movement defects of *unc-45* thermosensitive (*ts*) mutants are suppressed in animals lacking CHN-1 or UFD-2 most likely due to stabilization of the corresponding UNC-45 (*ts*) proteins. Interestingly, analysis of body-wall muscle cells by polarized light microscopy showed that the muscle structure of *chn-1* and *ufd-2* knockout worms is comparable to that of wild-type; however, overexpression of transgenic *unc-45* leads to strong sarcomeric assembly defects ([70]; PC Janiesch, J Kim and T Hoppe, unpublished data). Therefore, the amount of UNC-45 protein present in the muscle cells is critical for proper thick filament development.

CHN-1 and UFD-2 form a complex that apparently regulates UNC-45 protein levels and the assembly of myosin into striated muscles both *in vitro* and *in vivo*. Indeed, Northern blot analysis recently identified an upregulation of both *ufd-2* and *chn-1* transcripts during larval stages, in which body-wall muscle development mainly occurs (PC Janiesch, J Kim and T Hoppe, unpublished data). This indicates that the degradation of UNC-45 might be regulated *in vivo* by muscle-specific co-expression of both *ufd-2* and *chn-1* in a developmentally regulated manner. Conceptually, besides the regulation of sarcomere assembly, these findings support a new model in which two E3 enzymes, UFD-2 and CHN-1, team up to achieve E4 function.

Several lines of evidence support this speculation that a similarly conserved CHIP/Ufd2a/UNC45 mammalian complex may exist and mediate an equivalent ubiquitin-dependent regulation on processes that require myosin assembly. First, expression studies of human CHIP showed that it is highly expressed in adult human striated muscles as well as in a developmentally and spatially regulated manner in the mouse embryo, particularly during the course of cardiac and skeletal myogenesis [75]. However, the functional significance of the tissue-specific ex-

pression pattern of CHIP is presently unclear. Second, both human and mouse genomes contain two isoforms of UNC-45, which have separate, but possibly overlapping, functions in striated muscle differentiation [76]. Third, like CHIP, human and mouse UFD2 are highly expressed in skeletal muscle, and muscle atrophy leads to transcriptional upregulation of mouse Ufd2a [77–79]. Finally, both CHIP and two mouse homologues of UFD-2, Ufd2a and Ufd2b, collaborate with the same mammalian E2 enzymes Ubc4 and UbcH5c [80–82]. Thus, the regulatory role of the CHIP orthologue CHN-1 in *C. elegans* could indicate similar functions for CHIP in myosin assembly during the development of mammalian muscle.

2.5 UPS and Muscle Destruction or Degeneration

Maintaining the cytoskeletal architecture is necessary for muscle to perform its contractile function. Muscle tissue is particularly exposed to degeneration as a consequence of disuse, eccentric exercise, muscle injury or diseases affecting muscle either directly or indirectly, which in many cases result in atrophy.

Whereas hypertrophy is immediately associated with an increase in protein synthesis, atrophy occurs when protein synthesis rates are overtaken by an increase in muscle protein breakdown. UPS-mediated destruction of muscle proteins has been shown to occur via the N-end rule pathway. In addition, muscle wasting has recently been associated with the activation of an Akt-1-dependent transcriptional program, which in essence induces FOXO-mediated transcription of the muscle-specific ubiquitin ligase MuRF-1 and the aforementioned F-box protein, Atrogin-1/MAFbx. A considerable amount of progress has been made in elucidating the underlying mechanisms that induce the activation of these E3 enzymes.

2.5.1 N-end Rule and Muscle Atrophy

As early as 1986, Goldberg and co-workers provided direct evidence that the UPS is involved in muscle protein breakdown [83]. Later it was shown that starvation- and denervation-induced atrophy leads to an increase of UPS-associated mRNA levels including that of ubiquitin, several proteasomal subunits and, importantly, the E2 enzyme $E2_{14K}$/UBC2 [84, 85]. The findings that mRNA levels of the E3 enzyme E3α/UBR1 are upregulated and that proteasomal inhibition can abolish ATP-dependent proteolysis in atrophying muscle further substantiated the involvement of the UPS in muscle wasting, specifically via the N-end rule pathway [86, 87].

E3α, also known as Ubr1, belongs to the RING family of ubiquitin ligases and is broadly expressed, with highest levels in skeletal muscle and heart. Together with $E2_{14K}$, E3α is believed to recognize substrate proteins that begin with unblocked hydrophobic or basic amino acids at the N-terminus and to concomitantly mediate ubiquitination on an internal lysine residue. This process, known as the N-end rule pathway, has been defined as the relationship between the *in vivo* half-life of a

protein and the identity of its N-terminal residue. Proteins that are degraded via the N-end rule pathway depend on an N-degron which consists of a destabilizing N-terminal residue and an internal lysine residue [19]. Destabilizing N-terminal residues have been classified into groups: type I, which comprises basic amino acids, and type II, which comprises bulky, hydrophobic amino acids. E3α/UBR1 binds to both type I and type II N-terminal residues, although via different sites, and the binding can be selectively blocked by dipeptide inhibitors [19].

Indeed, specific blocking of E3α/UBR1 dramatically suppresses accelerated ubiquitin conjugation in atrophied muscle extracts derived from tumour-bearing and septic rats. This inhibition also suppresses ubiquitination in muscle extracts of healthy rats, indicating that the N-end rule pathway is also relevant in maintaining protein level homeostasis under physiological conditions [88]. Similarly, Goldberg and co-workers showed that blocking the N-end rule pathway by a dominant negative form of $E2_{14K}$ results in a reduction of ATP-dependent proteolysis in healthy muscle extracts by up to 50% [88]. Interestingly, Kwak et al. [89] found that a homologue of E3α, designated E3α-II, is dramatically upregulated during the progression of muscle atrophy in two different animal models of cancer cachexia. Consequently, activation of both E3α and E3α-II in skeletal muscle was accompanied by increased rates of N-end rule ubiquitination in atrophying muscle.

Clearly, the N-end rule pathway plays a decisive role in the breakdown of proteins during atrophy. However, $E2_{14K}$ and E3α inhibition does not fully abolish protein turnover, suggesting that other proteolytic mechanisms are involved in the atrophying process. The fact that the vast majority of proteins begin with stabilizing methionine or are acetylated *in vivo*, and that intact myofibrils cannot be degraded by the proteasome, supports this notion. Exactly how substrates could thus be accessible to N-end rule degradation via $E2_{14K}$ and E3α remains a major question. It has been suggested that cytosolic endopeptidases could initiate the proteolytic process by cleaving myofibrillar proteins, thereby generating free destabilizing N-terminal residues. For example, it has been shown that the major myofibrillar proteins actin and myosin are released from the sarcomere by a Ca^{2+}/calpain-dependent mechanism before they undergo ubiquitination and degradation [90–93].

Recent data suggest that caspases play an essential role in atrophy and also in a more general context, in the generation of free N-terminal residues and thus in the initiation of N-end rule-mediated degradation. In *Drosophila*, caspase-mediated cleavage of the stable apoptosis inhibitor protein DIAP1 results in the generation of a fragment with an N-terminal destabilizing asparagine residue, which subsequently triggers the recruitment of E3α/UBR1 and its associated E2 UbcD2 [94, 95]. Caspase-3 has been shown to be capable of cleaving myosin light chain [96], and more recently it was shown that caspase-3 can also cleave purified actomyosin complexes *in vitro* [97].

Taken together, these data suggest that both calpains and caspases could be the determining initiators in priming myofibrillar proteins for proteasomal degradation in the atrophying process. However, it remains unclear if these generated fragments are recognized by E3α/UBR1. Identification of the N-terminal residues

combined with *in vitro* ubiquitination assays of these fragments would certainly corroborate the involvement of the N-end rule pathway in muscle protein breakdown.

2.5.2 MuRFs, E3 Enzymes in Atrophying Muscles

As mentioned before, blocking the N-end rule pathway only suppresses ATP-dependent proteolysis in muscle by 50% suggesting that additional ubiquitin ligases are involved in muscle protein breakdown [88]. Indeed, in an attempt to identify potential markers of atrophy, Bodine et al. made use of a genomic differential display approach and reported that MuRF-1 is dramatically upregulated in atrophying muscles, owing to denervation, immobilization and hindlimb suspension; under atrophying conditions, MuRF-1 mRNA levels increase more than tenfold [98]. More recently, the substantial role of MuRF-1 in muscle atrophy has been underlined by the finding that MuRF-1 expression is upregulated in a diabetic atrophy model and also in space-flown rats subject to zero gravity [99, 100]. Most strikingly, MuRF-$1^{-/-}$ mice show a strong resistance towards the development of denervation-induced skeletal muscular atrophy compared to wild-type by 36% and this finding manifested a critical role of MuRF-1 in mediating the atrophic process [98].

MuRF-1 is a member of a gene family that includes the closely related members MuRF-2 and MuRF-3. All three are specifically expressed in cardiac and skeletal muscle [101]. Diverse functions have been ascribed to the different MuRFs, including the regulation of microtubule dynamics [102–104], regulation of gene expression [105, 106], myocardial contractility and also the regulation of the structural scaffold at the M-line of the sarcomere [103]. All three MuRFs contain an N-terminal RING finger domain, an adjacent zinc-binding domain and two coiled-coil domains in the C-terminal part, which are thought to mediate the homo- and heterodimerization between the different MuRFs [101]. *In vitro*, MuRF-1 demonstrated self-ubiquitination together with the E2 enzyme Ubc5c and this activity depends on the RING domain providing solid biochemical evidence for its functioning as a ubiquitin ligase [98].

Kedar et al. recently identified troponin I as the first bona fide ubiquitination target for MuRF-1 in cardiomyocytes [107]. However, this interaction has not been shown in skeletal muscle and, thus, it is not clear if this specific interaction could contribute to the development of muscle atrophy. New promising candidate targets for MuRF-1 have been suggested by Labeit and co-workers [108]. They performed a yeast two-hybrid screen with MuRF-1 as bait, and identified two distinct sets of muscle proteins that interact with MuRF-1. The two sets of proteins are either involved in ATP generation or they belong to a variety of myofibrillar proteins. These interactors include, for example, ATP synthase, creatine kinase, MLC (myosin light chain), Myotilin and the giant sarcomeric kinase Titin [108]. The finding that MuRF-1 locates to myofibrils and interacts with Titin, specifically in the M-line region, strongly suggests that MuRFs could target components of the myofibrillar apparatus for degradation in atrophying muscle [101, 103].

The interaction of MuRF-1 with Titin is of particular interest as it has been shown that Titin plays a role in the assembly of muscle thick filaments and in muscle elasticity by forming an elastic connection between one end of the thick filament and the Z-line [109]. It is attractive to suggest that MuRF ligase activity could be reciprocally regulated by the kinase function of Titin. However, phosphorylation of the MuRFs by the kinase domain of Titin and/or ubiquitination of Titin by MuRF-1 remains to be experimentally proven in future studies. Despite the finding that ubiquitin can be detected in the sarcomeric M-line region as well as at the periphery of the Z-line [107], exactly how Titin could be ubiquitinated is difficult to imagine since it is rigidly embedded in the sarcomere. Similarly to the notion that calpains and caspases could play an initiating role in N-end rule-mediated protein degradation, it has been suggested that MuRF-1-dependent ubiquitination could depend on a related proteolytic step upstream of MuRF-1 activity, for example by the activation of the site-specific endoproteases calpain-3 and caspase-3 [97, 108, 110]. Moreover, the existence of additional factors that mediate ubiquitin-dependent breakdown of muscle proteins is confirmed by the finding that the myofibrillar proteins Titin, nebulin and MLC-2 are still ubiquitinated in MuRF-1-deficient mice [108]. This could be due to functional redundancy between the different MuRFs or to the existence of other ubiquitin ligases that are involved in myofibrillar ubiquitination.

In addition to the interaction with myofibrillar proteins, MuRF-1 has been found to interact with the transcriptional regulator GMEB-1 (glucocorticoid modulatory element binding protein-1) [105]. It was shown that glucocorticoids can induce upregulation of MuRF-1 and concomitantly enhance muscle protein breakdown [98]. Consistent with its putative function in transcriptional control, MuRF-1 can be found in the nucleus of muscle cells [105]. It is attractive to speculate that MuRFs play a more general role upstream of an atrophy process and that they might directly govern the maintenance of muscle mass and energy homeostasis in response to environmental changes such as an increase of glucocorticoids.

Specific physiological targets of MuRF-1 during muscle wasting are yet to be identified and it is clear that more knowledge is needed about the interaction on MuRFs with their target substrates before their importance, not only in the atrophying process but also in physiological states, is fully understood.

2.5.3 Atrogin-1/MAFbx Function in Muscle Atrophy

Apart from MuRF-1, Glass and colleagues found another gene to be upregulated more than tenfold under muscle atrophying conditions, which they termed MAFbx (muscle atrophy F-box) [98]. In an independent study, Goldberg and co-workers identified the same gene, which they named Atrogin-1, by cDNA microarray technology comparing transcriptional profiles in atrophying muscles of fasted mice to littermate controls [111]. Consistent with the study of Bodine et al., they found that Atrogin-1 transcript is dramatically induced in atrophying muscle from fasted

mice [111, 112], rats with chronic renal failure, cancer cachexia and uncontrolled diabetes [99].

These initial studies showed that expression of Atrogin-1/MAFbx is specific to striated and cardiac muscle, with induction at least 12 h before significant muscle atrophy occurs [111]. As shown for MuRF-1, Atrogin-1/MAFbx expression is upregulated in muscles atrophying because of denervation, immobilization or hindlimb suspension. Strikingly, mice deficient for Atrogin-1/MAFbx are even more resistant to denervation atrophy than MuRF-1-deficient mice [98]. Taken together, these data strongly indicate a role for Atrogin-1/MAFbx in the early phases of the muscle atrophy process. Later it was shown that expression of Atrogin-1 is also significantly increased in atrophying muscle of septic rats [113] and Dehoux et al. reported upregulation of Atrogin-1/MAFbx during muscle wasting in their studies on rats under fasting and diabetic conditions [114].

Since the initial discovery of Atrogin-1/MAFbx, much research has focussed on finding specific substrates and on elucidating the exact mechanism by which Atrogin-1/MAFbx recognizes specific substrates. F-box proteins exert their function in recognizing specific substrates through protein–protein interaction domains to target them for ubiquitin-dependent degradation [37, 38]. Unlike many other F-box-containing proteins, Atrogin-1/MAFbx lacks typical domains, for example WD40 or leucine-rich repeats, that have been implicated as being important for the recognition of substrates [111]. This initially made it difficult to predict potential interactors. However, Atrogin-1/MAFbx does contain other known structural motifs that could help to elucidate its functional role in the muscle cell. The F-box domain of Atrogin-1/MAFbx is located in its C-terminal part and it is known that F-box proteins bind to Skp1, Cul1 and Roc1, which are all components of the SCF family of ubiquitin ligases [98, 111]. In its N-terminus, Atrogin-1/MAFbx contains a leucine zipper, a leucine-charged residue-rich domain (LCD) and possesses two potential nuclear localization sequences [21, 45]. This is consistent with the finding that Atrogin-1/MAFbx has also been shown to localize to the nuclei of muscle cells [115]. Additionally, Atrogin-1/MAFbx contains a cytochrome c family heme-binding site and a C-terminal PDZ-binding domain, which are both known to be necessary for protein–protein interactions [111, 116].

The presence of these various protein–protein interacting domains has led to the suggestion that Atrogin-1/MAFbx can potentially recognize different substrates and may play a role in the regulation of a variety of cellular processes. It has been suggested that Atrogin-1 targets key nuclear regulatory proteins such as transcription factors for degradation, which in turn might lead to a decrease in protein synthesis in atrophying muscle [115, 117, 118]. Alternatively, additional myofibrillar components might be directly subject to ubiquitin-mediated degradation similar to MuRF-1-dependent Titin ubiquitination (see above) [118]. Indeed, several binding partners for Atrogin-1/MAFbx have recently been identified. Tintignac et al. [41] demonstrated that Atrogin-1/MAFbx interacts with the myogenic transcription factor MyoD via the LCD. Together with a recombinant SCF complex, consisting of purified Skp1, Cul1 and Rbx1, Atrogin-1/MAFbx mediates MyoD ubiquitination

and hence targets MyoD for proteasome-dependent degradation (see above) [41]. So far, it is not clear whether the degradation of MyoD plays a role in the atrophy process. As mentioned above, the importance of MyoD degradation during myogenesis is evident. However, muscle atrophy occurs in fully differentiated adult muscle and animals lacking Atrogin-1/MAFbx develop normal muscle [98].

Since it was known that expression of Atrogin-1/MAFbx is not restricted to skeletal muscle but is also present in cardiac muscle, Patterson and co-workers screened a human heart cDNA library for potential interactors using a yeast two-hybrid approach [119]. They identified calcineurin A and the Z-disc component α-actinin as two novel interactors of Atrogin-1/MAFbx and affirmed the interaction with GST pulldown assays. They showed that Atrogin-1/MAFbx is able to ubiquitinate calcineurin A *in vitro* and to regulate endogenous protein levels of calcineurin A *in vivo*. Moreover, overexpression of Atrogin-1/MAFbx prevents calcineurin-mediated cardiac hypertrophy after aortic banding in transgenic mice, which indicates that Atrogin-1/MAFbx acts as a calcineurin signalling repressor [119]. It is not yet known if calcineurin A is a target of Atrogin-1/MAFbx in skeletal muscle. Li et al. showed that all three proteins Atrogin-1/MAFbx, α-Actinin and calcineurin A localize to the Z-disc of cultured cardiomyocytes [119]. In addition, calcineurin signalling pathways seem to affect muscle fibre type more than muscle fibre size [120]. Together, these data suggest that Atrogin-1/MAFbx is a key regulator in determining muscle mass of differentiated muscle of both striated muscle types.

2.5.4 Activation of Muscle-atrophy Pathways

The ubiquitin ligases E3α, MuRF-1 and Atrogin-l/MAFbx all play a substantial role in the breakdown of myofibrillar proteins at the endpoint of the muscle atrophy process. This gives rise to the suggestion that common signalling mechanisms exist to initiate or stimulate their expression. For example, hormones such as insulin, IGF-1, glucocorticoids and thyroid hormone are known to influence protein degradation [121, 122]. Additionally, rodents undergoing muscle atrophy due to fasting or induced diabetes show an increased expression of ubiquitin [123], $E2_{14k}$ [124], MuRF-1, Atrogin-1/MAFbx [99], UBR1, UBR2 and UBR3 [87, 125].

Indeed, activation of the IGF-1/PI3K/AKT-1 pathway has been identified as playing a key role in regulating the expression of both Atrogin-1/MAFbx and MuRF-1 via FOXO transcription factors during the progression of skeletal muscle atrophy [115, 117]. In essence, under conditions of muscle atrophy, it was shown that signalling through the IGF-1/PI3K/AKT-1 pathway is suppressed, which in turn maintains FOXO transcription factors in an active unphosphorylated state. FOXO factors are potent activators of Atrogin-1/MAFbx transcription by binding directly to the atrogin-1 promoter. Specifically, FOXO3 seems to be the key factor that regulates the expression of Atrogin-1/MAFbx [115]. The role of the IGF-1/PI3K/AKT-1 pathway in muscle protein breakdown in disease states is evident from the finding that insulin and IGF-1 signalling have also been shown to attenuate muscle wasting by inhibiting caspase-3 [97]. As mentioned before, it is thought that caspase-3

cleaves actomyosin to facilitate its destruction by the ubiquitin proteasome system via the N-end rule [4, 97].

Another major signalling system involved in the regulation of muscle mass includes the NFκB (nuclear factor κB) pathway. Cytokines such as TNFα can activate the NFκB transcription factor by stimulating IκB-kinase-β to phosphorylate IκBα, the inhibitor of NFκB. Phosphorylation of IκBα promotes ubiquitination and degradation of the inhibitor and hence activation of NFκB-mediated transcription [126, 127]. Mice overexpressing activated IκB-kinase-β in skeletal muscle show an increased expression of MuRF-1 and this results in severe muscle atrophy [128]. Consequently, overexpression of a nonactivatable form of IκBα prevents muscle wasting in these mice as well as in a mouse model of denervation atrophy [129]. Cai et al. [128] demonstrated that activation of the NFκB pathway results in significant atrophy by specifically inducing MuRF-1 but not Atrogin-1/MAFbx expression. This indicates that Atrogin-1/MAFbx upregulation is not required for NFκB-induced muscle loss. This finding provided the first functional dissection of MuRF-1 and Atrogin-1MAFbx signalling and it thus appears that FOXO-dependent expression of Atrogin-1/MAFbx is mediated via the IGF-1/PI3K/AKT-1 pathway, whereas MuRF expression is mediated by TNFα-mediated induction via the NFκB pathway.

Recently it was shown that the MAP kinase p38 can also trigger the upregulation of Atrogin-1/MAFbx in mouse C2C12 myotubes [130]. p38 has recently been identified as a potential regulator of muscle catabolism and its activity is increased in several models of catabolic myopathy [131–134]. TNFα acts via p38 to increase Atrogin-1/MAFbx gene expression in skeletal muscle since Atrogin-1/MAFbx upregulation upon TNFα exposure can be blocked by p38 inhibitors [130]. However, it is not clear if p38 has any effect on MuRF-1 gene expression or on the NFκB pathway.

2.6 Concluding Remarks

At first glance, it appears logical that upregulation of the proteasomal breakdown machinery in muscle will consequently result in enhanced breakdown of muscle proteins. It is becoming clear that the mechanisms that coordinate the activation of muscle protein synthesis are intricately balanced against a genetic program that is responsible for the degradation of muscle proteins, not only in disease states but also in conditions of health. Intriguingly, the UPS proves to play an active and substantial role in the initial development of muscle and the organization of myofibrillar proteins, as well as in their maintenance, remodelling and breakdown. Therefore, because the UPS is involved in different processes, it is obvious that disruption of the balance in the system could be detrimental and could be responsible for the pathogenesis of muscle disease. Loss of muscle mass accompanies, for example, cancer, sepsis, kidney disease, diabetes and heart failure. The many diseases associated with skeletal muscle mass, in addition to muscle injury

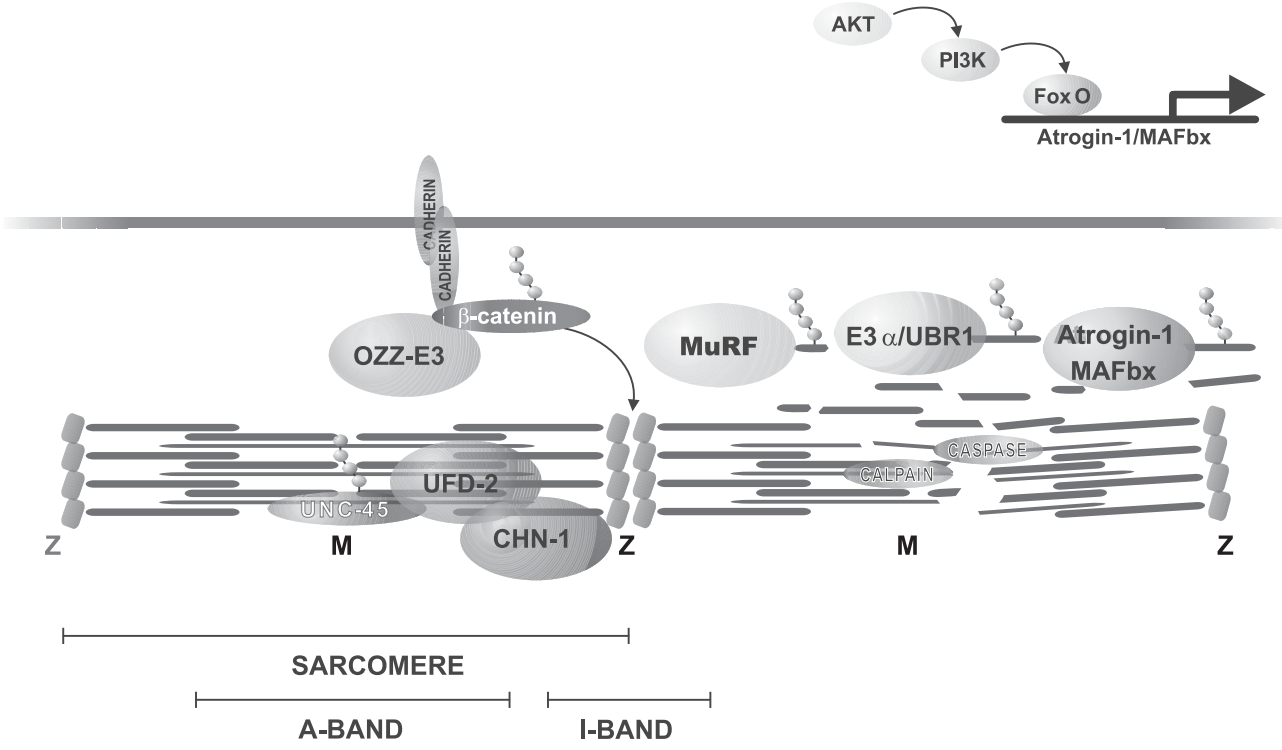

Fig. 2.3. Ubiquitin ligases are involved in the assembly, remodelling and breakdown of the sarcomere. The E3 ligases Ozz-E3 and the E3/E4 complex UFD-2/CHN-1 regulate sarcomere organization and myosin assembly by regulating protein levels of β-catenin or UNC-45, respectively (left). Caspases or calpains could initiate myofibrillar breakdown to provide subsequent access for E3 ubiquitin ligases. Akt signalling induces an atrophy programme resulting in the expression of the F-box component of the muscle specific E3–SCF ubiquitin ligase complex, Atrogin-1/MAFbx. Atrogin-1/MAFbx, MuRFs and E3α–UBR1 ubiquitinate muscle proteins, targeting them for terminal degradation by the 26S proteasome (right) (see text for details).

and normal aging processes, make it obvious that the identification of the key regulators in the different muscle degradation pathways will be a promising step in the discovery or development of therapeutic treatments.

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3
The COP9 Signalosome: Structural and Biochemical Conservation and Its Roles in the Regulation of Plant Development

Vicente Rubio and Xing Wang Deng

3.1
Introduction

The COP9 signalosome was first identified in plants as a multiprotein complex required for the repression in darkness of photomorphogenesis, or light-induced development of seedlings (Chamovitz et al. 1996; Wei and Deng 1996). Upon exposure to light, plants follow a de-etiolated or photomorphogenic developmental pattern characterized by a short hypocotyl (primary stem at the seedling stage), expanded cotyledons (first leaves) and increased chloroplast production, in contrast to the skotomorphogenic or etiolated phenotype of plants grown in darkness (Sullivan and Deng 2003). This distinct morphological pattern was used by different research groups to identify mutants with modifications in pathways controlling the light-triggered development of plants. Several mutant screens performed using *Arabidopsis* (*Arabidopsis thaliana*) as a plant model species led to the identification of a group of loss-of-function *cop* (*constitutive photomorphogenic*) and *det* (*detiolated*) mutants (reviewed in Wei and Deng 1996). These mutants share a constitutive photomorphogenic phenotype even when grown in darkness. It has been found that some of these mutants are allelic to a group of mutants characterized by accumulation of high levels of anthocyanins (purple pigments) and are therefore designated *fusca* (from the Latin word meaning "purple"). Cloning of the gene corresponding to the *cop9* mutant, followed by biochemical purification of a protein complex including the protein encoded by *COP9*, allowed the isolation of an eight-subunit complex initially named the COP9 complex, now known as the COP9 signalosome (abbreviated CSN) (Chamovitz et al. 1996). Identification of the genes responsible for several other *cop*/*det*/*fus* mutations and biochemical characterization of the proteins they encode revealed that five additional COP/DET/FUS proteins form part of the same complex. Each of the other two subunits of the CSN are encoded by two distinct genes present in the *Arabidopsis* genome and, therefore, were not identified in previous genetic screens (Kwok et al. 1998; Peng et al. 2001b). Conservation of the CSN has been reported in most eukaryotic model organisms, such as humans, mice, nematodes (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), fission yeast (*Schizosaccharomyces pombe*)

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and filamentous fungi (*Aspergillus nidulans*) (Busch et al. 2003; Freilich et al. 1999; Mundt et al. 1999; Seeger et al. 1998; Wei et al. 1998). In the case of budding yeast (*Saccharomyces cerevisiae*), the CSN does not appear to be fully conserved, but a CSN-related complex has been identified (Maytal-Kivity et al. 2002a; Wee et al. 2002). As the number of species known to posses the CSN has grown, recognition of the CSN's role in many biological processes has likewise increased. Today, the CSN's known functions span many different cellular processes, such as cell cycle progression, transcriptional regulation and signal transduction (reviewed in Schwechheimer 2004; Wei and Deng 2003).

In plants, it has been found that CSN function is not restricted to photomorphogenesis, but is also related to hormone signaling, disease resistance and floral development (Schwechheimer 2004; Serino and Deng 2003). The basis for such a diverse functionality stems from the CSN's range of biochemical activities. Remarkably, all of these activities involve components of the ubiquitin–26S proteasome pathway, the molecular machinery responsible for regulated proteolysis of substrates specifically tagged with polyubiquitin chains. The evidence for interactions between the CSN and the latter was strengthened by the finding that striking homology exists between all components of the CSN and the lid subcomplex of the 26S proteasome in a one-to-one relationship (Glickman et al. 1998). Moreover, multiple physical interactions were identified between subunits of the CSN and the 26S proteasome (Huang et al. 2005; Kwok et al. 1999; Peng et al. 2003). This evidence suggested that the CSN could interact with the proteasome to somehow replace the lid subcomplex in a manner that provides additional enzymatic activity to the proteasome and/or regulates its existing activities (Li et al. 2003). This hypothesis, known as the ''lid hypothesis'', though very interesting, still needs to be corroborated.

As we have mentioned, there is significant evidence to suggest that the CSN is involved in mechanisms controlling the regulated proteolysis of proteins through the ubiquitin–26S proteasome pathway in different organisms. This chapter discusses those involved in the regulation of plant development.

3.2 The Plant COP9 Signalosome

Inmunoaffinity and biochemical purification of the CSN complex from cauliflower (a species closely related to *Arabidopsis*) using a specific antibody to the COP9 protein allowed the identification of CSN core components and several other associated proteins (Chamovitz et al. 1996). Purified CSN contains eight core subunits named CSN1 to CSN8 according to protein size (Deng et al. 2000), though domain composition is another common way to distinguish them (Table 3.1). Six of the CSN subunits contain a signature domain known as PCI/PINT (Proteasome, COP9 signalosome, Initiation factor 3/Proteasome subunits, Int6/eIF3e, Nip1, Trip-15) in their C-terminal region (Aravind and Ponting 1998; Hofmann and Bucher 1998; Wei et al. 1998). The PCI/PINT is an alpha-helix-rich domain and

Table 3.1. *Arabidopsis* CSN subunit composition and identity with 19S lid and human CSN.

Subunit[a]	*Arabidopsis* locus[b]	MW (kDa)[c]	Motif	Identity with human CSN subunit (%)[d]	19S lid homologue[e]	Identity with lid subunit (%)[d]
CSN1	*COP11/FUS6*	50	PCI	44.7	Rpn7	22
CSN2	*COP12/FUS12*	51	PCI	61.1	Rpn6	21
CSN3	*COP13/FUS11*	47	PCI	42	Rpn3	20
CSN4	*COP8/FUS4*	45	PCI	49.5	Rpn5	19
CSN5	*CSN5a/AJH1* *CSN5b/AJH2*	40	MPN	62	Rpn11	28
CSN6	*CSN6a* *CSN6b*	35	MPN	39.8	Rpn8	22
CSN7	*COP15/FUS5*	25	PCI	34.4	Rpn9	15
CSN8	*COP9/FUS7/FUS8*	22	PCI	32.3	Rpn12	18

[a] *Arabidopsis* CSN subunits are named according to the unified nomenclature described in Deng et al., 2000.
[b] Correspondence between CSN subunits and *COP* and *FUS* genes is shown.
[c] Molecular weight of *Arabidopsis* CSN subunits.
[d] Percentages represent amino acid identity.
[e] Correspondence between CSN subunits and 19S lid components of *Arabidopsis*.

several studies have highlighted its importance in mediating interactions between the CSN subunits and in facilitating CSN complex assembly (Freilich et al. 1999; Hofmann and Bucher 1998; Tsuge et al. 2001). Cloning of several *COP* genes demonstrated that six of them each encode one of the PCI domain-containing CSN subunits (see Table 3.1 for correspondence between CSN subunits and *COP* genes). The other two subunits, CSN5 and CSN6, possess an MPN (MOV34, Pad1 N-terminal) domain at their N-terminus (Hofmann and Bucher 1998; Wei et al. 1998). In the *Arabidopsis* genome, these proteins are encoded by two genes each, explaining why they were not identified in previous genetic screens. In the case of CSN5, but not in CSN6, the MPN domain contains a metalloprotease motif, known as a JAMM or MPN+ motif, that seems to be responsible for the two major biochemical activities reported for the CSN complex: Nedd8 (or RUB1)-cullin deconjugating activity (deneddylation) and ubiquitin-substrate deconjugating activity (deubiquitination) (Cope et al. 2002; Groisman et al. 2003; Maytal-Kivity et al. 2002b). Although the catalytic center of these activities seems to be located in the MPN+ domain of CSN5, it appears that such activity still requires the rest of the CSN subunits, at least in the case of deneddylation, in which free CSN5 was shown to be inactive in cell-free assays (Cope et al. 2002). Point mutation analysis of the MPN+ motif in the *Arabidopsis CSN5A* gene has allowed the identification of three key metal-binding residues as well as two other amino acids outside the catalytic centre that play a critical role in CSN-mediated deneddylation activity in plants

(Gusmaroli et al. 2004). These results are in agreement with those previously obtained by point mutation analysis of *CSN5* in fission yeast (Cope et al. 2002).

A common feature of all plant CSN subunits is that a null mutation in any of their encoding genes leads to the death of seedlings at an early developmental stage. A null mutation in any CSN subunit is also accompanied by destabilization of the whole complex (Schwechheimer 2004; Serino and Deng 2003). However, recent data suggests that CSN subcomplex(es) might be formed in *Arabidopsis csn5a;csn5b* mutants. Nonetheless, this incomplete CSN complex is not functional and therefore *Arabidopsis csn5a;csn5b* plants exhibit few differences in phenotype compared with the rest of the *Arabidopsis* null mutants, presumably because of a general defect in CSN function (Dohmann et al. 2005). This feature highlights the importance of every subunit in maintaining the integrity of the plant CSN for proper functionality. CSN complex integrity is also essential for viability in other systems such as fruit fly and mice (Doronkin et al. 2003; Freilich et al. 1999; Lykke-Andersen et al. 2003; Oron et al. 2002; Yan et al. 2003). A high degree of conservation of the CSN during evolution possibly reflects the essential nature of the CSN function shared by different organism. Thus, more than 60% identity is maintained for some subunits, such as CSN2 and CSN5, between animal and plant homologues (Table 3.1). However, exceptions to the correlation between CSN conservation and the necessity for CSN functionality have been reported. For example, the absence of CSN3, CSN4 or CSN5 in fission yeast has little effect on cell viability and morphology. Only in the case of fission yeast do *csn1* and *csn2* mutants have defects in growth rate and cell shape, as well as in susceptibility to UV and gamma radiation (Mundt et al. 2002).

The generation of antibodies against all eight CSN subunits has helped to reveal the architecture, expression and localization of the CSN in *Arabidopsis*. In this matter, gel filtration studies and immunoblotting analysis using *Arabidopsis* protein extracts showed that the plant CSN complex elutes as a major peak in fractions corresponding to approximately 450 to 550 kDa (Wei et al. 1994). Similar studies also demonstrated the existence of smaller complexes containing only a subset of CSN subunits. A complex of about 250 to 300 kDa containing CSN4 and CSN7 and another of about 100 to 150 kDa containing CSN3 and CSN5 were detected in *Arabidopsis* (Gusmaroli et al. 2004; Karniol et al. 1999; Serino et al. 1999; Wang et al. 2002). Additionally, gel filtration profiles for CSN5 and CSN7 showed accumulation of their respective free forms (Karniol et al. 1999; Kwok et al. 1998; Wang et al. 2002). This situation is not specific to plants; the presence of free CSN subunits has been reported in fruit fly, fission yeast and mammalian cells (Mundt et al. 2002; Oron et al. 2002; Yang et al. 2002; Zhou et al. 2001). Until now, the function of such small complexes as well as free CSN5 and CSN7 remains an open question. Moreover, the precise composition of the small complexes and whether or not they interact with a set of proteins different from that of the CSN holo-complex remains unknown. Studies involving the subcellular localization of plant CSN8, a subunit that exclusively forms part of the CSN holo-complex, revealed that, as in other organisms, plant CSN localizes in the nucleus (Chamovitz et al. 1996). However, it has been reported that free CSN5 localizes in both the nucleus

and the cytosol of plant and mammalian cells and that, based on studies in mammalian cells, such distribution seems to be tightly regulated (Kwok et al. 1998; Tomoda et al. 2002). It has been shown that redistribution of CSN5 to the cytoplasm occurs as a result of contact inhibition and overexpression of growth receptor tyrosine kinase *Her2*/neu, a proto-oncogene related to human cancers (Caballero et al. 2002; Yang et al. 2000). Moreover, it is known that CSN5 contains a nucleus export signal (NES) in its C-terminal region (amino acids 233 to 242 and 237 to 240 in mammalian and plant CSN5, respectively). Point mutation analysis revealed that the integrity of the NES in CSN5 is essential for its activity involving p27, one of the substrates reported for mammalian CSN5 (Tomoda et al. 2002). Although there is growing evidence for the importance of controlling the subcellular localization of mammalian CSN5, little is known about the mechanisms operating in the case of its plant homologue and other smaller plant CSN complexes. Further studies here could shed light on a process that may represent a key step in the regulation of the function of independent CSN components by controlling their cellular compartmentalization.

3.3 CSN Involvement in the Ubiquitin–Proteasome Pathway

Among the earliest evidence pointing to a relationship between the plant CSN and regulated proteolysis mediated by the ubiquitin–26S proteasome pathway was the observation that reduction-of-function lines for subunits CSN1, CSN3, and CSN6 in *Arabidopsis* accumulated high levels of ubiquitinated proteins (Peng et al. 2001a; Peng et al. 2001b; Wang et al. 2002). Furthermore, impaired degradation of substrates targeted to the 26S proteasome was observed in plants partially deficient in CSN1 and CSN5 function. These lines showed accumulation of HY5 and PSIAA6, respectively, where HY5 is a positive regulator of photomorphogenesis and PSIAA6 is a repressor of responses to the plant hormone auxin (Schwechheimer et al. 2001; Wang et al. 2002). In addition, gel filtration studies using *Arabidopsis* extracts showed co-fractionation of CSN1 and CSN6 with Rpn6 and Rpt5, components of the lid and the base subcomplexes of the 26S proteasome, respectively, indicating molecular association of the CSN with the latter (Peng et al. 2003). The same study demonstrated, using co-inmunoprecipitation assays, that physical interaction *in vivo* occurs between components of these two complexes and members of the SCF (Skp1, Cullin/Cdc53, F-box) complex, which belongs to the Ring finger class of E3 ubiquitin–protein ligases (E3 ligases) (Deshaies 1999). These results suggest that plant cells contain a conglomerate consisting of at least these three different complexes involved in the ubiquitination and targeted degradation of a given substrate without releasing any intermediate once the substrate has been recruited (Peng et al. 2003). It has also been reported that plant CSN co-purifies with components of eIF3 (eukaryotic translation Initiation Factor 3), the multiprotein complex involved in the loading and subsequent scanning of the 40S ribosomal subunit on the 5′ leader of mRNAs during translation (Karniol et al.

1998). Further physical interaction between CSN, the 26S proteasome, and eIF3 components from plants has been obtained through yeast two-hybrid assays. Kwok et al. (1999) reported the interaction of *Arabidopsis* CSN1 with Rpn6. Using a similar approach, it has been shown that *Arabidopsis* CSN1 and CSN8 bind eIF3c/Nip1 and that *Arabidopsis* CSN7 interacts with eIF3e/Int6 (Karniol et al. 1998; Yahalom et al. 2001). These results are in agreement with those obtained in other organisms (reviewed in Kim et al. 2001). For example, eIF3e/Int6 has been found to strongly interact in yeast two-hybrid assays with mammalian CSN components, such as CSN3, CSN6, and CSN7, and with the 26S proteasome subunit Rpt4 (Hoareau Alves et al. 2002). Association of eIF3 with the 26S proteasome has also been reported in fission yeast, where direct interaction of eIF3i/Sum1 and eIF3e/Int6 with the 26S proteasome subunits occurs (Dunand-Sauthier et al. 2002; Yen et al. 2003). More recently, data on *in vivo* interaction between CSN and 26S proteasome components have been obtained from immunoprecipitation studies using mouse fibroblast cells (Huang et al. 2005).

It is noteworthy that, similar to the CSN, several components of the eIF3 complex and all subunits of the 26S proteasome lid subcomplex contain either a PCI/PINT or an MPN domain. Thus, out of the eleven subunits constituting the eIF3 complex in plants and mammals, three contain a PCI/PINT domain and two have an MPN domain (Hofmann and Bucher 1998; Kim et al. 2001). In the case of the lid subcomplex, the number of PCI/PINT- and MPN-containing subunits is the same as in the CSN, i.e. six and two, respectively, out of a total of eight subunits (Glickman et al. 1998). Furthermore, this similarity extends to the point that the lid subcomplex and the CSN components are paralogous to each other in a one-to-one relationship (see Table 3.1) and that the pattern of interaction among paralogous subunits is similar, suggesting that both complexes have similar architecture (Fu et al. 2001; Wei et al. 1998). Strikingly, both the CSN and the lid subcomplex share common structural features. They lack symmetry in subunit arrangement and both have a central groove, possibly suitable for scaffolding functions, according to structural studies using electronic microscopy (Kapelari et al. 2000). The lid and the base subcomplexes constitute the 19S regulatory particle (RP) located at either end of the 20S core proteolytic particle (CP) of the 26S proteasome. The 19S RP lid recognizes ubiquitinated proteins targeted to the 26S proteasome, whereas the base is in charge of the unfolding and funnelling of substrates into the 20S CP, which is responsible for their degradation. In order to recycle the ubiquitin moieties, the 19S RP lid also possesses a deubiquitinating activity to remove polyubiquitin chains from proteasome substrates (Verma et al. 2002). It has been reported that fission yeast and mammalian CSN also possess deubiquitinating activity (Groisman et al. 2003; Zhou et al. 2003).

The growing evidence of similarities between the CSN and the 19S RP lid suggests that the former could act as an alternative lid for the 26S proteasome. In this scenario, the CSN could replace the lid at one or both ends to form a new CSN–proteasome complex with functions distinct from those of each separated complex. On the other hand, the CSN could simply interact with the whole 26S proteasome as a means of regulating proteasome activities or conferring new functionality on

the combined complex (Li et al. 2003). A recent report on this matter supports the first of these hypotheses, showing evidence that the CSN competes with the 19S RP lid for 26S proteasome binding (Huang et al. 2005). An added complication, however, is the need to account for the aforementioned relationships between the CSN and the 26S proteasome with the eIF3 complex. Remarkably, many of the interactions between these three complexes involve the promiscuous PCI/PINT-containing eIF3e/Int6 protein. In addition to its role in mRNA translation, fission yeast eIF3e/Int6 seems to be responsible for shuttling the 26S proteasome subunit Rpn5 from the cytoplasm to the nucleus, where it is required for protein degradation. However, the relationship between eIF3e/Int6 and the CSN is poorly understood. It has been proposed that eIF3e/Int6 began as an accessory protein to the CSN, where it may have been required for the regulation of E3 ligases, and was later adopted by other protein complexes (von Arnim and Chamovitz 2003).

E3 ligases constitute the last step in the enzymatic cascade that attaches polyubiquitin chains to protein targets. This cascade begins by transference of a ubiquitin moiety from an E1 ubiquititin-activating enzyme to an E2 ubiquitin-conjugating enzyme. E3 ligases are responsible for bringing together the E2 ubiquitin-conjugating enzyme and the protein target that is then ubiquitinated at a lysine residue. Consecutive cycles of ubiquitination result in polyubiquitination and subsequent recognition by the 26S proteasome for degradation (Hershko and Ciechanover 1998). Accordingly, E3 ligases provide substrate specificity in the ubiquitination enzymatic cascade. Since many of the E3 ligases have been shown to be key regulators in different biological processes, from cell cycle progression to signal transduction and transcriptional regulation, malfunction of a particular E3 ligase generally results in alteration of several cellular processes, and causes severe defects in the development of plants and other organisms.

E3 ligases can be classified into two major groups: The HECT (Homologous to E6-AP carboxyl terminus) class and the Ring finger class (Deshaies 1999). So far, CSN has been involved in the regulation of the activity of two different E3 ligase types belonging to the Ring finger class, the SCF complexes and COP1. The latter is a repressor of photomorphogenesis, which controls the abundance of light-response activators in plants (Deng et al. 1992; Osterlund et al. 2000). As mentioned before, SCF complex subunits have been found to interact with CSN components directly. The SCF complex is composed of four subunits: a Cullin 1/Cdc53 protein that, together with Rbx1, constitutes the core of the complex, a protein adaptor Skp1 (S-phase kinase-associated protein 1) and an F-box protein that brings the substrate to the complex (Deshaies 1999). Among the SCF subunits from both plant and mammalian cells, direct interaction of Rbx1 with CSN6 and of Cullin 1 with CSN2 has been shown (Lyapina et al. 2001; Schwechheimer et al. 2001). In the case of COP1, although direct interaction with the CSN has not yet been reported, genetic evidence from *Arabidopsis cop1* and *csn* mutants strongly suggests that proper functionality of COP1 or CSN is required for each other's function in controlling photomorphogenesis (Holm et al. 2002).

As we will describe in the following sections, CSN operates through different mechanisms to control SCF and COP1 activities in plants. In the case of SCF,

CSN primarily mediates deconjugation of the ubiquitin-like protein Nedd8/RUB1 from cullins, whereas in the case of COP1, CSN is necessary for its light-dependent nucleocytoplasmic distribution (Chamovitz et al. 1996; Cope et al. 2002; Lyapina et al. 2001; Schwechheimer et al. 2001). The latter also requires the presence of COP10, an E2 ubiquitin-conjugating enzyme variant, and DET1, a repressor of photomorphogenesis that has recently been implicated in regulation of gene expression via chromatin remodelling (Benvenuto et al. 2002; Schroeder et al. 2002; Suzuki et al. 2002). A recent study on the characterization of COP10 showed that it belongs to a complex (named CDD) that also contains DET1 and DDB1 (UV-damaged DNA binding protein 1). The same study reported *in vivo* interaction of COP10 with several CSN and 26S proteasome components, with COP1 and with a UbcH5-related E2 conjugating enzyme (Yanagawa et al. 2004).

Looking at the complex map of interactions of the CSN, we can depict the CSN as a scaffold for many other protein complexes involved in ubiquitination and degradation of specific substrates (Figure 3.1). Close proximity of distinct components of the ubiquitin–26S proteasome pathway, possibly forming a supercomplex, would allow high efficiency and rapidity in processing many different substrates. The idea that such a supercomplex, responsible for localized and regulated proteolysis, may exist has been previously suggested by studies reporting interaction of the CSN, the 26S proteasome and SCF, and the presence of nuclear foci (or speckles) associated with COP1 (Huang et al. 2005; Peng et al. 2003; Wang et al. 2001).

3.4 Plant CSN Biochemical Activities

Since the late 1990s, several biochemical activities have been assigned to the CSN in different systems. The list includes two different isopeptidase activities: Nedd8/RUB1-cullin deconjugating activity (deneddylation) and ubiquitin-substrate deconjugating activity (deubiquitination), as well as phosphorylation of protein substrates and control of nucleocytoplasmic partitioning of protein targets (reviewed in Schwechheimer 2004; Serino and Deng 2003; Wei and Deng 2003). In most cases, the CSN employs more than one of these activities to control a particular biological process, e.g. control of cell cycle progression, where deneddylation and nucleocytoplasmic partitioning play pivotal roles (Tomoda et al. 1999; Yang et al. 2002). For some of these activities, the CSN subunit responsible has been identified. This is the case for deneddylation, in which the catalytic center has been located at the MPN+ domain in CSN5 (Cope et al. 2002; Gusmaroli et al. 2004). The latter is also responsible for one of the deubiquitinating activities associated with the CSN in mammalian cells, the cleavage of polyubiquitin chains from substrates. However, CSN5 does not mediate the CSN-associated depolymerization of polyubiquitin chains (Groisman et al. 2003). Nevertheless, the specific catalytic subunit(s) responsible for other CSN activities, such as the regulation of nucleocytoplasmic partitioning of plant COP1, has not been determined (von Arnim and Deng 1994).

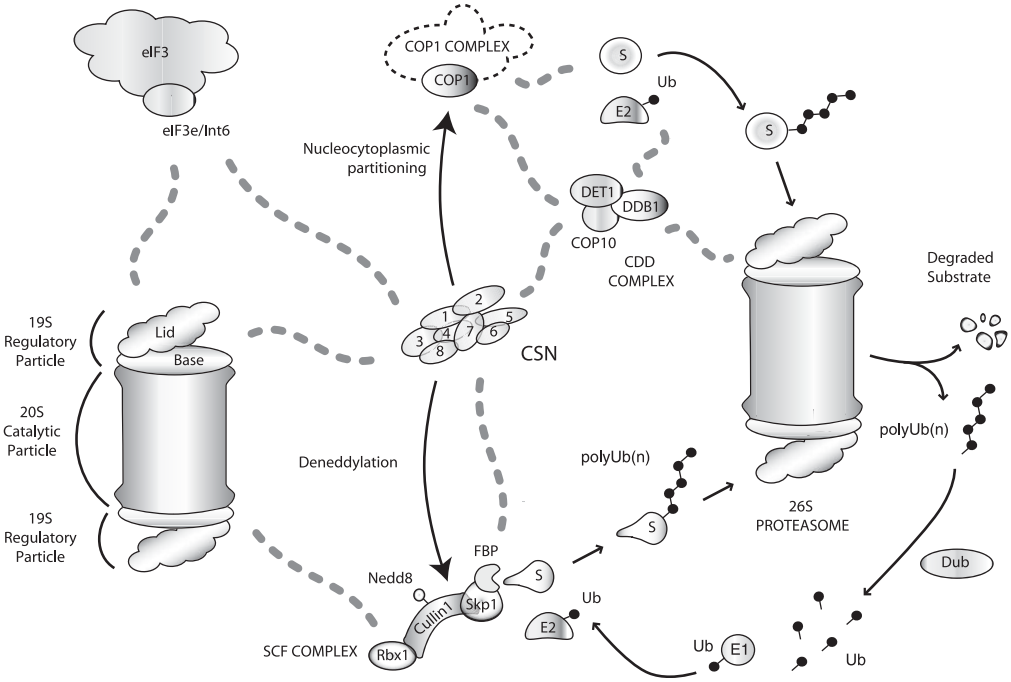

Fig. 3.1. *Arabidopsis* CSN biochemical activities and map of interactions with components of the ubiquitin/26S proteasome pathway. *Arabidopsis* CSN shows two different biochemical activities, represented with large headed arrows, directed towards the E3 ubiquitin–protein ligases: Nedd8–Cullin deconjugation (deneddylation) and nucleo-cytoplasmic partitioning control. Regulated protein degradation consists of cycles of substrate (S)-specific polyubiquitination, mediated by an E1 ubiquitin-activating enzyme (E1), an E2 ubiquitin-conjugating enzyme (E2) and an E3 ubiquitin-protein ligase (E3). Polyubiquitinated substrates are targeted for 26S proteasome-dependent degradation. Upon protein degradation, deubiquitinating enzymes (Dub) catalyze polyubiquitin chain (polyUb(n)) reduction to monomers of free ubiquitin (Ub) (reviewed in Hershko and Ciechanover, 1998). Two types of CSN-regulated E3 ligases are depicted: the SCF complex (composed of Rbx1, Cullin 1, the adaptor protein Skp1, and an F-box protein (FBP)), and COP1. The latter is represented as a complex according to gel filtration results obtained in *Arabidopsis* (Saijo et al., 2003). Wavy dashed lines represent interactions between components of the ubiquitin–26 S proteasome system. The CSN occupies a central position in the map of interactions, representing its possible function as a scaffold to many other protein complexes involved in ubiquitination and degradation of specific substrates. CSN is depicted according to its subunit contacts based on yeast two-hybrid assays (Serino and Deng, 2003). The distinction between the 19S regulatory particle (base and lid) and the 20S catalytic particle in the 26S proteasome is shown. The eIF3e/Int6 subunit has been delineated in the eIF3 complex to highlight its prominent role in mediating eIF3–CSN–proteasome interactions.

Among the CSN activities listed above, only deneddylation and control of nucleocytoplasmic partitioning have been reported in plants, and discussion of these two activities in plants will therefore be the aim of this section. Nonetheless, we can expect that new findings associated with some other, perhaps novel, activities of the plant CSN may emerge in the near future. For instance, it has been reported that casein kinase II co-purifies with mammalian CSN (Uhle et al. 2003). Interestingly, casein kinase II has been shown to phosphorylate HY5 and, in this way, to prevent its degradation mediated by COP1 and the CSN following transference of plants to darkness (Hardtke et al. 2000). Hence, it is reasonable to think that casein kinase II may also associate with CSN in plants in order to regulate substrate stability via phosphorylation.

3.4.1 Deneddylation

Cullins are the only known proteins that are susceptible to Nedd8/RUB1 modification (neddylation, also known as rubylation). Nedd8/RUB1 is a ubiquitin-like polypeptide that, contrary to ubiquitin, does not form chains and seems not to target the protein substrate for degradation, but most likely has a regulatory function similar to other posttranslational modifications such as phosphorylation (Liakopoulos et al. 1998). Neddylation of cullins requires an enzymatic cascade analogous to that responsible for ubiquitination, which is composed of Nedd8-specific E1 activating enzymes, Nedd8-specific E2 conjugases and Rbx1 as an activator of Nedd8 ligation to cullins (Gong and Yeh 1999; Kamura et al. 1999; Lammer et al. 1998). Although this process results in the conjugation of a single Nedd8 molecule to a conserved lysine in the cullin, hyperneddylation of Cullin 4A and 4B has been previously observed in human cells (Dias et al. 2002). In relation to deneddylation of cullins, only two plant biochemical entities have been linked to this enzymatic activity: the CSN and the plant orthologue for DEN1/NEDP1/SENP8 (Deneddylase 1/Nedd8-specific protease *1*/SUMO-sentrin specific protease 2). The latter was first identified in human cells and is characterized by a Nedd8 C-terminal hydrolytic activity, an important activity not present in the CSN that regenerates Nedd8 after cleavage from cullins. Orthologues of DEN1 have been identified in yeast, mice, fruit fly and *Arabidopsis*. However, in the case of the *Arabidopsis* orthologue, demonstration of intrinsic deneddylating activity has not yet been accomplished (Gan-Erdene et al. 2003; Mendoza et al. 2003; Wu et al. 2003). Regarding the CSN, several pieces of evidence have suggested that CSN controls the neddylation level of cullins. Thus, analysis of *csn* mutants in *Arabidopsis* and fission yeast revealed accumulation of mononeddylated Cullin 1 almost exclusively, while in the wild-type lines both neddylated and unmodified isoforms coexist (Lyapina et al. 2001; Schwechheimer et al. 2001). Moreover, *in vitro* assays using highly purified CSN demonstrated that it has an intrinsic deneddylating activity. In contrast to DEN1/NEDP1/SENP8, the CSN can only efficiently process mononeddylated cullins, not hyperneddylated cullins. It has been proposed that DEN1/NEDP1/

SENP8 may act to revert spurious hyperneddylation of cullins, yielding mononeddylated cullins that can therefore be processed by the CSN.

The significance of CSN deneddylation activity has been the subject of a number of studies. It is well known that neddylation and deneddylation of cullins is a highly dynamic process (Yang et al. 2002). In addition, it has been demonstrated that neddylation of cullins is necessary for cullin-containing SCF E3 ligase activity (Furukawa et al. 2000; Morimoto et al. 2000; Read et al. 2000). Apparently, neddylation of cullins promotes recruitment of E2 conjugases and increases the affinity of the SCF for the F-box protein that brings the substrate into the complex, thus allowing ubiquitination of the substrate (Kawakami et al. 2001; Osaka et al. 2000; Read et al. 2000). In this context, CSN-mediated deneddylation of cullins should inhibit the E3 ligase activity of SCF. Accordingly, addition of purified CSN to cell-free assays impairs ubiquitination and subsequent degradation of human p27, a cyclin-dependent kinase inhibitor, by SCF^{SKP2} (superscript indicates the name of the specific F-box protein) (Yang et al. 2002). The inhibitory activity of CSN is not limited to SCF complexes since other cullin-containing ubiquitin ligases distinct from SCF, such as CSA (Cockayne syndrome protein) and a DDB2-containing complex, are inhibited by the presence of the CSN (Groisman et al. 2003). These results contradict genetic studies in budding yeast and *Arabidopsis* where it has been demonstrated that CSN deneddylating activity is required for proper functionality of SCF complexes. Thus, *Arabidopsis* reduction-of-function lines for CSN5 showed impaired degradation of PSIAA6, a substrate of SCF^{TIR} that functions as a repressor of auxin responses (Schwechheimer et al. 2001). In budding yeast, mutations in CSN5 aggravated the defects caused by an SCF mutation that impaired Sic1 turnover and cell growth (Cope et al. 2002). One proposed solution to this paradox suggests that proper SCF functionality could require cycles of neddylation/deneddylation (Wei and Deng 2003). Evidence involving CAND1 (Cullin-associated neddylation dissociated 1) has been reported in support of this hypothesis. CAND1 is a 120-kDa protein, also known as TIP120 (TBP-interacting protein 120 kDa), which binds only deneddylated SCF cullins and promotes dissociation of the adaptor protein Skp1 from the Cullin 1–Rbx1 complex. Dissociation of the SCF complex avoids ubiquitination of any substrate attached to the Skp1-F-box protein subcomplex (Liu et al. 2002a; Oshikawa et al. 2003; Zheng et al. 2002). The crystal structure of the complex comprising Cand1–Cullin 1–Rbx1 has been solved and it shows that CAND1 adopts a highly sinuous superhelical structure that clamps around Cullin 1 and partially blocks the Skp1 binding site on Cullin 1, inhibiting its interaction with the Skp1 adaptor and the substrate-recruiting F-box protein. CAND1 also hides the conserved lysine residue on Cullin 1 that is susceptible to neddylation (Goldenberg et al. 2004). Additionally, CAND1 has been shown to enhance CSN-mediated deneddylation of Cullin 1 *in vitro*, possibly to favor its binding to Cullin 1 (Min et al. 2005). On the contrary, neddylation of Cullin 1 is able to block CAND1–Cullin 1 association and therefore facilitates SCF complex reassembly and E3 ligase activity (Goldenberg et al. 2004). Remarkably, CAND1 knockdown resulted in reduced levels of the human substrate receptor Skp2 and only a moderate increase in the levels of the Skp2 target p27 (Zheng et

al. 2002). Similarly, mutation of the CAND1 counterpart in *Arabidopsis* showed that it affects many different developmental processes, including flowering time, photomorphogenesis, floral organ formation and leaf patterning, as well as responses to plants hormones such as auxin and gibberellins. Molecular analysis of the *Arabidopsis cand1* mutant revealed that many of these defects are a consequence of the reduced activity of a set of SCF complexes (Cheng et al. 2004; Chuang et al. 2004; Feng et al. 2004). These findings suggest that, similar to CSN, CAND1 is also important for optimal SCF E3 ligase activity. As a result of this positive effect of CAND1 on SCF function, it has been proposed that CAND1 promotes dissociation of the SCF complex to avoid SCF self-ubiquitination and degradation and also makes the Cullin 1–Rbx1 core available to other charged Skp1–F-box-substrate complexes (Wei and Deng 2003). The CSN deneddylation activity also appears to have a positive effect on E3 ligase activity as observed in a study using *Drosophila csn5* null mutants where stability of Cullin 1 and Cullin 3 is severely compromised (Wu et al. 2005).

From the above-mentioned results, it has been inferred that the deneddylating activity of the CSN towards SCF complexes underlies many of the CSN roles in regulating biological processes, explaining why depletion of the CSN produces pleiotropic defects in different organisms (Lyapina et al. 2001; Schwechheimer et al. 2001; Zhou et al. 2001). This assertion might be especially meaningful in the case of plants, where the possible SCF combinations number in the hundreds. Indeed, approximately 700 F-box proteins have been identified in *Arabidopsis* and at least 700 SCF complexes could be potentially regulated by the CSN in this organism (Gagne et al. 2002). Moreover, in addition to SCF, other complexes containing a cullin–Rbx1 core are present in plants and other organisms. Cullin 3–Rbx1–BTB/POZ complexes displaying E3 ubiquitin ligase activity have been involved in plant embryogenesis, flowering and control of ethylene biosynthesis (Dieterle et al. 2005; Figueroa et al. 2005; Gingerich et al. 2005; Thomann et al. 2005a; Wang et al. 2004). In these complexes, the function of the adaptor and the substrate-recognizing protein (performed in the SCF complexes by Skp1 and the F-box protein, respectively) are combined in the BTB/POZ (Bric a brac, tramtrack and broad complex/pox virus and zinc finger protein) protein (Geyer et al. 2003; Pintard et al. 2003; Xu et al. 2003). As in the case of Cullin 1 in SCF complexes, neddylation and deneddylation of Cullin 3 seems to be required for the complex E3 ligase activity. Thus, it has been reported that inactivation of the CSN in nematodes causes accumulation of neddylated Cullin 3 and impairs targeting of MEI-1/katanin for degradation, resulting in defects on cytokinesis and spindle formation (Pintard et al. 2003). In agreement with these results, *Arabidopsis csn5a;csn5b* double mutants also display accumulation of neddylated Cullin 3 (Dohman et al. 2005). Two related *Cullin 3* genes, *Cullin 3A* and *Cullin 3B* have been identified in the genome of *Arabidopsis*. A genomic search also revealed the existence of at least 76 BTB/POZ proteins in *Arabidopsis*, and for many of them there is evidence that they are able to physically interact with both Cullin 3A and Cullin 3B, indicating that multiple Cullin 3–Rbx1–BTB/POZ complexes exist in *Arabidopsis* (Dieterle et al. 2005; Figueroa et al. 2005; Gingerich et al. 2005; Weber et al. 2005). Thus, there is the possibility

that the number of CSN-regulated ubiquitin ligases extends to all these potential Cullin 3–Rbx1–BTB/POZ complexes, and that the CSN therefore represents a key regulator of the developmental processes they mediate.

3.4.2 Subcellular Partitioning

COP1 plays a central role in the regulation of key light-response activators in plants (Osterlund et al. 2000; Holm et al. 2002). In *Arabidopsis* several targets have been identified for COP1 E3 ubiquitinating activity, such as the photoreceptor Phytochrome A (PhyA) and transcription factors HY5, LAF1 and HFR1 (Jang et al. 2005; Saijo et al. 2003; Seo et al. 2003; Seo et al. 2004; Yang et al. 2005). Interestingly, COP1 activity is regulated at the level of its nucleocytoplasmic distribution (von Arnim and Deng 1994). Thus, in dark conditions, COP1 is enriched in the nucleus and is able to ubiquitinate its substrates and target them for degradation by the 26S proteasome. Under these conditions, the plant follows a skotomorphogenic pattern characterized by a long hypocotyl, apical hook and close cotyledons, as well as absence of photosynthetic plastids, typical of plants grown in darkness. Upon illumination, the protein levels of COP1 are maintained but the protein remains excluded from the nucleus, allowing accumulation of positive regulators of photomorphogenesis in the nucleus and the transcription of light-induced genes that switch on a photomorphogenic plant developmental pattern (von Arnim and Deng 1994). Interestingly, a set of COP/DET/FUS proteins is necessary for the control of the light-dependent subcellular partitioning of COP1. Thus, it has been reported that single null mutations in *CSN8*, *COP10* and *DET1* impair accumulation of COP1 in the nucleus in darkness (Chamovitz et al. 1996; von Arnim et al. 1997). The finding that the complex containing COP10 and DET1 (CDD) physically interacts *in vivo* with CSN and COP1 supports a model in which both the CSN and the CDD complex cooperate to mediate the darkness-induced COP1 relocation in the nucleus (Yanagawa et al. 2004). Unfortunately, the molecular basis that underlies this regulatory mechanism is poorly understood, as there is no evidence regarding what specific CSN subunit(s) or biochemical activity might be responsible for this particular CSN function.

3.5 CSN Functions in Plant Development

It is now assumed that the control of E3 ligase activities accounts for many of the CSN's roles in diverse biological processes (Schwechheimer 2004; Serino and Deng 2003; Wei and Deng 2003). This can be exemplified in the case of the plant CSN, where reports of CSN association with cullin-containing complexes and its implications in many plant biological processes have flourished recently. Indeed, it is difficult not to find a link between a particular plant developmental trait, or a plant response to phytohormones or to a specific stress, that cannot be some-

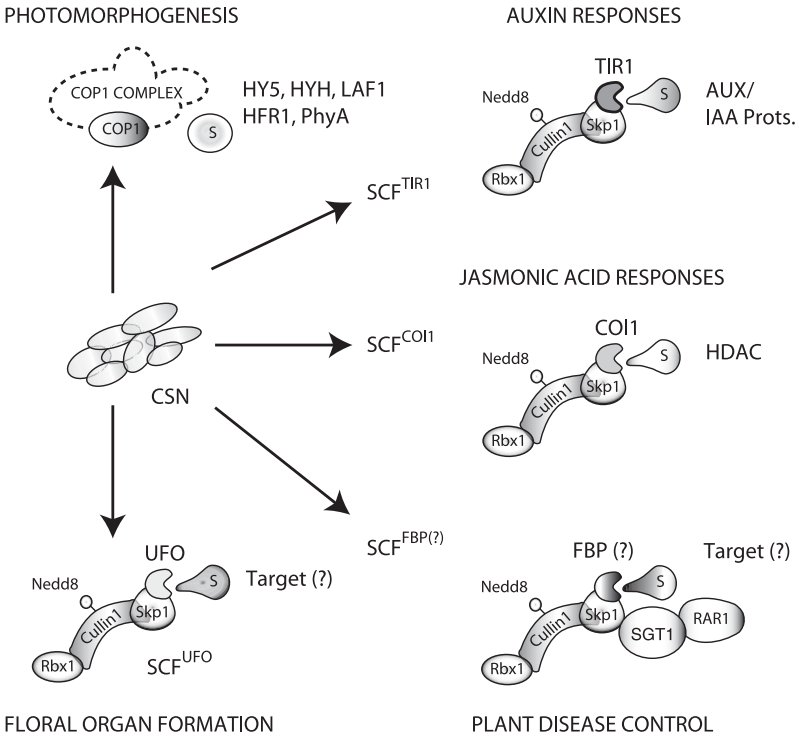

Fig. 3.2. *Arabidopsis* CSN functions in plant developmental processes. The CSN regulates E3 ubiquitin–protein ligases mediating different aspects of plant biology, including floral development, photomorphogenesis, and plant response to pathogens and hormones. The mechanism of action of the CSN involves deneddylation of SCF-type E3 ligases and control of the subcellular distribution (in the case of COP1). The latter is represented as a complex according to gel filtration results obtained in *Arabidopsis* (Saijo et al., 2003). A cullin-containing COP1 complex has been reported in mammals, suggesting that CSN-mediated dennedylation might also play a role in the control of photomorphogenesis (Wertz et al., 2004). Despite evidence suggesting that the CSN controls responses to numerous plant hormones, direct association of the CSN with cullin-containing E3 ligases has been reported only in the case of SCF complexes mediating auxin and jasmonic acid responses (depicted). The F-box proteins (FBP, in SCF complexes) and the targets for the E3 ligase specific to each developmental process are shown.

how related to the CSN through its potential to regulate cullin-containing complexes. In this section, we will discuss CSN function in controlling floral development, photomorphogenesis and plant responses to hormones and pathogens (Figure 3.2). Most studies on CSN function in plants treat the CSN as a whole and thereby preclude analysis of the independent contribution of each subunit. However, this makes little sense given that analysis of *Arabidopsis* reduction-of-function lines for different CSN subunits has shown that there are unique phenotypes associated with the specific CSN subunit that is silenced. Partial deletion of *Arabidopsis*

CSN1, CSN3 or CSN4 yields plants with altered symmetry in their flowers or abnormal development of meristems. However, in the case of partial silencing of the *CSN5* gene in *Arabidopsis*, floral development is unaffected, although phenotypes common to other CSN reduction-of-function lines, such as loss of apical dominance, still occur (Peng et al. 2001a; Peng et al. 2001b; Schwechheimer et al. 2001; Wang et al. 2002). The example of CSN5 further underscores the importance of each subunit function, owing to the fact that the two different isoforms present in *Arabidopsis*, CSN5A and CSN5B, play unequal roles in plant development. Thus, mutations on the MPN+ domain of *CSN5A*, which is expressed at a much higher level than *CSN5B*, cause a very strong pleiotropic phenotype, while mutations in the same positions on *CSN5B* produce little, if not zero, effect on plant development (Gusmaroli et al. 2004). It could be argued that CSN5A is the major functional isoform incorporated into the complex whereas CSN5B is not. However, it has been shown that distinct CSN complexes containing either CSN5A or CSN5B coexist in *Arabidopsis*, suggesting that the CSN may have different functionality depending on which isoform it contains (Gusmaroli et al. 2004).

3.5.1 Floral Development

Identification of the genes involved in floral organ specification has been the subject of several genetic studies in *Arabidopsis* and snapdragon (*Antirrhinum majus*). Only after analysis of the complex genetic interactions among them could these genes be classified on the basis of the organ they determine, and a functional model, the so-called ABC model, was proposed (Coen and Meyerowitz 1991; Weigel and Meyerowitz 1994). In this model, floral organ identity in dicotyledonous plants is specified by different combinations of the activities of A, B and C class homeotic genes. If expressed alone in the floral primordium, the A class genes determine sepal formation; however, when combined with B class genes, the result is petal production. In a similar way, the class C genes alone specify for carpels, but together with the B class genes produce stamens. *AP3* (*Apetala 3*) and *PI* (*Pistillata*) are B class genes encoding transcription factors that bind DNA as heterodimers to control the expression of the genes required for petal and stamen production. Consistent with this role, plants carrying null mutations on these genes display flowers that have sepals in the third floral whorl instead of petals, and carpels in the third floral whorl instead of stamens. The level of AP3 and PI proteins is positively controlled by the F-box protein encoded by *UFO* (*unusual floral organs*), and by *LFY* (*leafy*), which corresponds to a transcriptional regulator also required for the expression of A and C class genes (reviewed in Ng and Yanofsky 2000). Reports have shown that UFO physically associates with *Arabidopsis* Skp1 homologue and Cullin 1 as part of an SCF complex (SCF^{UFO}) (Samach et al. 1999; Wang et al. 2003). UFO also co-immunoprecipitated with *Arabidopsis* CSN components and this *in vivo* association seemed to be required for proper *AP3* expression. As further evidence of this interaction between the CSN and SCF^{UFO},

immunolocalization assays demonstrated that the CSN is enriched in flowers and its localization greatly overlaps with that of UFO in areas corresponding to the inner whorls primordia. Moreover, analysis of two independent *Arabidopsis* CSN1 reduction-of-function lines revealed that these plants have decreased levels of AP3 protein, possibly due to a defect in SCF^{UFO} function that impairs *AP3* expression. Surprisingly, the pattern of neddylated and deneddylated Cullin 1 in the partially deficient *csn1* plants was almost unaffected compared to the wild-type, suggesting that the CSN may control SCF^{UFO} activity by means other than its deneddylation activity (Wang et al. 2003). So far, the identity of the UFO target(s) remains to be unveiled. It has been suggested that UFO targets for degradation a repressor of *AP3* expression, possibly a negative regulator of LFY (Sullivan et al. 2003). While this hypothesis needs to be tested, other questions regarding CSN function in floral development have arisen. Thus, down-regulation of *AP3* expression alone is not enough to explain defects in floral development associated with partial silencing of CSN subunits, indicating that SCF^{UFO} and/or the CSN must regulate, in addition to *AP3*, other factors controlling floral morphogenesis (Peng et al. 2001a; Peng et al. 2001b; Schwechheimer et al. 2001).

3.5.2 Responses to Plant Hormones

Plant hormones (phytohormones) play a critical role in establishing many developmental programs in plants. Although phytohormones retain some specificity in their mechanism of action, in some cases their effects on plant developmental processes greatly overlap. For example, auxin and brassinosteroids are involved in the control of a broad range of responses in plants, including seed germination, stem and root elongation, vascular differentiation, leaf expansion and apical dominance, suggesting that there is a considerable interplay between these two hormones in the control of development (reviewed in Halliday 2004). Auxin affects gene expression by controlling the protein level of members of the AUX/IAA family. AUX/IAAs are repressors of the activity of the auxin response factors (ARFs), which modulate transcription by directly binding to the promoter region of auxin-regulated genes (Ulmasov et al. 1999). The abundance of AUX/IAA proteins is tightly regulated by the auxin-induced E3 ligase activity of SCF^{TIR1} (Gray et al. 2001; Yang et al. 2004). Two landmark studies have demonstrated that auxin directly interacts with TIR1 and, as a result, increases TIR1 affinity for AUX/IAA proteins, indicating that TIR1 is the auxin receptor (Dharmasiri et al. 2005; Kepinski and Leyser 2005). Interestingly, it has been shown that *Arabidopsis* CSN binds *in vivo* to components of the SCF^{TIR1} complex, including TIR1, and is required for proper degradation of PSIAA6, an AUX/IAA family member. In addition, *Arabidopsis* reduction-of-function lines for CSN5 showed decreased auxin responses similar to loss-of-function SCF^{TIR1} mutants, possibly as a consequence of impaired degradation of AUX/IAA proteins in these plants. These results suggest that the

CSN plays a key role in control of auxin signalling and represent the first evidence that the CSN affects the function of a plant hormone receptor (Schwechheimer et al. 2001).

As in the case of auxin, plant responses to other phytohormones, such as gibberellins, ethylene and jasmonic acid, also involve control of the abundance of critical regulators via specific SCF E3 activities (for reviews, see Schwechheimer and Villalobos 2004; Thomann et al. 2005b). Gibberellins (GA) are known to modulate development throughout plant life cycle, exhibiting important functions in stem growth and apical dominance control, seed germination, floral development and flowering time regulation. Members of the DELLA protein family, which includes RGA (repressor of *ga1-3*) and GAI (GA-insensitive) in *Arabidopsis* and SLR1 (slender rice 1) in rice, are known to repress GA-modulated gene expression, possibly by directly binding to DNA. In response to GA, the DELLA proteins are degraded via the ubiquitin–26S proteasome pathway, a process that involves the E3 activity of SCF^{SLY} (sleepy) and SCF^{GID2} (GA-insensitive dwarf 2) in *Arabidopsis* and rice, respectively (reviewed in Fleet and Sun 2005). It has been proposed that the DELLA proteins are subjected to posttranslational modifications or stable conformational changes that favor their recognition by the SCF. The identification of GID1 (GA-insensitive dwarf 1) in rice, a soluble GA receptor with homology to hormone-sensitive lipases, has shed light on this process. Thus, upon GA-GID1 binding, GID1 attains the ability to interact with SLR1 and, as a consequence, SLR1 becomes susceptible to ubiquitination by SCF^{GID2} (Ueguchi-Tanaka et al. 2005).

In the case of jasmonic acid, a phytohormone controlling pollen development, plant growth, wound responses and defence against pathogens, the formation of a specific SCF complex containing COI1 (Coronatine insensitive 1) is involved (Xie et al. 1998; Xu et al. 2002). Remarkably, COI1 binds to HDAC (Histone deacetylase), an enzyme associated with chromatin remodelling. Therefore, it is reasonable to think that SCF^{COI1} modulates jasmonic acid-dependent gene expression by triggering proteasome-mediated degradation of HDAC (Devoto et al. 2002). In the case of ethylene, two different types of cullin-containing E3 ligases participate in the response to this hormone in *Arabidopsis*: a Cullin 3–BTB/POZ complex and two redundant SCF complexes. Ethylene is a gaseous hydrocarbon molecule implicated in seed germination, fertilization, fruit ripening, seed dispersal, hair root production and leaf and fruit abscission (Alonso and Stepanova 2004). Responses to ethylene are modulated by controlling the abundance of EIN3 (ethylene insensitive 3), a transcriptional activator of gene expression in response to ethylene. EIN3 is expressed constitutively but it is unable to accumulate in the absence of ethylene because it is targeted for degradation by two F-box proteins, EBF1 and EBF2 (EIN3 binding factor 1 and 2). Upon detection of ethylene, EIN3 is stabilized and EIN3-dependent genes can be expressed (Guo and Ecker 2003; Potuschak et al. 2003). Additionally, ACS5, a component of the enzymatic cascade responsible for ethylene synthesis, is a target for regulated proteolysis. Thus, it has been shown that ACS5 stability decreases as a result of binding to ETO1 (ethylene overexpressor 1), a BTB/POZ protein that interacts with Cullin 3 in *Arabidopsis* (Wang et al. 2004). Contrary to the case of auxin and SCF^{TIR1}, there is no evidence showing di-

rect interaction between the corresponding hormone and any of the components of the cullin-containing complexes mediating responses to gibberellins, ethylene or jasmonic acid. Furthermore, current data shows that only the activity of SCF^{COI1} appears to be controlled by CSN. Feng et al. (2003) demonstrated that expression of most COI1-dependent genes requires CSN function, and that CSN abundance is important for jasmonic acid-induced responses. In agreement with these results, *Arabidopsis* CSN reduction-of-function plants exhibited a jasmonic acid-insensitive root elongation phenotype and absence of jasmonic acid-induced gene expression.

Regulated proteolysis of mediators of the brassinosteroid and absicic acid signalling pathways has been also proposed. Thus, BRZ1, a positive regulator of brassinosteroid responses, is dephosphorylated and accumulates in the nucleus in the presence of brassinosteroids (He et al. 2005; Wang et al. 2002). However, in the absence of brassinosteroids, BRZ1 is phosphorylated and degraded in a proteasome-dependent manner (He et al. 2002). Although evidence points to a function for cullin-containing complexes in the regulation of responses to brassinosteroids, the nature of these complexes has not yet been determined and a link with CSN is missing. The plant hormone abscisic acid (ABA) is involved in many aspects of plant development, such as stomatal aperture, and adaptation to drought, low temperature and salinity. More recently, an additional role in plant disease resistance has been assigned to ABA (reviewed in Mauch-Mani and Mauch 2005). Expression of ABA-responsive genes is mediated by ABF2 (ABRE-binding factor 2), a transcription factor that directly interacts with ARIA (arm repeat protein interacting with ABF2). ARIA is a BTB/POZ protein that modulates ABF2 transcriptional activity and positively regulates ABA responses (Kim et al. 2004). Interestingly, it has been demonstrated that proteins belonging to the same BTB/POZ class as ARIA interact with Cullin 3A and Cullin 3B, suggesting that this might be true also for ARIA (Dieterle et al. 2005; Figueroa et al. 2005; Gingerich et al. 2005; Weber et al. 2005). Since CSN most likely regulates Cullin 3–BTB/POZ E3 ligases by controlling their neddylation state, it is logical to assume that CSN might also control the E3 activity of a putative Cullin 3–ARIA in mediating ABA responses.

3.5.3 Disease Resistance

The defence response of plants against microorganisms begins with recognition of pathogen-encoded ligands by plant disease resistance (*R*) gene products. Upon recognition, a hypersensitive response or localized cell death is initiated at the site of the pathogen invasion. The hypersensitive response is characterized by a rapid oxidative burst, cell wall modifications, production of antimicrobial compounds (phytoalexins), and activation of several defence genes (Hammond-Kosack and Jones 1996; McDowell and Dangl 2000). Studies carried out in *Nicotiana benthamiana* (a plant species closely related to tobacco) have demonstrated that the *RAR1* (*required for barley Mla resistance 1*) gene is critical for the function of the *N* gene product, a member of the TIR–NBS–LRR class of *R* genes that confers resistance to tobacco mosaic virus (TMV) (reviewed in Jones and Takemoto 2004). Interestingly, it has

been shown that *Nicotiana benthamiana* RAR1 (NbRAR1) interacts *in vivo* with SGT1 (supressor of the G2 allele of *skp1–4*), a protein involved in plant pathogen responses and cell cycle control in yeast (Azevedo et al. 2002). Evidence that SGT1 associates with SCF complexes has been obtained from studies reporting physical interaction of SGT1 with the Skp1 adaptor protein in plants and yeast (Azevedo et al. 2002; Kitagawa et al. 1999). In accordance with a role in defence response control, suppression of *NbSGT1* and *NbSkp1* shows that these genes play an important role in the *N*-mediated resistance response to TMV (Azevedo et al. 2002). Interestingly, both NbRAR1 and NbSGT1 associate *in vivo* with the CSN, indicating that the CSN may influence the control of the hypersensitive response. Silencing of the CSN in *N. benthamiana* plants leads to a reduced *N*-mediated resistance response to TMV, similar to that observed in the case of silencing of *NbSGT1* and *NbSkp1* (Liu et al. 2002b). Although evidence suggests that the CSN exerts its action on the SCF complex associated to SGT1 via deneddylation of Cullin 1, confirmation of this hypothesis has yet to be obtained. Another open question concerns the identity of the targets for the SGT1-interacting SCF activity. In this regard, repressors of the activity of R proteins may represent an obvious set of candidates. However, it has been postulated that removal of defective, and perhaps dangerous, R proteins capable of causing inappropriate cell death may be an additional function of regulated proteolysis in plant disease control (Jones and Takemoto 2004).

3.5.4 Photomorphogenesis

The control of photomorphogenesis widely relies on the transcriptional regulation of light-responsive genes, as shown by genome-wide transcriptomic data estimating that expression of approximately one-third of the *Arabidopsis* genome changes in response to light (Ma et al. 2001; Tepperman et al. 2001). COP1 plays a key role in negatively regulating the protein levels of light signal receptors (PhyA) and photoresponsive transcriptional activators in darkness (HY5, HYH, LAF1 and HFR1, among others) (Holm et al. 2002; Jang et al. 2005; Osterlund et al. 2000; Saijo et al. 2003; Seo et al. 2003; Seo et al. 2004; Yang et al. 2005). Regulation of COP1 activity thus represents a critical step in photomorphogenesis control. Although it is generally assumed that control of subcellular partitioning is the main molecular switch affecting COP1 activity in plants, the finding that a COP1 homologue in humans is part of a multiprotein complex that includes Cullin 4A has triggered reconsideration (Wertz et al. 2004). This complex also contains Rbx1, DDB1 and DET1, and is involved in the ubiquitination and degradation of c-jun, a proto-oncogenic transcription factor. Interestingly, homologues for all the components of this complex, named $DCX^{hDET1\text{-}hCOP1}$, are present in *Arabidopsis*, suggesting that a similar complex could exist in plants. Interestingly, COP1 has been found to form part of a protein complex in *Arabidopsis*, as shown in gel filtration assays, although the composition of this complex is unknown (Saijo et al. 2003). Moreover, analysis of *Arabidopsis csn5a;csn5b* mutants has shown that the plant form of Cullin 4 is a

target of CSN deneddylation activity (Dohmann et al. 2005). Altogether, these data suggest that the CSN could control COP1 activity, and thus photomorphogenesis, by acting upon a putative plant $DCX^{DET1\text{-}COP1}$ complex.

Another possible role of the CSN in controlling photomorphogenesis comes from the study of *Arabidopsis cand1* mutants. CAND1 regulates the activity of SCF E3 ligases through binding to deneddylated Cullin 1 and promotion of disassembly between the Skp1–F-box–substrate subcomplex and the Cullin 1–Rbx1 core (Liu et al. 2002a; Oshikawa et al. 2003; Zheng et al. 2002). Also, CAND1 promotes CSN-mediated deneddylation of Cullin 1 (Min et al. 2005). It has been shown that mutant *cand1* plants hyperaccumulate HY5 in darkness as a result of impaired regulated proteolysis. Hence, *cand1* plants display light responses in the dark, such as elongated hypocotyls and partially expanded cotyledons (Feng et al. 2004). This evidence leads us to suggest that by controlling the neddylation state of SCF E3 ligases, the CSN may influence plant photomorphogenic responses. Further studies should indicate the composition of the SCF complexes involved in the regulation of plant responses to light. In this context, *EID1* (*Empfindlicher im dunkelroten Licht 1*, "hypersensitive to far-red light") may represent one of the first candidates associated with this function. *EID1* was identified in *Arabidopsis* as a repressor of PhyA-mediated light signalling (Büche et al. 2000). Cloning of the *EID1* gene revealed that it encodes an F-box protein that interacts with *Arabidopsis* Skp1 and Cullin 1, although the identity of the target(s) for EID1 activity remains elusive (Dieterle et al. 2001).

3.6 Conclusions

The wide range of potential substrates for the deneddylation activity of the CSN would be enough to explain its central role in regulated proteolysis and its involvement in a broad variety of cellular processes in plants and other organisms. However, this may not be the only manner in which CSN controls the activity of cullin-containing complexes. The newly identified deubiquitination activity of CSN may inhibit E3 ligase activity by removing polyubiquitin chains from protein targets (Groisman et al. 2003; Zhou et al. 2003). Thus, this CSN activity could act independently or together with that controlling the neddylation state of E3 ligases to regulate protein degradation. Based on this hypothesis, the CSN-mediated deubiquitination of E3 ligase substrates would represent an additional level of control over proteolysis at the CSN (Schwechheimer 2004). Further experimentation will be required to test the applicability of this hypothesis in the case of the plant CSN and to analyze its contribution to the regulation of plant developmental programs.

Finally, it is necessary to note that the list of proteins that reportedly interact with the CSN in different organisms also includes proteins that are not obviously related to the ubiquitin–26S proteasome pathway, either as regulators or as substrates (for reviews, see Schwechheimer 2004; Wei and Deng 2003). Therefore, we must be aware that the number of known CSN activities could increase in a near

future and that new roles for the CSN in other plant biological processes may yet be elucidated.

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4
Ubiquitin and Protein Sorting to the Lysosome

John McCullough, Michael J. Clague, and Sylvie Urbé

4.1
Introduction

The eukaryotic cell contains two major proteolytic systems that mediate protein turnover. The 26S proteasome governs the degradation of most intracellular proteins, whereas the lysosome is responsible for downregulation of cell surface receptors and for the degradation of exogenous proteins that have been internalized. The discovery of ubiquitin in the 1970s led to the identification of the 26S proteasome, which supplanted the notion of the lysosome as the principal player in intracellular protein degradation (Ciechanover 2005). There is therefore a nice irony that, as the molecular mechanisms of lysosomal sorting have been unravelled, covalent attachment of ubiquitin has been shown to play a key role in routing proteins through the endomembrane system for lysosomal degradation.

Appendage of K48-linked polyubiquitin chains targets proteins for proteasomal destruction. However, polyubiquitin chains linked through any of six other internal lysine residues are also represented within eukaryotic cells and their functions are largely unknown (Peng et al. 2003). Emerging roles for ubiquitin have been accompanied by an appreciation of ubiquitination as a dynamic modification, which can be used to govern the assembly and disassembly of macromolecular complexes, much like phosphorylation, through interaction with specific ubiquitin-binding domains. Accordingly, additional roles can be proposed for ubiquitin modification at endosomal membranes, such as cell signalling.

A receptor normally enters the endosomal system through incorporation into Clathrin-coated vesicles (CCVs) and delivery to a tubulo-vesicular compartment known as the early or sorting endosome (Figure 4.1) (Clague 1998). From here receptors may recycle to the plasma membrane, or be selected for lysosomal sorting, by incorporation into small vesicles that bud away from the cytosol into the vacuolar lumen to generate multi-vesicular bodies (MVBs). MVBs may then fuse directly with lysosomes or deliver material to late endosomes, both of which contain acid-dependent proteases. Classic electron microsocopy studies established this paradigm, largely by following the itinerary of epidermal growth factor receptors (EGFR), subsequent to ligand-induced internalization (Felder et al. 1990; Gor-

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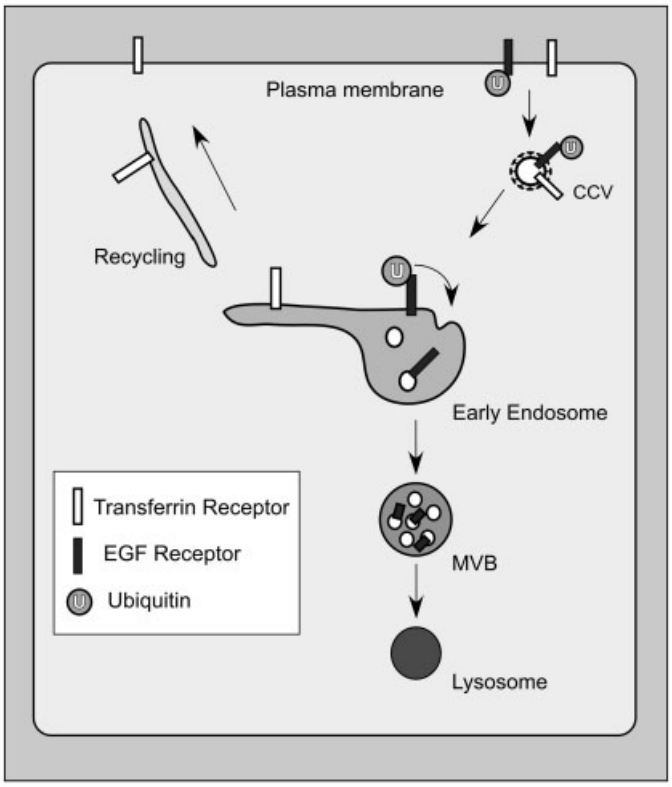

Fig. 4.1. Protein sorting in the endocytic pathway. Upon ligand activation (not shown), growth factor receptors (e.g., EGF receptor) are internalized via Clathrin-coated vesicles (CCV) and delivered to the tubulo-vesicular early endosome. Here, ubiquitinated receptors are sequestered into the lumenal vesicles of the multi-vesicular body (MVB), which delivers its content to the lysosome. Other plasma membrane proteins that are not ubiquitinated (e.g., Transferrin receptor) enter the pathway via the same route but are recycled back to the plasma membrane.

den et al. 1978; Haigler et al. 1979). This endocytic pathway has been conserved in yeast, with the yeast vacuole being functionally equivalent to the lysosome. Thus, much of our recent knowledge has been driven by yeast genetic studies of mutants defective in vacuolar transport (*vps* mutants; Bankaitis et al. 1986), which has led to the identification of the MVB sorting machinery.

In this chapter we shall focus first on the work that led to an appreciation of a role for ubiquitin on the endocytic pathway, before discussing the complex machinery that engages with ubiquitin for sorting towards the lysosome. We shall then consider the various E3-ligases and de-ubiquitinating enzymes (DUBs), which have been mooted to regulate the ubiquitin status of endosomal proteins, before finally considering the role of different polyubiquitin chains at the endosome.

4.2 Identification of Ubiquitin as an Endosomal Sorting Signal

The first hints that ubiquitin might play a role in endocytosis emerged in the mid-1980s, when it was found that a number of plasma membrane receptors, including PDGF receptor and GH receptor, were modified with ubiquitin (Leung et al. 1987; Yarden et al. 1986; reviewed in Bonifacino and Weissman 1998). In the early 1990s, studies revealed that ubiquitination of cell surface receptors that signal through tyrosine kinase activation occurs in response to binding of ligands. The first example of such was the T-cell receptor (TCR). Multiple TCR subunits were found to be ubiquitinated on cytosolic lysine residues in response to receptor occupancy (Cenciarelli et al. 1992).

It required yeast studies to clarify a function for conjugation of ubiquitin to membrane proteins. A link between ubiquitin and endocytosis was established using three different approaches. Firstly, Kolling and Hollenberg analysed the downregulation of Ste6p, the ATP-binding cassette (ABC) transporter for secretion of the pheromone a-factor (Kolling and Hollenberg 1994). They observed the accumulation of ubiquitinated forms of Ste6p in plasma membrane fractions prepared from a mutant *Saccharomyces cerevisiae* strain with impaired endocytosis. Second, by analysing yeast strains lacking ubiquitin-conjugating enzymes, Hicke and Riezman showed that ubiquitination of the yeast G protein-coupled α-factor receptor Ste2p marked this plasma membrane protein for proteolysis (Hicke and Riezman 1996). Using mutant strains, defective for either vacuolar hydrolases or proteasome function, they were able to establish that ubiquitination of Ste2p was required for the vacuolar degradation pathway. A third line of evidence for a link between ubiquitin and endocytosis in yeast was obtained by genetic analysis of the ammonium-induced downregulation of amino acid permeases. Npi1 was isolated as a nitrogen permease inactivator gene in 1983 (Grenson 1983); Hein et al. subsequently showed that the gene encodes an E3-ligase, Rsp5p (Hein et al. 1995).

In contrast to proteasomal degradation pathways, appendage of polyubiquitin chains to substrate proteins is not required for ubiquitin-dependent endocytosis in yeast. Ste2p is efficiently internalized under conditions where polyubiquitin chain formation is suppressed by the use of ubiquitin mutants lacking internal lysines (Terrel et al. 1998). Fusion of a single ubiquitin in-frame to the stable plasma membrane protein Pma1p stimulates endocytosis of this protein (Shih et al. 2000). For other proteins, specifically yeast permeases, such as uracil permease and the general amino acid permease, Gap1p, maximal internalization rates require the formation of diubiquitin chains linked through Lys63 of ubiquitin (Galan and Haguenauer-Tsapis 1997; Springael et al. 1999).

In mammalian cells, the smeary appearance of ubiquitinated receptors on Western blots was initially interpreted as reflecting polyubiquitination. However, it was not clear how polyubiquitinated receptors would escape proteasomal targeting, and instead be degraded in the lysosome. This issue has since been resolved by using a combination of monoclonal antibodies, which allow discrimination between mono- and polyubiquitinated proteins. EGFR, PDGFR and Met tyrosine kinase receptors

are all in fact monoubiquitinated at multiple lysine residues within the receptors (Haglund et al. 2003; Mosesson et al. 2003; Carter et al. 2004). Furthermore, Haglund et al. showed that fusion of ubiquitin to the C-terminus of EGFR results in constitutive endocytosis, which cannot be further enhanced by EGF stimulation. However, data from the Sorkin laboratory has shown that the dopamine transporter can be polyubiquitinated and contains a mixture of K11, K48 and K63-linked ubiquitin (Miranda et al. 2005).

In addition to promoting the internalization of receptors from the plasma membrane, ubiquitination also promotes sorting of receptors towards late endosomes and the vacuole. Mutation of lysine 6 in the cytoplasmic tail of the yeast vacuolar protein Phm5p inhibits its sorting to lumenal vesicles, but this can be restored by the biosynthetic addition of a single ubiquitin to create a Ub-Phm5p fusion protein (Reggiori and Pelham 2001). In mammalian cells, transferrin receptor normally recycles back to the plasma membrane after internalization to sorting endosomes (Hopkins 1983). Fusion of ubiquitin to the C-terminus of this receptor prevents its recycling, through interaction with the endosomal protein Hrs (Section 4.3.2; Raiborg et al. 2002).

4.3 Ubiquitin-mediated Sorting at the Endosome: The MVB Sorting Machinery

Our appreciation of the complexity of the molecular machinery responsible for MVB formation and receptor sorting to the lysosome, owes everything to the comprehensive characterization of *vps* mutant strains in yeast (Katzmann et al. 2002). These *vps* mutants can be subdivided into classes based on their characteristic phenotype, and it is Class E mutants that present with an enlarged, swollen pre-vacuolar compartment, indicative of a defect at the stage of MVB formation and inward vesiculation. The sorting events at the pre-vacuolar or early endosome that lead to the translocation of receptors into internal vesicles are thought to be mediated by a succession of at least four multiprotein complexes, which each have the ability to recognize ubiquitinated cargo through ubiquitin-interacting domains: the Hrs/STAM complex and the ESCRT complexes I, II and III. A fifth component that is essential for this process is an ATPase of the AAA family called Vps4 or SKD1.

4.3.1 Endosome-associated Ubiquitin Interacting Domains: Structure and Function

Several of the class E *vps* genes contain domains that are predicted to interact with ubiquitin. A feature common to all ubiquitin-binding motifs is their low affinity (100–500 μM, see Table 4.1). This makes sense in the face of the high concentration of free ubiquitin in the cytosol which has been estimated at 10 μM (Haas and Bright 1987); too high an affinity would mean that these modules would be permanently plugged with ubiquitin and unable to dynamically interact with ubiquiti-

Table 4.1. Ubiquitin-binding domains in endocytic proteins.

Name	Length	Examples	K_d (μM)	Structure
UIM	~20	Hrs/Vps27, STAM/HseI, Eps15, Epsin	Hrs: ~300 Vps27: ~100–300 STAM: ~200	α-helix
UBA	~40	Cbl	n.d.	Triple α-helix
CUE	~45–50	Tollip	Vps9: 20 μM	Triple α-helix
UEV	~60	Tsg101/Vps23	Tsg101: ~500	α-helix/β-sheets
VHS	~150	STAM	n.d.	Eight α-helices
GAT	var.	GGA3	yGGA: 100–400	Triple α-helix
NZF	~25–30	Vps36	Vps36: ~200	Zn finger

A large variety of ubiquitin-binding motifs are found in proteins involved in endocytic membrane traffic. The average length of the motif is given in amino acid residues. K_d values correspond to measured affinities for free ubiquitin.
n.d.: not determined.
Refer to text for references and abbreviations.

nated cargo. The small size of these domains has made them amenable to structural analysis.

The ubiquitin-interacting motif (UIM) was first identified in an unbiased bioinformatic screen based on homology to the ubiquitin-interacting region in the regulatory subunit S5a (Rpn10 in yeast) of the 26S proteasome (Hofmann and Falquet 2001). It became immediately clear that proteins involved in endocytic trafficking were highly represented amongst the emerging list of UIM proteins. UIM domains are found in Hrs, STAM and Epsin as well as in their yeast counterparts Vps27, HseI and Ent1/2. The UIM is characterized by a short 20 amino acid motif with a highly conserved stretch øxxAxxxSxxAc, where ø denotes a large hydrophobic, and Ac an acidic, residue, and which is preceded by a block of four mostly acidic residues (Hofmann and Falquet 2001). Solution structures of the UIMs of Hrs and Vps27, as well as a crystal structure of the second UIM of Vps27, are now available and indicate that the motif folds as a short amphipathic helix and interacts with the Leu8–Ile44–Val70 hydrophobic patch in ubiquitin (Fisher et al. 2003; Shekhtman and Cowburn 2002; Swanson et al. 2003). The structures indicate that UIM binding to a monoubiquitinated protein would occlude Lys48 of ubiquitin, rendering it unavailable for ubiquitin chain extension through this residue. This may provide one mechanism that protects ubiquitinated receptors from proteasomal degradation.

Ubiquitin-associated (UBA) domains (Hofmann and Bucher 1996; Vadlamudi et al. 1996) and CUE-domains (named after the founding member Cue1: coupling of ubiquitin conjugation to ER degradation) span 40 to 50 amino acids and share a three-helix bundle structure (Kang et al. 2003; Mueller and Feigon 2002; Prag et al. 2003). Two high-resolution crystal structures of the CUE domain show that binding to ubiquitin, as in the case of the UIM, is through hydrophobic surfaces, and that Lys48 of ubiquitin is likewise occluded by the interaction (Kang et al. 2003; Prag et al. 2003).

Ubiquitin E2 variant (UEV) domains have homology to the E2 conjugating enzymes that ligate ubiquitin to substrates, but they lack the catalytic cysteine. In contrast to UIM, UBA and CUE domains, the crystal structure of the ESCRT I component Tsg101 UEV domain complexed with ubiquitin indicates that both Lys 48 and Lys 63 of ubiquitin remain fully accessible (Pornillos et al. 2002; Sundquist et al. 2004).

Many UIM, UBA and CUE domains promote self-ubiquitination of their host protein. This can promote a network of proteins held together by ubiquitin interactions, or may regulate the availability of the ubiquitin-binding domain in the host protein (Di Fiore et al. 2003; Hicke and Dunn 2003).

4.3.2
The Hrs–STAM Complex and the Endosomal Clathrin Coat

Initial engagement between the sorting machinery and ubiquitinated cargo is mediated through interaction with UIM domains in hepatocyte growth factor tyrosine regulated substrate (Hrs) and signal transducing adapter molecule (STAM), also called Hrs-binding protein (Hbp). In yeast these proteins correspond to Vps27 and Hse1, respectively. Mutations in the UIM of either of these proteins result in specific defects in the sorting of ubiquitinated proteins into the vacuole lumen (Bilodeau et al. 2002; Shih et al. 2002). Association of Hrs with endosomal membranes is mediated through binding of its FYVE domain to the inositol lipid PtdIns3*P* (Gillooly et al. 2000; Urbé et al. 2000). Hrs fulfils an adapter function through direct interaction with ubiquitinated cargo and with the terminal domain (TD) of clathrin heavy chain (Clague 2002; Raiborg et al. 2001). Both Hrs and clathrin are components of an unusual coat structure that assembles on the vacuolar surface of sorting endosomes (Sachse et al. 2002). Typically, the coat presents as an extended flat surface, which gives the impression of opposing the natural curvature of the membrane. It comprises two relatively electron-dense layers separated by a thin electron-lucent layer. This particular clathrin coat does not form clathrin-coated vesicles, but instead may provide a matrix capable of trapping and concentrating ubiquitinated receptors such as EGFR (Clague 2002). On the other hand, the recycling transferrin receptor, whilst free to diffuse into this region, is not retained within it (Sachse et al. 2002).

A central role of Hrs/Vps27 in both receptor sorting and MVB formation has been proposed. Vps27 is a class E *vps* mutant defective in lumenal vesicle formation and Hrs knock-out in *Drosophila* provides a similar phenotype and inhibits

EGFR downregulation (Lloyd et al. 2002). siRNA knock-down of Hrs in mammalian cells partially inhibits Met receptor and EGFR downregulation (Bache et al. 2003b; Hammond et al. 2003). A role for Hrs in both receptor sorting and lumenal vesicle formation suggests that the two processes may be tightly coupled, with consequent advantages with respect to the loading efficiency. Both receptor sorting and lumenal vesicle formation can also be inhibited by overexpression of Hrs (Urbé et al. 2003). This inhibition of internal vesicle formation is contingent on an intact Hrs UIM domain, suggesting that it may play both positive (receptor sorting) and negative (vesicle formation) roles in the pathway leading to lumenal vesicle budding.

Yet another UIM-domain-containing protein, Eps15, has been reported as an additional subunit of the Hrs–STAM complex (Bache et al. 2003b). Eps15 has previously been shown to play a role in the CCV-mediated internalization of activated EGFR (Confalonieri et al. 2000; Torrisi et al. 1999). The fact that Eps15 clearly functions early on in endocytosis has made it difficult to address whether it is an essential component at a later stage of endosomal sorting. All three proteins, Hrs, STAM and Eps15, are tyrosine phosphorylated in response to growth factors, which could conceivably regulate further associations with components of the sorting machinery (Clague and Urbe 2001; Fazioli et al. 1993; Urbé et al. 2000) or with other signalling pathways (Row et al. 2005).

4.3.3
GGA and Tom1: Alternative Sorting Adapters?

Hrs and STAM are founder members of the family of proteins with a VHS (Vps27p/Hrs/STAM) domain. Striking similarities have now been found with other members, which share the ability to link ubiquitin and Clathrin: GGA (Golgi-associated g-adaptin homologues, Arf-binding) and Tom1/Tom1L1 (target of Myb1) (Figure 4.2) (Bilodeau et al. 2004; Puertollano 2005; Scott et al. 2004; Yamakami et al. 2003). GGA proteins have previously been described as monomeric adapters that are involved in clathrin-coated vesicular transport between the Golgi and the endosome as well as the endosome and the vacuole/lysosome (Bonifacino 2004). Ubiquitin binding is conferred by the GGA and Tom (GAT) domain and by the VHS domain, whereas clathrin is recruited by the Hinge-region (Bilodeau et al. 2004; Puertollano et al. 2001; Shiba et al. 2004; Zhu et al. 2001). The GAT domain is also responsible for binding the small GTPase Arf (Dell'Angelica et al. 2000). In the case of Tom1 and Tom1L1, the GAT domain binds ubiquitin and a CUE-domain protein called Tollip in a mutually exclusive way (Katoh et al. 2004; Yamakami et al. 2003). Tom1, but not Tom1L1 also binds to a FYVE-domain protein, Endofin, strengthening the analogy with the Hrs–STAM complex (Seet et al. 2004). Endofin or Tollip co-expression are required for recruitment of Tom1 to endosomes (Katoh et al. 2004; Seet et al. 2004). Finally, all three VHS-domain sorting complexes, Hrs–STAM, GGA and Tom1L1, can independently recruit the internal vesicle formation machinery through a P(S/T)AP motif which interacts with the ESCRTI component tumour suppressor gene 101 (Tsg101, see below) (Pornillos

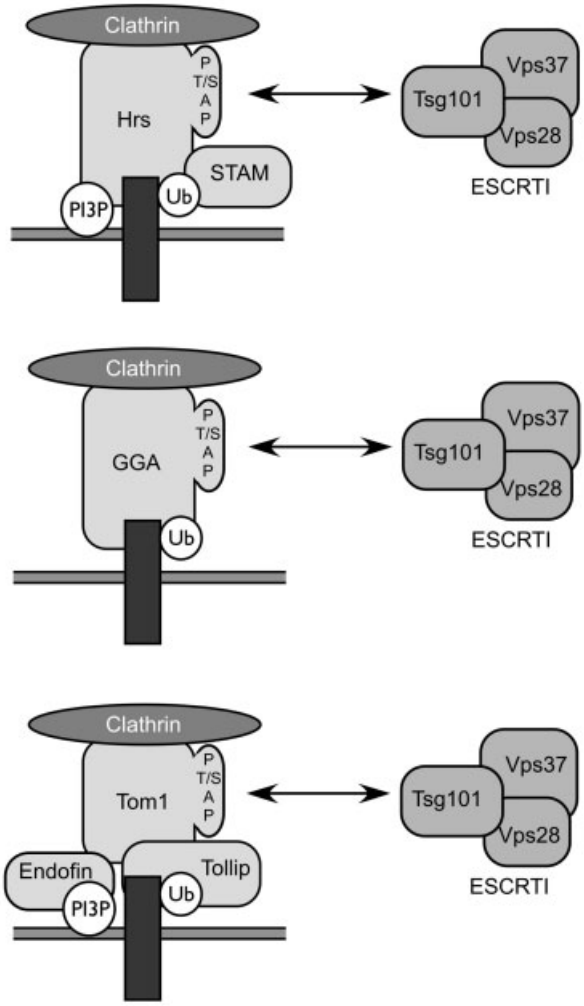

Fig. 4.2. Ubiquitin-binding adapter complexes implicated in MVB sorting. Three endosomal adapter complexes have been implicated in the sorting of proteins from the endosome to the lysosome. Each of these complexes interacts with clathrin, which decorates the endosomal membrane in discrete patches. Ubiquitin binding is conferred by UIM (Hrs, STAM), VHS (STAM, GGA), or GAT domains (GGA, Tom1) and might provide a means to concentrate ubiquitinated receptors in the endosomal clathrin-coated microdomains. The PS/TAP motif in the Hrs, GGA and Tom1 subunits may then recruit the ESCTI complex via direct interaction with Tsg101. It is as yet unclear whether these sorting complexes act independently or in concert with each other. Black rectangle: receptor cargo; PI3P: PtdIns3*P*; Ub: ubiquitin.

et al. 2003; Puertollano 2005; Puertollano and Bonifacino 2004). A yeast two-hybrid assay has also suggested that the VHS domains of GGA1 and 3, as well as of Tom1 are capable of binding to Hrs (Puertollano 2005).

It is unclear if the existence of multiple sorting complexes is an indication of redundancy in lysosomal sorting or whether the multitude of adapters provides a higher level of cargo selectivity to the sorting process. An analogy may be made with the clathrin-coated vesicle internalization pathway where a variety of adapters operate to provide both redundant and specific sorting mechanisms (Traub 2003).

4.3.4 The ESCRT Machinery

Yeast studies have defined three distinct multimeric Vps protein complexes, named endosomal sorting complex required for transport (ESCRT) I, II and III, which are proposed to be sequentially recruited and activated at the endosome (Figure 4.3) (Katzmann et al. 2002). These proteins constitute the core of the MVB formation machinery downstream of the Hrs–STAM complex. At least two of these complexes bind ubiquitin, and the ubiquitinated receptors may be passed along from one complex to another. The first complex, ESCRTI (~350 kDa) is composed of Vps23, Vps28 and Vps37. The ubiquitin-binding site of this complex is found in Vps23, which has a UEV domain. The mammalian homologue of Vps23, Tsg101 (tumour suppressor gene 101) was originally identified in a tissue culture screen for genes whose disruption causes cell transformation (Li and Cohen 1996). Tsg101 as well as hVps28 disruption by RNAi and antibody injection, respectively, clearly interfere with EGFR downregulation in human cells and cause a marked accumulation of ubiquitin on endosomes (Babst et al. 2000; Bishop et al. 2002). The mammalian homologue of Vps37 has only recently been identified and is the least conserved member of ESCRTI (Bache et al. 2004; Stuchell et al. 2004).

ESCRTI is recruited to endosomal membranes through binding of the UEV domain of TSG101/Vps23 to a conserved PT/(S)AP motif within Hrs–Vps27 (Bache et al. 2003a; Lu et al. 2003; Pornillos et al. 2003). Hrs and TSG101 are present in both cytosolic and membrane fractions, but only associate at the membrane (Bache et al. 2003a). How can this ordered ESCRT complex assembly be attained specifically at the membrane? Hrs is localized in part at the membrane through interaction of its FYVE domain with the inositide lipid PtdIns3*P* that is concentrated at early endosomes (Gillooly et al. 2000; Urbé et al. 2000), where it can then bind to ubiquitinated receptors. It is possible that PtdIns3*P* or ubiquitin binding could induce a conformational change in Hrs that unmasks a TSG101 binding site. TSG101 itself has a PTAP motif, which may interact with its own UEV domain – competitive binding by Hrs may then release TSG101 into a relaxed conformation permissive for ESCRT II recruitment (Clague and Urbe 2003; Lu et al. 2003; Pornillos et al. 2003).

ESCRTII was described as a cytosolic complex (~155 kDa) composed of the class E vps proteins Vps22/Eap30, Vps25/Eap25 and Vps36/Eap45 that transiently associates with endosomal membranes in an ESCRTI-dependent manner (Figure 4.3).

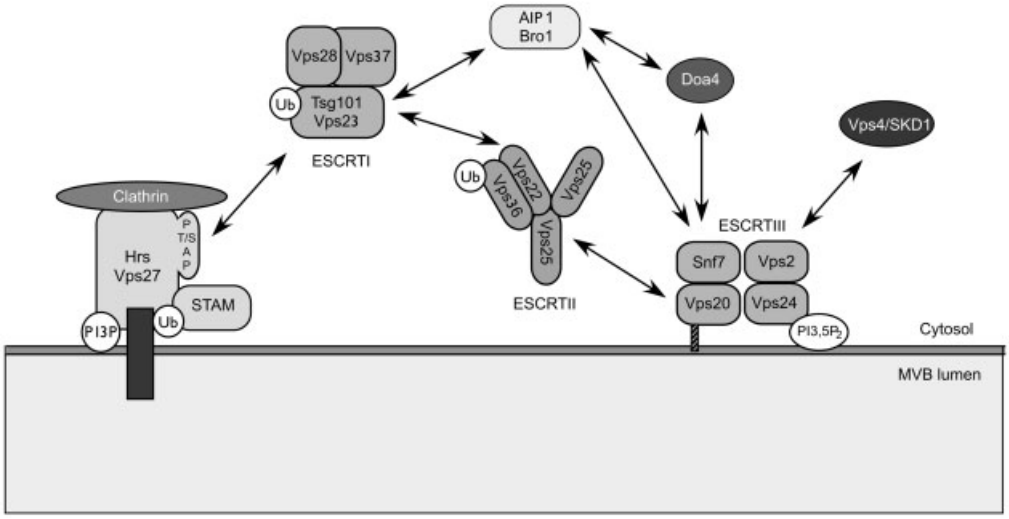

Fig. 4.3. Interactions of the MVB sorting machinery. The first point of engagement of ubiquitinated proteins with the MVB sorting machinery is the Hrs–STAM complex. Hrs (Vps27 in yeast) associates with the endosomal membrane through interaction of its FYVE-domain with PtdIns3*P*. Both Hrs and STAM (HseI in yeast) bind ubiquitin via their UIM domains. Hrs–Vps27 recruits the ESCRTI complex (composed of Vps23–Tsg101, Vps28 and Vps37), through interaction of its PS/TAP motif with Tsg101–Vps23, which in turn binds ESCRTII (Vps22, Vps25 and Vps36; Eap30, Eap25 and Eap45 in mammalian cells). Both ESCRTI and ESCRTII complexes are able to bind ubiquitin via the UEV domain in Tsg101, and the GLUE and NZF domains in Eap45 and Vps36, respectively. ESCRTII recruits and activates the last complex in this cascade, ESCRTIII. An additional connection between ESCRTI and ESCRTIII complexes is mediated through AIP1, which in yeast (Bro1) has also been implicated in recruiting the DUB Doa4 to the prevacuolar compartment. ESCRTIII is composed of two subcomplexes, Snf7–Vps20 and Vps2–Vps24. These proteins belong to a family of highly charged proteins called CHMPs in mammalian cells. Membrane association of the ESCRTIII complex may be mediated by binding of Vps24 to PtdIns(3,5)P_2 and a myristoyl moiety attached to Vps20. The ESCRTIII complex interacts with the AAA-ATPase Vps4 (SKD1 in mouse), which is thought to dissociate the components of the sorting machinery. Black bar: receptor, e.g., EGFR; PI3P: PtdIns3*P*; PI3,5P_2: PtdIns(3,5)P_2; Ub: ubiquitin.

Two 3.6-Å resolution crystal structures of an ESCRTII complex containing one molecule of Vps22, the carboxy-terminal domain of Vps36 and two molecules of Vps25 suggest that the complex has the shape of a capital letter 'Y', of which the sub-complex Vps22 and Vps36 form one branch. A flexible linker extending from the tip of this branch would lead to two consecutive NZF motifs, the second of which is believed to bind ubiquitinated cargo (Alam et al. 2004; Hierro et al. 2004; Teo et al. 2004). However, this domain was not solved owing to proteolysis during crystallization. The authors suggest that this structure could provide a ''long swinging arm'' for transfer of cargo over substantial distances. The NZF motif is

lacking in Eap45, the mammalian orthologue of Vps36, but this is compensated for by the inclusion of a GLUE domain, which has been found to have ubiquitin-binding properties (Slagsvold et al. 2005).

The ESCRTII complex is required for the membrane recruitment of the Snf7–Vps20 ESCRTIII sub-complex via an interaction between Vps25 and Vps20 (Teo et al. 2004; von Schwedler et al. 2003), and this in turn is a prerequisite for the recruitment of the other two ESCRTIII proteins Vps24 and Vps2 to the membranes (Babst et al. 2002a; Babst et al. 2002b). These last four class E Vps proteins belong to a structurally related "family" of small, highly charged, coiled-coil proteins that in mammalian cells are referred to as CHMPs (charged multivesicular proteins) (Howard et al. 2001). Membrane association of this complex may also be conferred by myristoylation of Vps20–CHMP6 and the ability of Vps24–CHMP3 to bind to the phosphatidyl-inositol lipid PtdIns(3,5)P_2 (Whitley et al. 2003). The mammalian homologues of Vps2 and Snf7 are called CHMP2(a and b) and CHMP4(a, b and c), respectively.

One highly connected protein is AIP1 or ALIX (ALG2-interacting protein) previously implicated in apoptosis. AIP1 shows interaction with both ESCRTI (Tsg101) and ESCRTIII complexes (Snf7–CHMP4(a, b and c)) and may therefore act as a bridge between these two (Katoh et al. 2003). The yeast homologue of AIP1, Bro1, plays a role in recruiting the deubiquitinating enzyme Doa4 (Luhtala and Odorizzi 2004; Odorizzi et al. 2003), possibly through stabilization of Snf7 at the endosome, with which the enzyme interacts directly (Bowers et al. 2004). Doa4 is not essential for MVB formation or sorting (Reggiori and Pelham 2001; Urbanowski and Piper 2001), but acts to recycle ubiquitin from cargo that has progressed beyond ubiquitin-dependent steps in the MVB sorting pathway (Amerik et al. 2000).

4.3.5 Vps4–SKD1

The ESCRT-III component Snf7, recruits a Class E Vps protein belonging to the AAA-ATPase family, called Vps4 in yeast, and SKD1 in mouse cells (Katzmann et al. 2002; Lin et al. 2005; Perier et al. 1994; Yoshimori et al. 2000). The last resolved step in the MVB formation cascade is the dissociation of the ESCRT-machinery powered by Vps4–SKD1 hydrolysis of ATP. This is inferred from the dramatic phenotype of ATPase-defective Vps4–SKD1 mutants in yeast and mammalian cells, respectively. Vps4 yeast deletion strains show a typical Class E swollen prevacuolar compartment on which the entire upstream sorting machinery accumulates (Amerik et al. 2000; Babst et al. 2000; Babst et al. 1998; Odorizzi et al. 2003). Overexpression of a catalytically inactive Vps4 mutant in mammalian cells recapitulates this phenotype by promoting the formation of enlarged endosomes on which an "Hrs-clathrin coat", as well as the ESCRTI machinery accumulates (Bishop et al. 2002; Fujita et al. 2003; Sachse et al. 2004; Yoshimori et al. 2000). At the ultrastructural level, cells expressing mutant Vps4 also show a depletion of internal vesicles, and membrane proteins destined for the lysosome accumulate at the peripheral membrane of this abnormal compartment (Sachse et al. 2004). This

suggests that dissociation of the sorting machinery and the formation of internal vesicles are tightly coupled to allow efficient recycling of ESCRT proteins.

4.4 Ubiquitin Ligases and Endosomal Sorting

The specificity of ubiquitination is largely due to cognate interactions with E3 ligases, of which there are probably in excess of 600 in mammals. By recruiting specific E2 enzymes they may also determine the topology of the ubiquitin extension. E3s are generally split into two major classes: the RING (really interesting new gene) ligases have a catalytic domain based on a double zinc-finger whilst the HECT (homologous to E6-AP carboxyl terminus) ligases contain a 350 amino acid C-terminal domain within which lies a conserved catalytic cysteine. HECT ligases recognize their substrate via WW domains that interact with various proline-rich sequences. They form a thiolester intermediate with ubiquitin, whereas RING ligases promote the direct transfer of ubiquitin from the E2 to the substrate (Dupre et al. 2004). RING ligases can be further subdivided into those in which the substrate binding site and the RING domain are encoded within a single polypeptide (e.g. c-Cbl) and those in which they are contained within different proteins of a larger complex (e.g. SCF and APC/Cyclosome). We will briefly review the major E3 ligases associated with endocytic trafficking.

4.4.1 Nedd4 Family

HECT domain proteins of the Nedd4 family regulate the trafficking of a variety of biosynthetic and endosomal cargo. They have a common architecture consisting of several WW domains and a C-terminal HECT domain. Most members also have an amino terminal C2 domain, which commonly bind to phosphoinositides (Ingham et al. 2004). The sole member of the Nedd4 family in *S. cerevisiae* is Rsp5, which seems to be the only ligase required for ubiquitination of cell surface proteins. Early studies demonstrated its involvement in constitutive ubiquitination of the uracil permease Fur4p (Galan et al. 1996) and the general amino acid permease Gap1p (Springael and Andre 1998). It is located and functions at multiple sites on the endocytic pathway (Wang et al. 2001). Accumulation at the prevacuolar compartment in *vps4*Δ cells suggests a function in the MVB sorting pathway. Mutation of the C2 domain disrupts this localization and inhibits sorting of the biosynthetic pathway-derived cargo carboxypeptidase S, but not the endocytosed receptor Ste2, for which ubiquitin can be appended at the plasma membrane (Dunn et al. 2004; Morvan et al. 2004). Some transporters such as Fur4p are downregulated both by endocytosis from the cell surface and by diversion from the biosynthetic pathway at the Golgi to the MVB pathway, without reaching the surface. Both pathways require Rsp5 function at the plasma membrane and prevacuole respectively (Blondel et al. 2004).

Drosophila Nedd4 regulates endocytosis of Notch and suppresses its ligand-independent activation (Sakata et al. 2004). In mammalian cells several family members, including Nedd4 and AIP4/Itch, have been implicated in endocytic trafficking. Nedd4 directly mediates ubiquitination of the epithelial Na^+ transporter ENaC, targeting it for downregulation (Rotin et al. 2001). AIP4/Itch is disrupted in nonagouti lethal or itchy mice, which are characterized by abnormal immune responses and constant itching (Perry et al. 1998). It interacts with and ubiquitinates mammalian Notch and the G-protein-coupled chemokine receptor CXCR4 (Marchese et al. 2003; Qiu et al. 2000). Salient substrates also include components of the endocytic machinery. Eps15 and Hrs ubiquitination are mediated by Nedd4 or by AIP4 (Angers et al. 2004; Katz et al. 2002; Marchese et al. 2003; Polo et al. 2002). Ubiquitination of these proteins depends on their UIM and it is thought that the HECT ligase is partially recruited through an interaction between its covalently attached ubiquitin and the UIM domain. Once the protein is ubiquitinated, its UIM may be occupied by its own ubiquitin and no longer able to recruit another HECT ligase. In this way, polyubiquitination and subsequent targeting of the endocytic machinery to the proteasome may be prevented (Di Fiore et al. 2003).

4.4.2 c-Cbl

In mammalian cells, the major E3 ligase implicated in endocytic trafficking of RTKs is the cellular proto-oncogene Cbl (Thien and Langdon 2001). It is recruited via its SH2 domain to phosphorylated RTKs. The viral oncogene v-cbl lacks the RING finger motif, and displaces endogenous Cbl, to allow growth factor receptors to escape from downregulation. Similarly, loss of Cbl binding ability through mutation is a recurring theme in oncogenic deregulation of RTKs (Peschard and Park 2003). The ubiquitination of the Met receptor is a well-characterized example. Cbl is recruited to activated Met via Grb2, then subsequent binding of its TKB domain to the juxtamembrane autophosphorylated Tyr1003 is proposed to elicit a conformational change within Cbl necessary for activation. A Y1003F mutation inhibits ligand-dependent ubiquitination of Met and leads to cell transformation (Peschard et al. 2001).

Overexpression of c-Cbl, but not mutants lacking ligase activity, dramatically stimulate the degradation of EGFR, apparently without affecting the rate of EGFR internalization (Levkowitz et al. 1998; Thien et al. 2001). This observation was interpreted to reflect ubiquitin-dependent lysosomal sorting (Levkowitz et al. 1998). Further evidence that Cbl might be required for a later step than internalization has come from work done in $Cbl^{-/-}$ mouse embryonic fibroblasts. This work indicated that EGFR is internalized to endosomes at a normal rate in the absence of Cbl, but its degradation was inhibited (Duan et al. 2003). However, the exact site of Cbl-action is still much debated and various reports have since proposed a positive role for Cbl-dependent ubiquitination on the EGFR internalization step (de Melker et al. 2004; Huang and Sorkin 2005).

Cbl also acts as a multivalent adapter for at least 40 proteins and some of its

roles correspond to this adapter function rather than to its ligase activity. For example, a Cbl–Cin85–endophilin complex positively regulates both Met receptor and EGFR endocytosis independently of E3 ligase activity (Petrelli et al. 2002; Soubeyran et al. 2002).

4.5 Endosomal DUBs

Deubiquitinating enzymes (DUBs) regulate the ubiquitin status of endosomal proteins in opposition to E3 ligases. Deubiquitination is not considered to be an obligate step on the MVB sorting pathway as biosynthetic production of chimeric proteins incorporating ubiquitin results in efficient targeting to MVBs (Reggiori and Pelham 2001). DUB activity at the endosome may be necessary to maintain the pool of free ubiquitin required for endosomal sorting. On the other hand it can negatively regulate lysosomal protein degradation if it acts on ubiquitinated receptors prior to their commitment to the lysosomal pathway (Figures 4.4 and 4.5). DUBs implicated in endosomal sorting include the yeast proteins Ubp1, Ubp2 and Doa4, and the mammalian proteins UBPY (USP8) and AMSH.

4.5.1 Ubp1 and Ubp2

Overexpression of a soluble form of Ubp1 is able to stabilize the ABC-transporter Ste6 and the α-factor receptor Ste2, which are transported to the vacuole for degradation (Schmitz et al. 2005). The stabilization effect of Ste6 was shown not to be due to deubiquitination of Ste6 itself, suggesting that the target of Ubp1 may be a component of the protein-transport machinery. Ubp2 shows specificity for K63 over K48-linked polyubiquitin chains and can antagonize Rsp5 E3 ligase activity (Kee et al. 2005). Interestingly Ubp2 co-purifies with Rsp5 and is physically linked to Rsp5 through an adapter protein Rup1.

4.5.2 Doa4

The yeast protein Doa4 was originally shown to interact with the 26S proteasome and proposed to promote proteolysis through removal of ubiquitin from proteolytic intermediates on the proteasome (Papa et al. 1999; Papa and Hochstrasser 1993). It has also been proposed to maintain free ubiquitin levels by recycling ubiquitin from cargo molecules that have been committed to the lysosomal sorting pathway, prior to their sequestration away from the cytosol (Amerik et al. 2000; Swaminathan et al. 1999). In common with ESCRT complex components, Doa4 accumulates on the endosome following inactivation of the ATPase Vps4 (Amerik et al. 2000). Deletion of ESCRT III-complex components blocks this localization (Amerik et al. 2000; Luhtala and Odorizzi 2004) and a direct interaction with the ESCRTIII component Snf7 has been shown through two-hybrid screens (Bowers et al. 2004).

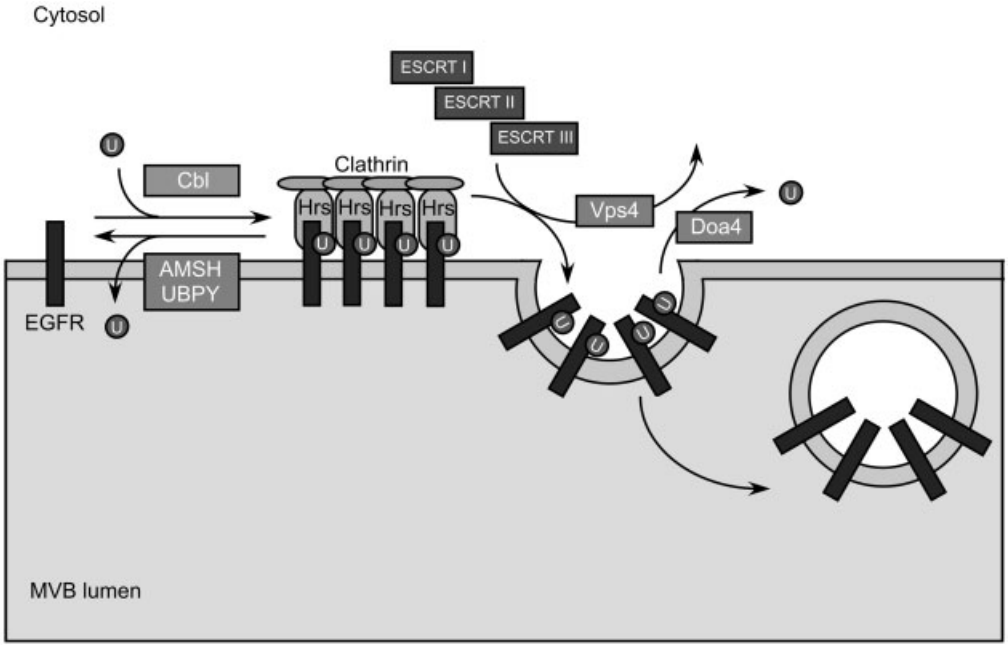

Fig. 4.4. Sorting of ubiquitinated receptors at the endosome. Ubiquitination of growth factor receptors (e.g., EGF receptor (EGFR)), by an E3 ligase (e.g. Cbl) promotes their interaction with Hrs in a Clathrin-coated microdomain. DUBs (e.g., AMSH and UBPY) may counteract Cbl activity and oppose MVB sorting and favour recycling. Hrs recruits the ESCRTI complex, which activates and assembles ESCRTII and III. This induces the translocation of the ubiquitinated receptor into internal vesicles of the MVB. Ubiquitin itself is recycled by a DUB (e.g., Doa4) just before, or in parallel with, the disassembly of the ESCRT-machinery by the AAA-ATPase Vps4, which irreversibly seals the sorting process. Note that this illustration combines elements from yeast and mammalian cells.

4.5.3 UBPY

UBPY (USP8), a member of the ubiquitin-specific processing protease (UBP) family, displays the highest similarity to Doa4 amongst mammalian DUBs. It accumulates upon growth stimulation of starved human fibroblasts and downregulates in response to growth arrest induced by cell–cell contact (Naviglio et al. 1998). A link to endosomal protein sorting was first suggested when UBPY was identified in a far-Western screen for Hbp–STAM binding partners (Kato et al. 2000). Mutagenic analysis identified a consensus sequence P*X*(V/I)(D/N)R*XX*KP as a binding module for interaction with the Hbp–STAM-SH3 domain-binding motif (Kaneko et al. 2003; Kato et al. 2000). This represents a novel SH3 binding motif lacking the canonical P*XX*P sequence. UBPY can hydrolyse both K48- and K63-linked chains as well as monoubiqitinated EGFR. Overexpression of UBPY retards EGFR degrada-

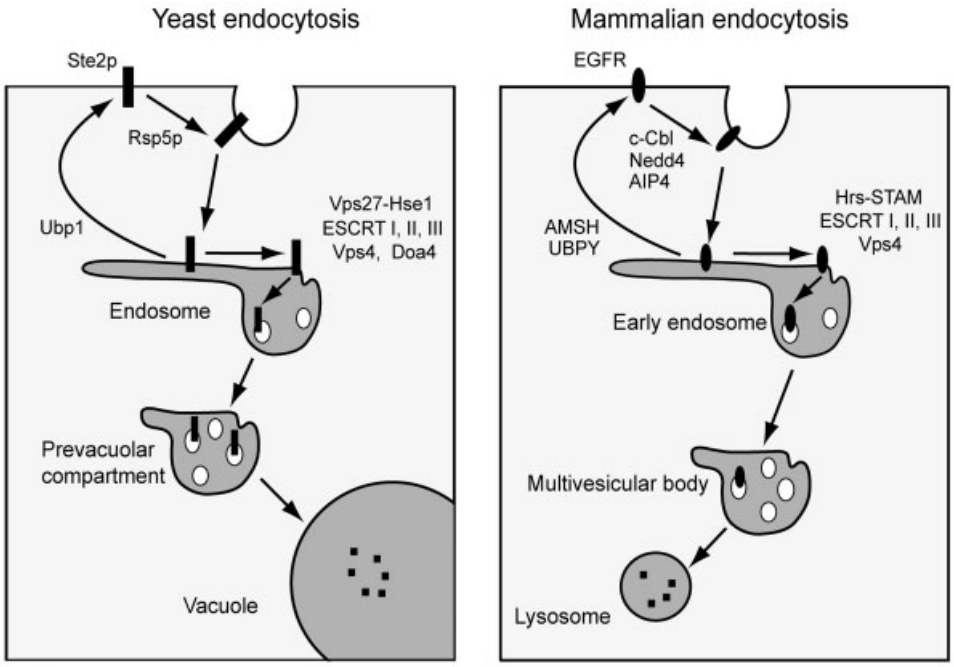

Fig. 4.5. The endocytic pathway in yeast and mammalian cells. Endocytosed proteins (e.g., Ste2 and EGFR) are sorted at the early endosome by multiple components of the multivesicular body (MVB) sorting machinery (Hrs–STAM, Vps27–HseI, ESCRTI–III). From here, cargo can be either recycled back to the plasma membrane or sorted to the MVB or prevacuolar compartments. Sorting to the lysosome and vacuole respectively is enhanced by the activity of E3-ligases (Rsp5, Cbl, Nedd4, AIP4), which may be opposed by DUBs (Ubp1, AMSH, UBPY).

tion whilst conflicting data have been reported for the effect of siRNA-mediated UBPY knock-down, which was found to either enhance (Mizuno et al. 2005) or inhibit EGFR downregulation (Bowers et al. 2005; Row, P.E., McCullough, J., Clague, M.J. and Urbé, S., unpublished observation).

UBPY can exist as a trimolecular complex with Otubain 1, a member of the OTU family of DUBs, and GRAIL, an E3 ligase crucial for the induction of CD4 T cell anergy (Soares et al. 2004). UBPY was shown to deubiquitinate GRAIL *in vitro* and Otubain 1 opposed this action by limiting the DUB activity of UBPY. UBPY-dependent deubiquitination has also been suggested to prevent degradation of the E3 ligase Nrdp1, which plays a role in regulating steady-state levels of ErbB3 and ErbB4 (Wu et al. 2004). Gnesutta et al. (2001) demonstrated an *in vitro* interaction between mouse UBPY and the N-terminal half of CDC25Mm, a Ras nucleotide exchange factor. This interaction may be functional in cells, since ubiquitination of CDC25Mm in HEK293 cells is diminished following co-expression of mUBPY.

4.5.4 AMSH

AMSH was originally isolated as a novel adapter molecule from human T cells that interacts with the SH3 domain of STAM (associated molecule with the SH3 domain) (Tanaka et al. 1999). Endogenous STAM was pulled down when AMSH was immunoprecipitated from IL-2 cell lysates in the presence of cross-linkers and both proteins were shown to interact when co-transfected into cells. Intriguingly, AMSH shares the P*X*(V/I)(D/N)R*XX*KP STAM/Hbp-SH3 domain-binding motif of UBPY (Kato et al. 2000). Thus, AMSH presumably competes with UBPY for binding to the SH3 domain of STAM.

AMSH belongs to the JAMM (JAB1/MPN/Mov34) metallo-enzyme family of deubiquitinating enzymes, of which the Rpn11–POH1 subunit of the 19S proteasome lid was the first described representative (Maytal-Kivity et al. 2002; Verma et al. 2002). No DUB activity was observed when Rpn11 was isolated from its multisubunit complex. In contrast, purified AMSH cleaves ubiquitin chains *in vitro*, and is hence the first JAMM-domain DUB to exhibit activity in isolation (McCullough et al. 2004). In common with Ubp2, AMSH displays specificity for K63- over K48-linked polyubiquitin chains (McCullough et al. 2004).

AMSH localizes to endosomes and an inactivating mutation in the JAMM domain of AMSH was shown to promote the accumulation of ubiquitin on endosomes (McCullough et al. 2004). Concomitantly, this mutant stabilizes an ubiquitinated form of STAM, which is contingent on an intact UIM within STAM. This led us to suggest that ubiquitin, which is appended to STAM in a UIM-dependent fashion and which would normally be removed by either AMSH or UBPY, may provide an additional binding site for enzymatically inactive AMSH. Hence, the inactive mutant of AMSH could act as a ''substrate trap'' mutant.

Ubiquitinated EGFR provides a substrate for AMSH *in vitro* and siRNA-mediated knock-down of AMSH enhances the degradation rate of EGFR (McCullough et al. 2004). We have proposed a model for the role of AMSH on endosomes in which AMSH can counteract the E3 ligase activity of c-Cbl on EGFR, before the ubiquitinated receptor has been committed to the lysosomal sorting pathway (Figure 4.4). AMSH activity will therefore favour recycling of the receptor. Note that existing data suggest that either AMSH or UBPY can fulfil this role (Figure 4.5).

AMSH also associates with a number of signalling molecules. Signalling defects in cells derived from AMSH-deficient mice were not obvious, although the mice die at about three weeks of age (Ishii et al. 2001). It was originally hypothesized that AMSH may play a role in cytokine-mediated signalling through its interaction with STAM (Tanaka et al. 1999). A novel Grb2 family member, Gads/Grf40, also associates with AMSH (Asada et al. 1999). Gads has been shown to be involved in T-cell receptor (TCR) signalling; Gads knock-out or Gads ΔSH2 transgenic mice show impairment in pre-T-cell development. In addition, AMSH also interacts with inhibitory Smads (I-Smads), and this association negatively regulates their function, and thereby promotes bone morphogenetic protein (BMP)-mediated signalling (Itoh et al. 2001).

Li and Seth (2004) have demonstrated that AMSH itself is ubiquitinated by the E3 ligase Smurf2. This is contingent on both proteins binding to the adaptor molecule RFN11 and leads to a reduction in steady-state levels of AMSH by proteasomal degradation. We can now see that association of E3 ligase activity with DUB activity is a recurring theme common to UBP2, UBPY and AMSH.

4.6 Polyubiquitin Linkages and Endocytosis

Although monoubiquitination may represent a minimal requirement for endosomal sorting and RTKs do not seem to incorporate polyubiquitin chains, there is a large body of work suggesting polyUb involvement in some sorting events.

4.6.1 Proteasome Involvement in Endocytic Sorting

The downregulation of a sub-set of RTKs and other receptors requires proteasomal activity. In most cases studied so far this requirement does not reflect proteasomal degradation of the receptor *per se*. Rather, this activity is permissive for receptor sorting towards the lysosomal degradation pathway. Thus receptor downregulation can be sensitive to both proteasome inhibitors (e.g. lactacystin) and inhibition of lysosomal acidification (e.g. concanamycin). K48-linked polyubiquitin chains specify proteasomal degradation and are therefore indirectly implicated in the lysosomal sorting process. Well-characterized examples include interleukin-2 receptors (Rocca et al. 2001), Growth Hormone Receptor (GHR) (van Kerkhof et al. 2000) and the RTK Met (Hammond et al. 2003; Hammond et al. 2001). In each case, inhibition of the proteasome promotes recycling of internalized receptors at the expense of sorting to lysosomes. Interestingly, ubiquitination of GHR itself appears to be dispensable for downregulation, which has led to a model in which polyubiquitination and proteasomal degradation of an unidentified accessory factor is required (van Kerkhof et al. 2000). An example of such a scenario may be found in neurons, where the endocytosis of AMPA-type glutamate receptors also requires ubiquitination and proteasomal degradation of the scaffolding protein PSD-95 (Colledge et al. 2003).

Proteasome inhibition could reduce free ubiquitin levels leading to a block in endocytosis. In the case of Met receptor the inhibitory effect of lactacystin can be overcome by overexpression of ubiquitin, but not by a form of ubiquitin unable to form K48 linkages (K48R) (Carter et al. 2004). It is baffling that a K48-linkage dependence is observed in the presence of a proteasomal inhibitor. Does this represent an entirely novel function for K48-linked ubiquitin, which has previously been uniquely associated with a proteasomal targeting signal?

One scenario may be that the critical step for lysosomal sorting consists of the sequestration of a polyubiquitinated accessory factor away from Met, which is followed by incidental proteasomal degradation. In this model, proteasome activity is

only required to generate free ubiquitin (possibly locally), which can contribute to K48-chain formation.

4.6.2 K63-linked Ubiquitin

Pioneering studies in yeast have provided evidence for a role of K63-linked polyubiquitin in vacuolar sorting. Gap1p and Fur4p have both been shown to be modified with short (2–3) K63-linked polyubiquitin chains (Blondel et al. 2004; Galan and Haguenauer-Tsapis 1997; Springael et al. 1999). In *doa4*Δ cells, which show low levels of endocytosis due to limited availabilty of ubiquitin, the endocytosis rate can be restored by overexpression of wild type or K48R ubiquitin but not by K63R ubiquitin, which can participate in monoubiquitination events but cannot form K63-linked chains. K63-linked chains are well represented in the pool of total cellular polyubiquitin (Peng et al. 2003). It may well be that in the excitement over monoubiquitination, we have underestimated the role of K63-linked chains in lysosomal sorting. After all, these can provide higher affinity interactions with ubiquitin-binding domains, whilst still avoiding proteasomal degradation.

4.7 Future Directions

Now that most of the core components of the ubiquitin-dependent MVB sorting pathway have been identified, the challenge lies in elucidating the choreography underlying this complex process. How is cargo passed between ESCRT complexes or is the whole process more co-operative? The significance of the ubiquitin-chain topology is likely to receive more attention and more ubiquitin-binding domains with distinct specificities remain to be identified. Allied to this one may consider that ubiquitin may not simply be a tag for sorting at the endosome but may coordinate spatially segregated signalling events through recruitment of adapter proteins and enzymes with ubiquitin-binding domains. The endosome may thus provide a ''hot spot'' for ubiquitin-dependent signalling.

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5
ISG15-dependent Regulation

Arthur L. Haas

5.1
Introduction and Overview

Cells regulate their short-term responses to internal and external signals from the environment through a repertoire of post-translational modifications that alter protein function. This regulatory strategy is ancient in origin, remarkably conserved across phyla, and evolutionarily robust – allowing novel biological applications to arise in response to new challenges. The emergence of eukaryotes with their more complex organizational demands is marked by the appearance in the genomic record of new regulatory strategies involving post-translational modifications that, for the first time, involved low-molecular-weight proteins as modifying groups rather than small inorganic/organic molecules. Ubiquitin represents the first example of this new class of post-translational modifying proteins identified [1–3]. Based on sequence and structural conservation, the bacterial molybdopterin cofactor synthase complex presumably served as the evolutionary template from which ubiquitin and its essential activating enzyme diverged [4–6]. Functional aspects of ubiquitin-dependent regulation must have been established relatively soon after radiance of eukaryotes since the polypeptide and most components of the requisite ligation pathways are remarkably conserved across phyla [7]; however, the absolute conservation of the ubiquitin sequence among higher eukaryotes suggests that the polypeptide has continued to acquire new roles, evidence of which is seen in the phylogeny of the E2/Ubc superfamily [8].

The regulatory advantage of this strategy cannot be over-emphasized. Because ubiquitin has a larger and more varied water-accessible surface than small-molecule modifiers, ubiquitin possesses a greater inherent information content. Moreover, the driving forces of gene speciation and natural selection can be exploited to adapt and mould this signalling molecule in ways not possible with immutable smaller post-translational modifying groups such as phosphate. In practical terms these advantages allow ubiquitin to serve as a reversible transposable binding element to alter target protein structure and/or target protein ligand interactions. Ubiquitin molecules can also be linked together to form repeating chains in order to amplify the ubiquitin signal and provide additional diversity. Because

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ubiquitin chains of defined linkage specificity must pack into distinct structures, the total repertoire of signalling potential is greatly expanded compared to simple monoubiquitination.

The success of ubiquitination as a genetically plastic regulatory strategy is best appreciated by considering the rapid evolutionary divergence and protein speciation of the polypeptide into a family of ubiquitin-like modifiers that includes SUMO/Smt3 [9, 10], Nedd8/Rub1 [11, 12], Hub1 [13], Apg12 [14], Aut7/GATE16 [15, 16], URM1 [17], FAT10 [18, 19], and ISG15 [20, 21], among others. Interestingly, the last three ubiquitin-like proteins exist only among higher eukaryotes, with no readily identified orthologs obvious among plants or fungi. This immediately suggests that these ubiquitin-like proteins arose late in evolution, as components of newly emerging functional pathways not required of the more widely expressed members of the ubiquitin-like protein superfamily. The ISG15 family represents the first example of a ubiquitin-like protein identified [20], predating the discovery of SUMO, Nedd8, and other members of this superfamily. Sequence conservation between ISG15 and ubiquitin, particularly in the canonical LRLRGG carboxyl terminal sequences of the two polypeptides, immediately suggested that the biological role(s) of ISG15 were expressed through its conjugation to specific cellular protein targets [20]. Our rudimentary understanding of ISG15 signalling currently reveals a complexity not fully anticipated from earlier predictions.

5.2 The Discovery of ISG15

The protein subsequently identified as ISG15 (GIP2/IFI15) was initially described by Farrell et al. as a constitutively expressed 14.5-kDa polypeptide whose protein and mRNA levels were markedly induced in Ehrlich ascites tumour cells in response to murine interferon [22]. Pulse-chase studies provided no indication of a precursor–product relationship in the interferon-induced accumulation of the nascent 15-kDa polypeptide; however, inhibition of ISG15 accumulation by actinomycin D suggested transcriptional regulation of ISG15 mRNA, which could be detected within seven hours of interferon treatment – the earliest gene product induced by the cytokine [22]. Subsequent observations by Knight and coworkers established ISG15 as an important primary response to interferon induction [23–25], providing later investigators with a robust genetic marker for monitoring early events in the interferon signalling pathway [26]. Korant et al. reported induction of the same 15-kDa protein in Daudi (human lymphoblast) and MDBK (bovine kidney) cells at concentrations of Type 1 (IFNα/β) but not Type 2 (IFNγ) interferons that elicited an antiviral phenotype, the first apparent evidence of a cytokine-specific response for ISG15 induction [23]. Purification of small amounts of human ISG15 from cell culture [23] and later refinements in the protocol by Blomstrom and coworkers allowed direct sequencing of the amino terminal 85% of the polypeptide, which was used to validate the inferred sequence derived by cDNA sequencing [24]. Rudimentary sequence comparisons using the limited

databases and search algorithms available at the time suggested that ISG15 represented a unique protein not previously identified [24].

Contemporaneously, Haas et al. independently identified ISG15 as a 15 kDa band by SDS-PAGE that was recognized by affinity-purified rabbit polyclonal antibodies against human ubiquitin while examining the effect of viral infection on steady-state ubiquitin pools within selected cell culture lines [20]. This ubiquitin cross-reactive protein (UCRP) was strongly induced by Type 1 interferons at concentrations as low as 3 IU ml^{-1} ($K_{1/2}$ for induction = 150 IU ml^{-1}), but much less so by Type 2 interferons, in human A549 (human lung carcinoma) cultures and could be detected as early as two hours after addition of IFNβ [20]. Temporal studies demonstrated that induction of ISG15 protein quantitatively paralleled the appearance of an antiviral response, suggesting a direct causal relationship [20]. The ability of ubiquitin-specific antibodies to recognize ISG15 was rationalized by noting the remarkable but cryptic sequence similarities between ISG15 and ubiquitin that had not been previously appreciated, as well as a symmetric pattern of conserved residues that predicted the adoption by ISG15 of a tandem ubiquitin-like fold (Figure 5.1). Paradoxically, the carboxyl terminal epitope recognized by the ubiquitin-specific antibodies was absent from the published sequence of the ISG15 protein [27]; however, Haas et al. recognized that a base substitution at the second nucleotide of codon 146 transformed the predicted STOP codon into a Ser codon and allowed read-through to generate the canonical LRLRGG ubiquitin carboxyl terminal motif linked to an extension peptide, the latter feature now recognized as a hallmark of all Class 1 ubiquitin-like proteins [7, 20].

5.3
Structure and Properties of the ISG15 Protein

The mature human ISG15 protein is composed of 157 amino acids (17 171 Da) arranged into two ubiquitin-like domains that exhibit symmetry in the pattern of residues that are conserved with ubiquitin [20] (Figure 5.1). Each domain possesses a pattern of six conserved large aliphatic residues that constitute the nearly immutable defining sequence motif for the β-grasp fold and the α-helix/β-sheet interface that forms the hydrophobic core of all ubiquitin-like proteins. The domains are connected through a poorly conserved linking peptide corresponding in position to the LRLRGG ubiquitin carboxyl terminus of the amino terminal domain (Figure 5.1). Sequence divergence in the linking peptide from that of the paralogous ubiquitin carboxyl terminus presumably serves to block the activity(ies) responsible for the rapid post-translation processing of carboxyl terminal extensions from ubiquitin-like proteins that would otherwise result in cleavage and inactivation of the two ISG15 domains [20]. A prolyl residue (Pro^{81}, human numbering) positioned between the linking peptide and the carboxyl terminal domain, originally noted in human ISG15, was thought to assist in blocking domain cleavage [20, 21]; however, this residue is not well conserved among subsequent additions to the seven extant sequences (Figure 5.1).

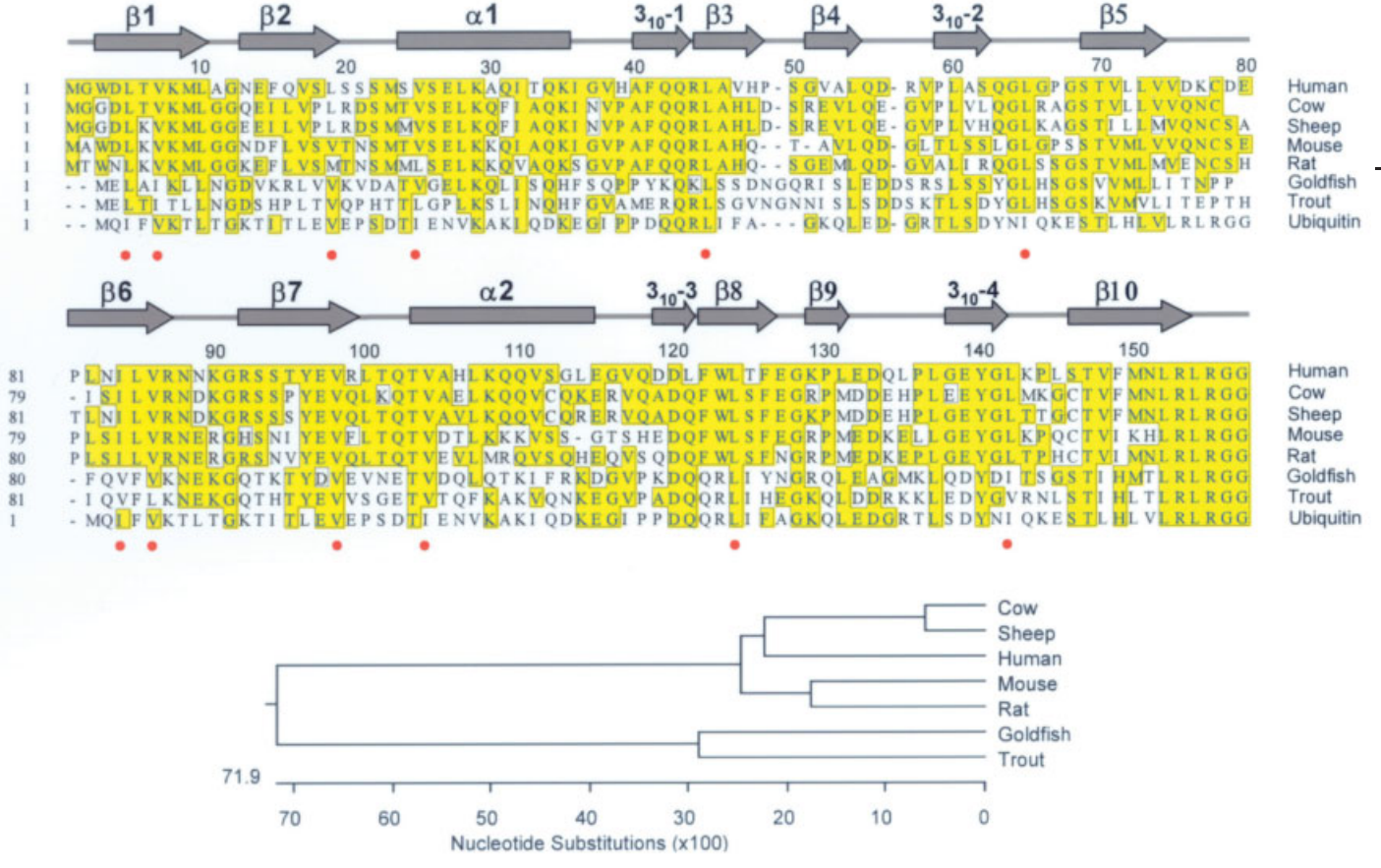

Fig. 5.1. Sequence comparison of ISG15 orthologs to ubiquitin. Upper panel: Sequences for the extant ISG15 orthologs are compared to human ubiquitin using Clustal W and a PAM250 residue weight table. Sequences are segregated into the two ubiquitin-like domains based on sequence homology. Secondary structural elements based on the 2.4 Å crystal structure are schematically shown above the sequences [28]. The six conserved aliphatic hydrophobic residues that constitute a structural motif for the β-grasp fold are denoted by dots. Lower panel: Phylogenetic comparison of the ISG15 orthologs.

The ISG15 orthologs exhibit considerably less sequence conservation than is found among ubiquitin orthologs, which are absolutely conserved among vertebrates [8]. As expected, ISG15 sequence conservation decreases with evolutionary distance from humans (Figure 5.1). The lower sequence conservation is typical of a pattern observed with other ubiquitin-like proteins and likely reflects the greater selective pressures on ubiquitin, due to a more diverse repertoire of functional roles, than required of the more specialized ubiquitin-like polypeptides. The carboxyl terminal domains of ISG15 exhibit significantly more sequence conservation than the amino terminal domains, even when adjusted for the contribution of the canonical LRLRGG sequence, indicating greater selective pressure on the carboxyl terminal domain that must arise from constraints imposed by protein interactions with downstream ISG15-conjugating enzymes [20]. The sequence of ISG15 is otherwise unremarkable, containing neither recognizable interaction nor phosphorylation motifs. However, one residue of note is Cys^{78}, which readily forms a homodimeric ISG15 disulfide bond [28]. The resulting disulfide-linked ISG15 homodimer is a thermodynamically metastable structure that rapidly and irreversibly denatures [28]. Mutation of Cys^{78} to serine stabilizes the ISG15 structure by 3.3 kcal mol^{-1} and produces a polypeptide whose overall stability is more typical of β-grasp fold structures and approaches that of ubiquitin [28], allowing large-scale expression and purification of the intact protein.

Inherent instability of the wild-type ISG15 polypeptide accounts for early observations that recombinant protein spontaneously precipitated from solution at concentrations above ca. 100 μg ml^{-1}, thwarting efforts to crystallize the polypeptide or to study *in vitro* conjugation [21, 23, 24]. Early efforts to study *in vitro* conjugation of ISG15 were also confounded by the rapid proteolytic inactivation of recombinant ISG15 through cleavage of the carboxyl terminal glycine dipeptide from the mature protein by a bacterial periplasmic carboxypeptidase, a tendency exhibited by all recombinant ubiquitin-like proteins possessing an RGG carboxyl terminus [29]. However, strategies have been developed that allow quantitative expression of intact recombinant mature polypeptide using either an arginine cap to protect the glycine dipeptide, followed by carboxypeptidase B processing to remove the cap residue [28], or expression in an *Escherichia coli* AR58 strain that lacks the periplasmic carboxypeptidase responsible for inactivation (J. M. Klein and A. L. Haas, unpublished observation).

The 2.4 Å crystal structure for the mature human ISG15 protein confirms the tandem ubiquitin-like domain architecture predicted from the symmetric pattern of conserved residues; formally, the protein assumes tandem β-grasp folds [28] (Figure 5.2). Within this context, it is more appropriate to refer to the domain structure as a β-grasp fold rather than a "ubiquitin fold" since the former is one of thirty highly populated metafold families identified in proteins while ubiquitin is only one member of the larger β-grasp protein family [30]. The carboxyl terminal RGG segment is not resolved in the structure, reflecting the marked structural mobility of this highly water-accessible region that is typical of all ubiquitin-like protein families [28]. The overall fold of each domain is remarkably conserved with that of ubiquitin and is unaffected by mutation of Cys^{78}. The two β-grasp domains

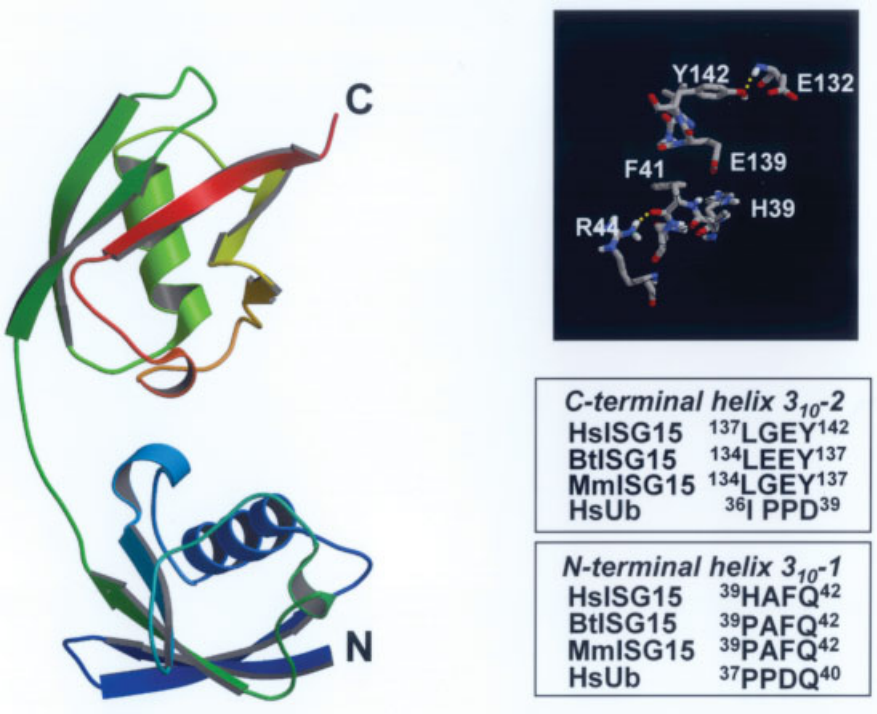

Fig. 5.2. Crystal structure of human ISG15. Left panel: The 2.4 Å crystal structure for Human ISG15 harbouring a C78S point mutation to enhance solution stability against spontaneous denaturation [28]. Colours for the ribbon diagram range from dark blue for the amino terminus to red for the carboxyl terminus. Residues 155–157 are disordered and not represented in the figure. Upper right panel: Structural details of the interactions between the 3_{10} helices at the interdomain interface. Lower right panel: Sequence conservation in the interacting 3_{10} helices for human (HsISG15), bovine (BtISG15), and mouse (MmISG15) compared to human ubiquitin (HsUb).

are set at an angle and this orientation is stabilized by hydrophobic interactions between two conserved 3_{10} helices and other stabilizing hydrogen bonds (Figure 5.2). The extensive buried contact surface between the two domains and conservation in the sequences of the interacting 3_{10} helices, which are distinct from that of ubiquitin, suggest that there is little interdomain flexibility in solution [28]. Observed deletions in the linking peptide segment among ISG15 orthologs (Figure 5.2) requires subtle adjustments in domain packing, suggesting that the functional contributions of the two domains may be largely independent. The computed charge distribution of human ISG15 reveals a pronounced ridge of acidic residues extending down the long axis of the structure; in addition, nearly half of the water-accessible surface of the amino terminal domain represents a conserved apolar region of indeterminate function [28].

Human ISG15 protein is expressed as a precursor bearing an eight-residue

carboxyl terminal extension peptide [20, 26]. Expression of nascent polypeptides having a carboxyl terminal extension is a common feature of ubiquitin and all ubiquitin-like proteins examined to date, the function of which is uncertain since processing occurs nearly co-translationally. The carboxyl terminal extensions of ISG15 orthologs are not well conserved, suggesting that the overall sequence is irrelevant to folding or structural stability [28, 31]. This conclusion is supported by the consistently good yield in expression of recombinant mature protein [28]. Biochemical studies demonstrate that proISG15 processing is catalyzed by the ubiquitin-specific protease Ubp1, for which the propeptide serves as a low-affinity alternative substrate [32]. Processing of proISG15 to the mature active form is stimulated 12-fold by physiological concentrations of free ubiquitin, common for ubiquitin-specific proteases involved in disassembling polyubiquitin chains. Intracellular Ubp1 exists in soluble and membrane-anchored forms transcribed from the same gene, presumably resulting from alternative splicing, and normally functions to regulate turnover of the ATP-binding cassette-transporter Ste6 in the endocytic pathway [33]. A second proISG15-processing activity of 30 kDa representing ca. 1% of total ubiquitin-stimulated Ubp1 activity suggests overlapping functions in processing [32]. The alternate activity is not the putative ISG15-specific protease Ubp43/Usp18 (USP18), which is considerably larger than 30 kDa and is catalytically inactive in processing proISG15 [34]. More likely, the alternative processing activity is contributed by members of the ubiquitin carboxyl-terminal hydrolase (UCH) family of ubiquitin-specific proteases that serve a recycling function by cleaving low-molecular-weight peptides from the carboxyl terminus of ubiquitin [35].

5.4 The ISG15 Conjugation Pathway

The biological effects of intracellular ISG15 are mediated through its covalent ligation to cellular proteins [21]. Mass spectrometric-based proteomics have been exploited to identify a number potential targets for this post-translational modification [36, 37]. Conjugation of ISG15 occurs through an enzyme pathway distinct from that of ubiquitin [29]; however, the conjugation of ubiquitin and other ubiquitin-like proteins, including ISG15, share a common mechanism requiring three classes of components that are highly conserved but specific for their cognate polypeptide [8, 38, 39]. The overall reaction of ISG15 conjugation formally belongs to the ligase enzyme class. As is typical of all enzymes of this class, the mechanism of ISG15 conjugation proceeds through two half-reactions: an ATP-coupled activation step generating a high-energy intermediate and a ligation step in which cleavage of the high-energy intermediate is coupled to new bond formation. By analogy to the mechanism for ubiquitin, and also shown experimentally to be conserved for Nedd8 activation [40–42], an ISG15 activating enzyme (E1) couples ATP hydrolysis to the activation of the carboxyl terminus of ISG15 to generate a ternary complex composed of covalently bound ISG15 thiolester and a noncova-

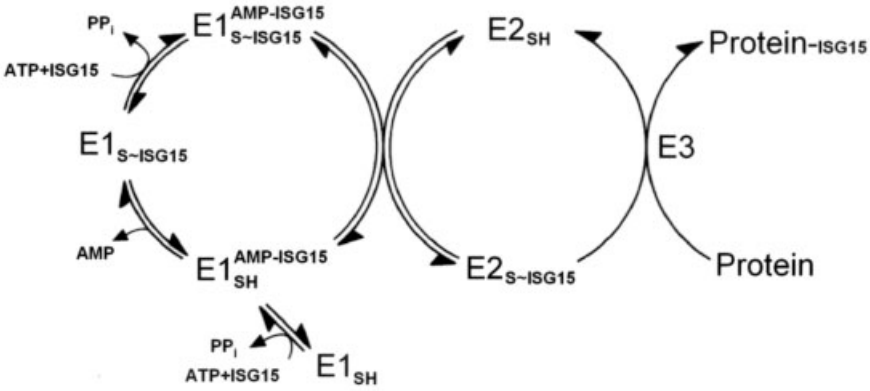

Fig. 5.3. Mechanism of ISG15 conjugation. The mechanism of ISG15 conjugation is composed of three enzymes as described in the text. E1: ISG15 activating enzyme; E2: ISG15 carrier protein (UbcH8); E3: ISG15 isopeptide ligase.

lent but tightly bound ISG15 adenylate intermediate which serves as the immediate precursor for the thiolester (Figure 5.3). Aminolysis of the high energy ISG15 thiolester is ultimately coupled to ligation of the polypeptide to free ε-amino lysyl residue(s) on the target protein in a step catalyzed by ISG15 isopeptide ligase(s), E3. The two half-reactions are functionally linked through an E2 carrier protein (Ubc) that shuttles activated ISG15 between the two half reactions as an E2–ISG15 thiolester.

5.4.1 Activation of ISG15 by UbE1L

The obligatory ATP-coupled activation step for ISG15 is catalyzed by the late interferon-inducible enzyme UbE1L (UBE1L), a ca. 112-kDa paralog of the Uba1a (UBE1) ubiquitin-activating enzyme. The UbE1L protein was identified as the activating enzyme for ISG15 by Yuan et al. while examining the ability of the influenza B protein NS1B to block ISG15 conjugation, the mechanism of which involves specific binding to and sequestering of ISG15 from activation by UbE1L [43]. Kok et al. originally identified human UbE1L as a Uba1-like protein of undetermined function that was virtually absent from lung tumour-derived cell lines, due to a deletion in the 3p21 chromosomal region [44], but widely expressed among other tumour and non-tumour cell lines, suggesting UbE1L functioned as a tumour suppressor [45]. Subsequent immunological studies confirmed that UbE1L protein levels were below the limit of detection in lung cancer-derived cell lines and tissues but abundant in normal cells and tissues [46]. Human UbE1L (1012 amino acids) and Uba1a (1058 amino acids) exhibit significant overall sequence conservation (49% identity) that is typical of other activating enzyme paralogs including those for SUMO and Nedd8, suggesting they diverged from Uba1a;

however, the 40-residue amino terminal nuclear localization peptide present on Uba1a is absent from the activating enzymes for UbE1L, SUMO, and Nedd8 [28, 47]. Interestingly, immunohistochemical localization in human lung tissue sections reveals abundant UbE1L expression in bronchial macrophages and bronchial epithelium, presumably reflecting the role of ISG15 in cellular innate immunity [48]. Within the bronchial epithelial cells, UbE1L is distributed within the cytoplasm, nucleus, and apical membrane [48].

Each activating enzyme is absolutely specific for its cognate polypeptide and the structurally paralogous positions corresponding to Arg^{72} of ubiquitin appear to be particularly critical in allowing the activating enzymes for ubiquitin, SUMO, and Nedd8 to discriminate among cognate and noncognate polypeptides [42, 49–51]. However, Arg^{72} of ubiquitin is conserved in the paralogous position within the carboxyl terminal domain of ISG15 (Figure 5.1), indicating that another residue(s) must direct specificity. The significant sequence conservation among the various E1 paralogs for the ubiquitin-like proteins allows one to infer a great deal about UbE1L. Structural conservation in the adenylate active site between the heterodimeric AppBp1–Uba3 activating enzyme for Nedd8 [50, 52] and the heterodimeric Sae1–Sae2 activating enzyme for SUMO [51] permits one to use these datasets to model a structure for UbE1L [28]. The nearly identical folds for ISG15 and Nedd8 provide the basis for a docking simulation of ISG15 bound to UbE1L, in which the structure for the carboxyl terminal domain of ISG15 is superimposed on that of Nedd8 bound within the adenylate active site of AppBp1–Uba3 [28]. The adenylate active site easily accommodates the carboxyl terminal domain of ISG15 without physically engaging the amino terminal domain [28]. The docking simulation suggests candidate side-chain interactions that allow UbE1L to distinguish ISG15 from noncognate ubiquitin-like proteins, including (in order of predicted importance) $Lys^{90} > Trp^{123} > Phe^{149} > Arg^{87}$ on ISG15 [28].

Parsimony between the mechanisms for UbE1L and Uba1 has recently been empirically confirmed (A. L. Haas and J. M. Klein, in preparation), including the predicted stoichiometry of the UbE1L ternary complex shown in Figure 5.3 and binding affinities for ATP·Mg^{2+} (17 μM) and ISG15 (0.5 μM) that are comparable to those found for human Uba1 and human AppBp1–Uba3 [42, 53]. In the pathways responsible for ubiquitin conjugation, the Uba1-catalyzed activation step is generally never rate limiting [8]. This may not be the case with UbE1L since its k_{cat} for transthiolation to the cognate UbcH8 ISG15-conjugating enzyme (see below) is 100-fold lower than that for the Uba1-catalyzed reaction with ubiquitin (A.L. Haas and J.M. Klein, in preparation). It remains an open question whether some additional regulatory or allosteric step is required in order to enhance the unusually low activity of UbE1L; however, this attenuated activity is consistent with the requirement for additional UbE1L expression in order to observe enhanced *in vivo* ISG15 conjugate pools in cultured cells [36, 54]. Human UbE1L and its cognate E2 isoform UbcH8 are constitutively expressed at low levels in normal cells but are significantly induced by Type 1 interferons [43, 55, 56]. Together with the induction of ISG15 in response to Type 1 interferons, this represents a coordinated upregulation in the ISG15 ligation pathway that appears to

drive the accumulation of ISG15 conjugates. Considering the significantly lower k_{cat} for recombinant UbE1L noted earlier, substrate recognition may be a passive step in ISG15 conjugation so that targeting of all available substrates increases in concert. Marked similarities between the distribution of ISG15 conjugates prior to and following interferon induction have been noted previously, suggesting simple upregulation rather than a generalized ligation of novel proteins in response to the cytokine [21]. Human UbE1L is also induced as an early gene product in response to all-*trans* retinoic acid treatment of various cultured cell lines [57]. Retinoic acid induction of UbE1L, ISG15, and ISG15 conjugates in NB4 promyelocytic leukaemia cells signals degradation of the PML/RARα repressor and triggers subsequent apoptosis, a response proposed to account clinically for retinoic acid-induced remission [57, 58]. As noted earlier, the UBE1L gene product also appears to function as a tumour suppressor in lung cancer [44]. The short arm of chromosome 3 within the 3p21 region has long been assumed to harbour a tumour suppressor, since this region is consistently deleted in both small-cell and non-small-cell lung carcinomas [44, 48, 59]. A ubiquitously expressed candidate tumour suppressor gene from this region, later recognized for its homology to Uba1 [45], was identified whose mRNA and protein were consistently undetectable in various lung cancers [44, 48]. That UbE1L functions as a *bona fide* tumour suppressor is supported by the chemiopreventive effect of all-*trans* retinoic acid treatment in blocking transformation of immortalized human bronchial epithelial cells for which subsequent microarray analysis implicates UbE1L as a candidate target gene [48]. However, it is unclear in lung carcinogenesis whether UbE1L similarly functions to downregulate PML/RARα.

5.4.2 UbcH8 is an ISG15-specific Conjugating Enzyme

The E1-catalyzed activation step constitutes the principal point of specificity for ensuring the fidelity of target protein modification by the ubiquitin-like modifiers, since rigorous empirical evidence indicates that these activating enzymes are absolutely specific for their cognate ubiquitin-like proteins [29, 42], as discussed previously [8]. In the ubiquitin-conjugation pathways, transthiolation to yield an E2-ubiquitin thiolester in turn represents the first step for partitioning activated polypeptide among contemporaneous signalling events that are distinguished by the specificity of the relevant ligases for their cognate E2 paralogs and targets. Therefore, the E2 step represents a potential point for regulating the repertoire of ligation pathways available to the cell. Overt regulation of an E2 isoform by phosphorylation has been shown for Ubc2/Rad6-dependent histone ubiquitination in cell cycle progression and transcriptional control [60]. More frequently, regulation occurs as a cellular ''change of state function'' by alterations in the intracellular concentrations of specific E2 isoforms. Thus, identifying the cognate E2 for a ligation pathway represents an important step in understanding functionality.

Studies by Zhao et al. [56] and Kim et al. [54] demonstrate that conjugation of ISG15 absolutely requires the E2 isoform UbcH8 (UBE2L6). Members of this

E2 family were first identified in humans, and database screens demonstrate that UbcH8 orthologs are found only among higher eukaryotes [8, 61], a pattern that mirrors the phylogenetic distribution of ISG15 and UbE1L. The 153-residue polypeptide (17 767 Da; human isoform) is distinct from the similarly named Ubc8 (UBE2H) family of E2 ubiquitin-conjugating enzymes, which is distributed among all eukaryotes and which functions in a set of distinct regulatory pathways unrelated to those of ISG15 [8, 62, 63]. Since higher eukaryotes frequently express functionally indistinguishable isozymes belonging to the same E2 family [8], UbcH8 was initially thought to represent an isozyme of UbcH7 (UbE2L3) because of considerable sequence identity between the polypeptides and their identical distribution among higher eukaryotes [8, 64, 65] (Figure 5.4). However, UbcH8 is distinguished from UbcH7 in being a late interferon- and retinoic acid-inducible gene whereas UbcH7 expression is unaffected by both agents [54, 56]. In addition, UbcH7 orthologs show much higher sequence conservation than those of UbcH8 and contain a characteristic pattern of conserved residues that suggests the former represents a distinct family within the Ubc4/5 clade of the E2 superfamily [8] (Figure 5.4). Finally, microarray analysis reveals that UbcH7 and UbcH8 exhibit somewhat different expression patterns in human tissues and cell lines [66].

The UbcH8 protein was originally identified as a ubiquitin-conjugating enzyme based on the ability of Uba1 ternary complex to catalyze UbcH8 transthiolation [67]. In turn, UbcH8–ubiquitin thiolester is proposed to support various ubiquitin-conjugation pathways including the Hect-domain ligase E6AP responsible for p53 and E6TP1 targeting in human papilloma virus-mediated cervical cell transformation [61, 68–70], the RING finger ligase Parkin whose loss-of-function mutation is responsible for some forms of familial juvenile Parkinsonism [71, 72], the centrosome-associated RING finger ligase Dorfin, which is thought to target superoxide dismutase-1 (SOD1) for proteasomal degradation and which displays a protective effect in amyotrophic lateral sclerosis (ALS) [73], and the RING finger ligase Staring, which targets syntaxin-1 in regulating neurotransmitter release [74], among others. More recent studies show that transient ablation of the BRCA2 tumour suppressor expression in human mammary epithelial cells and human breast carcinoma cells results in downregulation of ISG15 and UbcH8 expression, suggesting a role for UbcH8-mediated ISG15 ligation in breast tumour promotion [75].

Definitive empirical evidence for assigning an E2 to a specific conjugation pathway ideally should be predicated on carefully designed functional studies in which the concentrations of components are known, since members of the E2 superfamily exhibit considerable sequence similarity and the subset of residues that allow E1 paralogs to recognize their cognate E2 isoforms is not well established. Experiments in which the concentrations of active components are not considered risk creating conditions in which the *in vitro* or *in vivo* levels may drive otherwise unfavourable binding interactions and lead to erroneous conclusions regarding specificity, as discussed previously [8]. This issue is particularly critical for UbcH8 since it is proposed to represent a point at which the ubiquitin and ISG15 ligation pathways converge [54, 56], a hypothesis that violates the principle of parallel but

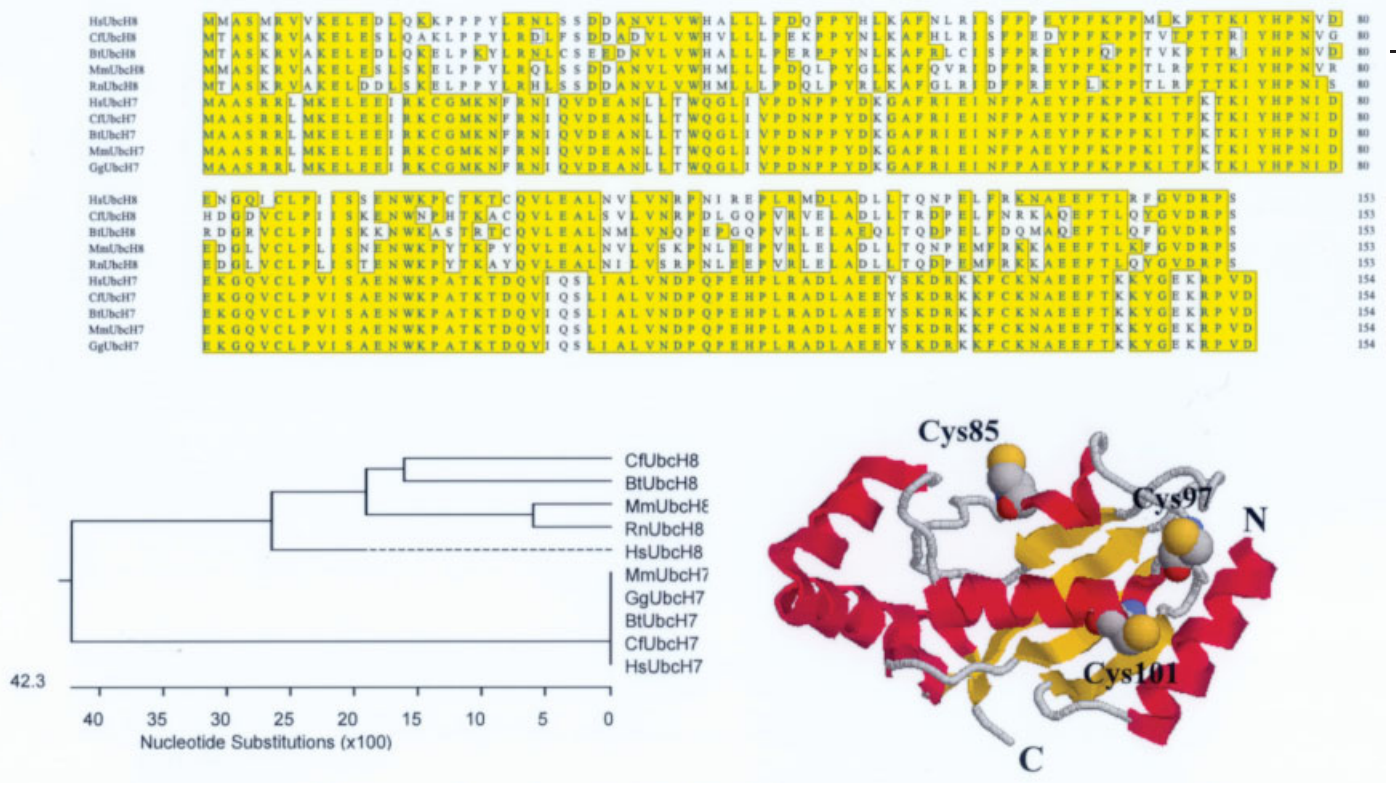

Fig. 5.4. Sequence comparison of UbcH7 and UbcH8 orthologs. Top panel: Sequence comparison of extant UbcH7 and UbcH8 orthologs by the Clustal W method using a PAM250 residue weight table. Lower left panel: Phylogenetic comparison of UbcH8 and UbcH7 orthologs. Lower right panel: Crystal structure of human UbcH8 showing the relative position of the active site Cys^{85} and the other non-catalytic Cys^{97} and Cys^{101}. Cf: dog; Bt: cow; Mm: mouse; Rn: rat; Hs: human Gg: chicken.

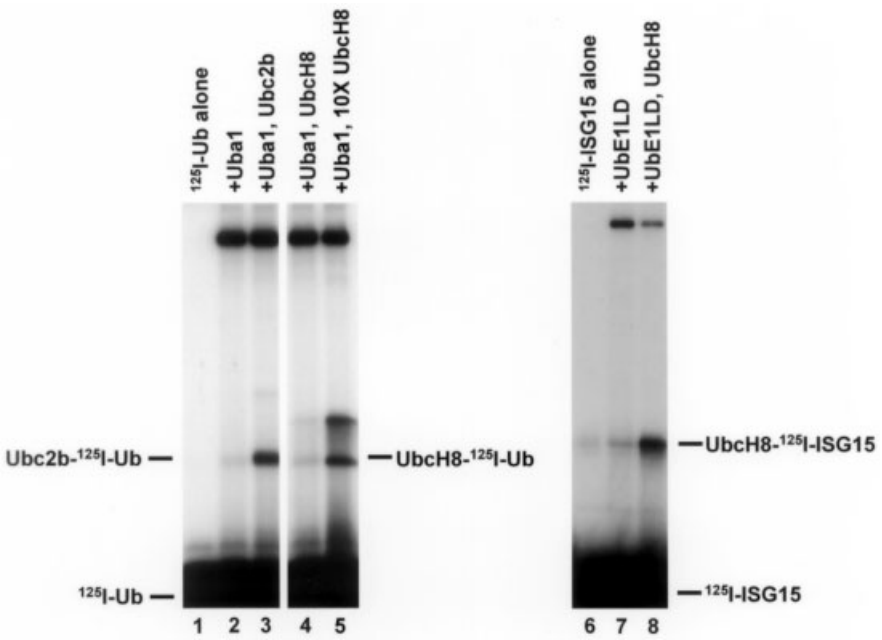

Fig. 5.5. Functional analysis of UbcH8. Autoradiogram of end-point functional thiolester assays of Uba1- (left panel, lanes 1–5) or UbE1L- (right panel, lanes 6–8) catalyzed ^{125}I-ubiquitin (left panel) or ^{125}I-ISG15 transthiolation to recombinant UbcH8. Assays were conducted as described previously then resolved by nonreducing SDS-PAGE [76].

distinct ligation pathways for ubiquitin and ubiquitin-like proteins [29]. The importance of considering basic enzymological precepts in functional assays is dramatically illustrated by recent studies that provide definitive evidence that UbcH7 and UbcH8 represent functionally distinct E2 families based on such biochemically defined *in vitro* assays (A.L. Haas, and J.M. Klein, in preparation).

Figure 5.5 shows an autoradiogram from a representative functional assay for the human Uba1-catalyzed formation of E2–^{125}I-ubiquitin thiolester [40, 76]. Within 1 min the human Uba1 ubiquitin-activating enzyme rapidly reaches end-point and forms stoichiometric ^{125}I-ubiquitin thiolester with the *bona fide* ubiquitin-specific carrier protein Ubc2b, the human ortholog of *S. cerevisiae* Rad6 [77, 78] (lane 3 of Figure 5.5). An equivalent amount of UbcH8 (as judged by end-point thiolester formation) forms a negligible amount of UbcH8–^{125}I-ubiquitin thiolester within 1 min (lane 4 of Figure 5.5). Subsequent time-course studies revealed that Uba1 required at least 30 min to reached completion in loading UbcH8 with ^{125}I-ubiquitin, indicating significantly slower kinetics. In contrast, UbE1L readily forms stoichiometric UbcH8–^{125}I-ISG15 thiolester within 1 min under nearly equivalent conditions (lane 8 of Figure 5.5), indicating that the kinetics for UbE1L transthiolation are significantly faster than those of Uba1. This result can be reconciled with

earlier published evidence purportedly supporting UbcH8 as a ubiquitin-specific E2 by increasing the concentration of UbcH8 10-fold (lane 5 of Figure 5.5) or either increasing the incubation time under the conditions of lane 4 or increasing the amount of Uba1 (not shown). The latter conditions serve to force an apparently unfavourable interaction between Uba1 and UbcH8 or to enhance detection of a minor side reaction that is subsequently misinterpreted as the actual product in qualitative assays. It is also apparent from the autoradiogram that Uba1forms two distinct UbcH8–^{125}I-ubiquitin thiolester adducts (Figure 5.5, lane 5) while UbE1L forms only a single UbcH8–^{125}I-ISG15 thiolester species (Figure 5.5, lane 8). Because the former bands differ by a relative molecular weight of ca. 2 kDa, they do not represent a stoichiometry resulting from formation of two ^{125}I-ubiquitin thiolesters per UbcH8 molecule but more likely result from nonenzymatic exchange between the active site Cys^{88} and one or more of the other two cysteines present within the human UbcH8 ortholog (Figure 5.4). Nonideal mobility resulting from positional effects of thiolesters has been noted previously in nonreducing SDS–PAGE detection of E2–^{125}I-ubiquitin thiolesters [76].

We have previously shown that by determining the E2 concentration dependence of the initial rate of E2 transthiolation, it is possible to accurately estimate the affinity, K_d (as K_m), for E2 binding to the E1 ternary complex [42, 53]. Such studies reveal that UbcH7 binds to human Uba1 ternary complex with a K_m of 100 nM, comparable to the value of 123 nM for human Ubc2b binding to Uba1 [53] and of 43 nM for human Ubc12 binding the heterodimeric AppBp1–Uba3 Nedd8-activating enzyme [42], while UbcH8 exhibits a K_m of 43 μM for binding to Uba1 under identical conditions (A.L. Haas and J.M. Klein, in preparation). The 430-fold difference in binding affinity suggests UbcH7 is a ubiquitin-specific E2 while UbcH8 probably does not normally function in a ubiquitin ligation pathway. Conversely, parallel ^{125}I-ISG15 transthiolation kinetics reveal that UbcH8 binds to UbE1L ternary complex with a K_m of 100 nM while UbcH7 binds with a K_m of 1.8 μM (A.L. Haas and J.M. Klein, in preparation). The 18-fold difference in affinities suggest that UbcH8 is an ISG15-specific E2 while UbcH7 probably does not normally support ISG15 ligases.

Therefore, UbcH7 and UbcH8 represent functionally distinct E2 families among a total of four such E2 families (including E2$_{epf}$ and UbcH6) that are unique to higher eukaryotes [8], indicating that new roles have continued to evolve through divergence of the E2 superfamily. Recognition that UbcH7 and UbcH8 support post-translational modifications by distinct ubiquitin paralogs forces us to reinterpret the role(s) of UbcH8 and ISG15 conjugation among ligases that interact with this E2 paralog.

5.4.3
Candidate ISG15-specific Ligases

The E3 ligases direct the specificity of target protein conjugation and catalyze transfer of the ubiquitin paralog thiolesters from their cognate E2 isoforms to specific lysine residue(s) on the target proteins. Both qualitative and quantitative as-

Table 5.1. Candidate ISG15-dependent ligases.

Name	Gene code	UbcH8 binding	UbcH7 binding	Function
Dorfin	DORFIN	+	+	Centrosome-associated; SOD1 degradation
Efp	EFP	+	+	Oestrogen-induced cell growth
E6AP	UBE3A	+	+	Targeted p53 degradation in human papilloma virus cell transformation
HHARI	ARH1	+	+	Human Ariadne ligase ortholog
Herc5	HERC5	+	?	p53/Retinoblastoma protein-inhibited cyclin-dependent kinase regulator
Parkin	PARK2	+	−	Targeting of synaptic vessel associated CDRel
Siah-1A/2	SIAH-1A/2	+	−	Synaptophysin targeting in neurotransmitter release
Staring	RNF40	+	+	Syntaxin1 targeting in neurotransmitter release
p53RFP	IBRDC2	+	+	Mediates caspase-independent 53-dependent apoptosis

+: binding occurs; −: binding does not occur; ?: binding uncertain.

says demonstrate that ligases are specific for orthologs only within their cognate E2 family. Since defined kinetic studies such as those described in Section 5.4.2 indicate unequivocally that UbcH8 is an ISG15-specific E2, candidate ISG15 ligases can be functionally defined by their ability to interact with UbcH8 at physiological concentrations.

Table 5.1 lists all E3 ligases currently documented to interact with UbcH8, many of which also interact with the closely related but functionally distinct UbcH7. One can reasonably anticipate that additional ligases will be identified whose catalytic cycles are supported by UbcH8, since the human genome is estimated to contain several hundred potential ligases for ubiquitin paralogs. With the exception of E6AP and Herc5, all of the candidate enzymes are RING finger ligases, defined by the presence of a specific Cys_3/His Zn^{2+}-coordinated RING finger motif that serves as an E2-binding domain [79, 80]. Significantly, four of the enzymes (Parkin, Siah-1A, Siah-2, and Staring) are associated with endosomal trafficking and neurotransmitter release [74, 81, 82] while another four of the candidate ligases (Dorfin, Efp, E6AP, and Herc5) are associated with mitotic progression and cell growth [83–87]. This distribution is consistent with recent proteomic studies that identified an array of cellular targets for ISG15 conjugation that span nearly all functional classifications in eukaryotes [36, 37].

The interferon-inducible Efp and Herc5 are the only directly validated ISG15-dependent ligases within the list of UbcH8-interacting ligases. Designation of Efp and Herc5 as ISG15-specific ligases is based on independent *in vivo* studies using siRNA against UbcH8 expression that significantly inhibits the ability of Efp and Herc5 to modify their specific intracellular targets with ISG15 [84, 86]. Ablation of UbcH8 expression by siRNA has emerged as the method of choice for demonstrating ISG15 specificity, since it obviates technical problems associated with over-expressed ligation components and the concomitant potential for driving otherwise thermodynamically unfavourable interactions. Interestingly, Efp is an oestrogen-induced ligase that mediates oestrogen-dependent cell proliferation and organ development by targeting the G2-checkpoint cell cycle inhibitor 14–3–3σ for ubiquitin-mediated proteasomal degradation [85]. Recent clinical studies demonstrate that Efp is routinely elevated in breast cancer biopsy samples and quantitatively correlates with a poor prognosis [88]. Following interferon induction, Efp-dependent conjugation of 14–3–3σ with ISG15 rather than ubiquitin can be demonstrated [86]; however, the effect of substituting ISG15 for ubiquitin has not been characterized. Herc5 is less well characterized but belongs to a family of six Hect domain ligases (Herc1–6) that exhibit two different domain architectures [89]. Herc5 and Herc6 are the most closely homologous in sequence within the Herc family and both are well-documented late interferon-induced proteins but only Herc5 exhibits ISG15 conjugating activity [84]. Co-transfection of Herc5 with UbcH8 but not UbcH7 stimulates the accumulation of ISG15 conjugates in HeLa cells, consistent with evidence that UbcH8 is an ISG15-specific E2 [84]. The ability of Herc5 to interact with UbcH7 or to catalyze UbcH7-dependent ubiquitin conjugation has not been examined to date.

The preponderance of candidate ligases that bind both UbcH7 and UbcH8 is paradoxical since members of this enzyme class generally exhibit absolute specificity only for orthologs within a single E2 family. This apparent dual functionality has led others to suggest that UbcH8 shares two ubiquitin paralog ligation pathways [54, 56]. More likely, duality of function resides at the level of the ligases functioning as conjugating enzymes for both ubiquitin and ISG15, depending on the substrate availability of UbcH7–ubiquitin versus UbcH8–ISG15 thiolesters. A number of different empirical observations regarding these ligases are satisfied by a model in which the enzymes normally function as UbcH7-dependent ubiquitin ligases in the absence of interferon or other conditions that signal the coordinated induction of the ISG15 ligation pathway. Upon the concerted induction of ISG15, UbcH8, and UbE1L, the enzymes become ISG15-dependent ligases as the concentration of UbcH8–ISG15 increases to levels that effectively compete with UbcH7–ubiquitin for binding. The dual nature of Efp conjugation is consistent with this model [86] and there is ample evidence in the literature for antagonist effects of ligation by different ubiquitin paralogs at identical or overlapping sites [39, 90, 91]. The model requires that dual function ligases not be capable of effectively discriminating between UbcH7–ubiquitin and UbcH8–ISG15 thiolesters, a hypothesis easily tested by quantitative kinetic assays [53]. Because Uba1 can charge UbcH8 with ubiquitin in spite of the substantially unfavourable catalytic specificity

against such a reaction (Section 5.4.2), UbcH8 can be forced to support ubiquitin-dependent targeting under nonphysiological conditions, accounting for published *in vitro* observations that UbcH8 is a ubiquitin-specific E2. Desai et al. have shown that the elevated expression of ISG15 in tumour cells interferes with ubiquitin-mediated proteasomal degradation, consistent with an antagonistic role for ISG15 compared with ubiquitin modification for dual function ligases [92]. Interestingly, a similar proposal was advanced by Liu et al. as an alternative interpretation of data showing that proteasomal inhibitors caused an increase in ISG15 adducts [93].

Many of the candidate ligases listed in Table 5.1 have interesting functional roles in cells for which an antagonistic role for ISG15 ligation can be easily rationalized. Dorfin is a short-lived protein, subject to ubiquitin-dependent proteasomal degradation, which was originally cloned from human spinal cord and is localized to the centrosome where it is suggested to function in microtubule organization [83]. Dorfin is known to function in the targeted degradation of synphilin-1, but not α-synuclein, and is a component of Lewy-body neuronal inclusions that characterize Parkinson disease [94]. Dorfin also co-localizes within inclusion bodies characteristic of ALS, where it binds to and targets mutant SOD1 for ubiquitin-mediated proteasomal degradation in a UbcH7/UbcH8-dependent reaction, based on *in vitro* assays [73]. *In vivo* overexpression of Dorfin delays neuronal cell death, presumably by targeting the degradation of mutant SOD1, which would otherwise accumulate and trigger caspase-dependent apoptosis [95]. Dorfin has not been tested to date as an ISG15-dependent ligase; however, if Dorfin represents a dual function ligase then an antagonistic effect of ISG15 conjugation is predicted to stabilize SOD1 and promote Lewy-body formation, potentially accounting for clinical observations that interferon therapy induces a reversible cognitive decline in ALS patients [96].

The recently identified p53RFP shares with Parkin and Parc (PARC) a conserved carboxyl terminal architecture consisting of two RING finger domains separated by an in-between RING finger (IBR) domain [97–99]. The p53RFP ligase interacts with p53 and is involved in triggering apoptosis through a p53-mediated caspase-independent pathway [97]; in addition, p53RFP catalyzes the ubiquitination and targeted proteasomal-dependent degradation of p21WAF1, a cyclin-dependent kinase inhibitor responsible for G1 checkpoint arrest [100]. Parc interacts with p53 to localize the latter to the cytoplasm in order to suppress p53-dependent apoptosis [101]. The E2 specificity of Parc has not been examined, but p53RFP and Parkin qualitatively exhibit similar binding affinities for UbcH7 and UbcH8 [81, 97]. None of the three RING–IBR–RING proteins have been tested for intrinsic ISG15 ligase activity even though p53RFP and Parkin (and presumably Parc) interact with UbcH8.

E6AP and Herc5 are E3 ligases belonging to the HECT domain (homologous to E6AP carboxyl terminus) family of conjugating enzymes [102]. The HECT domain ligases are defined by the presence of a highly conserved 250-residue carboxyl terminal domain that serves as an E2 interaction domain and protein conjugation module that contains an absolutely conserved active site cysteine to which cognate E2 thiolesters transfer their ubiquitin paralog as part of their catalytic cycle [102]. E6AP was originally identified as a 100-kDa protein that, in complex with the E6

oncoprotein encoded by the human papilloma virus type 16 (HPV16), targeted the degradation of p53, believed to be a requisite step in cervical cell transformation and immortalization [69, 87, 102, 103]. More recent work suggests that E6AP targeting of E6TP1 rather than p53 is probably responsible for transformation and immortalization of cervical cells infected with HPV16 [70]. Interestingly, the E6 protein redirects the specificity of E6AP toward p53 and E6TP1 targeting, since the ligase normally conjugates a different subset of cellular proteins in the absence of viral E6 protein [87]. Two-hybrid studies demonstrated that UbcH7 and UbcH8, but not Ubc5 or UbcH6, interact strongly with E6AP [61]; thus, E6AP may also serve as a dual-function ligase. Given the central role of E6AP in HPV16-mediated cell transformation, an antagonistic role for E6AP-catalyzed ISG15 conjugation is easily appreciated.

5.5 Regulation of Intracellular ISG15 Pools

Ubiquitin is an abundant protein in eukaryotes, with total intracellular pools generally in the range of 100 pmol/10^6 cells and representing an intracellular concentration corresponding to ca. 25 μM [104, 105]. In contrast, estimates of total ISG15 pools in unstimulated cells represent approximately 5% to 10% of total ubiquitin pools based on immunochemical assays [21]. Following Type 1 interferon stimulation, total ISG15 pools increase to approximately half that of total ubiquitin, the latter of which is unaffected by addition of the cytokine [21, 84]. In the absence of interferon stimulation, cells contain a low but measurable constitutive level of ISG15 conjugates [21]. Biphasic induction of free and conjugated ISG15 pools in A549 human lung carcinoma cultures is typical of that observed in other cell lines and is typical of other early immediate primary responses to interferon, such as the induction of the antiviral MxA protein (IFI78) (Figure 5.6). Increased ISG15 mRNA and protein can be detected within two to four hours after addition of IFNβ at 10^3 IU ml^{-1}, after which the polypeptide accumulates over the next 12–18 hours [21]. New ISG15 conjugates begin to appear at 12–14 hours and continue to accumulate thereafter (Figure 5.6). The late interferon accumulation of ISG15 conjugates results from induction of UbcH8 and UbE1L as late interferon-induced proteins [84] and can be blocked by the addition of inhibitors of protein synthesis at 12 hours following addition of interferon (J. Narasimhan and A.L. Haas, unpublished observation). The biphasic time course for intracellular ISG15 pools following interferon addition is unique among ubiquitin paralogs. Under conditions of ubiquitin induction, free and conjugated pools of the polypeptide increase in parallel owing to coordinated co-induction of ubiquitin, Uba1, and the requisite E2 isoforms [105–107]. The biphasic induction of ISG15 may serve to provide an early pool of free polypeptide for noncanonical secretion prior to sequestration of the ubiquitin paralog as nonsecreted target protein adducts.

ISG15 mRNA and protein is widely distributed in the tissues of humans [66, 108]. Within cells, ISG15 adducts have been shown in part to localize in a punctate

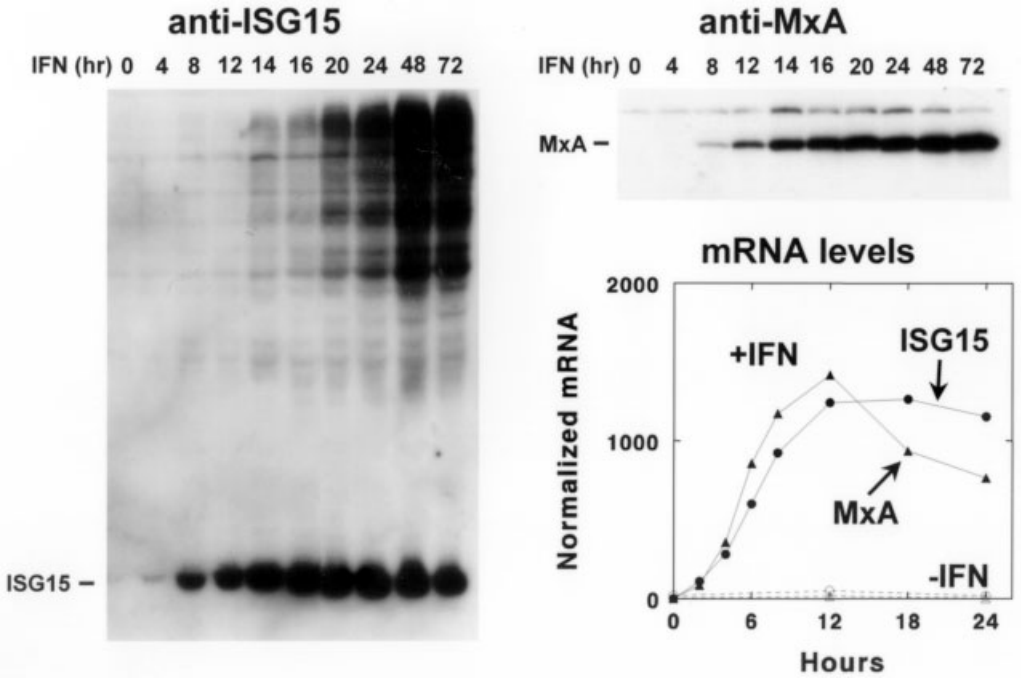

Fig. 5.6. Biphasic induction of ISG15 pools. Left panel: Western blot showing the biphasic induction of free and conjugated ISG15 pools following addition of IFNβ (10^3 IU ml^{-1}) to confluent A549 cultures [21]. Upper right panel: Western blot showing the induction of antiviral MxA protein in the same samples. Lower right panel: Normalized Northern blot results for ISG15 versus MxA mRNA in the absence (dashed line) or presence (solid line) of interferon induction.

cytoskeletal pattern in cultured A549 cells and paraffin-embedded human tissues by immunospecific histochemical co-localization [66, 109]. Differential extraction of confluent A549 cells demonstrates that the cytoskeletal distribution represents co-localization with intermediate filaments [109]. Ectopic expression of a stable ISG15–chloramphenicol acetyltransferase (CAT) fusion protein in A549 cells, but not CAT alone, also localized in a cytoskeletal pattern on intermediate filaments when probed with an anti-CAT antibody, demonstrating that such localization results from binding interactions with ISG15 and not by direct ligation of the polypeptide to intermediate filament proteins (S. Twigger and A.L. Haas, unpublished observation). In fixed human tissue sections, ISG15 immunoreactivity also localizes to neuromuscular junctions, where it presumably has a role in stimulus–secretion coupling [108]. Interestingly, several candidate ISG15 ligases are involved in endosomal pathways associated with neurotransmitter release (Section 5.4.3). Depletion of ATP in cultured cell lines and cell-free extracts by addition of 2-deoxyglucose and 2,4-dinitrophenol leads to the rapid depletion of ubiquitin conjugates due to disassembly of the polypeptide from the adducts by ubiquitin-specific

isopeptidases, as monitored by SDS-PAGE and Western blotting [21, 104, 106, 110]. Similar ATP depletion of interferon-treated cell cultures or of untreated cells consistently exhibit no loss of stimulated or constitutive ISG15 conjugate pools while ubiquitin conjugate pools are rapidly lost, suggesting the ISG15 adducts are stable and that cells do not contain ISG15-specific isopeptidase activities [21]. In contrast, Zhang and her colleagues have cloned an interferon- and LPS-induced putative ISG15-specific isopeptidase termed Ubp43 (USP18) that is identical to the previously identified ubiquitin-specific protease Usp18 [34, 111, 112]. Human Ubp43 maps to chromosome 22q11.2 in a region identified as the DiGeorge syndrome critical region since a 2-Mb deletion at this locus results in DiGeorge syndrome, characterized by thymic and parathyroid aplasia or hypoaplasia and cardiac abnormalities [113]. Transgenic Ubp43$^{-/-}$ mice show increased ISG15 conjugates, hypersensitivity to interferon resulting in enhanced resistance to a spectrum of viral and bacterial challenges, deregulated STAT1 signalling, and developmental neuronal abnormalities that have been interpreted to indicate that ISG15 conjugation is required for a spectrum of cellular and developmental processes [114–117]. However, the Ubp43 knockout studies have recently been questioned by subsequent results. Transgenic ISG15$^{-/-}$ mice do not exhibit the rescue phenotypes predicted from the results, which assumes elevated ISG15 conjugates with Ubp43$^{-/-}$ mice [118]. Additionally, independent studies with ISG15 single- or ISG15/Ubp43 double-knockout mice failed to replicate the results of the earlier work [118, 119]. Finally, more recent studies with UbE1L$^{-/-}$ transgenic mice suggest that the earlier results with Ubp43 knockouts must represent ISG15-independent phenotypes [120].

5.6 Functional Roles for ISG15

Unlike other ubiquitin-like proteins, the functional roles for ISG15 can be divided between extracellular and intracellular effects. The earliest roles ascribed to ISG15 relate to its remarkable properties as a secreted immunomodulatory cytokine. Subsequent recognition of the sequence and structural relationship between ubiquitin and ISG15 shifted the focus to that of ISG15 as an intracellular signalling molecule functioning through its ATP-dependent conjugation to cellular targets. Thus, there are many areas for future investigation in which the fundamental groundwork and the central questions have been circumscribed.

5.6.1 ISG15 as an Extracellular Cytokine

Early investigations by Knight and Cordoba demonstrated the rapid IFNβ-stimulated secretion of mature ISG15 from isolated human lymphocytes and monocytes [25], an unexpected response that was subsequently replicated in cultured human immune and nonimmune cells including monocytes (THP-1), B

lymphocytes (Raji), T lymphocytes (Jurkat), primary corneal keratocytes, lung epithelial carcinoma (A549), and ovarian epithelial adenocarcinoma (OVCAR-3) lines [21, 121, 122]. Interferon-stimulated secretion of ISG15 within the first 24 hours of treatment constitutes a significant fraction of the total intracellular pool of the mature free polypeptide [25], which may represent stimulation of the slow basal rate of release noted earlier [21]. In human subjects, low circulating levels of free ISG15 are detected by ELISA, which exhibit a dose-dependent elevation during therapeutic IFNβ treatment, demonstrating that secretion is not an artifact of *in vitro* experimental conditions [121]. The mechanism of ISG15 secretion has not been explored but is of some interest since the nascent polypeptide does not harbor an amino terminal signal sequence, Figure 5.1. The inability concurrently to detect intracellular markers such as ubiquitin in the cell culture medium following IFNβ stimulation suggests that extracellular ISG15 results from cytokine-stimulated secretion through a noncanonical pathway rather than from cell lysis [21]. Notably, there is recent precedent for cytokine-stimulated secretion of bioactive peptides derived from proteolytic fragments of specific aminoacyl tRNA synthetases that follow a noncanonical signal sequence-independent pathway, suggesting a possible mechanism for ISG15 secretion in response to IFNβ stimulation [123, 124].

Free ISG15 exhibits remarkable properties as an extracellular cytokine that are unique among the ubiquitin-like proteins. In the first such study, Recht et al. demonstrated that addition of nanomolar concentrations of mature recombinant ISG15 to human peripheral blood monocytes induced interferon γ (IFNγ) secretion in a population of $CD3^+$ lymphocytes [125]. That human recombinant ISG15 had no similar effect on murine lymphocytes presumably reflects species-specific sequence differences in the polypeptide [126] (Figure 5.1). More recent studies reveal that extracellular ISG15 functions in concert with IFNγ as an immunomodulatory cytokine [121, 126]. Thus, stimulation of B cell-depleted human lymphocytes with recombinant ISG15 triggers secretion of IFNγ specifically from $CD3^+$ T lymphocytes, synergistically acts with the nascent IFNγ to trigger $CD56^+$ natural killer cell proliferation, and induces nonmajor histocompatibility complex-restricted cytolysis of tumour cell targets by natural killer cell-derived lymphokine-activated killer cells in the absence of detectable IL-2 or IL-12 secretion, which are independently capable of natural killer cell expansion [126]. Interestingly, the immunomodulatory effects of recombinant ISG15 required intact mature polypeptide since proISG15 containing the octapeptide carboxyl terminal extension was inactive and ISG15 from which the carboxyl terminal glycine dipeptide had been cleaved during expression and purification exhibited greatly diminished efficacy [126].

Other studies demonstrate constitutive secretion of ISG15 by specific melanoma cell lines in response to autocrine induction by IFNβ; significantly, the resulting extracellular ISG15 induced E-cadherin expression on the surface of immature monocyte-derived dendritic cells, a response known to impair tumour infiltration by increasing cell adhesion [127, 128]. Other studies indicate a role for ISG15 in neutrophil-mediated mechanisms associated with innate immunity. Experimental induction of malaria in mice by infection with *Plasmodium yoelii* results in release of ISG15 from murine erythrocytes [129]. The resulting extracellular ISG15 exhib-

its specific chemotactic activity toward neutrophils and activated neutrophils to induce the release of eosinophil chemotactic factors [129]. Thus, ISG15 may act as a critical extracellular first messenger that signals cell damage or invasion in order to recruit a focused and localized immune response.

Obviously, the immunomodulatory effects of ISG15 imply the presence of a specific plasma membrane receptor on the surface of target lymphocytes and neutrophils that probably also exists on other cell types. Early data by Recht et al. are consistent with the presence of an ISG15-specific receptor exhibiting a nanomolar affinity for the polypeptide [125]. However, there has been little subsequent work to identify and isolate the ISG15 receptor, which remains a major point of interest. Interestingly, ubiquitin was first identified as a polypeptide with lymphocyte and granulocyte differentiating activity with efficacy in the nanomolar range [130, 131] that functioned through activation of adenylate cyclase [132]. The biological activity of purified ubiquitin could be replicated in part by a synthetic peptide corresponding to residues 59–74 of ubiquitin [133]. Given that ISG15 is documented to exhibit many of the same cytokine responses, there is a compelling argument that the initially reported pharmacological effects of ubiquitin in fact arise from contamination of the ubiquitin preparations with ISG15, particularly since the two peptides share almost identical physicochemical properties and co-purify from cell lysates (A.L. Haas, unpublished observation).

5.6.2 Role of ISG15 in the Antiviral Response

Interferon production is a well-established consequence of viral infection and ISG15 induction is the earliest response of interferon-sensitive cells to even low levels of the cytokine [20, 21]. Induction of interferon synthesis and secretion in response to viral infection signals host cell invasion in order to propagate an antiviral response in neighbouring cells. Parsimony between the appearance of the antiviral phenotype in selected cell lines and the temporal or interferon concentration-dependent induction of ISG15 represents the earliest evidence for a role of ISG15 in the antiviral activity of interferon [20]. Cells also possess a primary line of defence against viral and parasitic invasion that relies on the specific induction of a subset of cellular proteins that allow the infected cell to mount a viral response. Among this cohort of innate host defence proteins, ISG15 is one of the earliest and most strongly induced in response to viral [134–139] and microbial [140, 141] infection. However, the specific viral inducibility of ISG15 appears to be highly cell type specific, as discussed previously [142]. The observation that Lipofectamine and similar agents induce interferon production and ISG15 [143] is of technical concern for transient transfection experiments.

The precise antiviral mechanism(s) for ISG15 has not been determined but almost certainly requires conjugation of the polypeptide to cellular or viral targets since UbcH8 [54, 56] and several candidate ISG15-specific ligases including Efp [86] and Herc5 [84, 144] are also interferon- and lipopolysaccharide-inducible. Also, the ability of the influenza B virus NS1B protein to block ISG15 conjugation

by binding to and sequestering the polypeptide, as well as the abrogation of ISG15 protein expression by influenza A virus NS1A protein, provides strong circumstantial evidence for the antiviral action of ISG15 adducts [43, 145]. Kunzi and Pitha have demonstrated that overexpression of ISG15 alone mimicked the interferon effect and led to sequestration of unspliced nuclear HIV transcripts and inhibition of HIV protein synthesis by blocking nuclear export [146]. Subsequent studies have shown that ectopic expression of ISG15 targets the endosomal trafficking pathway exploited by HIV for release of assembled virions [147]. Specifically, ISG15 expression ablates the required ubiquitination of Gag and Tsg101, which prevents the heterodimerization that is required for the endosomal pathway; conversely, blocking ISG15 expression by siRNA obviates the anti-viral effect of interferon in preventing HIV virion release [147]. Other anti-viral mechanisms may depend of the ability of ISG15 to serve as a transposable binding element operating in *trans* to localize antiviral proteins to the cytoskeleton [109]. For many viruses the cytoskeleton is critical for viral trafficking from the plasma membrane to the nucleus and as sites for replication and assembly, reviewed in Ref. [148]. Specific ISG15 adducts bound to the cytoskeleton might function directly to block viral protein trafficking or viral assembly.

The best evidence to date for the role of ISG15 adducts as intracellular antiviral agents comes from the recent work of Lenschow et al. using IFNα/β receptor (IFNα/βR) knockout mice incapable of mounting an interferon-induced antiviral response [139]. By using a recombinant chimeric Sindbis virus construct to express different interferon-stimulated genes in IFNα/βR$^{-/-}$ mice, the authors identified ISG15 as having intrinsic antiviral activity. Over expression of intact ISG15 significantly reduced Sindbis virus lethality and ablated Sindbis virus replication; however, overexpression of ISG15 in which the carboxyl terminal glycines of the canonical LRLRGG sequence were mutated to alanine failed to show anti-viral effects, indirectly implicating the requirement for ISG15 conjugation [139].

5.6.3
ISG15 and Early Events of Pregnancy

Edometrium is a complex and dynamic tissue that is normally subject to cyclic remodelling and regeneration in adult mammals during oestrous; however, the greatest tissue alterations occur immediately during and after implantation of the conceptus and extend through the first and second trimesters of pregnancy [149]. Following fertilization, the conceptus invades the receptive uterine epithelium and induces a decidual response that is characterized by inflammation and activation of angiogenesis to increase blood supply for the developing placenta. Much of the remodelling accompanying conceptus implantation and the transition from oocyte to embryo involves targeted degradation by ubiquitin-mediated proteasome-dependent degradation [150]. However, early post-implantation events in mammals also include production of Type 1 interferon by the conceptus and the macrophage-enriched placenta [149, 151–155]. Hansen and coworkers have demonstrated that ruminant conceptus elaborates a specific Type 1 interferon (IFNτ)

in early pregnancy that initiates a programmed Type 1 interferon response resulting in the robust induction of ISG15, UbE1L, and the accumulation of ISG15 conjugates [156–159]. A fraction of the IFNτ-induced ISG15 is secreted by the conceptus into the surrounding space, presumably to mediate its extracellular cytokine responses [151].

Conceptus implantation in mammals is accompanied by induction of cell surface molecules such as E-cadherin that account for the enhanced cell–cell adhesion required for anchorage [160]. Secretion of ISG15 by the conceptus is likely required for induction of E-cadherin [127]. However, ISG15 conjugates may also participate in cell–cell adhesion or signalling since ISG15 conjugates accumulate at the uterine–placental interface [161]. In humans, ISG15 continues to be elaborated by decidual cells of pregnant tissue during the first and second trimesters [149]. In spite of considerable circumstantial evidence, knockout studies suggest that the role(s) of ISG15 in the early events of conceptus anchorage and embryo development is not essential since ISG15$^{-/-}$ transgenic mice develop to full term and qualitatively display no obvious abnormalities [118].

5.7 Perspective

Since its initial discovery as a ubiquitin paralog, the function of ISG15 has been provocative, in part because of its limited phenotypic distribution and concomitant implications for novel functions not found in the more broadly expressed members of the ubiquitin-like protein superfamily. Although progress in understanding the role of ISG15 in eukaryotic regulation has developed slowly, largely because the absence of this signalling pathway in yeast precluded the exploitation of established genetic approaches for functional gene characterization, progress in this area is now rapid on several fronts. The immediate future holds significant promise for new insights into the role of this polypeptide in vertebrates.

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6
The Role of the Ubiquitin–Proteasome Pathway in the Regulation of the Cellular Hypoxia Response

Koh Nakayama and Ze'ev Ronai

Abstract

Changes in oxygen concentration in tissues and cell types govern each of the physiologically important regulatory responses. Key players in the cellular response to altered oxygen concentration include prolyl hydroxylases and the transcription factor HIF1α, both of which are subject to regulation by E3 ubiquitin ligases, Siah and pVHL, respectively. The emerging role of the ubiquitin proteasome pathway in the regulation of cellular hypoxia is discussed.

6.1
Overview of the Hypoxia Response

Organisms are constantly exposed to oxygen, which is utilized primarily for efficient metabolism and energy production. Mammals regulate oxygen delivery and utilization through a series of cellular and systemic processes, which were developed to cope with O_2 concentrations ranging from less than 1% to 21%. Changes in oxygen concentration reflect physiological body homeostatic processes, given the different concentrations of oxygen in various tissues. Certain levels of oxygen are required for development and differentiation as well as for cell growth and division and programmed cell death. Since conditions that could lead to lack of oxygen at both the macro (within the surrounding environment) and micro (within tissues or cells) levels recur frequently, specific pathways have evolved to adjust and allow physiological cellular metabolism and growth under low levels of oxygen as well as in response to changes in its levels.

Hypoxia is defined as an environment (atmospheric or cellular) below the ambient oxygen condition (<21%). As the range is quite broad, certain cellular processes can be activated under conditions of relatively mild hypoxia (5% to 15%), whereas other pathways are activated in severe hypoxia (1% to 5%) or in response to anoxia (0%). In general, compared to surface tissues, internal organs are constantly in a state of mild hypoxia (estimated within the range of 4% to 6% oxygen), which requires them to adapt by means of metabolic and cellular processes.

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In such circumstances, cells activate signalling pathways that lead to changes in metabolism, respiration, and energy production that eventually alter the cell cycle, cell survival, and cell differentiation status. The hypoxia response is an active state of coping with lower oxygen conditions rather than a passive state of adaptation. In response to hypoxia, cells decrease metabolism, increase respiration, and turn off general transcription and translation activities [1]. Significantly, however, hypoxia upregulates specific sets of proteins such as enzymes and growth factors, primarily via the transcription factor HIF-1α, which initiates a unique transcriptional pattern to enable cells to maintain their functions under low oxygen concentrations [2].

Hypoxia also affects mitochondrial electron transport and oxidative phosphorylation. Alteration of these pathways in mitochondria during hypoxia lessens the efficiency of energy production and triggers formation of reactive oxygen species (ROS) [3]. Intriguingly, ROS also forms in response to high levels of oxygen radicals [4], indicating that similar cellular mechanisms come into play under high as well as low oxygen levels. ROS have been implicated as having an important role in HIF-1α expression under both normoxic and hypoxic conditions [5–7], as well as in the hypoxia response involving HIF-1α-dependent and -independent mechanisms. ROS enhances stress signalling by stress kinase activation [8], cytokine production [9], alterations in gene expressions and cell motility [10], and adipocyte differentiation and the like [11].

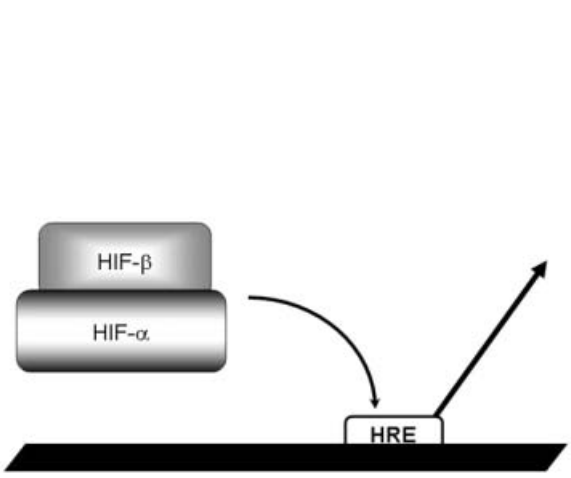
Hypoxia results in reduced oxidative phosphorylation and a shift towards glycolysis as the primary means of ATP production. To facilitate this change, cells upregulate expression of genes that encode glycolytic enzymes and glucose transporters [12, 13]. Tissues and cells that experience reduced oxygen supply exhibit increase in VEGF (vascular endothelial growth factor) [14, 15] which is implicated in wound healing, stroke, heart attack, and cancer [13]. The oxygen delivery systems can become dysregulated, leading to hypoxia response, which can occur in various settings such as high altitude, ischemia, organ structure, and tumorigenesis before the blood vessel network is fully established. For example, in a tumour mass (i.e., solid tumour), the oxygen concentration decreases from the surface inward, making for an extremely low oxygen concentration at the center. Under those conditions, depending on the degree of hypoxia and cell types, cells activate both common and cell-specific signalling pathways to respond in ways that meet physiological needs. In this chapter, we will focus on the signalling molecules activated in hypoxia with a special emphasis on the role of the ubiquitin–proteasome pathway in achieving and maintaining the physiologically appropriate responses to the hypoxic condition.

6.2
Players in the Hypoxia-response Signalling Pathway

6.2.1
Hypoxia-inducible Factors

Work since the mid-1990s has revealed the important role of dioxygenases, which belong to the Fe(II) and 2OG (2-oxoglutarate)-dependent oxygenases, in direct-

ing the activity of the key transcriptional regulator in hypoxia, termed hypoxia inducible factor (HIF). The HIF family of transcription factors consists of two major players, the α and β subunits, which are regulated differently but operate in concert to activate transcription [16]. HIF-1α contains bHLH-PAS domains and forms a heterodimer with HIF-1β to become transcriptionally active [17]. The transcriptionally active heterodimeric complex binds to a core DNA sequence (G/ACGTG) in hypoxia response elements (HREs) coupled to target genes [18], through which they activate more than 50 genes, including erythropoietin (EPO), VEGF, and glucose transporters (GLUT), which are involved in multiple processes including glucose metabolism, angiogenesis, cell growth, and cell death (Figure 6.1). This transcriptional network is activated only in conditions that enable stabilization of the HIF-1α component, which is usually undetected under normoxia conditions. Thus, only reductions in oxygen concentration to below threshold levels, or certain other



Erythropoiesis
EPO
Angiogenesis
VEGF
Flt-1
Flk-1
plasiminogen inhibitor-1
Cell growth, differentiation
cyclin G2
cyclin D1
IGF2
IGF-BP1
c-Met
TGF-β3
Endoglin
TERT
Wilms' tumor suppressor
Cell death
Nip3
Noxa
Motility
SDF-1
CXCR4
Matrix metalloproteinase 2
Metabolism
GLUT-1
hexokinase-1,2
GAPDH
PGK1
Leptin
LDL-related protein 1
Enolase 1
Aldolase A
Transcriptional regulation
DEC1, 2
ETS-1

Fig. 6.1. HIF upregulates multiple genes. Once an HIF-α subunit is stabilized, it forms a heterodimer with an HIF-β subunit to transactivate various genes which are regulated by the hypoxia-responsive element (HRE) on their promoter. This transactivation engages all cellular regulatory processes, including erythropoiesis, angiogenesis, glycogenesis, cell growth, cell death, motility and transcriptional regulation. Examples of HIF-inducible genes are shown in the table.

physiological conditions (see below), allow the stabilization of HIF-1α, making it available to function as a transcription factor.

The HIF family of transcription factors includes other isoforms, HIF-2α and HIF-3α, that are also regulated in an oxygen-dependent manner [19]. HIF-2α also plays a major role in hypoxia-dependent upregulation of genes, although the repertoire of genes upregulated by HIF-2α appears to differ in part from that of HIF-1α [19–21]. This difference could reflect variations in cell types, whose mechanistic basis is yet to be identified. The importance of HIF-3α was revealed through its ability to inhibit HIF-1α-dependent transcription through competition for the β subunit [22]. It was also shown that HIF-3α forms a splice variant in response to hypoxia [23]. The β subunit is common to the three HIF isoforms and is constitutively expressed in cells. It is implicated in stabilization of HIF-α proteins when a dimer has been formed between the two [24].

Increased PI3K/AKT, or decreased PTEN, activity activates the HIF pathway in various tumours [25–28]. HERs overexpression, which results in constitutively active AKT, turns on HIF-1α independently of hypoxia [29]. Such activation is weaker under hypoxia. Interestingly, AKT was shown to mediate its effects on HIF activity via interaction with – and phosphorylation of – HIF-β, which results in enhanced binding between HIF-1α and β subunits [29].

6.2.2
Prolyl-hydroxylase Domain-containing Enzymes and FIH

PHD (prolyl-hydroxylase domain-containing) is an enzyme that hydroxylates human HIF-1α on its proline residues 402 and 564 [30–32]. PHD belongs to the prolyl-4-hydroxylase family and shares homology with collagen hydroxylases [31, 32]. PHD-dependent prolyl hydroxylation of HIF-1α leads HIF-1α to degradation through the ubiquitin–proteasome pathway [33–35]. The three identified PHD proteins are PHD1, PHD2, and PHD3 [31, 32]. One report suggests the possible existence of PHD4, based on a database search [36]. Expression of PHD2 and PHD3, but not of PHD1, is induced by hypoxia, pointing to a possible negative feedback loop mechanism in the regulation of HIF-1α availability [37–39]. Differences among the PHDs have been noted in their subcellular localization pattern [40, 41]. *In vitro* analysis indicates that the three PHDs are equally active in their ability to hydroxylate HIF, whereas cell-based analysis, using an siRNA approach, suggests that in certain cell lines it is predominantly PHD2 that is required to maintain low levels of HIF-1α in normoxia [31, 38]. Multiple studies clearly demonstrate that PHD3 as well as PHD2 is important in the cellular response during as well as following hypoxia (i.e., re-oxygenation that restores normoxia) [38, 39].

The precise determinants of PHD1 function are yet to be determined. PHD activity requires co-factors; iron ion, 2OG, ascorbate, and oxygen. Each could be the limiting factor in the PHD activity, highlighting the tight regulation of this activity by environmental cues. Their requirement for oxygen is one of the characteristic features of these enzymes. Like procollagen prolyl and lysyl hydroxylases the HIF hydroxylases belong to the 2OG-dependent oxygenase superfamily, whose

members require the Fe(II) co-factor and uses the 2OG as a co-substrate [42]. Mechanistically, an enzyme–Fe(II) complex first binds 2OG and then primes its substrate, which consequently displaces a water molecule to trigger a reaction with molecular oxygen, resulting in oxidative decarboxylation of 2OG to produce succinate, CO_2, and a ferryl species at the iron centre. This highly reactive intermediate oxidizes the prime substrate. Because of its requirement of an oxygen molecule for this activity, it has been suggested that PHDs may serve as the oxygen sensors that regulate HIF-1α in normoxia but not in hypoxia.

The PHD enzymes are evolutionary conserved from *Caenorhabditis elegans*, where they were first identified as EGLN1, 2, and 3 [31] to *Drosophila* [43] and vertebrates. Using transgenic flies that express an oxygen-dependent degradation domain (ODD) fused to a marker protein GFP (ODD–GFP), the hypoxia cascade has been analyzed in different embryonic and larval tissues; hypoxia accumulation of the reporter protein has been found in the entire tracheal tree but not in the endoderm. Hypoxic stabilization of ODD-GFP in the ectoderm is restored on altering pVHL expression, suggesting that Drosophila tissues exhibit a different pattern of tissue sensitivity to hypoxia.

Of note is that the PHD homologue in *C. elegans*, EGLN1 (PHD2) [44], has been found to affect not only HIF hydroxylation and stability but also transcription [44]. The latter finding suggests that accumulation of EGLN1 in hypoxia may trigger a negative feedback mechanism to modulate HIF-1α target gene expression.

PHD activity fostering hydroxylation of HIF-1α could be also induced by nitric oxide (NO), under hypoxia conditions that otherwise prevent PHD activity [45]. Free radical scavengers such as NAC are able to attenuate the effect of NO on PHD activity. Indeed, low levels of ROS PHD activity [45]. The formation of NO donors in response to ROS formation constitutes an alternative mechanism for regulation of PHD activities.

FIH, another member of the prolyl-4-hydroxylase family, has been shown to hydroxylate HIF-1α at asparagine residue 803 [46]. Similar to PHDs, it requires oxygen and co-factors to be active as an enzyme. Such hydroxylation decreases the transcriptional activity of HIF-1α by inhibiting interaction with p300, a transcriptional co-activator, without affecting the stability of HIF-1α.

6.3 pVHL-dependent Degradation of HIF-1α

Von Hippel–Lindau protein (pVHL) is an F-box type of E3 ubiquitin ligase containing protein that recognizes certain targets, and is thus able to assemble the complex of SCF ligases that allows ubiquitination-dependent proteasomal degradation of the bound target [47, 48]. It is common to F-box proteins that they only recognize their substrates after their post-translational modification, usually by means of phosphorylation [49, 50]. In the case of pVHL recognition of HIF-1α, the modification is by means of prolyl hydroxylation (see below). pVHL, consisting of 213 amino acids, is known to be mutated often in cancer patients, including those with kidney

or pancreas tumours or clear cell renal carcinoma [51]. To function as an E3 ligase, pVHL associates with elongin B,C and Cullin2 (the VBC complex) to form a functional SCF–E3 ligase complex [47]. In this complex, pVHL serves as a bridge accepting the substrate HIF-1α, whereas Cullin2-bound Rbx1/Roc1 acts as a catalytic protein to form a ubiquitin polychain. Once HIF-1α is recognized by pVHL, HIF-1α is efficiently polyubiquitinated by the E3 ligase complex and degraded by the proteasome (Figure 6.2) [52]. pVHL consists of two domains; α and β. The α domain, consisting of amino acids 157–189, interacts with elongin C and is required for VBC complex formation, whereas the β domain, consisting of amino acids 64–

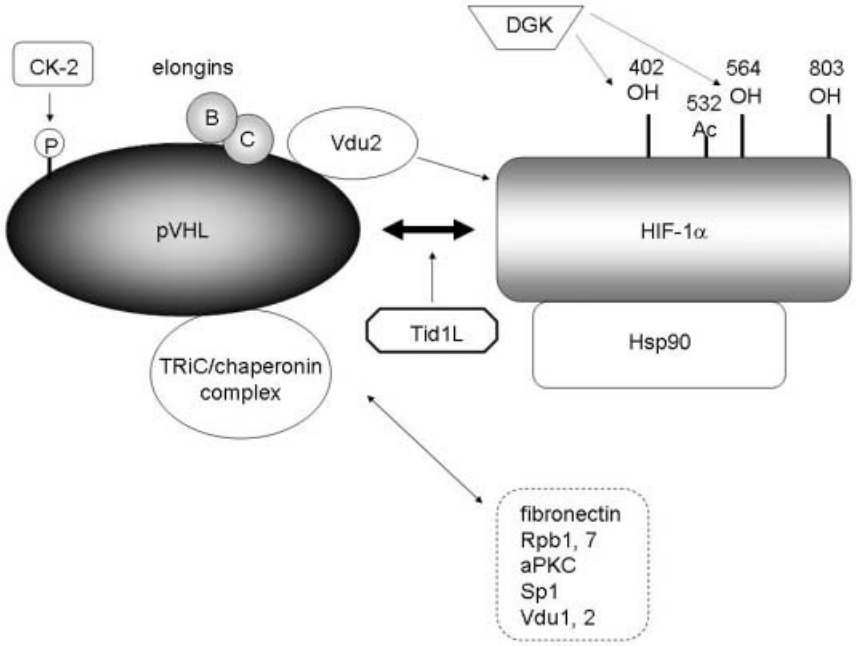


Fig. 6.2. Regulation of the pVHL–HIF pathway by multiple molecules. HIF-1α is post-translationally modified by hydroxylation on proline residues at positions 402 and 564. This hydroxylation serves as a docking site for the pVHL which will lead to its degradation. It also appears that acetylation on lysine 532 is involved in HIF-1α stability. Hydroxylation of an asparagine residue at position 803 by FIH is known to inhibit the transcriptional activity of HIF. Alternatively, HIF-1α can be stabilized through interaction with Hsp90, which is independent of pVHL. pVHL is known to be unstable if it is not properly folded and some chaperone-like proteins including elongin C and the TRiC–chaperonin complex, binds to it to support the stability. Groups of proteins have been found to interact with pVHL, including fibronectin, RNA polymerase II subunits (Rpb1, 7), and aPKC, some of which might serve as a substrate for the pVHL complex. Tid-L is known to promote the interaction between pVHL and HIF-1α, and Vdu2, a deubiquitinating enzyme, which could remove the ubiquitin from HIF-1α and thus provide an alternative way to stabilize/destabilize HIF-1α. pVHL is phosphorylated on its N-terminal acidic domain by casein kinase-2 (CK2) and affects its interaction with pVHL and fibronectin. Diacyl glycerol kinase has been shown to enhance the PHD activity, thus rendering HIF-1α more degradable.

156, interacts with HIF-α. There are five β-strands in the β domain that form a pocket to capture HIF-1α [53, 54]. pVHL recognition of HIF-1α requires PHD enzyme-catalyzed modification of HIF-1α on its proline residues at positions 402 and 564. Such proline hydroxylation of HIF-1α forms a site that is structurally recognized by the pVHL pocket, resulting in formation of a hydrogen-bond with residues 111 (serine) and 115 (histidine) [53, 54]. Since PHD enzymes hydroxylate HIF-1α mainly in normoxia, in which oxygen molecules are abundant, pVHL-dependent degradation of HIF-1α is highly dependent on the ambient oxygen level. The half-life of HIF-1α has been shown to be 5 min in normoxia [55], which well reflects the efficiency of this degradation.

Observations of pVHL mutants derived from tumours, including renal clear cell carcinoma cells, indicate that the mutation frequency is nearly 50% at the β domain regions between residues 60 and 153 which is involved in recognition of the hydroxylated proline [53]. In these cells, expression of HIF-1α is increased and mostly coincides with upregulation of its target genes, causing the cells to be tumorigenic [51]. Reintroduction of pVHL in these cells results in reversing the phenotype transformed to a benign [56, 57]. Other groups of pVHL mutants are modified on the α domain that are capable of binding to HIF-1α but not to elongin C or other components in this ligase complex, including the chaperonines [58–60]. pVHL is a relatively unstable protein unless it is appropriately folded and bound to elongin B, elongin C, or the chaperonines, and it is known to be regulated in a proteasome-dependent manner [61]. The phenotypes of the α domain mutants are similar to those of the β domain mutants, suggesting that pVHL–HIF-1α interaction and formation of VBC complex play key roles in HIF-1α degradation.

Interaction of pVHL and HIF-1α is enhanced by the protein Tid1L. Originally identified in the fly as a tumour-suppressor gene *l(2)tid*, and also shown to be present in its mouse homologue Tid-1L, this protein potentiates destabilization of HIF-1α through enhanced interaction with pVHL [62]. These findings suggest that Tid1L may offer another layer in the regulation of pVHL activity.

pVHL activity towards HIF-1α is also regulated by the deubiquitinating enzyme VDU. Through its ability to deubiqutinate HIF-1α, VDU can antagonize the stabilizing effect of pVHL on the protein. The balance between pVHL and VDU offers an independent layer for regulation of HIF availability [63] although the conditions for VDU activity are yet to be identified.

It is of interest that VHL was reported to be in the static state within the nucleolus and yet released from its ''detention'' following stimulation. A protein surface region of the pVHL β–sheet domain was identified as a discrete (H^+)-responsive nucleolar localization signal that targets the VHL/Cullin2 ubiquitin ligase complex to nucleoli in response to environmental fluctuations in pH [64].

It is also noteworthy that phosphorylation of the N-terminal acidic domain of pVHL by casein kinase 2 is essential for its tumour-suppressor function. Although this modification does not affect levels of HIF-1α, it changes binding of pVHL to other binding partners, such as fibronectin. Consequently, inappropriate fibronectin matrix deposition attenuates tumour formation in mice [65].

HIF-1α accumulation in hypoxia is impaired by the inhibitor of diacylglycerol kinase R59949. Binding of pVHL to HIF-1α is enhanced in the presence of R59949, even under hypoxia, as a result of its ability to stimulate prolyl hydroxylase activity (regardless of the level of oxygen) [66].

Intriguingly, pVHL expression is inhibited in anoxic conditions in neuronal progenitor cells [67], suggesting that expression of HIF-1α (as well as of other pVHL targets) is required in certain phases of differentiation. The role of specific oxygen tension in cell fate determination as well as organ differentiation has been demonstrated [68–72].

pVHL is also capable of affecting the stability of the atypical PKC group member lambda. The active form of PKC-lambda is preferentially ubiquitinated by pVHL. Given the role of aPKC in the regulation of cell polarity, a possible role of pVHL in such changes in an HIF-independent pathway has been proposed [73].

Intriguingly, PI3K/AKT has been associated with upregulation of HIF-1α expression. A possible mechanism that would explain how this comes about pointed to the role of this signalling pathway in the regulation of heat shock proteins 70 and 90 expression. In turn, Hsp90 was found to associate with HIF-1α in a manner that protects it from pVHL ubiquitination and degradation. Thus, changes in Hsp70 and Hsp90 were proposed to affect the extent of their ability to "protect" HIF-1α from pVHL-dependent degradation [74]. In the presence of the Hsp90 antagonist geldanamycin A (GA), HIF-1α is efficiently ubiquitinated and degraded, suggesting that an Hsp90 interaction with HIF-1α has a role in HIF-1α stabilization [75]. Hsp90 inhibition-dependent degradation of HIF-1α was observed in both normoxia and hypoxia, indicating that escape from pVHL-dependent degradation is not sufficient for HIF-1α to be stabilized in hypoxia. More interestingly, HIF-1α degradation by GA was also observed in pVHL-deficient renal carcinoma cell lines, suggesting the possibility of an alternative HIF-1α degradation mechanism independent of pVHL that is inhibited by Hsp90 in the steady state [76].

Another component of the SCF complex has recently been shown to be subject to prolyl hydroxylation. Skp1, which serves as the adaptor for the SCF–ubiquitin ligase complex, was shown to be subject to 4-hydroxylation at proline 143, followed by O-glycosylation by α-linked GlcNac [77].

6.4 Siah-dependent Regulation of PHD

Siah is an N-terminal RING finger protein, having two isoforms, Siah1 and Siah2 (mice have two variants of Siah1, called Siah1a and Siah1b) [78]. It was originally identified in *Drosophila* as Sina, which targets the Tramtrack receptor for degradation, to promote eye differentiation [79, 80]. In mammals, Siahs target multiple proteins involved in the cell cycle (DCC, PEG10) [20, 81], apoptosis (Pe1/Peg3) [82], transcription (N-coR, C-Myb, BOB.1/OBF.1, PML, Numb, CtIP) [83–89], splicing (T-STAR) [90], cytoskeletal organization (Kid) [91], neuronal function

(synaptophysin, glutamate receptor, α-synuclein, synphilin-1, Af4) [92–97], and energy production (α-ketoglutarate dehydrogenase) [98]. It has been structurally demonstrated that Siah forms a homodimer on its C-terminal domain and forms a pocket-like structure with its N-terminal portions [99, 100]. Siah-interacting proteins, such as SIP and phyllopod, are considered to serve as adaptors between E3 ligase and substrates [79, 80, 101].

Among Siah substrates are PHD proteins which interact with Siah2, and are actively degraded in a ubiquitin–proteasome-dependent manner mediated by Siah [102]. In mouse embryonic fibroblast (MEF) cells, comparing wild-type and Siah2-null backgrounds, expression of PHD3 was elevated in Siah2 KO cells, which thus showed less HIF-1α stabilization in hypoxia. Therefore, Siah2 is thought to play an important role in the cellular hypoxic response. Siah2 KO mice develop and survive normally with limited phenotypes [103]. However, when those mice are subjected to hypoxic conditions, they show an impaired ventilatory response to the acute phase of hypoxia and deficiency in hypoxia-induced polycythemia (lesser increase in red blood cells). These phenotypes could be due to less stabilization of HIF-1α, although the possibility that Siah2 has additional roles in the hypoxia adaptation step besides those in the HIF-1α pathway can not be ruled out.

Importantly, activity of Siah2 in degrading PHD3 increases in hypoxic conditions. One of the mechanisms increasing such activity is the upregulation of Siah2 transcription. Identification of the mechanism of this upregulation and determination of whether it is dependent on HIF-1α are under investigation. Another possible mechanism is post-translational modification of Siah2 (e.g., ubiquitination, SUMOylation, phosphorylation) that either makes Siah2 resistant to its self-ubiquitination or more active as an E3 ligase. The possibility that Siah2 ligase activity is affected by hypoxia is currently under investigation (see below; Figure 6.3).

6.5 Other Examples of Altered Ubiquitination During Hypoxia

6.5.1 p53/Mdm2

Although more than 500 ubiquitin ligases may be present in the entire human genome, knowledge of changes in general ubiquitin proteasome activity in hypoxia is limited. However, given the changes in key factors in hypoxia, both global and specific changes in ligases are expected. Some examples of such changes have already been described, and more are expected.

One of the short-lived proteins that undergoes a different mode of ubiquitination in hypoxia is p53. In hypoxia, p53 is stabilized and expressed well in some cell types, including MCF7 [104]. The stabilization mechanism appears to be partly HIF-1α-dependent, which could be due to the interaction of HIF-1α with p53 or of proteins induced by HIF-1α or p53 [105]. In addition, two of the well-characterized

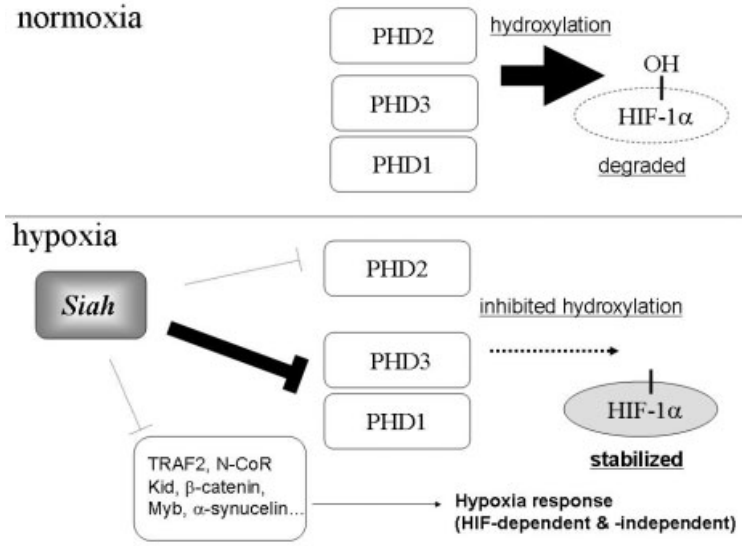


Fig. 6.3. Siah2-dependent regulation of PHDs leads to HIF-1α stabilization in hypoxia. In normoxia PHDs hydroxylate HIF-1α to enable pVHL association which results in its degradation. In hypoxia Siah2 is induced and actively targets PHD3 and 1 for degradation, therefore limiting the availability of PHD to hydroxylate HIF-1α, resulting in the stabilization of HIF-1α. Regulation of PHD1/3 by Siah offers new understanding for the regulation of general hypoxic responses in both HIF-1α-dependent and -independent manners.

ubiquitin E3 ligases for p53, i.e., MDM2 and E6/E6AP, have been shown to exhibit reduced ability to degrade p53 in hypoxia [106]. HPV-infected cells exhibit weaker interaction between p53 and E6 in hypoxia than in normoxia, thereby further explaining how p53 becomes stabilized and expressed in hypoxia. Additionally, in the same cell type, MDM2 expression was found to be decreased in hypoxia, pointing to another mechanism for how p53 is stabilized. The nature of the altered p53 affinity towards E6 or the reduced expression of MDM2 in hypoxia remains to be determined: the possible implications could be of great significance for hypoxia-dependent stabilization of p53, as for its availability.

It should be noted that MDM2 itself is regulated by hypoxia. Activation of the PI3K/Akt pathway by hypoxia leads to increase in MDM2 and HIF-1α [107, 108]. Overexpression of MDM2 increases HIF-1α expression even in normoxia. Intriguingly, p53/MDM2 double KO cells show impaired stabilization of HIF-1α in response to IGF1, suggesting that MDM2 is a positive regulator of HIF-1α. MDM2 is transcriptionally induced in hypoxia independent of p53; not surprisingly, MDM2 has positive roles in adaptation to hypoxia that are independent of p53 regulation. Furthermore, it has been shown that MDM2 associates with HIF-1α [109, 110]. The effect of MDM2 on HIF-1α remains elusive yet it has been established that MDM2-dependent degradation of p53 is remarkably inhibited when MDM2

is bound to HIF-1α in hypoxia, suggesting another possible explanation for stabilization of p53 in hypoxia.

6.5.2 MyoD

MyoD, a transcription factor involved in muscle differentiation, is actively degraded through the ubiquitin–proteasome pathway under hypoxic conditions in myoblasts, thus inhibiting differentiation into muscle cells [111]. The E3-ligase for this ubiquitination is yet to be identified, although this phenomenon represents a good model, indicating the importance of the UPS system in cell differentiation in hypoxia.

6.5.3 CREB

The cAMP response element binding protein (CREB) is involved in the induction of pro-inflammatory genes in response to cytokines such as TNFα. CREB has been shown to be hyperphosphorylated on one of the serine residues that shares a homology with IκBα and β-catenin during hypoxia, as the protein phosphatase 1γ (PP1γ), known to dephosphorylate CREB, is downregulated [112]. This hyperphosphorylation leads to the ubiquitination and degradation of CREB by a yet to be identified ligase. The physiological relevance of CREB downregulation in hypoxia remains elusive.

6.5.4 SUMOylation

Expression of SUMO, a ubiquitin-like protein, has been found to be upregulated in brain and heart exposed to hypoxia [113, 114]. This finding was connected to co-localization of SUMO with HIF-1α, suggesting its role in HIF-1α stabilization and activity. Another report indicates the possible role of SUMO in hypoxia, showing that CREB is actively SUMOylated to stabilize CREB and changing the subcellular localization of CREB to be retained in the nucleus [114]. As CREB has also been shown to be degraded in a ubiquitination-dependent manner, the precise regulation of CREB in hypoxia is not clear, and a relationship between the ubiquitination and SUMOylation of specific substrates may be present in hypoxia. In the same report, IκBα is also shown to be SUMOylated at the late stage of hypoxia (48–72 h), but the role of SUMOylation in relation to IκBα remains unknown.

SUMOylation of ARNT, a β subunit of HIF, has also been identified [115]. A decrease in the transcriptional activity of both HIF-1 and AHR transcription factors is noted upon changing the subcellular localization of ARNT in the nucleus. Although the connection to hypoxia is not well established, another interesting possibility is that HIF-1 transcriptional activity is regulated by a mechanism independent of its stability.

6.6 Ischemia Model

Ischemia caused by obstruction in blood flow also exposes cells to limited oxygen, which could be understood as a pathological hypoxia. The major difference between ischemia and hypoxia is that ischemia deprives cells of nutrients from the environment, causing a more complex response that cannot be explained by the hypoxia response. In an ischemia model of rat brain, such as forebrain and hippocampus, an increase in the level of ubiquitin as well as ubiquitin-conjugated proteins was observed [116, 117]. It is currently understood that this change in the ubiquitin level is part of the stress response of appropriately removing the unfolded protein that would accumulate in the ischemic condition. However, this understanding raises the questions of how ubiquitin conjugation is regulated, and of whether there is any substrate specificity in this context. Interestingly, the ischemic-dependent induction of ubiquitinated proteins was not seen in hypoxia treatment, suggesting that other factors (e.g., nutrients) are involved in this more complex form of response.

6.7 Regulation of the Ubiquitin System in Hypoxia

As described above, there are certain ubiquitin-related proteins that are either activated or inhibited during the hypoxia response. There are multiple possibilities for ubiquitin–proteasome system alteration in different oxygen concentrations since hypoxia activates a number of signalling cascades known to change the cellular status. The possible mechanisms for altering ubiquitin ligase activities are: expression level, as seen for Siah2; post-translational modification of substrate (e.g., CREB, IκBα); affinity between the ligase and the substrate; and localization of ligase and substrate. pVHL, one of the key proteins regulating HIF-1α, is also well characterized as a protein requiring appropriate folding by chaperones. Mutation in the α domain of pVHL which is crucial for the TRiC chaperonine complex and/or elongin C interaction, prevents pVHL from proper folding, thereby making it unstable and targeted to degradation via the UPS pathway [118, 119]. This mutation also results in VHL disease, as seen in the HIF-1α interacting β domain mutants. Since pVHL is an unstable protein if not properly folded, complex formation with elongin B,C is essential for pVHL to retain its activity in the cells. Expression of pVHL itself is not thought to be affected by hypoxia; however, as hypoxia reduces protein synthesis and could alter chaperone expression, it is an interesting notion that pVHL stability itself may be affected during hypoxia.

It has been shown that the pVHL–elongin B,C–Cullin2 complex is sequestered into the nucleoli during hypoxia [120]. Hypoxia-induced acidosis changes the pH in the cells to trigger the pVHL sequestration that will make this complex inactive and separate from HIF-1α. Interestingly, a similar observation was made about the

MDM2 protein. The role of MDM2 in hypoxia has been discussed above. It is an intriguing aspect that pVHL and MDM2 are regulated in similar ways.

6.8 Concluding Remarks

The hypoxia response is an essential system if an organism is to adapt to – and overcome – severe conditions that affect its metabolism, respiration, and energy production. Furthermore, investigations have demonstrated the significance of hypoxic conditions in the microenvironment (i.e., organ, tissue, and cell) during development, both for cell differentiation and in pathological conditions, such as tumorigenesis or ischemia. As the UPS system is also involved in multiple cellular responses, including cell cycle, signal transduction, cell differentiation, and so forth, it is almost certain that a growing number of ubiquitin/ubiquitin-related proteins will be found to be involved in the hypoxia response one also expects to gain a better understanding of the hypoxia response in both HIF-dependent and -independent ways.

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7
p97 and Ubiquitin: A Complex Story

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Abstract

p97 is an abundant, hexameric AAA ATPase, constituting 1% of the cytosol. It carries out diverse cellular roles, and is increasingly linked to ubiquitin in these processes. Ubiquitin modification determines the fate of many cellular proteins. Conjugation with a single ubiquitin molecule is a signal associated with altered protein trafficking whereas conjugation of a chain of ubiquitin can target a substrate protein to the proteasome for degradation, a function of the ubiquitin–proteasome system (UPS). Recent advances have established p97 (also known as VCP in mammals and Cdc48 in yeast) as a key part of the UPS, best characterized in the ERAD pathway.

The UPS is a nonlysosomal proteolytic system, in which a candidate protein (short-lived or misfolded) is identified, modified with a ubiquitin chain, escorted to the proteasome and then unfolded, deubiquitinated and subjected to proteolysis. This involves recognition of the substrate protein and the actions of a succession of proteins on it. p97 is of particular importance as it is able to interact with many different proteins in this series of events. Current evidence points to a role for p97 in the identification and possible subsequent partial unfolding or disassembly of a given protein or protein complex. In the UPS, for example, this could be the disassembly of ubiquitinated proteins from unmodified proteins, prior to capture by the following interacting protein. It appears that this functionality possibly extends to other cellular processes that p97 participates in, such as post-mitotic membrane fusion.

In this chapter we will give an overview of these p97 interacting proteins and detail how p97 targets ubiquitin-modified proteins in cellular processes such as ERAD.

7.1
Introduction

p97 was first identified in yeast as a cell-division-cycle gene, Cdc48, which when mutated led to cell cycle arrest with abherent spindles and microtubules [1]. In

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mammals, p97 was first observed as a protein that contained valosin peptide, VCP (valosin containing protein), an artifact of a screen for novel gut peptides [2–4]. The two homologues were linked by Fröhlich and coworkers who recognized the homology between these genes and that of NSF (*N*-ethylmaleimide-sensitive fusion), a protein implicated in Golgi vesicle transport, and suggested that they may carry out similar functions *in vivo* [5]. Also in 1991, the homology between many proteins with diverse function was delineated as an ATPase domain and named the AAA domain (ATPases associated with diverse cellular activities) [6]. It has been shown that p97 can indeed carry out a function similar to its fellow AAA ATPase, NSF, by mediating post-mitotic Golgi reassembly and is also implicated in ubiquitin-dependent proteolysis [7–10].

The AAA family of ATPases is a subfamily of Walker-type NTPases and typically form ring-shaped oligomers. They are defined as not only containing Walker A and B motifs crucial for ATP binding and hydrolysis, in common with Walker-type NTPases, but also contain a second region of homology (SRH, also known as AAA minimum consensus) which is key to communication within the oligomeric ring (reviewed in Refs [11, 12]). Many AAA proteins function within the UPS, although not as proteases as observed in bacteria. Recently, much work has firmly identified hexameric p97/Cdc48 as a member of the UPS and interestingly also implicate ubiquitin in p97's other roles such as membrane fusion.

Ubiquitin modification is widely used to target proteins to specific destinations within the cell. A single ubiquitin (monoubiquitin) is usually a sorting signal for altered protein trafficking whereas proteins tagged by multiple ubiquitin molecules joined in a chain (polyubiquitin) have different destinations, depending on the chain length and linkage residue. Ubiquitin is a small, 8.6-kDa protein that exhibits a β-grasp fold and can be covalently attached to a target protein through the ε-amino group of a lysine residue. Ubiquitin has seven lysine residues, of which five are known to be sites for sequential ubiquitin modification *in vivo* (Lys 6, Lys 11, Lys 29, Lys 48 and Lys 63) resulting in a number of different types of ubiquitin chain. Ubiquitin conjugation is a multistep process involving the E1 ubiquitin-activating enzyme, which in an ATP-dependent manner forms a thioester linkage between ubiquitin and itself, an E2 ubiquitin-conjugating enzyme which receives ubiquitin and forms a new thioester linkage and an E3 ubiquitin ligase that selects the substrate and facilitates ubiquitination (reviewed in Ref. [13]). As there are many E3 enzymes it is thought that these are crucial for substrate specificity in the proteolytic system. The E1, E2 and E3 enzymes are sufficient to modify a protein with one or two ubiquitin moieties. In some cases E4 polyubiquitin chain-conjugation factors have been identified to act alongside E1–E3 to elongate mono/diubiquitin to a polyubiquitin chain [14]. p97 has been shown to interact directly or indirectly with many E3 and E4 enzymes.

The polyubiquitin signal is recognized by the 26S proteasome, the point of convergence for all UPS functions, and results in the proteolysis of targeted proteins. Proteasomes are large multiple-protein complexes, which are present in the cytosol and the nucleus of eukaryotic cells. Each proteasome has a central cylinder (20S)

that degrades the captured protein and regulatory caps (19S) at each end that recognize the substrate and feed it into the central core to be degraded. The 19S regulatory cap may be replaced by other regulatory complexes but these do not recognize ubiquitinated proteins or use ATP [15]. Protein degradation occurs concurrently with ubiquitin cleavage from the substrate.

However, not all proteins modified by ubiquitin are targeted to the proteasome for degradation (reviewed in Ref. [16]). Only those with chains linked by Lys 48 and possibly Lys 29 have been shown to be targeted, although there is also evidence of nonubiquitinated proteins being degraded by the proteasome but this is outside the scope of this chapter. Chains linked by Lys 63 are indicative of nonproteolytic functions such as DNA repair, kinase activation, trafficking and translation, and Lys 6 chains have been reported to inhibit ubiquitin-dependent degradation and may function in DNA double-strand break repair [17, 18]. p97 has been shown to interact with monoubiquitin and Lys 48 polyubiquitin chains and is potentially linked to Lys 6 and 29 polyubiquitin chains through adaptor proteins.

p97 is implicated in many cellular processes including Golgi and nuclear envelope reformation post mitosis, spindle disassembly at the end of mitosis and processes involving the UPS. A uniting factor among many of these processes is the involvement of ubiquitin and ubiquitin-interacting proteins. In connection with the UPS, p97 has been shown to be involved in degradation of cytosolic proteins, in the degradation of ER luminal and transmembrane proteins via the ERAD pathway and also to act in the regulated processing of transcription factors [8, 19–21]. Many proteins involved in the UPS function in parallel fashions and appear redundant; however, p97 has been shown to be an essential factor. p97 is thought to be targeted to these specific functions by adaptor molecules that often bind ubiquitin [22].

The functions of the adaptors p47 and Ufd1–Npl4, are the best characterized: Ufd1-Npl4 directs p97's action to ERAD and mitotic spindle disassembly and p47 in higher eukaryotes targets p97 to post-mitotic homotypic membrane fusion events [23–25]. Interestingly, whilst it was initially thought that a specific adaptor targeted p97 to a specific cellular role, a more complicated picture has recently emerged. p47 in yeast cells also appears to associate with ubiquitinated substrates, possibly acting as a shuttling factor to escort them to the proteasome. p47 as well as Ufd1-Npl4 were also shown to be crucial to nuclear envelope reformation [26]. It seems possible that the adaptors may target p97 to a specific action rather than a particular cellular pathway.

A large body of work has been published about the involvement of p97 in ERAD (see Section 7.3.1). ERAD is the process by which ER luminal and transmembrane proteins are degraded as part of protein quality-control mechanisms or as regulated degradation. ERAD substrates are transported to the cytoplasmic side of the ER where they are polyubiquitinated (through Lys 48). The p97–Ufd1–Npl4 complex then plays a role in modulating the chain length and separating the substrate from the ER membrane [27–29]. The substrate is then targeted to the proteasome

either directly or by so-called shuttle factors before being degraded (reviewed in Ref. [30]).

Post-mitotic homotypic membrane fusion events allow the reformation of organelles through multiple cycles of SNARE-mediated fusion. Post-fusion the SNARE four-helical-bundle complexes are tightly bound together and must be separated to allow further rounds of fusion. The p97–p47 complex is essential for Golgi, transitional ER and nuclear envelope reformation and is thought to play a role in disassembling the t–t–SNARE complexes or removing a factor that contributes to the stability of the complex (reviewed in Ref. [31]) (see Section 7.3.2.4). In Golgi reformation, p97-p47 has been shown to also require a protein with deubiquitinating activity, VCIP135, so it seems likely that ubiquitin also plays a role in this p97 function [32].

p97 interacts with many other proteins for which, as yet, no functional pathway has been defined (e.g., Ufd3, see Section 7.3.2.3). Interestingly though, many of these proteins are connected with the UPS, and so may present undefined pathways for p97 to act to separate protein complexes.

The mechanisms by which p97 can interact with so many proteins are increasingly understood, but currently there is little information about the exact action of p97 in these complexes. Adaptor proteins are generally bound through the N domain of p97 (reviewed in Ref. [22]). The N domain is linked to two AAA domains, D1 and D2, and the three domains are connected by flexible linkers. It is thought that energy from ATP hydrolysis in the AAA domains is transmitted through linkers within the protein to cause N-domain movement. These conformational changes are then transmitted to adaptor proteins and consequently to bound substrates. This appears to result in the substrates becoming isolated or untethered from other protein complexes, aggregates or membranes, leading to p97 being described as a molecular chaperone or a segregase [20, 33] (see Section 7.4).

Consistent with its many cellular functions, p97 is essential for cell viability. Mutations in p97 lead to cellular abnormalities, such as cell cycle arrest, swollen ER and morphological changes such as formation of cytoplasmic vacuoles leading to cell death [5, 34–36]. Interestingly, human mutations of p97 are associated with inclusion body myopathy of Paget's disease of bone and frontotemporal dementia (IBMPFD), and malfunction of p97 is widely associated with other inclusion body neurodegenerative diseases characterized by neuronal aggregates of unfolded proteins (see Section 7.5) [37]. This is consistent with p97's role in the UPS and its putative function as a segregase or chaperone.

In this chapter we outline the links between p97 functions, ubiquitin and the proteasome. However, a detailed review of the UPS is beyond its scope. We examine the structural basis for ubiquitin and p97 recognition by the many proteins with which they interact, and discuss some common features. We review the role of p97 in pathways that involve ubiquitin, in particular ERAD in which p97 is well characterized. Finally, we suggest how p97 carries out these actions and discuss the phenotypes that arise when these proteins fail to function.

7.2
Interactions of Ubiquitin, p97 and Adaptors

Ubiquitin recognition is vital for many cellular processes. Many p97-interacting proteins are able to bind to ubiquitin and share certain common structural features. p97 is able to interact with ubiquitin and some ubiquitin-like domains (see Sections 7.2.2 and 7.2.3). In particular, many multi-domain p97 adaptor proteins contain ubiquitin-like, ubiquitin regulatory X (UBX) domains, such as the p47 adaptor (Section 7.2.4). In addition, many p97-interacting proteins contain not only ubiquitin-like folds but also ubiquitin-recognition domains or bind mutually to proteins that can recognize ubiquitin (see Section 7.2.1) [22]. p97 is also able to interact with some E3 ubiquitin ligases and deubiquitinating enzymes although these interactions are less well defined (Sections 7.2.5 and 7.2.6).

In the following sections we provide an overview of the basis of p97 and its interactions with adaptors and ubiquitin. Many of the proteins described in this chapter are highly conserved between yeast and higher eukaryotes. Table 7.1 provides a list of names commonly used for these orthologues and Figure 7.1 shows representations of the proteins. As many p97-binding proteins also interact with ubiquitin we will first outline the types of ubiquitin-interacting domains.

7.2.1
Ubiquitin-binding Domains and Motifs

Ubiquitin adopts a β-grasp fold, which is characterized by the presence of a β-β-α-β-β core. Other domains that adopt this fold are known as ubiquitin-like (UBL) domains, which can either act alone as modifiers similar to ubiquitin itself (such as the ubiquitin homologue SUMO (small ubiquitin-like modifier)) or exist as integral UBL domains within ubiquitin domain proteins (UDP) (such as Rad23) [38]. By covalently linking ubiquitin or UBL modifiers to proteins, the cell creates a diverse family of modified proteins. This provides signals that can be identified downstream by a plethora of protein receptors/interactors to control many pathways in a cell. UBL modifiers share a few characteristics that distinguish them from integral UBL domains. To date, all UBL modifiers contain a C-terminal double glycine extension with the exception of Hub1 (C-terminal di-tyrosine motif followed by a single variable residue) [38]. Additionally, the S3/S4 loop of all UBL modifier proteins known so far is the same length as the S3/S4 loop of ubiquitin [39]. Integral UBL domains do not contain this double glycine motif and exhibit variable lengths of S3/S4 loops.

The first indication of how ubiquitin might bind to other proteins came from insights into ubiquitin binding to the proteasome subunit S5a [40]. This identified a hydrophobic patch on ubiquitin consisting of Leu 8, Ile 44 and Val 70 [41]. S5a binds ubiquitin through a ubiquitin-interacting motif (UIM). Among many other UIM-containing proteins, endocytic factors Eps15 and Hrs (Vps27 yeast) bind monoubiquitin with very low affinities (dissociation constants: 200–300 μM).

Table 7.1. Proteins and their aliases.

Function	Homo sapiens	Saccharomyces cerevisiae	Schizosaccharomyces pombe
AAA ATPases	p97, VCP	Cdc48	Cdc48
	NSF	SEC18	SEC18
	PEX1	PEX1, PAS1	
		Hsp104	
	hVPS4B, SKD1	Vps4	
Proteasome subunits and shuttling factors	S5a, PSMD4	Rpn10	Pus1
	S6, PSMC4, TBP7, Rpt3	Rpt5	
	Rad23A, HhR23A	Rad23	
	Rad23B, HhR23B	Rad23	
	PLIC2, Ubiquilin 2, Chap1	Dsk2	
		Dsk2	
		Ddi1, Mud1	
p97/Cdc48 adaptors	p47	Shp1	Ubx3
	Ufd1L	Ufd1	
	Npl4, KIAA1499	Npl4, Hrd4	
E1 ubiquitin-activating enzyme	UBE1	UBA1	
E2 ubiquitin-conjugating enzymes	UBE2G2	Ubc7	
	Hip2	Ubc1	
	NCUBE1 and 2	Ubc6	
E3 ligase complex members	BRCA1		
	Bard1		
	Hrd1	Hrd1, Der3	
	Sel1	Hrd3	
	Dorfin		
	Gp78, AMFR		
	Parkin		
	Fbs1	Doa10, Ssm4	
	TEB4, MARCH-VI		
E4 polyubiquitination factors	CHIP, STUB1		
	Ufd2a, UBE4A	Ufd2	
ER membrane translocons	Derlin-1	Der1	
	Sec61 complex	Sec61 complex	
Hsp70 chaperones	BiP, Hspa5, Grp78	Kar2	
		Ssa1–4	
Membrane anchors	VIMP	Ubx2, Sel1	
		Cue1	
		Ubx2, Sel1	
		Cue1	

Table 7.1 *(continued)*

Function	Homo sapiens	Saccharomyces cerevisiae	Schizosaccharomyces pombe
Deubiquitinating enzymes	Ataxin 3 VCIP135		
SNARE proteins	Syntaxin 5, Syntaxin 5a Syntaxin 18, Stx18	Sed5 Ufe1	
Other p97/Cdc48 binding proteins with unknown function	HDAC6, KIAA0901 PLAA, PLAP	Ufd3, Zzz4, Doa1 Cui1, Ubx4 Cui2, Ubx6 Cui3, Ubx7	 Lub1 Ubx2

Many other small ubiquitin-binding domains exist, namely: the ubiquitin-associated (UBA) domain; the coupling of ubiquitin conjugation to ER degradation (CUE) domain; the ubiquitin E2 enzyme variant (UEV) domain, which lacks enzymatic activity; the GGA and Tom1 (GAT) domain; the Vps27, HRS, STAM (VHS) domain; the GRAM-like ubiquitin binding in Eap45 (GLUE) domain and zinc finger domains like the Npl4 zinc finger (NZF) domain and the HDAC6 (histone deacetylase 6) polyubiquitin associated zinc finger (PAZ) domain [42–55]. An overview of these domains can be seen in Table 7.2. (These ubiquitin-binding domains have been reviewed in Ref. [56].) We now know many structural characteristics of these complexes, although there is no structure or mapping data for the PAZ–ubiquitin, GLUE–ubiquitin or VHS–ubiquitin complexes. The p97 N domain adopts a double Ψ-β-barrel fold and is reported to bind ubiquitin. Interestingly, a similar domain in Ufd1, designated UT3, is also responsible for ubiquitin binding [26, 57]. To date, no structural details are available for the p97–ubiquitin interaction (see Section 7.2.2).

The common theme of these interactions is that they recognize the side of ubiquitin containing a hydrophobic pocket formed by Leu 8, Ile 44, His 68 and Val 70, which was first observed for S5a binding to ubiquitin (Figure 7.2). The way this particular surface recognition is achieved, however, varies between the different domains (Figure 7.3). Interestingly, another protein-interaction surface around Phe 4 of ubiquitin has been predicted [58]. In support of this, Dikic recently identified proteins that do not bind to ubiquitin through the Ile 44 surface (reported in ref [17]). There are three different main groups of ubiquitin-binding domains; α-helical, zinc finger and α/β proteins. The UIM is the simplest case of the α-helical group comprising just a single helix. S5a of higher eukaryotes contains two ubiquitin-interacting (UIM) motifs (UIM 1 and UIM 2) and both bind polyubiquitin *in vitro* albeit with very different affinities [59]. UIM-2 has higher affinity for ubiqui-

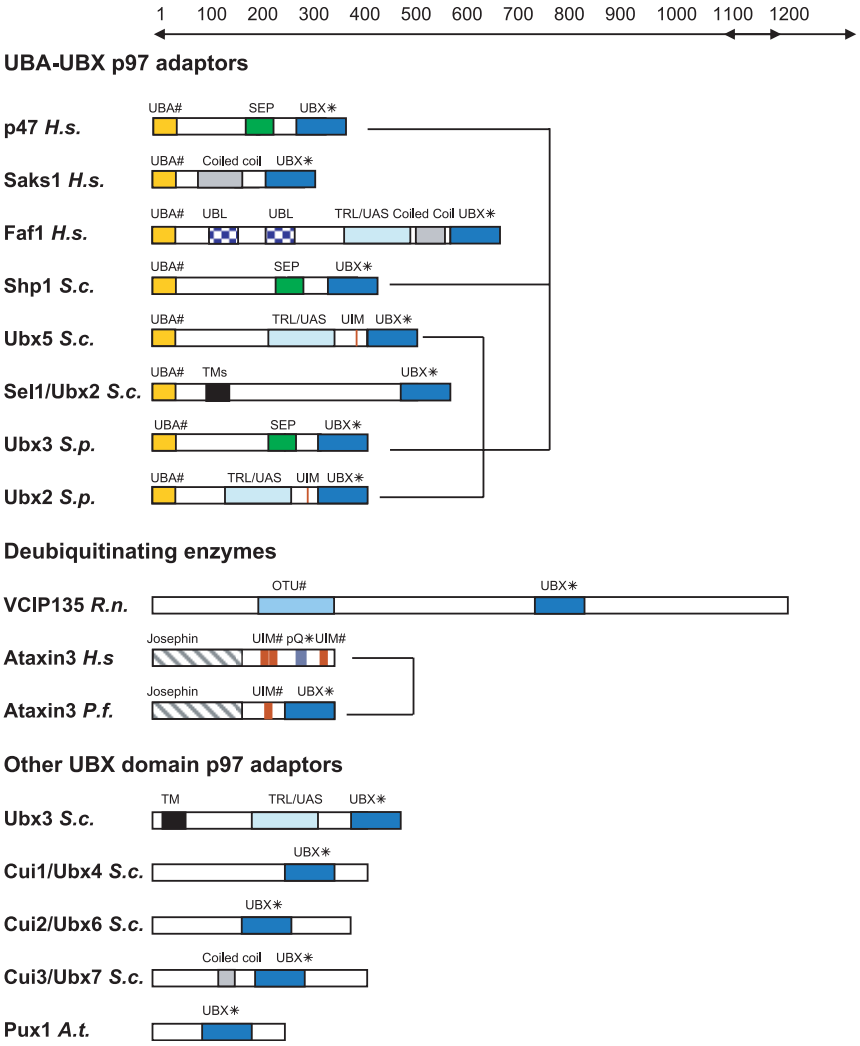


Fig. 7.1. Representations of different p97/CDC48/VCP interactors and assorted proteins important for the ubiquitin–proteasome system, showing their predicted and/or known domain structures. *: the part of the protein that was mapped to interact with p97/CDC48/VCP. #: ubiquitin-binding domains. Domain names and structural descriptions are mostly in agreement with the SMART database nomenclature or else with the current literature. Abbreviations: H.s., Homo sapiens; S.c., Saccharomyces cerevisiae; S.p., Schizosaccharomyces pombe; P.f., Plasmodium falciparnum; A.t., Arabidopsis thaliana.

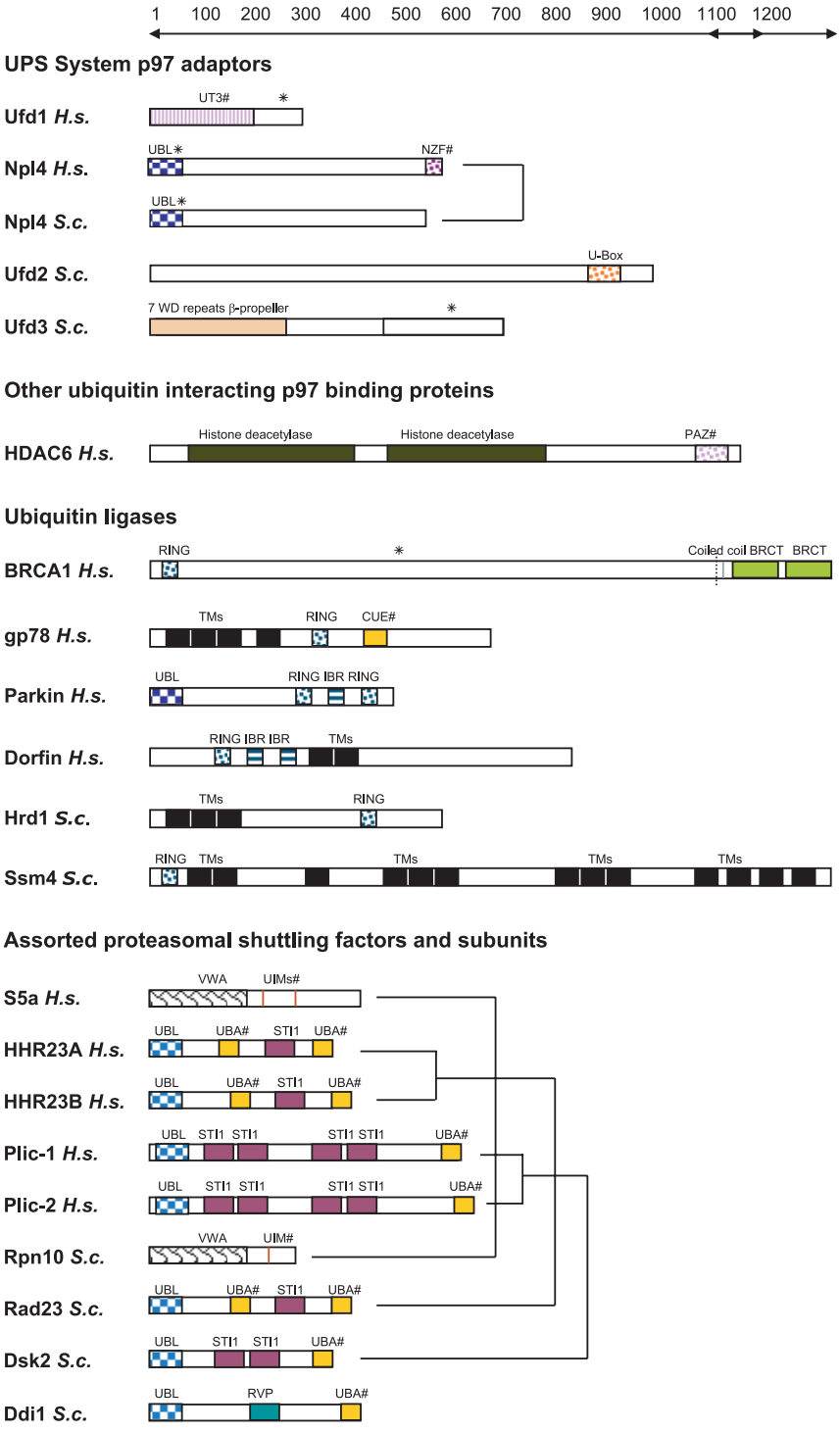


Fig. 7.1 (continued)

Table 7.2. An overview of ubiquitin-binding domains.

Domain	PDB codes	Structure	Ubiquitin preference	p97 binding proteins containing the domain
UIM	**1YX6**, **1YX5**, 1YX4, 1UEL, **1Q0W**, 1Q0V, 1O06	Helix	Mono- or polyubiquitin	Ataxin-3, Ubx2, Ubx5
UBA	1DV0, 1IFY, 1OAI, 1PGY, 1Q02, 1TR8, 1V92, 1VDL, 1VEG, 1VEJ, 1VEK, 1VG5, 1WGN, 1WHC, 1WIV, 1WJI, **1WR1**, 1ZV1	Three-helical bundle	Mono- or polyubiquitin	p47, Ubx2, Ubx5, Saks1, Faf1
CUE	1MN3, **1OTR**, **1P3Q**, 1WGL	Three-helical bundle	Monoubiquitin	
GAT	1J2J, 1NAF, 1NWM, 1O3X, 1OXZ, **1WR6**, 1X79, **1YD8**	Three-helical bundle	Monoubiquitin	
VHS	1DVP, 1ELK, 1JPL, 1JUQ, 1JWF, 1JWG, 1LF8, 1MHQ, 1PY1, 1UJJ, 1UJK	Eight helices in superhelix.	Not known	Ufd3
UEV	1KPP, 1KPQ, 1M4P, 1M4Q, **1S1Q**, **1UZX**	α/β fold	Monoubiquitin	
GLUE	–	PH domain predicted	Monoubiquitin	
NZF	1NJ3, **1Q5W**	Zn finger	Mono- or polyubiquitin	Npl4
PAZ	–	Zn finger	Not known	HDAC6
P97-N/UT3	1CZ5, 1E32, 1OZ4, 1QDN, 1R7R, 1S3S, 1YPW, 1YQ0, 1YQI, 1WLF, 1ZC1	Double-Ψ and β-barrel fold	Mono- or polyubiquitin	Ufd1

The structures of ubiquitin domain complexes are in bold type

tin and the UBL domain of HHR23A, a proteasome shuttling factor that participates in the ERAD pathway and the wider UPS. It was suggested that UIM-1 could have a shorter helix and hence display less affinity owing to a smaller surface area of interaction [60]. The yeast homologue of S5a, Rpn10, only has one UIM motif, corresponding to the UIM-1 in S5a; the yeast homologue of HHR23A, Rad23, binds to the proteasome via a different subunit (S2, Rpn1) [61]. The CUE, UBA

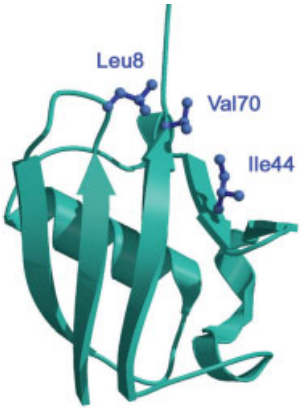


Fig. 7.2. The structure of ubiquitin. Key interacting residues are shown in ball and stick form.

and GAT domains form a three-helical bundle, and the VHS domain consists of eight helices arranged in a super-helical fold, but there is a variation in how these helical structures bind to ubiquitin (Figure 7.3) [56].

The second group of ubiquitin-binding domains, the zinc finger domains, consist of NZF and PAZ domains. The zinc finger of Npl4 (NZF domain) binds ubiquitin via a few residues clustered around the zinc binding site. It is not clear whether a similar PAZ–ubiquitin interface exists. In addition there are α/β-fold ubiquitin-binding domains. An interesting example of α/β-fold ubiquitin-binding domains is the UEV domain [62]. The tumour susceptibility gene 101 (TSG101) UEV contacts the Ile 44 surface and an adjacent loop of ubiquitin. Comparison

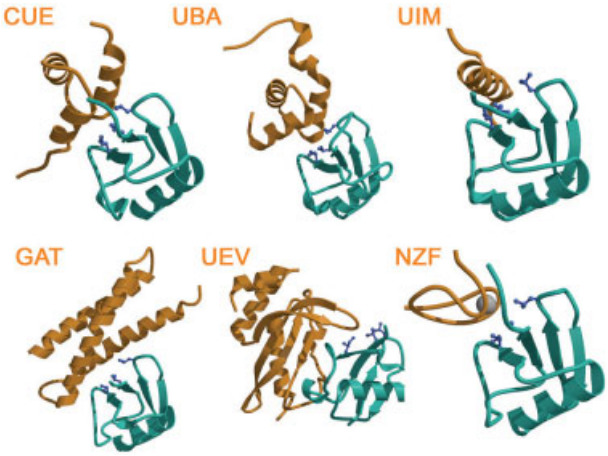


Fig. 7.3. Structure gallery of ubiquitin with residues of its hydrophobic patch in ball and stick representation (turquoise) and structures of its complexes with ubiquitin-binding domains (orange): CUE–ubiquitin (pdb code 1OTR), UBA–ubiquitin (pdb code 1WR1), UIM–ubiquitin (pdb code 1Q0W), GAT–ubiquitin (pdb code 1YD8), UEV–ubiquitin (pdb code 1S1Q) and NZF–ubiquitin (pdb code 1Q5W) [62, 161–165].

with mapping data from other UEV-domain and related E2 proteins suggests that the same fold can bind ubiquitin through different interfaces [62].

In some cases these domains are not exclusively ubiquitin-binding domains. For example, the GAT domain is a multifunctional module that interacts not only with ubiquitin but with various partners including the small GTPase ARF, the endosomal fusion regulator Rabaptin-5, and TSG101 [63]. On the other hand not all "ubiquitin-interaction domains" actually bind to ubiquitin. UBA domains can be classified into different groups according to their binding specificities towards different ubiquitin conjugates and it has been shown that some UBA domains lack ubiquitin-binding ability [64]. To what extent UBL domains can bind to ubiquitin-recognition domains *in vivo* is not yet fully understood. A group of proteins that contain a UBL domain are often described as proteasome shuttling factors or ubiquitin receptors [65]. They usually contain a UBL domain that can bind to the ubiquitin-recognition domains of the proteasome (for example UIM of S5a) and a UBA domain that binds to ubiquitinated substrates. Examples of these proteins include Rad23, Dsk2, and Ddi1. It is likely that more proteins and ubiquitin-binding motifs will emerge.

7.2.2
p97 Interacts Directly With Ubiquitin

The N-terminal domain of p97 comprises a double ψ-β-barrel structure that has been shown to interact with ubiquitin. p97, in the absence or presence of nucleotide, binds efficiently to tetraubiquitin (a mimic of polyubiquitin) through the N-terminal domains [66]. The binding of monoubiquitin, however, is still a matter of debate, probably because it is of very low affinity [20, 42, 66]. Competition experiments between ubiquitin and the UBX domain of p47 have established that tetraubiquitin could compete with the UBX domain for p97 binding, whereas monoubiquitin could not, in agreement with structural predictions [39, 67]. As UBX

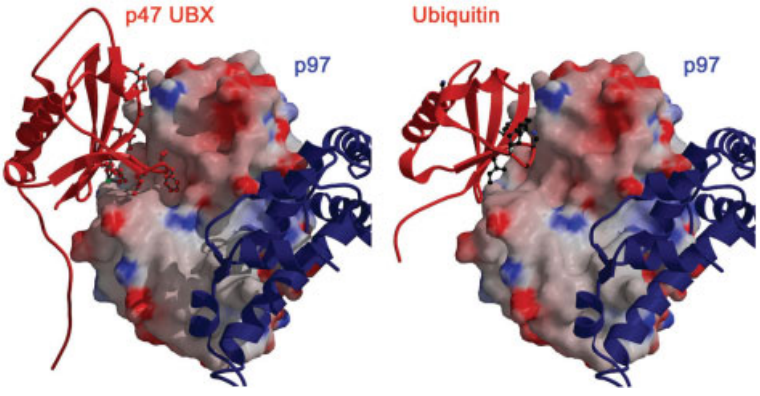


Fig. 7.4. Electrostatic surface representation of the p97 N domain in complex with p47 UBX (red) in comparison with ubiquitin binding modelled with a similar interface. Note the significantly shorter S3/S4 loop in ubiquitin.

domains have a similar fold to ubiquitin, ubiquitin can be modelled in the place of p47-UBX domain binding to p97-N domain (Figure 7.4). Whilst the interface could be similar, the S3/S4 loop is much shorter in ubiquitin, predicting a weaker interaction between ubiquitin and p97-N than p47-UBX and p97-N. However, low affinities of ubiquitin binding are not unusual and it is possible that p97 can transiently bind to monoubiquitinated proteins *in vivo* [56].

Polyubiquitin binding to p97 could be a consequence of a series of weak interactions synergistically making a stronger interaction. This could arise from more than one p97 N domain being involved in polyubiquitin binding, or a p97 N domain together with an adaptor protein strengthening the interaction. This could be the case for the p97–Ufd1–Npl4 complex that is essential for ERAD. As Ufd1 and p97 N domains have similar structures, it was suggested that they could have the same ubiquitin-binding sites. However, a superposition of Ufd1 with p97 N domain shows that residues responsible for ubiquitin binding in Ufd1 are not conserved in p97. The location of the Ufd1 monoubiquitin-binding site would largely be buried at the N–D1 interface and partially covered by the N–D1 linker of p97. For this proposed recognition site to be exposed, the p97 N domains would have to undergo a very large conformational change. The region attributed to polyubiquitin binding in Ufd1 is situated in p97 on the peripheral underside of the N domain, adjacent to the p97 N–p47 UBX domain-binding site. Despite a similar fold it is not conclusive whether Ufd1 and p97 bind to ubiquitin in similar ways. Indeed, to provide specificity it is more likely that recognition differs between the two domains. Whether the structurally related N domains of NSF or PEX1 can also bind to ubiquitin is currently unresolved.

In addition to ubiquitin itself, it has been shown that UBL domains can bind to the N domain of p97. An example of this is the Npl4 N-terminal UBL domain and the large family of UBX domains can be seen as a subfamily of UBL domains [68].

7.2.3
p97 Adaptor Proteins Can Also Interact With Ubiquitin

p97 interacts with various proteins and adaptors, many of which are known to bind ubiquitin (see Table 7.1 and Figure 7.5). A number of these proteins bind p97 via a UBX domain, namely VCIP135, p47 (Shp1 in *Saccharomyces cerevisiae* and Ubx3 in *Schizosaccharomyces pombe*), Ubx2, Ubx5, Faf1 and Saks1. UBX domain containing proteins are widespread in eukaryotes. There are at least two UBX-containing proteins in *S. pombe* (Ubx2 and Ubx3), seven in *S. cerevisae* (Shp1, Ubx2–Ubx7) and forty-one in humans, including the proteins p47, Rep-8, Socius and Faf1. It is not clear at present whether all of these also contain ubiquitin-binding domains. In particular, with the exception of VCIP135, those that contain UBX domains in the middle, such as Cui1, Cui2, Cui3 and Pux1, do not seem to contain any known ubiquitin-binding motifs. However, the Cui proteins bind to Ufd3 that recognizes Lys 29-linked polyubiquitin chains so these proteins may interact indirectly with ubiquitin [69, 70].

Proteins that contain UBA and UBX domains generally do so at the N- and the C-terminals, respectively, and most of the UBA domains seem to bind to mono-

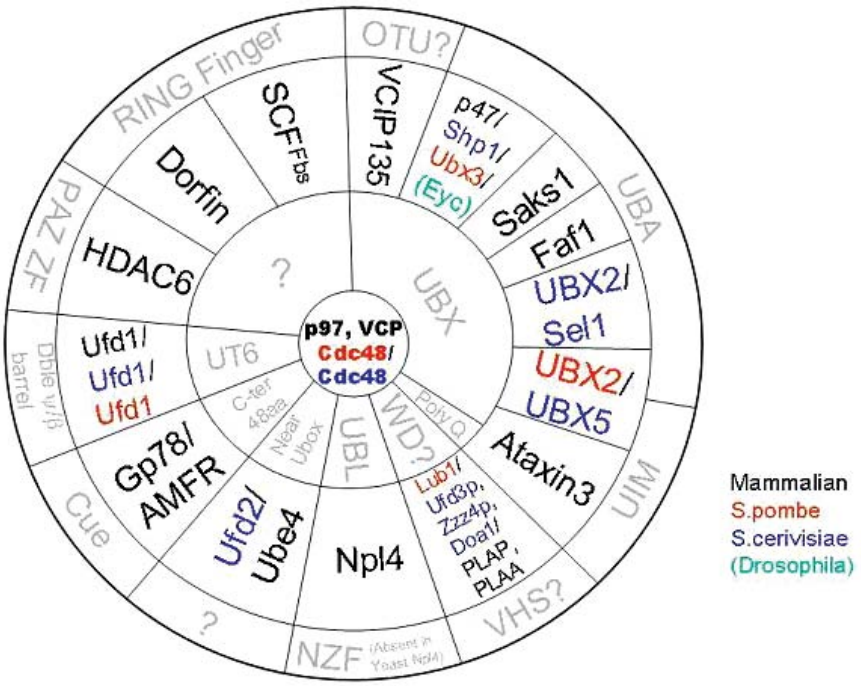


Fig. 7.5. Classification of proteins that interact with both p97 and ubiquitin according to their structural domain of interaction. From the centre (p97) are, first, the domains that interact with p97, then the proteins (colour-coded homologues according to origin), and finally the outer circle, which identifies the domain that is responsible for ubiquitin interactions.

and polyubiquitin (predominantly Lys 48- and Lys 63-linked chains) chains alike, although some of the results are conflicting [64]. It has been reported, for example, that p47 has a preference for monoubiquitin and can only bind it in the context of the p97–p47 complex [42]. In yeast, however, it was suggested that the p47 homologue Shp1 binds polyubiquitin as well as monoubiquitin [67, 71]. The "middle domains" of UBA-UBX p97 adaptor proteins vary: SEP domain (p47, Shp1), thioredoxin-like TRL/UAS domain (Ubx2, Ubx5, Faf1), UBL domain (Faf1), UIM domain (Ubx2, Ubx5), coiled-coil domain (Saks1, Faf1). A function of the SEP domain as a reversible competitive inhibitor of cathepsin L has been suggested; the function of the other "middle domains" of these adaptors is unknown at present [72].

HDAC6 and Npl4 bind ubiquitin via a zinc finger domain, although in yeast Npl4 this domain is not conserved. Both Ufd1 and Npl4 bind to both ubiquitin (through double ψ/β barrel or UT3 and NZF domains, respectively) and to p97 (through UT6 and UBL domains, respectively). Interestingly, two different binding sites for monoubiquitin and Lys 48-linked polyubiquitin have been mapped to the Ufd1 UT3 domain, indicating that the same domain can bind different ubiquitin conjugates in different ways [57].

Ufd3 contains a putative C-terminal VHS domain that is implicated in ubiquitin

binding. The C-terminus is also the p97 binding site, although in the fission yeast homologue Lub1 the WD domain was implicated in Cdc48 binding [70]. p97 interacts not only with ubiquitin-binding adaptor proteins but also with proteins that are involved in ubiquitin conjugation and processing (see Sections 7.2.5 and 7.2.6).

7.2.4 p97-p47 Structure as a General Model for UBX Domain Binding: A Level of Similarity Between UBX Domains

Currently a number of different UBX domain-containing proteins across a range of organisms have experimentally been shown to interact with p97/Cdc48, although p47-UBX domain bound to p97-ND1 is the only one for which the structure has been determined (Figure 7.7) [24, 67, 70, 73]. At present p47 and Ubx2 seem to be the only UBX-p97 interacting proteins that are conserved throughout these species. UBX domains share little overall sequence identity and can be found either at the C-terminus or in the middle of a protein. However, sequence alignment has revealed a few highly conserved residues within the UBX family, an arginine/lysine residue (Arg 301 in p47) and a ''hydrophobic residue followed by proline'' signature found in the S3/S4 loop (Phe 343 and Pro 344 in p47) (shown in Figure 7.6). These residues have been demonstrated to be involved in binding to the p97 N domain [39]. Mutation of either Phe 343 or Arg 301 results in reduced full-length p97 binding *in vitro*. Insertions and deletions in UBX domains are mainly restricted to loops after H1 (H2 in p47 C) and S4, which should not interfere with p97 binding [73]. This led to the proposal that UBX domains may generally act as binding modules for p97 and/or p97 homologues [39]. On a sequence level, the majority of

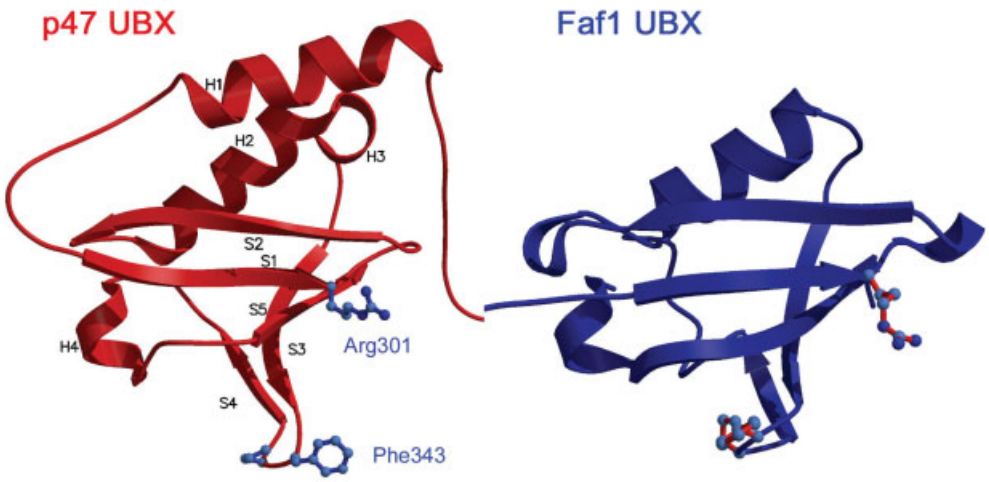


Fig. 7.6. Structures of the UBX domains of p47 (red, pdb code 1S3S, [39]) and FAF1 (blue, pdb code 1H8C, [73]). Conserved residues in the S3/S4 loop and the conserved arginine are represented in ball and stick form.

UBX domains that bind p97 have the "hydrophobic residue followed by proline signature" in their S3/S4 loop. There are however two exceptions, namely Cui1 which has a histidine instead of a hydrophobic residue and Ubx5 which lacks both (sequence alignments suggest an Asp and a His at these positions). Whilst an uncharged imidazole ring does have a certain degree of hydrophobicity (as in the case of Cui1), a UBX domain that has an S3/S4 loop containing Asp and His residues (as in the case of Ubx5) probably displays reduced affinity or binds in a slightly different manner to p97. The conserved arginine/lysine of UBX domain family members is conserved in all known p97 UBX interactors. It is well recognized that shape complementarities (for example, a specific fold that can bind to another) are only one determinant in protein–protein interactions. In the case of UBA domains, for example, the same three-helical-bundle fold can have different specificities for different ubiquitin conjugates and there are some that do not bind ubiquitin at all [64]. It would not be surprising if some of the UBX domains also showed different specificities.

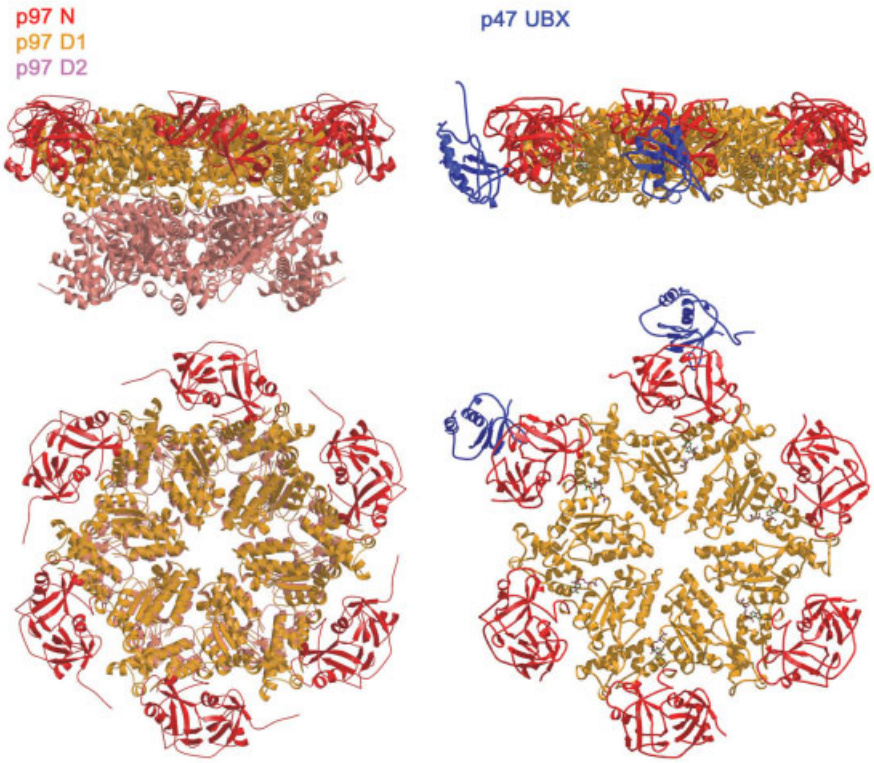


Fig. 7.7. Left: Ribbon representation (top and side views) of full-length p97 (pdb code 1R7R, [141]) (in red, orange and salmon). Right: Corresponding views of p97-ND1 complexed with p47-UBX (pdb code 1JRU, [39]).

7.2.5 The Interaction of p97 With Ubiquitin Ligases

E3 ubiquitin ligases are a large family of proteins that can be classified into three major structurally distinct types: N-end rule E3s, E3s containing the homology to E6AP C-terminus (HECT) domain and E3s with a really interesting new gene (RING) finger or its derivatives, the U-Box and the plant homeo-domain (PHD). E3 ubiquitin ligases exist as single polypeptide or multimeric complexes and they have an important role in substrate specificity. Members of the HECT family of E3s bind to E2s and form a ubiquitin thioester intermediate via a conserved cysteine in the HECT domain, before transferring the ubiquitin onto the substrate. Various RING fingers exhibit binding activity towards E2 ubiquitin-conjugating enzymes, but facilitate the transfer of ubiquitin from the E2 to the substrate rather than binding ubiquitin directly. Cullin–RING complexes compose the largest known class of ubiquitin ligases. Proteins containing a U-box (a 70 amino acid modified RING finger domain) generally interact with molecular chaperones [74].

Several E3s have been shown to play a role in ERAD: Hrd1, Doa10 and Gp78 are localized at the ER; Parkin and SCF^{Fbs} complex are localized in the cytosol (see also Section 7.3.1.4) [75–80]. p97 has been shown to interact with Gp78 and the SCF complex (Fbs1, Fbs2), but no direct interactions have been shown for the others. However, it has been shown that p97 may bind to Doa10 and Hrd1 via a mutual interaction with Ubx2 in *S. cerevisiae* [81, 82]. One ERAD enzyme with E3 and E4 characteristics is CHIP which can associate with Ataxin-3, a p97 interactor [83, 84]. p97 also interacts with non-ERAD ubiquitin ligases or ubiquitin ligase complexes. In higher eukaryotes, p97 was shown to interact with the breast- and ovarian-specific tumour suppressor protein, BRCA1, which, when associated with BARD1, is a ubiquitin ligase [85]. In addition, p97 also interacts with the ubiquitin ligase Dorfin, which is suggested to be an ERAD E3 [86].

Structural details of the interactions between p97 and these ubiquitin ligases are scarce. p97's interaction with the SCF^{Fbs} complex is probably indirect via Ufd1-Npl4 [87]. p97 often interacts with the C-terminal regions of ubiquitin ligases. In the case of Gp78, p97 interacts with the C-terminal 49 amino acids and this interaction enhances the polyubiquitin-binding affinity of Gp78 CUE domain [88]. Dorfin also interacts directly with p97 through its C-terminal region and p97's ATPase activity stimulates Dorfin E3 ligase activity [86]. In yeast, as well as mammals, p97 interacts with Ufd2a (Ufd2 in yeast), the E4 enzyme necessary for efficient polyubiquitination [14, 28, 89]. Ufd2 binds Cdc48 via a region proximal to the C-terminal U-box domain. Interestingly, Ufd2 binds to a region on Cdc48 that is not the N domain (208–835) which sets it apart from all other known adaptors [28]. The Cdc48–Ufd2 interaction seems to be stimulated by the Ufd1-Npl4 cofactors, allowing Ufd2 to bind ubiquitin strongly when in complex with Cdc48–Ufd1–Npl4. Ufd2 can also bind Cdc48 and Rad23 simultaneously. Finally, the p97 N domain binds to amino acid residues 303–625 in the BRCA1 protein, but no structure has been assigned to this protein region. In summary, p97 can interact directly or indirectly with RING E3s (Dorfin, Gp78, BRCA1, SCF^{Fbs}, Hrd1 and Doa10)

as well as to the U-box E4 enzyme Ufd2a. This allows p97 to be connected to different ubiquitin-based pathways such as ERAD and DNA-repair pathways.

7.2.6
The Interactions of p97 With Deubiquitinating Enzymes

Deubiquitinating enzymes specifically cleave the amide bond between the ubiquitin C-terminal glycine and the ε-amino group of a lysine residue. They perform a regulating function by removing ubiquitin from molecules no longer destined for a certain location or for proteolysis. They proofread ubiquitin conjugates and allow recycling of ubiquitin once a protein is being processed by the proteasome (reviewed in Ref. [90]). There are at least five distinct families of deubiquitinating enzymes according to their sequence and mechanism of action. Four of them are cysteine protease families: ubiquitin-specific processing protease group (UBP) (for example, HAUSP, Doa4 Faf1), ubiquitin carboxy-terminal hydrolases (UCH) (for example, Yuh1, UCH-L3), ovarian tumour-related proteases (OTU) (for example, Otubain1, Cezanne, VCIP135) and the Ataxin-3 family. A fifth family is formed by proteasome subunits and consists of zinc-dependent metalloproteases (for example, Rpn11, POH1, Csn5).

In higher eukaryotes, p97 has been shown to interact with two deubiquitinating enzymes from two of these families, namely VCIP135 (OTU family) and Ataxin-3 [32, 91]. The interaction of p97 N domain with VCIP135 is mediated by the UBX domain of VCIP135. An Ataxin-3 homologue in *Plasmodium falciparum* also interacts via a UBX domain, but in other species the p97 binding region lies within the polyQ stretch of the sequence [92]. While in VCIP135, an OTU domain in the middle of the protein is responsible for the deubiquitinating activity, in Ataxin-3 an N-terminal josephin domain fulfils this function. The josephin domain can also interact with the ubiquitin- and proteasome-binding factor HHR23B. Interestingly, HDAC6, a mammalian p97-binding protein, co-purifies with deubiquitinating enzymes [55].

7.3
The Cellular Roles of p97 and Ubiquitin

p97 is involved in many cellular processes including membrane fusion, mitotic spindle disassembly and ubiquitin-dependent degradation by the UPS. The role of p97 in the UPS is best characterized in the case of ERAD. Whether the proteasome is also involved in p97's role in membrane fusion events is not clear at present, but ubiquitin does seem to play a role in these processes. p97 and the proteasome are active in ubiquitin-dependent degradation of cytosolic proteins, as well as in regulated ubiquitin-dependent processing of transcription factors, and they are essential components of ERAD. Over the years much has been published on p97/Cdc48 functions in ERAD.

7.3.1 ERAD

It is vital for a cell's viability and proliferation to have the correct make up of proteins at every given time point during the cell cycle and for these proteins to be in a correctly folded, functional state. Quality control of proteins is essential and in eukaryotes is largely the responsibility of the UPS. One of the most studied pathways in this system is ERAD for which p97 and its adaptor complex Ufd1–Npl4 are essential [19].

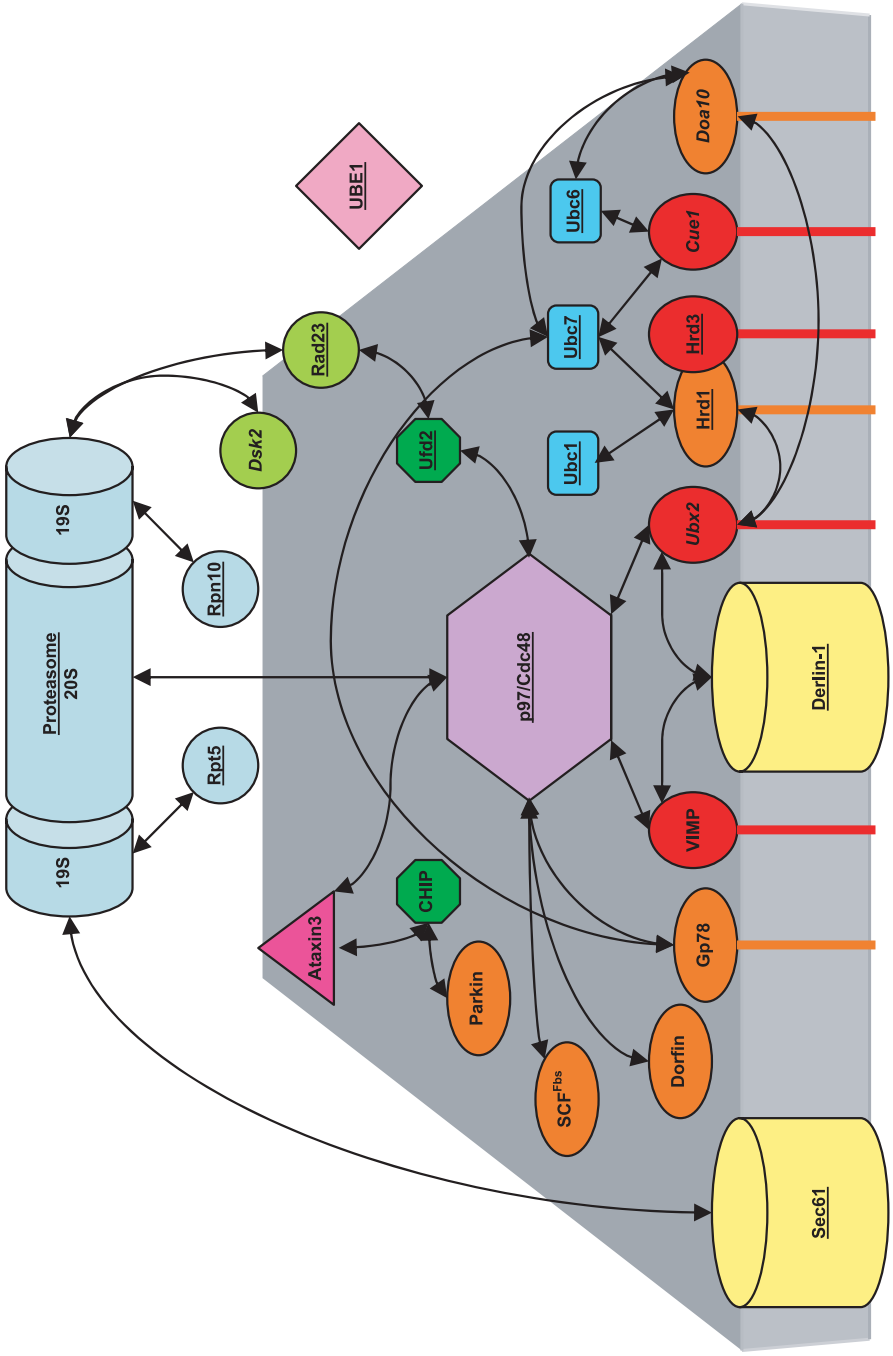
Accumulation of misfolded, aggregated polypeptides leads to toxic protein aggregation events, inactivation of functional proteins and ultimately cell death, all of which lead to many diseases (discussed in Section 7.5). Conversely, the premature degradation of key proteins can also be a cause of disease, such as the degradation of CFTR in cystic fibrosis. The regulated breakdown of a key enzyme of the mevalonate pathway, HMGCoA reductase, is also controlled by ERAD. ERAD is also utilized to infer short half-lives on certain ''normal'' proteins whose concentrations must change promptly with alterations in the state of the cell. Many are degraded rapidly at all times while others are stable until they are suddenly degraded at one particular point to enable cell cycle progression.

7.3.1.1 The ERAD Pathway

During protein synthesis, transmembrane and soluble proteins destined for the ER have an N-terminal ER signal peptide. This directs the ribosome to the ER and the polypeptide is threaded from the ribosome into the ER lumen through Sec61 translocon as it is synthesized (co-translationally). Transmembrane proteins are only partially translocated across the ER membrane and become embedded in it, whereas soluble proteins, destined for either the lumens of organelles or secretion, are fully translocated across the ER membrane.

The ER provides the environment and machinery for protein folding, assembly into larger multimeric complexes and for post-translational modifications such as covalent attachment of N-linked oligosaccharides. The ER also hosts a checkpoint system whereby polypeptides that cannot reach their correct conformation with the aid of ATP-driven chaperones (for example, Hsp60 and Hsp70) or are irreversibly misfolded are identified and retained in the ER. These proteins must be eliminated from the ER and destroyed; this is the role of ERAD. The journey of the ERAD substrate from the ER to the 26S proteasome, although probably quite short in terms of distance, involves a cascade of protein interactions that allow the substrate to travel in the correct direction and also shield it from further aggregation.

Proteins that are to be eliminated by ERAD are shuttled to the cytosolic side of the ER membrane where one or two ubiquitin moieties are covalently attached to lysine residues. p97 interacts with many components of the translocation and ubiquitination machinery (Figure 7.8). It is thought that p97 bound to the Ufd1–Npl4 adaptor participates in the subsequent ATP-dependent release of the substrate from the ER. Also, Ufd2 (E4) cooperates with p97–Ufd1–Npl4 and extends the ubiquitin chain up to six ubiquitin moieties. The size-restricted ubiquitin chain al-


UBE1
Rad23
Dsk2
Ufd2
Ubc6
Ubc7
Ubc1
Doa10
Cue1
Hrd3
Hrd1
Ubx2
19S
Proteasome 20S
19S
Rpn10
Rpt5
p97/Cdc48
Derlin-1
VIMP
Gp78
Dorfin
Sec61
SCF^Fbs
Parkin
CHIP
Ataxin3

lows the interaction of one subset of proteasome-binding factors (such as Rad23). In yeast, it has been shown that Cdc48 can interact with Rad23 and Ufd2 simultaneously, possibly allowing the polyubiquitinated substrate to be passed from the ubiquitination machinery to the proteasome shuttling factors. If the ubiquitin chain is further extended, evading these, it can interact with a proteasome subunit (Rpn10). These proteasome binding factors direct the substrate into the proteolytic chamber for degradation.

7.3.1.2 **Recognition of ERAD Substrates**

First, a protein must be recognized as a target for proteolysis. Denatured or misfolded proteins, are recognized in the ER, presumably by exposure of signals such as sequences or conformational motifs that are usually buried in a properly folded protein. Alternatively, when glycoproteins are irreversibly misfolded, the oligosaccharide on the glycoprotein can be trimmed in such a manner that generates a signal for the export from the ER.

Little is currently known about the way that substrates are directed out of the ER. Luminal substrates seem to require the binding and activity of BiP, an ER luminal Hsp70, which binds to exposed hydrophobic regions of folding intermediates and misfolded proteins and prevents aggregation. Further interactions with other proteins are also required such as protein disulfide isomerase (PDI), Eps1p, (a membrane-anchored PDI) to reduce disulfide bonds and J-domain proteins (for example, Jem1p and Scj1p) for their solubility (reviewed in Ref. [93]). Degradation of transmembrane substrates is independent of BiP probably because they are already at the ER membrane, but has been shown to require cytosolic Hsp70 chaperones Ssa1–4.

Before translocation across the ER membrane can occur, the ERAD substrate has to be soluble, with any disulfide bonds reduced and in a monomeric form. It is unclear whether or not the substrate needs to be fully unfolded to traverse the membrane, if the translocon was large enough for partially folded substrates to be passed through, or if the passage of the polypeptide through the translocon exerts sufficient mechanical stress for the substrate to become unfolded as it is passed

Fig. 7.8. A non-exhaustive summary of cytosolic protein interactions in ERAD with a focus on proteins that interact with p97 (purple hexagon). The grey plane represents the ER membrane, and those proteins which are tethered to the membrane by transmembrane domains are shown with tails implanted into the grey plane. The colours of shapes denote what role proteins carry out: yellow cylinder, translocon; rose diamond, E1; cyan square, E2; orange oval. E3; red oval, membrane anchor; green octagon, E4; lime circle, shuttling factor; cerise triangle, deubiquitinating enzyme; light turquoise circle, proteasomal polyubiquitin receptor or shuttling factor; light turquoise cylinder, proteasome (sizes/shapes are not representative of actual structures). Proteins identified in yeast are shown in italics, those in mammals in plain type and those in both yeast and mammals are in underlined plain type. For simplicity, the interactions that p97 makes with adaptor Ufd1–Npl4 whilst bound to it are excluded, as not all have been characterised in this respect.

through the narrow channel. Whilst p97 has been shown to bind and aid the folding of test proteins *in vitro*, it is not localized in the ER lumen, so does not participate in ERAD processes until the substrate is exposed to the cytosol.

7.3.1.3 Translocation into the Cytosol

In both yeast and mammals, substrate proteins were originally thought to be expelled from the ER via the same Sec61 channel that they enter, although recently other routes have been identified. Transmembrane substrates are not as reliant as luminal substrates on the Sec61 channel. BiP appears to also act as a gate to the Sec61 translocon [94].

An alternative translocation channel is Derlin-1 (Der1 in yeast), which was identified simultaneously by two studies observing the removal of MHC Class I heavy chains from the ER in human cytomegalovirus infected cells [95, 96]. This channel, in mammals, has been shown to bind to a transmembrane protein, VIMP, which is also able to bind to p97 [96]. In yeast, it was shown that Der1 interacts with Ubx2, an integral ER membrane protein that interacts with Cdc48 [82, 97]. Therefore, VIMP and Ubx2 provide means by which p97 is localized to the ER and linked to a translocon and may possibly assist in translocation and/or ubiquitination.

7.3.1.4 Mono/diubiquitin Conjugation

The substrate, still embedded in the ER membrane but with an exposed lysine residue at the cytosolic face, is first modified by the covalent fusion of one or two ubiquitin moieties by the action of E1, E2 and E3 enzymes. The E2 and E3 form a complex and are tethered to the membrane by one or more members of the complex. Their active sites rest on the cytoplasmic side of the ER membrane and they use the cytosolic E1 UBE1 (UBA1 yeast). There are two well-characterized RING finger E3 complexes, Hrd1–Hrd3 and Doa10. These are able to utilize ERAD E2 enzymes Ubc1, Ubc6 or Ubc7 [98]. Hrd1 and Hrd3 form a complex at the ER membrane through transmembrane domains on both proteins. It has been suggested that the cytosolic RING finger domain in Hrd1 may be regulated by the interactions of the luminal domain of Hrd3 and its interactions with luminal chaperones [99]. Ubc1 or Ubc7 can be paired with the Hrd1–Hrd3 complex [75]. Doa10 is a multispanning RING finger E3 and is partnered with either Ubc6 or Ubc7 E2s. Both of these E2s seem to bind to the ER membrane as a result of their interaction with Cue1 [100, 101]. It has been suggested that Cdc48 binds to Hrd1 and Doa10 through Ubx2 in *S. cerevisae* [81, 82].

The degradation of many different test substrates has been followed through the ERAD cycle to identify the required components, and a level of substrate specificity has emerged. Hrd1–Hrd3 complexes are generally associated with luminal substrates and Doa10 with transmembrane substrates with cytoplasmic lesions. However, it has been shown that Ole1p, a well-known yeast ERAD substrate, required neither of these E3s, so it is likely that other E3s are involved in ERAD [102].

Three E3 ligases, Gp78, Dorfin and SCF^{Fbs}, that act upon ERAD substrates have been shown to associate with p97. Gp78 (also known as AMFR) is a mammalian

ER-bound E3 that acts with E2 Ubc7 and targets ERAD substrate CD3-Δ for degradation. Gp78 binds p97 and enhances the affinity of p97 for polyubiquitin chains [77, 88]. Dorfin (double RING finger) is a cytosolic mammalian E3 ligase that interacts with p97 and has been shown to ubiquitinate mutated Cu/Zn superoxide dismutase (SOD1). Although SOD1 is not an established ERAD substrate, a lack of degradation of the mutant form appears to be key to the pathogenesis of familial amyotrophic lateral sclerosis, leading to the suggestion that Dorfin may function in ERAD [86]. Glycoproteins that are degraded through ERAD are translocated to the cytosol before being deglycosylated. SCF^{Fbs} is a cytosolic E3 complex composed of Cullin1/Cdc53, Skp1, Roc1/Rbx1 and an F-box protein and is recruited to the ER by its interaction with p97. Fbs1 and Fbs2 are F-box proteins that are able to recognize the inner chitobiose of high-mannose oligosaccharides. Fbs1 binding to test substrate, pre-integrin-β, was stimulated when active p97 was present [87].

7.3.1.5 **Polyubiquitination by E4 Factors**

The actions of E1, E2 and E3 allow the substrate to be mono- or diubiquitinated. However, to be recognized by the proteasome, typically more than four ubiquitin moieties are required [103]. Previously, it was thought that longer polyubiquitin chain lengths were formed spontaneously from multiple rounds of E1, E2, E3 enzyme cycles, but now a ''new'' class of enzymes, E4s, are thought to be responsible [14]. In *in vitro* experiments, E4s generally show E3 ligase activity, but when in conjunction with a full set of E1, E2 and E3 they can direct polyubiquitin chain extensions. As a result of this, ERAD E4 enzymes such as CHIP and Ufd2 have also been classified as E3s. It remains controversial whether E4s are simply a new family of E3s (the U-box family) or comprise a distinct enzymatic activity [104].

Ufd2 is the best characterized E4. In the presence of Ufd2, ubiquitin chains are extended to lengths of up to twenty ubiquitin molecules *in vitro* [14]. Ufd1–Npl4 is reported to enhance the binding of Ufd2 to p97 although no direct interaction between Ufd1–Npl4 and Ufd2 has been shown. Furthermore, the presence of Cdc48–Ufd1–Npl4 in *in vitro* ubiquitin-conjugation assays, containing E1, E2, E3, E4 and substrate, appeared to restrict the E4 activity such that the polyubiquitin chains formed were limited to a maximum of six ubiquitin moieties [28].

CHIP is another U-box ERAD E4, which experimentally shows E3 activity [105, 106]. CHIP has not been shown to bind to p97 but does bind to Hsc70 and is able to regulate its chaperone activity and ubiquitinate ERAD substrate CFTR and also proteins recognized by Hsc70 [74, 105]. The E4 activity of CHIP was revealed when the E3 Parkin, responsible for the familial juvenile version of Parkinson's disease, was shown to interact with it. *In vitro* assays showed that CHIP, with E1, E2 and Parkin as E3, is able to cause the dissociation of Pael-R Parkin substrate from Hsc70 and stimulate its polyubiquitination [107].

7.3.1.6 **Release from the ER Membrane**

In order for the ERAD substrate to be degraded, it must first be released from interactions with the ER membrane components [29]. It is unclear whether the

substrate must be mono-, di- or polyubiquitinated before it is released from the membrane.

Release of all substrates (luminal and transmembrane) from the ER requires ubiquitination, ATPase activity of p97–Ufd1–Npl4 and the 19S regulatory cap of the proteasome. Experimentally, in permeabilized yeast cells, this was observed as protease protection and membrane association of an ERAD test substrate in Cdc48, Npl4 or Ufd1 mutants. *In vivo*, expression of these mutants led to the initiation of the unfolded protein response (UPR). In permeabilized mammalian cells, ubiquitinated MHC class I heavy chains were observed predominantly in the soluble fraction and were digested by proteinase K when wild-type p97 was present. When ATP hydrolysis-deficient p97 was added, ubiquitinated MHC class I heavy chains were observed instead to be membrane associated and were more protected from protease digestion [108]. It was therefore suggested that p97 may assist in retrotranslocation of ERAD substrates [109]. Dissecting this further, Elkabetz and colleagues observed two stages requiring p97. Firstly, the passage across the membrane required active p97 because when p97 was mutated, the ERAD test substrate was protected from trypsin and salt washes. This implied that the majority of the test substrate was inside the lumen of the ER. Once the substrate reached the membrane face, it remained associated with the membrane and was ubiquitinated. A second step, release to the cytosol (dislocation), required ER membrane-associated factors including p97 [29]. Elkabetz and coworkers also noted that whilst p97 can recognize polyubquitinated chains, it also acts prior to polyubiquitination.

So how is retrotranslocation or dislocation achieved? p97 is thought to be anchored to the membrane, possibly via interactions with membrane proteins such as VIMP, Derlin-1, Ubx2 and ER-resident E3s (such as Gp78). The adaptor complex Ufd1–Npl4 also increases p97 affinity for membrane association as the ubiquitin tags attached to the ER-bound substrate are recognized at the cytosolic face by the p97–Ufd1–Npl4 complex. The energy produced by the ATP binding and hydrolysis of p97 causes conformational changes in p97, possibly transmitted as movements to the Ufd1–Npl4 adaptor, and the ERAD substrate is mechanically dislocated from the ER membrane.

Once polyubiquitination is complete and the substrate is free from membrane-bound proteins, the substrate is now capable of being recognized by the 19S regulatory particle of the proteasome.

7.3.1.7 **Transport to the Proteasome**

The ubiquitinated substrate is recognized by the 26S proteasome via the 19S regulatory domain. This is proposed to happen in a variety of ways and could involve a number of ''shuttling factors'' that escort the substrate to the proteasome. Proteins that have been proposed as shuttling factors are Rad23, Dsk2 and Rpn10. Multiple genetic studies in yeast have observed that individual knockouts of Rad23, Dsk2 and Rpn10 have little effect upon cell viability and only mild defects are observed under stress conditions. Combinations of double and triple knockouts led to increased sensitivity [28, 110]. In conjunction with biochemical evidence, this

strongly implies that Rad23, Dsk2 and Rpn10 form parallel redundant pathways to the proteasome. It has been suggested that p97 may pass polyubiquitinated proteins from the ubiquitin ligase complex to the proteasome via these factors. The E4 Ufd2 can bind Rad23 via its N-terminal region whereas Cdc48 binds to a different domain, proximal to the C-terminal U-box, thus allowing simultaneous binding and providing an important linkage between Cdc48–Ufd1–Npl4, Ufd2, Rad23 and the proteasome [28].

Whilst it has been suggested that Rad23, Dsk2 and Rpn10 act redundantly, more detailed work has revealed interesting differences between the three. Rpn10 exists in two populations: as a proteasomal subunit in the 19S regulatory particle and free in the cytoplasm. Rpn10 binds ubiquitin and UBL domains through UIM domains and the proteasome core particle via a VWA domain [111]. As a consequence, it is controversial whether Rpn10 should be viewed as a proteasomal ubiquitin receptor or a shuttling factor [30]. Rad23 and Dsk2 were identified as ERAD components downstream of Cdc48–Ufd1–Npl4 and are able to bind polyubiquitinated proteins through UBA domains and the proteasome through a UBL domain (Rad23 and Dsk2 are often referred to as UBL–UBA proteins) [112]. Interestingly, although ERAD is highly conserved between yeast and higher eukaryotes, the UBL of Rad23 binds at different positions in the proteasome, in yeast via the Rpn1 subunit and in mammals via the additional UIM domain in S5a, the mammalian orthologue of Rpn10 (also considered a shuttling factor) [61, 110, 113–115].

In order to understand the interaction of Rpn10 and Rad23 with the proteasome, Verma et al. purified intact 26S proteasomes from Rad23 and Rpn10 knockout strains of yeast [116]. These proteasomes were unable to degrade the test substrate Ub-MBP-Sic1, and activity was restored by respectively adding back Rad23 or Rpn10. Interestingly, adding Rad23 to proteasomes from Rpn10 knockout strains only partially restored degradation activity; however, if the VWA domain of Rpn10 was also added with Rad23, full degradation activity was restored. Identical findings were observed for Dsk2. The authors suggest that this indicates that the VWA domain acts as a facilitator of degradation downstream of Rad23, Dsk2 and Rpn10-UIM domain [116]. An alternative view is that the VWA acts in a more basic way, maintaining the correct interactions between the lid and base of the 19S complex. In support of this, it was reported that the lid and base dissociated more readily when Rpn10 was absent and Rpn10 has been purified associated with either the lid or the base [117, 118].

Whilst there is evidence that the three shuttling factors are able to act in each other's place, we are led to ask whether preferences exist between them for different substrates.

It has been suggested that Rad23 and Dsk2 may accept substrates targeted by p97. When Rad23 and Dsk2 were identified as members of ERAD by Medicherla et al., they tested two groups of substrates, well-characterized ERAD test substrates (CPY* and CTG*) and cytoplasmic-soluble proteasome substrates (ΔssCPY*–GFP and Deg1–GFP). The ERAD substrate degradation was shown to be dependent upon functional Ufd1 (part of the Cdc48–Ufd1–Npl4 complex) and Rad23 or Dsk2, whereas the cytosolic protein degradation was not dependent on either

Ufd1, Rad23 or Dsk2. They suggested that Dsk2 and Rad23 participate downstream in pathways that require Cdc48–Ufd1–Npl4 [112]. Verma and colleagues also studied the degradation of CPY* and Deg1-GFP. CPY* degradation was still functional in Rpn10 and Rad23 knockouts, suggesting that Dsk2 could replace them in agreement with the previous study [116]. They also showed that Deg1–GFP degradation was also independent of Rpn10, suggesting that Rad23, Dsk2 and Rpn10 do not act as proteasomal receptors for this protein. Deg1–GFP is a hybrid substrate as it is cytoplasmic but has been shown to be ubiquitinated by ER-tethered ligases (Doa10 and Ubc6 and Ubc7) [76]. The hypothesis that Rad23 and Dsk2 participate in pathways containing Cdc48–Ufd1–Npl4 is controversial in the case of Deg1–GFP as degradation was found to be independent of Ufd1 by Medicherla et al. and dependent on Ufd1 by Verma et al. [112, 116].

It has also been suggested that polyubiquitin chain length may act as a key determinant for which receptor recognizes the chain. Following their findings that Cdc48–Ufd1–Npl4, in conjunction with Ufd2 (E4), limited polyubiquitin chain length to six ubiquitin moieties, Richly et al. tested whether Rad23, Dsk2 and Rpn10 had different chain-length preferences. They found that when presented with a variety of chain lengths, Rpn10 preferentially bound to chain lengths greater than six ubiquitin moieties whereas Rad23 and Dsk2 bound to chains less than six in length [28]. This supports the idea that Rad23 and Dsk2 may indeed, as a first choice, collect ubiquitinated proteins downstream of Cdc48–Ufd1–Npl4. Interestingly, the domain architecture of Rad23 and Dsk2 differs; Rad23 has two UBA domains whereas Dsk2 has one. This could be reflected in differing affinities for polyubiquitin or even different length preferences within the three to six ubiquitin chain range [64].

It was also proposed that the E3 responsible for ubiquitinating the substrate may direct the chain to a specific receptor. In an attempt to elucidate whether Rad23, Dsk2 and Rpn10 receive specific polyubiquitinated proteins, a study of many cytoplasmic proteasomal substrates showed the preferences of different proteins for different receptors. Importantly, this was not correlated to the E3 responsible for polyubiquitination [116]. Furthermore, some substrates were not received by the proteasome by any of the receptors, suggesting that there are other, hitherto uncharacterized receptors.

There are several other putative polyubiquitin receptors. Experiments crosslinking polyubiquitin to the proteasome identified a component of the 19S regulatory unit of the proteasome, AAA ATPase, S6 (Rpt5) [119]. However, a study observing the degradation of many cellular substrates did not find that Rpt5 was a necessary receptor [116]. Other UBL–UBA proteins have been identified that are possibly capable of carrying out similar functions, including Ddi1, a yeast protein that is involved in Securin (Cut2) degradation alongside Rad23 prior to mitosis. Amongst other proteins that can interact with both the proteasome and polyubiquitin are Ubx3 (fission yeast), which appears to have a parallel function to Rpn10, and Ataxin-3 (mammalian), which, whilst it has been suggested to have proteasomal receptor functions, also potentially has deubiquitinating activity [91].

7.3.1.8 The Proteasome in ERAD

Proteasomes are numerous and are found in the cytosol and the nucleus, but are absent in other compartments of the cell. A subpopulation of proteasomes has been observed bound to the ER membrane and it is this population that interacts with the ERAD substrate [29]. This suggests that ERAD proteasomes are spatially close to the translocon. A further study went on to identify that the 26S proteasome (via the 19S regulatory cap) could actually immunoprecipitate Sec61 and shared a common binding footprint to the ribosome [120]. This suggests a mechanism by which a population of proteasomes could be maintained in close proximity to the translocon, but does not exclude the possibility of other proteins mediating the interaction [120].

The proteasomal AAA proteins promote substrate unfolding and threading through the narrow channel of the proteolyic core particle as a prelude to degradation. It has also been suggested that p97 may act as an unfoldase in a more conventional chaperone mode of action. Dai et al. proposed that p97 was a component of the 19S cap of the 26S proteasome, indicating that the p97 ring might dock onto the regulatory particle of the proteasome and replace a resident AAA protein to form a variant proteasome assembly [8]. Further confirmation and exact interactions of this association of p97 with the 26S proteasome have not yet been reported.

7.3.2 Other Ubiquitin-dependent Processes That Involve p97

A large body of work firmly identifies p97 as a member of the ERAD pathway machinery. However, p97 has been shown to interact with many proteins outside this pathway that also interact with ubiquitin and function within the UPS.

7.3.2.1 p97 and the Degradation of Cytoplasmic Substrates

Several studies have shown that p97 also acts in the degradation of cytoplasmic UPS substrates. For example, Cdc48 plays a role in the degradation of the test substrate Ub–Pro–β-gal and p97 is involved degrading cytosolic proteasome substrate IκBα (see Section 7.3.2.2) [7, 8]. Additionally, p97 is also required for the degradation of short-lived proteins such as cyclins [66]. Ubx3, the p47 orthologue in fission yeast, also has a role in the degradation of proteins independent of ERAD [67]. *S. cerevisiae* strains deficient in Shp1 and Ubx2 also show defects in the degradation of a ubiquitinated model substrate [71].

7.3.2.2 p97 and the Proteasome in Transcription-factor Processing

The availability of transcription factors to the nucleus must be tightly controlled to prevent inappropriate transcription. Two yeast transcription factors, Spt23 and Mga2, activate transcription of the OLE1 gene, encoding an ER bound Δ9 fatty acid desaturase that controls unsaturated fatty acid pools. Interestingly, these transcription factors exist as inactive precursors bound to the ER and possibly may

sense the lipid composition of this membrane. The inactive precursors are dimerized and activation is triggered by ubiquitination by Rsp5 ligase. This causes proteasomal processing of the inactive transcription factor to a shorter variant, which rapidly redimerizes with a full length factor. The active processed transcription factors are separated from their precursors by the action of the Cdc48–Ufd1–Npl4 complex [20, 21]. Cdc48–Ufd1–Npl4 is recruited to this complex by a monoubiquitin tag retained from the initial proteasomal processing step. Once the shorter form is released, it is then able to enter the nucleus and initiate transcription.

A distant homologue of Spt23 and Mga2 is NF-κB, a transcription factor that is maintained in a cytosolic pool by binding to a member of the IκB inhibitor family. Liberation of NF-κB can be triggered by hyperphosphorylation of IκBα leading to its ubiquitination. p97 is able to bind to this form of IκBα and is necessary but not sufficient for proteasomal degradation of IκBα [8].

Both processes are often referred to as ''regulated ubiquitin/proteasome-dependent processing'' or RUP [121]. p97 and Cdc48 appear to have a role in these processes as segregases of ubiquitinated proteins from nonubiquitinated partners (see Section 7.4).

7.3.2.3 p97 and Other Ubiquitin-binding Adaptors

Recently, other p97-binding proteins, namely Ufd3, HDAC6 and Ataxin-3, have been shown to interact not only with forms of ubiquitin but also with each other. Ataxin-3 is already implicated in ubiquitin-mediated proteolysis since it interacts with Rad23, p97 and polyubiquitinated proteins and can bind the proteasome [91]. It also contains a deubiquitinating josephin domain that has been proposed to function in editing polyubiquitin chains [122]. Additionally, it has been reported that Ataxin-3 associates with HDAC6 and Dynein. These proteins are implicated in the formation of the aggresomes and transport of misfolded proteins [123]. HDAC6 itself has been shown to bind polyubiquitin and also p97 [55, 124]. Immunopurification identified phospholipase A2 activating protein (PLAA), a mammalian homologue of yeast Ufd3, as an associated protein of p97 and HDAC6 [124]. Ufd3 has been shown to interact directly with Cdc48 and in a separate study with Lys 29-linked polyubiquitin chains [7, 69]. Cdc48 UBX-containing interactors 1, 2, 3 (Cui1–3) are yeast proteins shown to interact with Cdc48 and Ufd3. Knockouts of Cui1–3 are defective in degradation of a test substrate, suggestive of a role in UPS [70]. Although these proteins appear to be connected through interactions, they are currently unassigned to a specific pathway. Possibly, the acetylation/deacetylation processes may provide a link between them and ubquitination.

Additionally, many of the UBA–UBX p97 adaptors are poorly characterized in terms of function. Saks1 and p97 can be co-immunoprecipitated with the proteasome subunit S5a [125]. Saks1 is a substrate for stress-activated protein kinases (SAPK) suggesting a role for Saks1 in times of cellular stress. The knockouts of homologues of p47 in yeast, Shp1 and Ubx3, display phenotypes consistent with defects in the degradation of a ubiquitinated model substrate [71]. It is unresolved at present whether this represents a novel action of p47 in yeast polyubiquitination processes. It has also been hypothesized that Faf1, another p97 interactor with

UBA–UBX domains may be involved in the regulation of protein degradation by the UPS [126]. In fission yeast, Ubx2 binds Cdc48 but when deleted does not display any obvious protein-degradation phenotypes. Further details of these p97 adaptor functions in connection with the UPS await further investigation.

7.3.2.4 p97 and Ubiquitin in Membrane Fusion

p97 mediates telophase membrane-fusion events that result in the reformation of Golgi cisternae and the expansion and resealing of the nuclear envelope [10, 26]. During interphase, p97 mediates membrane-fusion events that lead to the formation of transitional ER [127]. These activities are regulated by specific p97 adaptor protein complexes that bind to p97 and confer functional specificity [128]. p47 is required for the p97-regulated membrane reassembly of the ER, the nuclear envelope and the Golgi apparatus [9, 10, 26, 127, 129]. It is thought to assist p97 in the dissociation of post-fusion Golgi t-t-SNARE complexes involving syntaxin 5, using the energy from p97 ATP binding or hydrolysis and prepare them for further rounds of membrane fusion [127]. In this process, p97 was thought to perform a function analogous to that of the highly structurally homologous protein NSF. p97 acts only in a restricted set of homotypic membrane-fusion pathways. p97–p47 activity is dependent on another cofactor, VCIP135, which resides on the membrane and can form a transient complex with p97–p47–syntaxin 5 via a putative UBX domain [32]. The deubiquitinating activity of VCIP135 is required for Golgi reformation, indicating that removal of a ubiquitin signal generated during Golgi fragmentation is an essential step in the p97-mediated mechanism that triggers membrane fusion [130]. The NSF-α-SNAP complexes and the p97–p47 complex aided by VCIP135 have been shown to act sequentially in cell cycle-dependent reformation of the ER network. These events also involve the t-SNARE syntaxin 18 but do not seem to implicate ubiquitin [131].

These data reveal a cycle of ubiquitination and deubiquitination regulating Golgi membrane dynamics during mitosis, suggesting that ubiquitin binding is a common feature of the p97-mediated activities. Localization studies suggest that proteasomal proteolysis mainly occurs at the nuclear envelope/rough ER [132]. However, many membrane trafficking processes are regulated by ubiquitination but do not involve the proteasome. p47 has one binding site for ubiquitin at its UBA domain and binds primarily to monoubiquitin [26], although in yeast the p47 homologue Shp1 was shown to interact with ubiquitinated proteins *in vivo*, linking p47 with proteasome-dependent protein degradation [71].

It has been proposed that monoubiquitin regulates internalization and endosomal sorting by interacting with modular ubiquitin-binding domains in core components of the protein-transport machinery. Therefore, an attractive comparison can be drawn for the p97-mediated function in membrane fusion because of the similarity to other vesicle fusion pathways associated with AAA proteins and ubiquitin-like molecules. For example, the AAA+ ATPase Vps4 and monoubiquitin interact, targeting proteins for nonproteasomal degradation in the lysosome. The function of ubiquitin as tag in the multivesicular body (MVB)-sorting pathway is quite well understood (reviewed in Ref. [133]). The cytosolic tails of proteins to be

sorted into the MVB are labeled with ubiquitin moieties. The yeast protein Vps27 binds ubiquitinated cargoes via its UIM domains and recruits the ubiquitin-binding complex ESCRT-I (endosomal sorting complex required for transport I). Two more ESCRT complexes (II and III) sequentially interact with the ubiquitinated cargoes and deliver them into budding areas to generate the MVB. The MVB fuses with the lysosome/vacuole and the vesicles and their contents are degraded. This pathway requires Vps4, which is believed to recycle the sorting factors from the membrane. It is possible, therefore, that p97 may be recruited to either SNARE proteins or a SNARE regulator by monoubiquitin in a process independent of the UPS but with similarities to the MVB sorting pathway.

7.4 The Action of p97

The ATPase cycle of p97 is linked to conformational changes. Both p97 AAA domains, D1 and D2, can hydrolyze ATP to some extent [109, 134]. Currently, there is little agreement in the field about how p97 transforms chemical energy into domain motions, and interpretation of results is clouded by the possibility that the D1 and D2 rings may or may not cooperate and may be fully or unequally occupied by nucleotide.

Many structural studies have looked at p97 at various stages of the ATPase cycle [135–138]. Cryo-EM studies, carried out in saturated quantities of different nucleotides have shown large global conformational changes. Whilst difficulties in assigning the domains and nucleotide-binding state have hampered the definitive interpretations of D1 and D2, some changes are consistently observed, such as dilations of the ring and central pore and differences in the N domain position. More rigid p97 N domains are visible in the presence of a non-hydrolyzable ATP analogue and transition state mimic, potentially representing a flexible-to-rigid transition transmitted to the bound adaptors [135, 137]. Shapes of the p97 hexamer observed by SAXS broadly resemble those seen by EM [139].

Crystal structures have shown some possible nucleotide-binding states, ADP bound to D1 and empty, ADP, ATP analogue (AMPPNP) and transition-state analogue (ADP–AlF_x) bound D2 domains. They have also identified possible unequal occupancy of nucleotide binding in the D2 domain [138]. However, accurate interpretation of these structural models (including side-chain detail) is limited by low resolution (3.5–4.4 Å) and high B factors. In all crystal structures, parts of the D2 and C-terminal domains are disordered. Different crystal forms may also result in localized changes due to crystal packing effects [138, 140–142]. Compared to the large global changes observed in cryo-EM studies, the changes in the crystal structures in the presence of different nucleotides are small, although one study does observe small internal rotations of the N domain within the hexamer and small rigid body motions in the N domain, the D1 α-helical domain and the D2 α/β domain between nucleotide states and order–disorder transitions in the D2 α-helical domain [138]. In contrast with cryo-EM studies, all crystal structures in all

nucleotide-bound states show N domains coplanar with the D1 domain. Possibly, the low amount of conformational change observed in the higher resolution crystal structures compared with the large conformational changes observed in low-resolution EM/SAXS structures reflects the constraint of a flexible molecule in the crystal lattice, and more defined physiologically relevant information will be gained from EM and SAXS structures of p97 bound to adaptors such as p47 and Ufd1–Npl4 and their putative substrates.

7.4.1 p97 as a Chaperone

The archeal homologue of p97, VAT, was found to have unfoldase/chaperone activity. *In vitro* assays using two well-characterized test substrates showed that VAT can, depending on experimental conditions, not only unfold test substrates but also assist the refolding of heat and chemically denatured proteins. The VAT N domain alone can also promote refolding [143]. The substrates selected are well characterized in folding/unfolding assays, although their biological relevance to the pathways that VAT/Cdc48/p97 are implicated in is, however, unclear at present. In denaturation experiments, yeast homologue Cdc48 was observed to prevent denaturation and aggregation of luciferase and rhodanese. However, as this is an ATP-independent effect, it was suggested that Cdc48 acts as a holding protein that prevents aggregation of unfolded proteins [33]. This apparent activity could represent the "residual" activity of VAT, the ancestor of p97.

A study observing the direct degradation of ubiquitinated substrates by proteasomes tested the possibility that p97 may unfold ubiquitinated substrates before presentation to the proteasome. This showed that p97 concentration had little effect upon rates of degradation of ubiquitinated UPS test substrate Ub_5DHFR [103]. So while p97 co-immunoprecipitates with the proteasome and polyubquitinated substrates, there is no direct evidence that p97 actively unfolds or presents this substrate to the proteasome.

7.4.2 p97 and NSF: SNARE Disassembly Machines

Originally, by homology, p97 was suggested to act like NSF, an AAA ATPase that coupled ATP hydrolysis to the mechanical process of post-membrane fusion SNARE disassembly [144]. NSF uses the adaptor protein α-SNAP to disassemble SNAREs involved in heterotypic membrane fusion, whereas p97 with the p47 adaptor is involved in the disassembly of SNAREs involved in homotypic membrane fusion, for example syntaxin 5. The adaptor-binding surfaces differ between the two AAA ATPase–adaptor complexes [39, 145], although the overall shape of the two complexes is strikingly similar [146]. The apparent individual magnitudes of ATPase activity of D1 and D2 domains also differs between NSF and p97 [130]. In order to disassemble the very stable four-helical bundle of a SNARE complex,

p97 and NSF have to exert some kind of unwinding force on the complex. How exactly this is achieved is unclear at present.

7.4.3 p97 Liberates Polyubiquitinated Substrates from the ER Membrane

Studies of the degradation of test substrates in cellular fractions containing ER microsomes have observed that when p97 is present and functional, the polyubiquitinated substrate is identified in the ''cytosolic fraction'', whereas nonfunctional or absent p97 leads to the substrate remaining at the ''ER membrane'' and inside the ''lumen''. p97 has been proposed to act in three modes to power liberation of the substrate protein from the ER membrane: first, as a translocase pulling the substrate through the translocon, second, in the extraction of the substrate's tail from the translocon and third as a dislocase, pulling the substrate out of complexes with the ER membrane components of the ERAD machinery.

Protease protection experiments showed that active p97 was necessary to release ubiquitinated MHC class I heavy chains from the ER microsomes. However, when a p97 hydrolysis-deficient mutant was used, MHC class I heavy chains had comparatively more protease protection, implying that they remained inside the ER or protected in a complex at the ER membrane [108]. This led the authors to propose that p97 acted as a motor, translocating unfolded substrates through its central pore [109]. The pore of p97 is large enough to accommodate an unfolded polypeptide chain (approx 9 Å in diameter) and alters throughout the ATPase cycle, although a Zn^{2+} ion in the pore of one crystal form may act to block it [93, 138, 142]. It seems unlikely, chemically, that the ion is bound stably enough to act as a plug. Protein retrotranslocation through Sec61 has been found to also involve the ER chaperone BiP, which acts to push the protein through the Sec61 pore (reviewed in Ref. [147]). There is little direct biochemical evidence to show that p97 is able to physically power translocation. Furthermore, p97 participates in many other cellular processes and translocation, in particular, is a highly specialized role. Whilst it seems unlikely that p97 is a translocase, it is entirely possible that p97–Ufd1–Npl4 binds polyubiquitinated substrates and exerts force on them to extract the tail from the translocon or liberate the polyubiquitinated substrate from a membrane-bound complex. This is supported by the observation that p97 and the proteasome are likely to participate in a further step releasing the polyubiquitinated substrate from the ER membrane. Only after this dislocation step was the substrate degraded [29].

7.4.4 p97 as a Segregase

In a more general sense, the role of large conformational change throughout the ATPase cycle has been rationalized as an ability to separate or disassemble protein complexes. The first data to support this was the selective removal and degradation of inhibitory IκBα from the NF-κB transcription factor. Polyubiquitinated IκBα was

released by p97 and degraded by the proteasome freeing the active transcription factor. The authors suggested that p97 may act as a form of chaperone that was able to release the ubiquitinated IκBα from an unmodified protein [8].

Activity consistent with this has been observed in the role of Cdc48 in the dissociation of Spt23 transcription factor heterodimer allowing the active form to pass into the nucleus. Cdc48–Ufd1–Npl4 complex in this scenario is able to separate ubiquitinated p90 Spt23 variant from a nonubiquitinated p120 Spt23 that is anchored to the ER. This is necessary for the p90 form to pass into the nucleus and initiate transcription [20].

Outside the UPS, the p97–p47 complex is proposed to play a role in the separation of SNARE complexes or the removal of a SNARE-complex regulator in membrane fusion (reviewed in Ref. [31]). p97–Ufd1–Npl4 also acts in mitotic spindle disassembly, binding spindle-assembly factors and modulating their interaction with microtubules [25]. A uniting view could be that p97 acts as a transfer factor or segregase that disassembles ubiquitinated proteins from complexes with nonubiquitinated proteins. The involvement of ubiquitin or ubiquitin-like modifiers in membrane fusion or spindle disassembly is yet to be proven directly, although it is an attractive proposition that they may play a role in recruiting p97.

7.5
When Things Go Wrong: p97 in Disease

We have discussed some of the vital roles that p97 plays in the cell, in particular those that involve ubiquitin. We now discuss briefly what can happen if these cellular processes fail, underscoring the importance of p97, ubiquitin and the proteasome functionality. Failure to eliminate denatured and misfolded proteins from the cell is a major cause of disease. Toxic aggregates build up, resulting in cellular apoptosis, which is a common feature in neurodegenerative diseases.

One class of inherited neurodegenerative diseases is caused by polyglutamine (polyQ) expansions in otherwise unrelated proteins. PolyQ diseases include Huntington's disease, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, spinocerebellar ataxia and Machado–Joseph disease [148]. When the polyQ repeats are larger than forty residues these proteins form insoluble, granular and fibrous deposits in the cytoplasm. These aggregates are possibly formed from self-associating β-sheets of the polyQ stretches [149]. The polyQ deposits have been shown to be ubiquitinated *in vivo* and *in vitro* and also have p97 bound *in vivo* [148, 150–152]. Immunohistochemical studies have shown p97 staining in ubiquitin-positive intraneuronal inclusions in motor neuron disease with dementia, ballooned neurons in Creutzfeldt–Jakob disease, dystrophic neurites of senile plaques in Alzheimer's disease, and Lewy and Marinesco bodies and Lewy neurites in Parkinson's disease. This suggests that p97 and ubiquitin interact with abnormal or misfolded proteins and play a role in accelerating the process of degeneration and cell death [34]. Additionally, Mallory bodies (aggregates found in the liver cells of alcoholic and chronic nonalcoholic liver disease) also contain p97 and ubiq-

uitin alongside cytokeratin, chaperones and proteasomal subunits [153]. It has been suggested that polyQ aggregates may resist degradation and prevent ubiquitin recycling thereby disrupting the proteasome function resulting in cell fatality [150].

Formation of the polyQ aggregates is partially suppressed by co-expression of p97 suggesting that p97 may either protect the polyQ from aggregation or disassociate them in order for them to be degraded [154]. Interestingly, the expanded polyQ repeats in several diseases may disrupt the protein's normal cellular function, which could further exacerbate the disease when the protein normally participates in the UPS. For example, in Parkinson's disease, Parkin is normally an E3 ligase and in spinocerebellar ataxia Ataxin-3 with expanded polyQ repeats can no longer bind to the proteasome.

Single point mutations in p97 have been shown to be associated with IBMPFD [37]. This complex disease syndrome results in distal muscle weaknesses, early onset bone disease and dementia, with inclusion bodies staining for p97 and ubiquitin observed in patient muscle cells. The mutations in p97 causing this disease are mainly in the N domain and as so could interfere with adaptor and/or ubiquitin binding, leading to inclusion bodies in the affected areas.

Finally many studies have discovered that the level of p97 expression is closely correlated with disease and recurrence rates of a variety of carcinomas [155–160]. This has been linked to the role of p97 in activation of NF-κB, a transcription factor linked with anti-apoptosis and cell proliferation (see Section 7.3.2.2). It has been suggested that p97 plays an important role in tumour invasion and metastasis but it is unclear if ubiquitin is also involved with this. It has been proposed that expression levels of p97 could be used as an independent indicator of the metastatic potential of tumours and help to predict the outcome for patients with cancer. It is feasible that p97's role in cancer may be linked with its association with DNA-repair proteins such as BRCA1.

It is clear that the functions of p97 are multiple and crucial for cell survival and this is emphasized by the involvement of p97 in so many disease states. The exact roles of p97 in healthy cells, and its exact contribution to the disease states mentioned above, are not clear at a molecular level. Future experiments can be expected to clarify what happens, biochemically, when things do go wrong and eventually help in conquering these horrific diseases.

7.6 Conclusions

p97 has many cellular roles, but currently most is known about its roles within the UPS. As a consequence of p97's diversity of cellular function, it interacts with a variety of adaptor proteins and enzymes, many of which interact with ubiquitin [22]. p97 is also able to interact with tetra/polyubiquitin and, with very low affinity, monoubiquitin. p47 and Ufd1–Npl4 adaptors are crucial factors in the activity of p97 and potentially enhance the affinity and provide the specificity for its interac-

tions with mono- or polyubiquitin. Almost all of p97's known interactions are through the N-terminal adaptor-binding domain, and often involve ubiquitin-like domains, such as the UBX domain.

In ERAD, p97, Ufd1 and Npl4 are crucial. p97 has been shown to interact with E3 and E4 ubiquitination enzymes (E3: dorfin, Gp78, SCF^{Fbs}; E4: Ufd2) and indirectly with Hrd1–Hrd3 and Doa10 through mutual binding to the membrane anchor protein Ubx2. p97/Cdc48 is closely physically associated with the ER translocon and binds indirectly to Derlin-1 (through VIMP in mammals and Ubx2 in yeast) although no interaction has yet been shown with Sec61. This suggests a model in which p97, E3 ligases and E4 polyubiquitination factors are clustered at the ER, spatially close to the translocon and E2 enzymes. This provides a hub at which the emerging substrate protein may be efficiently polyubiquitinated and then released from the membrane-bound cluster. A population of proteasomes is associated with the ER (possibly also through interactions with a translocon or p97) and could then accept the polyubiquitinated substrate in an interaction mediated by shuttling factors such as Rad23.

The story of p97's role in membrane fusion is less well defined, although it seems likely that ubiquitin is involved as the deubiquitinating enzyme VCIP135 is essential and the adaptors required, p47 and also Ufd1–Npl4 (in nuclear envelope reformation only) are able to bind mono- and polyubiquitin. The interactions of p97 with Ufd3, Ataxin-3, HDAC6 and the Cui proteins (Cui1, Cui2, Cui3) represent a link between p97 and Lys 29 polyubiquitin chains although a cellular function for these is yet to be determined. Furthermore, as Ufd3, Ataxin-3 and HDAC6 are each associated with the competing covalent modification of lysine acetylation, a more complex picture involving regulation of ubiquitination may emerge.

p97 undergoes large conformational changes throughout the ATPase cycle, causing bound adaptors to also undergo concomitant movement and conformational change. However, owing to substantial differences between the two best-characterized adaptors, p47 and Ufd1–Npl4 in terms of domains present, ubiquitin specificity and oligomerization state, the motions that p97 undergoes with these adaptors may be dissimilar. The observation of apparent cross-talk between the adaptors p47 and Ufd1–Npl4 (both are required for the nuclear envelope reformation and, in yeast, p47 interacts with ubiquitinated proteins), is suggestive that the adaptors may not target p97 to a specific cellular role but instead to different actions and/or movements.

How p97 fulfills its cellular roles is unclear and has not been demonstrated conclusively *in vitro*. Clues may be taken from the functional gap p97 fills in spindle disassembly, transcription-factor processing, homotypic membrane fusion and release of polyubiquitinated substrates from the ER membrane in ERAD. Current evidence points to conformational changes leading to, generically, disassembly and, specifically in ERAD, separation of protein complexes, possibly by extraction of ubiquitinated substrate from the translocon or dislocation of ubiquitinated substrates from ER membrane-bound complexes. The role of p97 in ubiquitin chain elongation through an interaction with Ufd2 remains an intriguing avenue and whether this is a consequence of a new function for p97 or resulting from steric

hindrance of formation of polyubiquitin chains greater than six moieties remains to be resolved.

There appears to be a degree of redundancy in the ubiquitination machinery and proteasomal receptors for the substrates, but there appears to be no other protein able to do p97's job. The role of p97 in the mitigation of inclusion bodies formed in neurodegenerative disease and the devastating effect of apparently conservative mutations of p97 in IBMPFD highlights how crucial p97 is.

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8
Cdc48 (p97) and Its Cofactors

Alexander Buchberger

8.1
Introduction

The AAA ATPase Cdc48 (also known as p97 or VCP in higher eukaryotes) is a chaperone-like essential protein that is highly conserved among all eukaryotes. Cdc48 is involved in a large variety of cellular processes, including protein degradation via the ubiquitin–proteasome system, homotypic membrane fusion, nuclear envelope reassembly, cell cycle progression, and others [1].* The molecular basis underlying these diverse functions is believed to be the conversion of chemical energy from ATP hydrolysis into mechanical force exerted to segregate substrate proteins from environments such as membranes or protein complexes.

While some nonubiquitinated putative substrates have been shown to interact with Cdc48, at least *in vitro* [2–4], Cdc48 in general appears to selectively recognize substrates after they have been ubiquitinated by specific E3 ubiquitin ligases. In the endoplasmic reticulum-associated protein degradation (ERAD) pathway, Cdc48 drives the dislocation of substrates through a proteinaceous pore to the cytosolic face of the ER membrane, and targets dislocated substrates for proteasomal degradation [5–9]. Similarly, in the OLE pathway, Cdc48 is required to liberate the active, processed p90 form of the transcription factor Spt23 from the tight interaction with the inactive, ER membrane-anchored p120 precursor form [10, 11]. In the ubiquitin fusion degradation (UFD) pathway [12], Cdc48 appears to render the ubiquitin moiety of the tetrameric ubiquitin–proline–β-galactosidase fusion protein accessible for polyubiquitination and subsequent degradation [13, 14]. In contrast, in the homotypic fusion of Golgi, ER, and nuclear envelope membranes, Cdc48 has been suggested to modulate SNARE complexes and/or their regulators in a process that requires monoubiquitination, but not polyubiquitination or degradation, of the still unknown substrate(s).

* For the sake of clarity, the term "Cdc48" is used throughout this chapter collectively for all eukaryotic Cdc48 orthologues including *Xenopus* and mammalian p97, even though most of the knowledge about its role in membrane fusion processes is based on studies performed in the mammalian system.

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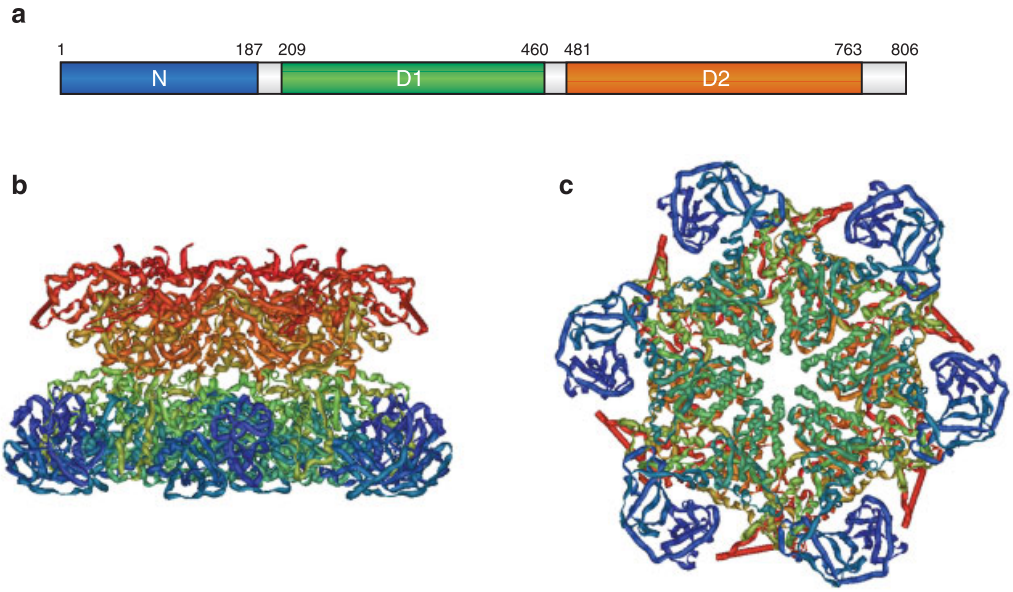

Fig. 8.1. Structure and domain composition of p97. (a) Schematic view of p97 domain composition. The domain borders of the N, D1, and D2 domains are indicated, and the domain colours were chosen to closely match the colour scheme in (b) and (c). (b) Side view of the three-dimensional structure of p97 in ribbon representation. Colour coding is from the amino-terminus (bottom, blue) to the carboxy-terminus (top, red). (c) Bottom view along the central pore.

Cdc48 is a ring-shaped complex of six identical subunits, which are composed of an amino terminal N domain and two ATPase domains, D1 and D2 (Figure 8.1). The different cellular functions of Cdc48 outlined above are specified by a large number of cofactors, most of which bind to the mobile N domain, while some others interact with the D1–D2 domains. The focus of this chapter is on the regulation of Cdc48 "segregase" activity on the levels of substrate recruitment and substrate processing by various cofactors. A detailed discussion of the structure and conformational changes of the mammalian Cdc48 orthologue p97 can be found in Chapter 7.

8.2 Cdc48 Cofactors

Most Cdc48 cofactors can be classified on the basis of their substrate-recruiting and substrate-processing activities. Before describing these activities in detail, I will give an overview of the increasingly large number of Cdc48 cofactors (Figure 8.2).

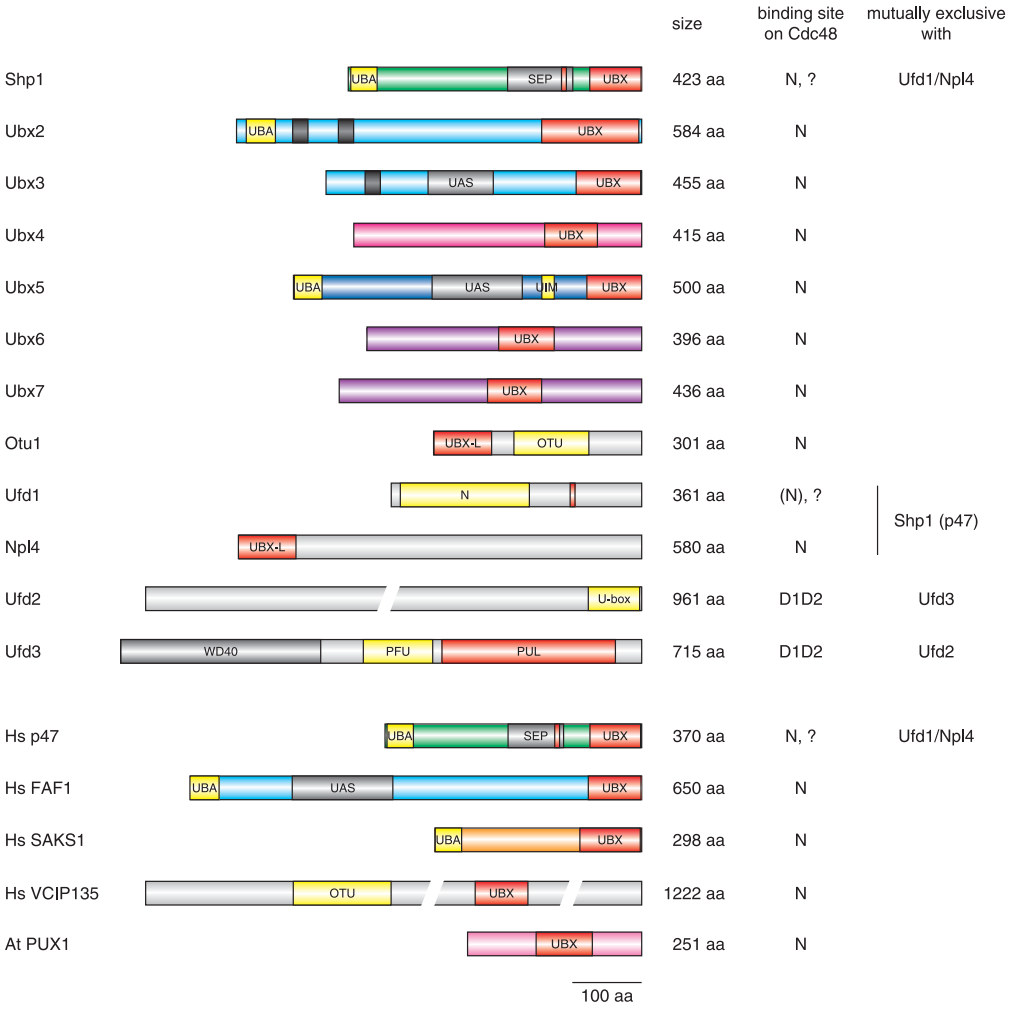

Fig. 8.2. Cdc48 cofactors. The figure shows all known cofactors from *Saccharomyces cerevisiae* (top) and selected cofactors from other species that are discussed in the text (bottom). Cdc48 binding modules including the UBX and UBX-like (UBX-L) domains, the PUL domain, and a short binding motif found in Shp1, Ufd1, and p47 (not labelled) are shown in red. The binding site on Cdc48 is indicated at the right. Ufd1 interacts via Npl4 with the N domain of Cdc48. Ubiquitin-binding modules including the UBA, OTU, PFU, and N domains, the UIM motif, and the U-box are shown in yellow. Transmembrane regions in Ubx2 and Ubx3 are shown in black. Further domains not mentioned in the text are shown in dark grey. Sequence homology between UBX domain proteins outside the defined domains is indicated by similar colours. Mutually exclusive binding of cofactors to Cdc48 is indicated at the right.

Cofactors are defined here as proteins that interact directly with Cdc48, are not Cdc48 substrates, and regulate some aspect of substrate turnover. This definition excludes a significant number of yet uncharacterized known Cdc48 interactors, and it would be no surprise if in the future some of them turned out to be regulatory cofactors as well.

8.2.1 Cofactor Families

8.2.1.1 UBX Domain Proteins

The mammalian UBX domain containing protein p47 was the first Cdc48 cofactor identified and was shown to be essential for the Cdc48-mediated post-mitotic fusion of Golgi vesicles [15]. Today, UBX domain proteins constitute the largest family of Cdc48 cofactors with seven members in budding yeast (Figure 8.2) and at least a dozen members in humans according to the SMART database. The UBX domain has been shown to be necessary and sufficient for interaction of several UBX domain proteins with Cdc48, and thus to be a general Cdc48 interaction module [16–20], even though p47 and its orthologues possess a second Cdc48-binding site (see below) [21, 22]. Interestingly, the three-dimensional structure of the UBX domain closely resembles that of ubiquitin [23, 24], perhaps reflecting an ancient gene-fusion event between ubiquitin and some archetypical Cdc48 cofactor. The structural basis for the binding of UBX domains to the N domain of Cdc48 has been elucidated in detail [25].

A number of UBX domain proteins possess known ubiquitin-binding modules, including an amino-terminal UBA domain and internal UIM motifs [16, 18, 23, 26–28] (Figure 8.2). Based on its suggestive domain architecture, this subgroup of UBX domain proteins has been proposed to represent substrate-recruiting cofactors of Cdc48 [29]. Indeed, several UBA/UBX proteins bind ubiquitin or ubiquitinated proteins [16, 18–20, 30], and some have been shown to recruit substrates to Cdc48 [16, 31], strongly suggesting that this is a general function of UBA/UBX proteins. However, several other UBX domain proteins do not possess any further known domains and are still awaiting their functional characterization.

8.2.1.2 Ufd1/Npl4

Ufd1 and Npl4 form a stable heterodimer *in vivo* and *in vitro*, and can thus be regarded as a single cofactor of Cdc48 [22, 32]. Both subunits of the Ufd1/Npl4 heterodimer interact with Cdc48 via distinct motifs. Npl4 possesses a UBX-like ubiquitin-fold domain that most likely interacts in the same way as classical UBX domains with the N domain of Cdc48 [22]. Similar UBX-like domains have also been identified in two Cdc48 cofactors possessing deubiquitinating activity: VCIP135 [21] and Otu1 [33]. In contrast, Ufd1 interacts with Cdc48 via a linear sequence motif (FxGzGQxb; x: any amino acid, z: hydrophilic, b: hydrophobic; [34]) that is also found in p47 and other proteins linked to the ubiquitin–proteasome

system [22, 25, 34]. Thus, Ufd1/Npl4 and p47, but not other UBX domain proteins, interact with Cdc48 through a bipartite binding mechanism [22]. This bipartite binding is probably the basis underlying the mutual exclusive binding of Ufd1/Npl4 and p47 to Cdc48 [32], while for example Ufd1/Npl4 and Ubx2 [31] or p47 and VCIP135 [21] can bind simultaneously to Cdc48.

In contrast to UBX domain proteins, neither Ufd1 nor Npl4 contain canonical ubiquitin binding domains. However, the amino-terminal domain of Ufd1 was shown to directly bind K48-linked ubiquitin chains [4]. Intriguingly, the structure of this domain is highly homologous to the N domain of Cdc48 itself [35, 36], and harbours two distinct ubiquitin-binding sites, one for monoubiquitin and one with higher affinity for ubiquitin chains, raising the intriguing possibility that Ufd1 employs different binding and/or delivery mechanisms for mono- and polyubiquitinated substrates [35]. In mammalian Npl4, a carboxyl-terminal NZF zinc finger domain [30] has been shown to directly bind K48- and K63-linked ubiquitin chains [4], but this domain is absent in yeast Npl4. While mammalian Npl4 lacking the NZF domain (Npl4ΔNZF) was impaired in ubiquitin binding, yeast Ufd1/Npl4 and mammalian Ufd1/Npl4ΔNZF exhibited specific binding to K48-linked ubiquitin chains [4]. These findings suggest that the evolutionary conserved ability of Ufd1/Npl4 to bind K48-linked chains resides exclusively in Ufd1, whereas the additional, more promiscuous, ubiquitin binding to the NZF domain of Npl4 evolved later.

8.2.1.3 **Other Cofactors**

Several other cofactors of Cdc48 that have been shown to bind directly to Cdc48 do not contain one of the Cdc48-binding motifs described above. Ufd2 and Ufd3 bind competitively to the same or two overlapping sites in the D1 and/or D2 domains of Cdc48 [33]. In Ufd3, the so-called PUL domain in the carboxy-terminal part of Ufd3 constitutes the Cdc48 binding site, and the central PFU domain was characterized as a novel type of ubiquitin-binding domain [37]. Ufd2 possesses a carboxy-terminal U-box catalyzing ubiquitin-chain elongation [13, 38], but it is not known whether this is also the binding site for ubiquitin chains. The Cdc48 binding site of Ufd2 has not been precisely mapped, even though a sequence stretch preceding the U-box is necessary for a yeast two-hybrid interaction between Ufd2 and Cdc48 [38].

VIMP is an ER membrane protein found in vertebrates, which binds predominantly to the N domain of Cdc48 in a manner that is not mutually exclusive with Ufd1 [39]. SVIP is a small membrane-associated protein found in higher eukaryotes that competes with Ufd1 and p47 for binding to Cdc48 and was proposed to be an alternative adaptor involved in the integrity of ER membranes [40]. Mammalian peptidyl-*N*-glycanase (PNGase), an enzyme catalyzing the removal of glycans from misfolded glycoproteins [41, 42], has been shown to bind directly to Cdc48 [19, 43]. The interaction has not been further analyzed, but appears to require the amino terminal PUB domain of PNGase [43] that is also found in proteins containing UBX and/or UBA domains [44].

8.2.2 Cofactor Functions

8.2.2.1 Substrate-recruiting Cofactors

Ufd1/Npl4 and p47 are prototypical substrate-recruiting factors essential for proteasomal targeting and membrane-fusion functions of Cdc48, respectively. While Ufd1/Npl4 is required for the recruitment of mono- or oligoubiquitinated substrates of the ERAD [31], OLE [11], and UFD [11, 12] pathways to Cdc48, the postulated monoubiquitinated p47 substrate critical in homotypic membrane fusion [30, 45] remains enigmatic. Interestingly, the UBX domain protein Ubx2 has also been shown to be important for the efficient recruitment of various ERAD substrates to Cdc48 [31, 46], most likely prior to or concomitant with Ufd1/Npl4 [31]. This finding suggests that additional regulatory levels of substrate recruitment may also exist in other pathways involving $Cdc48^{Ufd1/Npl4}$.

Besides the well-characterized substrate-recruiting cofactors p47 and Ubx2, further UBA/UBX domain proteins including Ubx5 [16, 18], SAKS1 [19], and FAF1 [20] have been implicated in substrate recruitment to Cdc48, even though physiological substrates and the exact mechanisms of recruitment are still unknown in these cases.

8.2.2.2 Substrate-processing Cofactors

Substrates recruited to Cdc48 are believed to be ubiquitin conjugates carrying few, or only one, ubiquitin moieties rather than a long polyubiquitin chain [38]. Because this mono- or oligoubiquitination is normally insufficient to target proteins for proteasomal degradation [47], Cdc48 has emerged as a crucial platform for the decision whether substrates are delivered to the proteasome or released as stable proteins (Figure 8.3). The fate of Cdc48-bound substrates is determined by several substrate-processing cofactors modulating their ubiquitination state. The first such cofactor identified was the E4 polyubiquitination factor Ufd2, which catalyzes ubiquitin-chain elongation on mono- or oligoubiquitinated substrates, thereby targeting them for proteasomal degradation [13]. Interestingly, the length of the ubiquitin chain assembled by Ufd2 is restricted by Cdc48 itself to a size compatible with efficient downstream proteasomal targeting and degradation [38].

The polyubiquitination activity of Ufd2 can be antagonized by two other substrate-processing cofactors, Ufd3 and Otu1 [33]. Competition of Ufd3 with Ufd2 for binding to the Cdc48 D1–D2 domains stabilizes the mono- or oligoubiquitinated state of substrates and effectively prevents their proteasomal degradation [33]. In addition, the deubiquitinating enzyme Otu1 may mediate an even stronger counterbalance to Ufd2-catalyzed substrate polyubiquitination. Otu1 has been shown to preferentially deconjugate K48-linked ubiquitin chains, and overexpression of Otu1, like that of Ufd3, stabilizes the Ufd2 target Spt23 [33]. Notably, Ufd3 and Otu1 bind to different domains of Cdc48, making cooperation of these inhibitory cofactors possible. Even though the range of cellular targets of Otu1 is still unknown, Otu1 may completely deconjugate erroneously ubiquitinated pro-

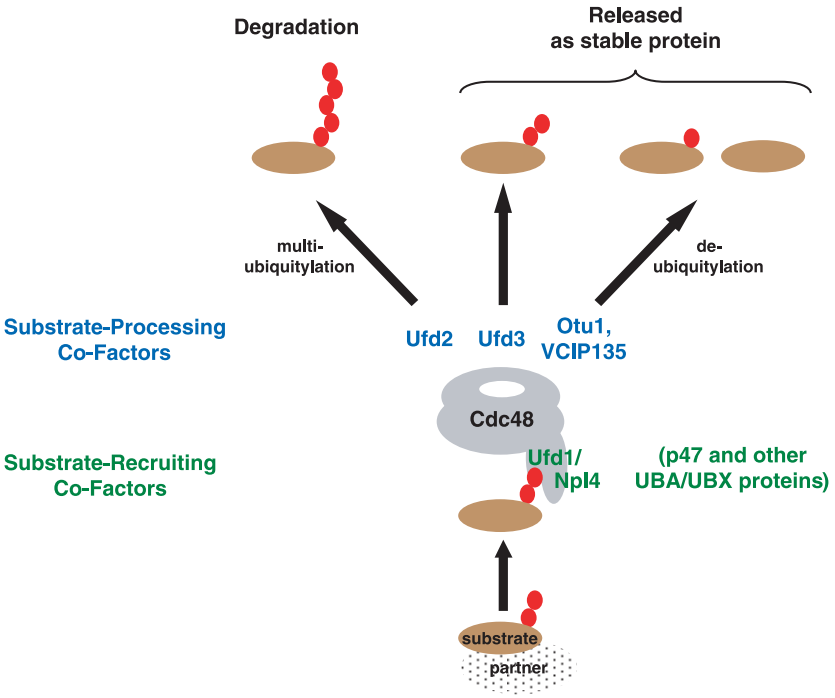

Fig. 8.3. Cdc48 cofactors determine the fate of substrates. Substrate marked by short ubiquitin chains (red circles) is recognized by substrate-recruiting cofactors including Ufd1/Npl4, p47, or other UBA/UBX domain containing proteins (green) and segregated from their partner protein(s). Substrate-processing cofactors (blue) catalyze multiubiquitylation that targets the substrate for Rad23- and/or Dsk2-mediated delivery to the 26S proteasome and subsequent degradation (left); inhibit multiubiquitylation to release the substrate in its oligoubiquitylated state (middle); or catalyze deubiquitylation to release mono- or nonubiquitylated susbtrate (right). The latter two options destine the released substrate for nondegradative pathways. Hypothetical model modified from Ref. [33].

teins, trim multi- or oligoubiquitinated proteins to monoubiquitinated species that are subject to nondegradative functions of the ubiquitin system, or both. A precedence for the latter activity is the function of VCIP135 in p47-mediated homotypic membrane fusion. Like Otu1, VCIP 135 is a deubiquitinating enzyme that interacts *via* its UBX domain with the N domain of p97 [21]. While critical cellular substrates of the fusion process remain to be identified, it is clear, at least *in vitro*, that their monoubiquitination is sufficient, and that VCIP135 deubiquitinating activity is required for membrane fusion [45]. This suggests that the trimming of polyubiquitinated substrates by deubiquitinating enzymes, and thus release of stable "products", is a conserved feature of the Cdc48 machinery.

In contrast to factors regulating the ubiquitination state of substrates, PNGase is a substrate-processing cofactor that is believed to assist in the dislocation and/or

proteasomal degradation of glycosylated ERAD substrates by removal of their glycan chains [41, 48, 49]. While a preference of PNGase for denatured and/or misfolded glycoprotein substrates has been shown [50, 51], the importance of PNGase activity for efficient ERAD is still controversial (discussed in Ref. [52]). In yeast, for instance, Png1 appears to be critical for the degradation of some, but not all, glycosylated ERAD substrates [52]. The significance of the direct interaction between mammalian PNGase and Cdc48 for ERAD remains to be demonstrated.

8.2.2.3 Additional Functions of Cofactors

While most Cdc48 cofactors function in the recruitment or processing of substrates, some regulate the localization and activity of Cdc48 itself.

The mammalian ER membrane protein VIMP recruits Cdc48 to the putative ERAD retrotranslocation pore component Derlin-1, thereby probably increasing the efficiency of Cdc48-mediated retrotranslocation of substrates [39]. Yeast Ubx2, also an integral membrane protein of the ER, appears to possess a dual function as a membrane anchor analogous to VIMP, and as a substrate-recruiting factor required for the efficient binding of ERAD substrates to $Cdc48^{Ufd1/Npl4}$ [31].

p47 provides another example of a substrate-recruiting factor with an additional function. p47 inhibits the ATPase activity of Cdc48 by up to 85% [53], suggesting that it is a major regulator of the functional cycle of Cdc48. No similar effects have so far been reported for other Cdc48 substrate-recruiting or -processing cofactors. The molecular basis of the inhibition and its implications in the mechanism of p47-mediated membrane-fusion processes are completely unclear at the moment.

Finally, another intriguing way of regulating Cdc48 is illustrated by the *Arabidopsis thaliana* UBX domain protein AtPUX1 [54]. Besides its UBX domain, AtPUX1 does not contain any known ubiquitin-binding or protein–protein interaction motif. Consistently, it appears to function as neither a substrate-recruiting nor a substrate-processing cofactor of Cdc48. Rather, AtPUX1 appears to shift the equilibrium between hexameric and mono- or dimeric subpopulations of Cdc48, which normally lies far in favour of the hexamer relative to the mono- or dimeric form. As a consequence, the overall ATPase activity of the Cdc48 population decreases in the presence of excess AtPUX1, probably reflecting the existence of positive cooperativity in the ATPase activity of the hexamer. AtPUX1 may function either by actively dissociating Cdc48 hexamers, or by preventing monomers and dimers from oligomerization. While the molecular mechanism of AtPUX1 function is still unclear, regulation of the oligomeric state of Cdc48 appears to be important, because *Arabidopsis* mutant plants lacking AtPUX1 exhibit aberrant, accelerated growth [54].

8.3 Cellular Functions

Most known cellular functions of Cdc48 involve one of the two mutually exclusive substrate-recruiting factors, Ufd1/Npl4 or Shp1 (p47 in higher eukaryotes).

8.3.1 Cdc48$^{Ufd1/Npl4}$

8.3.1.1 Protein-degradation Pathways

The first evidence implicating Cdc48 and its cofactors in protein degradation by the ubiquitin–proteasome system came from the dissection of the UFD pathway: a genetic screen revealed that Ufd1, Ufd2, and Ufd3, together with a specific E3 ubiquitin ligase, Ufd4, are required for the degradation of the model substrate, ubiquitin–proline–β-galactosidase (Ub–P–βGal) [12]. Subsequently, Cdc48 itself [14], Npl4 [11], and the proteasomal targeting factors Rad23 and Dsk2 [55, 56] were identified as further components of the UFD pathway. Intriguingly, these proteins turned out not only to participate in the same genetic pathway but in fact to physically escort Ub–P–βGal to the 26S proteasome. According to a current model, Ufd1/Npl4 recruits the substrate to Cdc48, where it is polyubiquitinated by Ufd2, and passed over to Rad23 and Dsk2 for proteasomal delivery [38]. However, although the necessity of its presence is well established, the exact function of Cdc48$^{Ufd1/Npl4}$ in the degradation of Ub–P–βGal is still unclear. In that respect, it is informative that related model substrates lacking the amino-terminal ubiquitin moiety, e.g. Arg-βGal and *Deg1*-βGal, are degraded through distinct pathways that do not involve Cdc48 [14, 57]. This makes it unlikely that Cdc48$^{Ufd1/Npl4}$ is simply required for the dissociation of the tight β-galactosidase tetramer. Rather, Cdc48$^{Ufd1/Npl4}$ may bind Ub–P–βGal after the Ufd4-catalyzed attachment of one to three ubiquitin moieties, and render the short ubiquitin chain accessible for elongation by the polyubiquitination factor Ufd2 [13]. Consistent with this hypothesis is the fact that the initial, Ufd4-catalyzed attachment of the first ubiquitin moieties occurs via the unusual K29-linkage, while Ufd2-catalyzed chain elongation proceeds via canonical K48-linkages [12, 13, 58]. After the Ufd2-catalyzed elongation of the ubiquitin chain, Ub–P–βGal is targeted for proteasomal degradation by the homologous proteins Rad23 and Dsk2 [55, 56]. Intriguingly, the latter two proteins bind directly to Ufd2, thus escorting substrates from the Cdc48-bound processing factor to the 26S proteasome [38, 59].

ufd3 mutants exhibit strongly reduced levels of free ubiquitin, leading to degradation defects that are not specific for the UFD pathway [12, 14]. Intriguingly, however, Ub–P–βGal was found to be stabilized not only upon inactivation, but also upon overexpression of *UFD3* [33]. The latter result reflects the competitive binding of Ufd3 and Ufd2 to Cdc48 and demonstrates that both substrate-processing cofactors possess antagonistic roles in the UFD pathway *in vivo*.

The role of Cdc48 and its cofactors in the UFD pathway can serve as a paradigm for their escort function in other cellular degradation pathways including the OLE and ERAD pathways [38]. In the OLE pathway, the inactive, ER membrane-bound p120 form of the transcription factor Spt23 is monoubiquitinated by the ubiquitin ligase Rsp5 and processed by the 26S proteasome into its active p90 form [10]. Monoubiquitinated p90 is recognized by Cdc48$^{Ufd1/Npl4}$ and segregated from the unprocessed p120 precursor [11]. The mobilized transcription factor is then transported to the nucleus, most likely in complex with Cdc48$^{Ufd1/Npl4}$ [38], where it can

activate expression of its key target gene, *OLE1*. Subsequently, one subpopulation of monoubiquitinated p90 is converted into a degradation target by Ufd2 activity and escorted by Rad23 or Dsk2 to the 26S proteasome for degradation, while another subpopulation appears to be degraded in a parallel pathway requiring Rpn10, but not Ufd2, Rad23, and Dsk2 [38]. Intriguingly, the function of Ufd2 in the OLE pathway can also be antagonized by Ufd3, as well as by Otu1, even though p90 is probably not their major cellular target under normal physiological conditions [33].

The Cdc48 escort is also in operation during ERAD in guiding substrate proteins from the ER membrane to the 26S proteasome [38, 60]. In addition to the cofactors involved in the UFD and OLE pathways, the ER-membrane protein Ubx2 is required for efficient degradation of ERAD substrates [31, 46]. Ubx2 not only recruits $Cdc48^{Ufd1/Npl4}$ to substrates but also interacts with the ERAD ubiquitin ligases Hrd1 and Doa10, and with the putative retrotranslocation pore component Der1 [31, 46]. Ubx2 thus probably interacts with ERAD substrates as soon as they emerge at the cytosolic face of the ER membrane, and would therefore be the most upstream cofactor of the Cdc48 escort pathway. Consistent with this view, Ubx2 is not involved in the Cdc48-independent degradation of soluble, cytosolic substrates of the ERAD ubiquitin ligase Doa10 [57].

Besides the well-defined degradation pathways described above, Cdc48 has also been implicated in the degradation of several other substrates of the ubiquitin–proteasome system, among them IκBα [20, 61] and cyclins [62].

8.3.1.2 **Cell Cycle Regulation**

Yeast *cdc48* conditional mutants arrest in G2/M as large budded cells [63]. Despite the fact that *CDC48* was identified in a screen for cell cycle mutants, the critical function of Cdc48 in this process has long been enigmatic, and it could not be excluded that the cell cycle defect of *cdc48* mutants is an indirect consequence of the pleiotropic defects these cells exhibit. However, several studies have revealed that $Cdc48^{Ufd1/Npl4}$ in fact has multiple functions in the cell cycle. A study in yeast using a tightly regulatable conditional *cdc48* mutant allele revealed that Cdc48 is involved not only in mitosis but also in the regulation of Start (the yeast equivalent to restriction point in mammals) at the G1/S transition [64]. The critical Cdc48 substrate in this process is the G1 cyclin-dependent kinase inhibitor Far1. Intriguingly, the Far1 target G1 cyclin Cln2 was identified in a proteomics approach as a Cdc48 substrate as well [65], suggesting that Cdc48-mediated degradation is an important regulatory principle in S phase entry, even though the degradation pathway of these targets and the involvement of Cdc48 cofactors have not been investigated in detail.

During mitotic exit, Cdc48 has been shown in yeast and in *Xenopus* egg extracts to be involved in spindle disassembly [66]. In this study, the spindle assembly factors XMAP215 and TPX2 (*Xenopus*) and Ase1 and Cdc5 (yeast) were postulated to be targets of $Cdc48^{Ufd1/Npl4}$ that are either sequestered or degraded by $Cdc48^{Ufd1/Npl4}$ in order to allow spindle disassembly to occur. Again, the exact mechanism of Cdc48-mediated regulation in this process remains to be elucidated.

In higher eukaryotes, $Cdc48^{Ufd1/Npl4}$ possesses a further role in mitosis by mediating correct chromosome alignment and segregation [67]. Both processes are regulated by the chromosomal passenger complex protein survivin. Modification of survivin by K63-linked ubiquitin chains, which do not target proteins for proteasomal degradation, was found to be crucial for its correct localization to the centrosome. Surprisingly, survivin ubiquitination with K63-linked chains was nearly abolished in the absence of Ufd1, providing the first example of an involvement of $Cdc48^{Ufd1/Npl4}$ in processes linked to this chain type. Whether $Cdc48^{Ufd1/Npl4}$ is actively involved in assembling a K63-linked chain on survivin, e.g., by recruiting a specific ubiquitin ligase, has not been addressed. Alternatively, in analogy to the functions of VCIP135 and Otu1 (Figure 8.3), the important function of $Cdc48^{Ufd1/Npl4}$ in this process could be the removal of K48-linked ubiquitin moieties through recruitment of a deubiquitylating enzyme, as a prerequisite for subsequent, perhaps even $Cdc48^{Ufd1/Npl4}$-independent, K63 chain assembly. In this context, it is interesting to note that in fission yeast, overexpression of separase/Cut1 suppresses the mitotic phenotype of *cdc48* temperature-sensitive mutants [68], suggesting that Cdc48 could also be involved in chromosome segregation in fungi.

8.3.2 $Cdc48^{Shp1}$

8.3.2.1 Membrane Fusion

Even before its central role in many protein degradation pathways was established, Cdc48 was identified as an essential factor in the homotypic fusion of Golgi fragments [21, 69, 70], ER membranes [71–73], nuclear envelope vesicles [74], and yeast vacuole membrane [75] (for recent reviews see Refs [76, 77]). In analogy to the well-established role of the AAA ATPase NSF in membrane-fusion processes, the critical function of Cdc48 in Golgi and ER membrane fusion was proposed to be the disassembly of SNARE complexes containing the SNARE syntaxin 5 (Ufe1 in yeast) [2, 21, 78]. More specifically, the substrate-recruiting cofactor p47 (Shp1 in yeast) is believed to recruit Cdc48 to syntaxin 5, which is part of stable, membrane-bound SNARE complexes after one round of membrane fusion, where the Cdc48 segregase activity releases (''primes'') syntaxin 5 for another round of membrane fusion. Indeed, syntaxin 5 and Ufe1 interact with Cdc48 *in vitro* [2, 78], and the efficient binding of syntaxin 5 to Cdc48 requires p47 [2]. Moreover, the UBX domain protein VCIP135 was found to form a stable complex with syntaxin 5 in the presence of p47, and to dissociate the otherwise stable ternary complex of syntaxin 5, p47, and Cdc48 [21]. The VCIP135-dependent release of syntaxin 5 from p47 and Cdc48 was postulated to be essential for Cdc48-mediated membrane fusion [21]. However, in contrast to the role of NSF in SNARE disassembly, a direct role of Cdc48 in SNARE disassembly has not generally been accepted so far (see Ref. [79] for a detailed discussion). Even though Cdc48 binds syntaxin 5 in a p47-dependent manner, there is no evidence that Cdc48, p47, and

VCIP135 are in fact involved in the disassembly of SNARE complexes containing syntaxin 5 *in vivo*. Instead, it has been argued that membrane fusion defects observed in cells with impaired Cdc48 function could be an indirect consequence of protein-degradation defects, in particular of the ERAD pathway [79].

An important new perspective regarding the role of Cdc48 in homotypic membrane fusion was opened by the finding that ubiquitination of a yet unknown factor, possibly representing the critical Cdc48 target, is essential for the fusion of mitotic Golgi cisternae *in vitro* [30, 45]. In this *in vitro* assay, the UBA domain of p47 was required for membrane fusion, suggesting that p47 recruits a ubiquitinated substrate(s) to Cdc48 [30]. Interestingly, the activity of Cdc48 in this process is apparently not linked to proteasomal degradation, because addition of proteasome inhibitors or a ubiquitin variant incapable of forming K48-linked chains had no effect [45]. In contrast, the deubiquitinating activity of VCIP135 was essential for membrane fusion, suggesting that substrate(s) carrying long ubiquitin chains need to be trimmed to a mono- or oligoubiquitinated species [45]. It is tempting to speculate that VCIP135, similar to Otu1, is a substrate-processing cofactor that prevents the downstream degradation of the substrate(s) critical in membrane fusion by antagonizing the action of ubiquitin ligases. Given the essential role of substrate ubiquitination in this assay, it is unclear whether syntaxin 5 is indeed a direct target of p47 and Cdc48 in homotypic membrane fusion, because syntaxin 5 binds p47 efficiently *in vitro* in the absence of any ubiquitin modification [2]. Alternatively, the critical *in vivo* target may not be the SNARE itself, but rather a regulatory protein of the fusion process that needs to be modulated and/or sequestered by Cdc48.

A less well-studied function of Cdc48 is its role in the post-mitotic reassembly of the nuclear envelope [74]. In *Xenopus* egg extracts, Cdc48 was found to be required for two consecutive steps of nuclear envelope reassembly: first for the formation of a closed nuclear envelope from an open, chromatin-attached membrane network, and second during nuclear expansion. Interestingly, the two steps involve the two different major Cdc48 cofactors. While p47 is required for nuclear expansion, a process that may closely resemble homotypic fusion of ER or Golgi membranes, formation of a closed nuclear envelope surprisingly requires Ufd1/Npl4, but not p47. This is so far the only example of the involvement of Ufd1/Npl4 in a membrane-fusion-related process. It is possible that this activity of $Cdc48^{Ufd1/Npl4}$ is linked to its functions during mitotic exit (see above) and reflects sequestration and/or degradation of proteins inhibitory for nuclear envelope formation rather than a membrane fusion activity itself.

8.3.2.2 **Protein Degradation**

Ufd1/Npl4 is clearly the major substrate-recruiting cofactor in Cdc48-mediated protein degradation, while the function of p47 in Cdc48-mediated membrane-fusion processes has been initially linked to nondegradative pathways. However, substantial evidence has accumulated suggesting that Shp1 and p47 are also involved in Cdc48-mediated protein degradation. The UBA domain of Shp1 has been shown to bind *in vitro* tetraubiquitin chains [18, 80] and oligo- and polyubi-

quitinated forms of a UFD substrate [16]. *In vivo*, Shp1 associated with polyubiquitinated cellular proteins and with the UFD substrate Ub–P–βGal [16]. Finally, deletion of *SHP1* resulted in the stabilization of Ub–P–βGal in budding yeast [16] and of the cell cycle regulator Rum1 in fission yeast [18], and knockdown of p47 in HeLa cells led to the accumulation of polyubiquitinated proteins [81]. The physiological relevance of these degradation-linked functions of $Cdc48^{Shp1}$ is, however, still unclear.

8.3.3 Further Functions

Besides its well-established functions in protein degradation and membrane fusion, Cdc48 has been implicated in a vast number of other cellular processes, whose detailed description is beyond the scope of this chapter. However, two important processes seem worth mentioning. First, Cdc48 interacts directly with WRN protein, a RecQ-type DNA helicase involved in DNA repair that is inactivated in patients suffering from the DNA damage accumulating disease Werner syndrome. *In vivo*, Cdc48 and WRN colocalize in the nucleolus, but the interaction is lost in the presence of the DNA damaging agent camptothecin, perhaps indicating an involvement of Cdc48 in some aspect of WRN-mediated DNA repair. Second, the $cdc48^{S565G}$ conditional allele has been shown to induce hallmarks of apoptosis in yeast at the nonpermissive temperature, including exposure of phosphatidylserine at the outer layer of the cytoplasmic membrane, DNA fragmentation, and chromatin condensation and fragmentation [82]. It will be interesting to identify the underlying cellular defect(s) caused by this particular *cdc48* mutant allele.

8.4 Outlook

Compared to other eukaryotic AAA ATPases, the highly abundant Cdc48 segregase complex interacts with a wider range of substrates and is involved in a greater variety of cellular pathways. This central role of Cdc48 is the consequence of relying on the versatility of ubiquitin conjugation as the major substrate recognition signal, and of employing a multitude of cofactors regulating recruitment and fate of ubiquitinated substrates. Although significant progress in understanding the functions of Cdc48 and several of its cofactors has been made, many open questions still remain. On the molecular level, the mechanism of Cdc48 segregase activity and its regulation by substrate-recruiting and substrate-processing cofactors needs to be clarified. Are substrates threaded through the central pore of Cdc48, or worked upon on the surface of the hexamer? Or are even distinct mechanisms operational in $Cdc48^{Ufd1/Npl4}$ and $Cdc48^{Shp1}$? On the cellular level, on the other hand, equally interesting questions are raised by the ever-growing number of Cdc48 cofactors whose specific functions in Cdc48-mediated processes and pathways are still awaiting discovery.

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9
Deubiquitinating Enzymes, Cell Proliferation, and Cancer

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9.1
Introduction

9.1.1
Ubiquitination

The ubiquitin pathway involves the enzymatic attachment of one or more molecules of the 76-amino acid protein ubiquitin to the free amino group of a lysine side chain (or occasionally to the amino terminus) of another protein, and the resulting effects this can have on protein localization, activity, function, and/or degradation. Research into this pathway has evolved over the last 30 or so years from being considered rather esoteric to now being central to many, if not all, processes in the eukaryotic cell. Many aspects of the ubiquitin–proteasome pathway have been covered in depth in other chapters in this series and in reviews [1–4], and therefore the pathway will be only briefly summarized here. Ubiquitin is translated from ubiquitin genes always as a precursor protein, consisting of linear fusions of ubiquitin to itself (polyubiquitin genes) or to one of two ribosomal proteins. These fusions are rapidly and precisely cleaved by deubiquitinating enzymes (DUBs) to produce free ubiquitin (and free ribosomal proteins, which are incorporated into nascent ribosomes [5]). The resulting free ubiquitin can be activated by a ubiquitin-activating enzyme (or E1), transferred to one of many ubiquitin-conjugating enzymes (E2s), and then finally transferred to its ultimate target protein in a covalent isopeptide bond linkage to the ε-amino group of a lysine side chain (or peptide bond to the N-terminal amine group), in most cases requiring the assistance of a ubiquitin–protein ligase (E3). Ubiquitin itself can then act as an acceptor for further ubiquitin molecules, by subsequent conjugation to lysine residues within ubiquitin, to form ubiquitin chains. While any of the seven lysines within ubiquitin can serve as acceptors (at least in yeast [6]), most functional information has been gathered on monoubiquitin conjugates, or on chains formed using Lys-48 or Lys-63 of ubiquitin. Discoveries since 2000 have revealed that monoubiquitination is generally involved in trafficking of proteins within the cell

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and endocytosis [7–9]. Ubiquitin chains of four or more ubiquitins linked by Lys-48 isopeptide bonds generally target proteins to the proteasome for degradation, while Lys-63-linked chains generally specify nonproteolytic fates, and are involved in DNA repair, activation of kinases (see Section 9.2.3 below), and also trafficking (reviewed in Ref. [10]). It has become clear in recent years that there are different ubiquitin-binding proteins that can discriminate between the different ubiquitin-chain topologies and monoubiquitinated proteins, thus directing the fate of a ubiquitinated protein.

9.1.2 Deubiquitination

Like most biochemical reactions, ubiquitination is reversible. Cleavage of ubiquitin from its isopeptide-linked conjugates is performed by deubiquitinating enzymes (DUBs), of which there are many enzymes in mammals. Some of these enzymes also cleave the linear ubiquitin-fusion proteins that arise from ubiquitin genes (see above) [11]. DUBs have been the subject of several reviews from which the reader can obtain more information, and they will not be summarized in detail here [12–17].

In this chapter, the term ''DUB'' (deubiquitinating enzyme) will be used to define any enzyme capable of hydrolysing an ester, thiol ester, or amide (peptide) bound to the carboxyl group of Gly-76 of ubiquitin [18]. This is not to be confused with the subset of mouse DUBs of the UBP/USP family, DUB1 and DUB2, which are discussed below (Section 9.2.8). DUBs include the ubiquitin carboxy-terminal hydrolases (UCH); ubiquitin-specific peptidases (UBP/USP); the cylindromatosis protein (CYLD, although this may be a variant USP-type sequence); the ovarian tumour proteins (OTU or Otubain); the Josephin or Machado–Joseph disease (MJD) proteins; and the Jab1/MPN domain-associated metalloisopeptidase (JAMM) proteins. The first five classes of DUBs are all cysteine peptidases (''peptidase'' being the systematic MEROPS term to replace ''protease'') while the JAMM proteins are zinc metallopeptidases. A recent analysis of the human genome and transcriptome predicted that while the human genome contains some 95 predicted DUB genes (58 USPs, 4 UCHs, 5 MJDs, 14 OTUs, and 14 JAMM), 5 were not supported by the transcriptome, and a further 11 did not contain sufficient conservation of catalytic residues to suggest that they would be active DUBs, resulting in a prediction of 79 active DUBs in humans [17]. Of course, some of these peptidases will cleave ubiquitin-like proteins, either additionally or exclusively, and there may be new types of DUBs yet to be discovered. This chapter will focus on the mechanisms of action of those DUBs that have been linked to cancer and cell transformation/proliferation in mammals, as these shed some light on the many different facets of both ubiquitination and deubiquitination.

9.2
DUBs, Oncogenes, and Cell Transformation

9.2.1
USP6/Tre-2/Tre-17

The isolation of the first three DUBs of the UBP family from the yeast *Saccharomyces cerevisiae* enabled the identification of two blocks of conserved sequence, which contain the (subsequently determined) catalytic triad Cys, and His and Asp/Asn residues, respectively [11]. These sequence domains have allowed the subsequent identification of homologous family members from many organisms, and gave the first compelling evidence of a role for DUBs in cancer. In this case, identification of the gene mutated in a yeast strain that was deficient in ubiquitin-dependent proteolysis led to the fourth yeast UBP family member, Doa4p (Ubp4p) [19]. Comparison of Doa4p to sequence databases revealed that it was more similar to a human oncogene, *tre-2*, that to any of the known yeast UBPs. *tre-2* is now known as *USP6* or *TRE-17*. It was originally isolated in a screen for DNA with oncogenic potential derived from a Ewing's sarcoma cell line, and was determined to contain DNA fragments from three separate human chromosomal loci that recombined during the transfection process, hence the original name transfection recombined-1 (*tre or tre-1*) [20, 21]. Subsequent studies revealed that the 3′ portion of *tre-1*, derived from chromosome 17, was sufficient to transform NIH3T3 cells in a nude mouse assay when expressed from a strong promoter, and this portion was named *tre-2* [22] or *TRE17*, reflecting its chromosome 17 origin [23]. It is this region that was similar to the catalytic core of yeast UBP proteins, and is referred to as USP6 in the remainder of this chapter. Notably, it was only the N-terminal half of USP6 (with the Cys-box but lacking the His-box) that was tumorigenic, whereas an open reading frame that included the full-length protein (with intact catalytic core) of USP6 was not tumorigenic [22]. The oncogenicity of the N-terminal half may be due to its containing a GTPase-activating protein homology domain (see below) acting in a dominant fashion, and/or that it confers substrate-binding specificity for ubiquitinated proteins, and could act as a dominant-negative mutant in this case. The same study found that *USP6* was expressed in a wide variety of human cancer cells representing many different tissues, but was not expressed in normal tissues [22].

Papa and Hochstrasser identified USP6 (*tre-2*) as a potential DUB, and demonstrated that it had DUB activity, which depended on the putative active-site cysteine, thus establishing for the first time that a DUB could be an oncogene [19]. These authors also identified other potential UBP-type DUBs by sequence similarity, including Unp (Usp4), which is discussed further below (Section 9.2.2).

Further studies have revealed more of the role of USP6 in cancer. USP6 has arisen from the chimeric fusion of two genes, TBC1D3 at the N-terminus, and a DUB, USP32, at the C-terminus [24]. TBC1D3 contributes a "TBC" GTPase-activating protein (GAP) homology domain, so named from its occurrence in the proteins Tre-2/Bub2/Cdc16. USP6 (Tre-2) is a founding member of this family

of some 50 GAPs. It has been found that the TBC domain of USP6 targets the Afr6 GTPase, which regulates plasma membrane-endosome trafficking [25]. This is very interesting, given the now well-established role for ubiquitination in vesicle trafficking [7–9], and more recent evidence of a role for DUBs in regulating this process [26, 27]. Interestingly, USP6 itself has recently been shown to be monoubiquitinated in a calcium/calmodulin-dependent manner, with possible implications in its own trafficking [28]. However, USP6 does not function as a GAP for Arf6, but rather promotes its activation, possibly by facilitating its access to membrane-associated guanine nucleotide exchange factors (GEFs) [25].

USP6 has been linked directly to human cancers, namely aneurysmal bone cysts (ABCs), which are locally aggressive bone tumours that often feature chromosome 17p13 rearrangements – the *USP6* or *TRE-17* locus. There are five known examples of chromosomal rearrangements that have positioned *USP6* downstream of a heterologous gene promoter that would force inappropriate *USP6* expression in a bone/mesenchymal context: *Osteomodulin; Collagen 1A1; TRAP150; ZNF9*; and *CDH11* [29–31]. High-level USP6 expression was also detected in four other human cancers with an origin from mesenchymal neoplastic cells in a bone context (one Ewings Sarcoma, two osteoblastomas, and one myofibroma), but not in 50 other non-ABC tumours, suggesting that USP6 may have a broader oncogenic role in mesenchymal tumours [31].

In all five cases mentioned above, it remains unclear whether the heterologous promoters cause overexpression of normal, full-length USP6 protein, or whether there have been further mutations, deletions, or alternate splicing within *USP6* to produce an altered, oncogenic USP6 protein.

9.2.2 Unp/Usp4/Usp15

Usp4 (Unp) was first noted to have high sequence similarity to the USP6 oncoprotein discussed above [32] and was subsequently demonstrated to have DUB activity [33, 34]. Prompted by its similarity to USP6, Gray and colleagues demonstrated that Usp4 was also an oncoprotein, causing tumours when overexpressed in nude mice [35]. Usp4 has been shown to bind to the retinoblastoma tumour-suppressor protein (pRB), as well as to two other members of the pRb family, p107 and p130, in humans and mice [36, 37]. It is unclear whether Usp4 binding to members of the Rb-family of proteins facilitates their deubiquitination or has other functional significance, and whether this feature contributes to Usp4's oncogenicity. In a study of primary human lung tumour tissue, expression of the *USP4* gene was shown to be consistently elevated in small cell tumours and adenocarcinomas of the lung, but not in squamous cell carcinomas or large cell carcinomas, suggesting a possible causative role for USP4 in the neoplastic process in specific cancers [38]. However, in a different study using cell lines rather than primary lung tissue, USP4 protein levels were shown to be slightly but consistently reduced in cell lines derived from small cell tumours, leading to the suggestion that *USP4* may in fact be a tumour-suppressor gene [39]. This discrepancy could result from the comparison

of primary cancer tissue with cell lines. Also, subsequent work has suggested that the antibody used in this study was probably also detecting the closely related DUB USP15 [40], and thus these results need reinvestigation with more specific antibodies. It should also be noted that the oncogenicity of USP15 has not yet been investigated, although given that it is approximately 70% identical to USP4, and also contains potential Rb-interacting motifs, it may well also be an oncogene. A recent transcription profiling study has found that *USP4* is significantly upregulated in adrenocortical carcinomas (but not adenomas) and forms part of a molecular signature for these cancers [41]. However, the biological importance of *USP4* upregulation in this case is yet to be determined.

Usp4 has recently been shown to be a nuclear-cytoplasmic shuttling protein [40], exhibiting different extents of nuclear localization in different cell lines, which may explain previous differing reports of either nuclear or cytoplasmic localization [38, 39]. Endogenous Usp4 exhibits dramatically different localization in different cell lines. Thus, in cancerous HeLa and Saos-2 cells, Usp4 is exclusively nuclear, while in nontransformed and nonimmortalized mouse primary fibroblasts, Usp4 was detected mostly in the cytoplasm [40]. Whether this difference is a cause or a consequence of the cancerous state of the cells remains to be determined, as does its dependence on the Rb-family status of particular cell lines.

Another intriguing feature of Usp4/Unp was revealed by Laroia and colleagues [42]. When overexpressed in mammalian cells, Usp4 can prevent degradation of mRNAs containing AREs (AU-rich elements), possibly by stabilizing the ARE-binding protein(s), which presumably prevent ARE-mediated mRNA degradation. Given that the expression of many cytokines (and other growth-regulatory proteins) is regulated to some extent by mRNA instability due to AREs in their mRNAs, the ability of Usp4 to stabilize such mRNAs could also contribute to its oncogenicity.

Recently, USP4 has been shown to interact with the protein Ro52, an autoantigen associated with the autoimmune disease Sjögren's syndrome [43], and which is known to be ubiquitinated [44]. More recent work has shown that, in transfected cells, USP4 can deubiquitinate Ro52, that Ro52 localizes to cytoplasmic rod-like structures, and that USP4 co-localizes to these structures with Ro52, keeping it in a nonubiquitinated state [45]. However, whether this explains or is linked to the oncogenic role of USP4 is presently unclear.

In addition, Usp4 has been found to interact with the A_{2A}-adenosine receptor [46], a G-protein-coupled receptor involved in a pathway that suppresses inflammatory responses of essentially all immune cells, presumably by modulating NFκB signalling [47]. Usp4 binds to the carboxyl terminus of this receptor and can deubiquitinate it. The authors propose that the DUB activity of Usp4 relaxes quality control in the ER during secretion of the receptor, allows more of the receptor to fold properly, and enhances its cell surface expression [46]. The A_{2A}-adenosine receptor pathway is involved primarily in suppressing immune responses in wound repair, and an immediate link to oncogenicity is not apparent. However, while Usp4 was proposed to be specific for the A_{2A}-adenosine receptor, only one other receptor was tested, and if Usp4 could increase cell surface expression of other types

of G-protein-coupled receptors, then a direct link between Usp4 overexpression and cell transformation could be easily envisaged.

9.2.3 DUBs and NFκB Signalling

NFκB signalling has been shown to be regulated by DUBs at several points, primarily by novel classes of DUBs as summarized below. The cylindromatosis protein (CYLD1) is a tumour suppressor, mutations of which are linked to familial cylindromatosis, an autosomal dominant predisposition to multiple neoplasms of the skin appendages. Analysis of its sequence revealed some sequence similarity to the Cys and His boxes of the UBP-type DUBs [48], and the ability of the CYLD1 protein to be labelled with a modified ubiquitin "probe" specific for active DUBs suggested that it was an active DUB [49]. CYLD1 has indeed since been shown to have deubiquitinating activity both *in vitro* and in whole cells; in the latter case this appears to be specific for non K-48-linked ubiquitin chains [50–52]. A clear role for ubiquitin-dependent proteolysis in the activation of NFκB has been established. Upon receptor activation, TRAF2, TRAF6 and the IκKinase (IKK) gamma subunit become ubiquitinated with K-63-linked ubiquitin chains, which are necessary to activate the IKK complex to phosphorylate IκB, which in turn becomes ubiquitinated with Lys-48-linked ubiquitin chains and is degraded, releasing NFκB transcription factors to translocate to the nucleus and activate target genes. CYLD can disassemble the K-63-linked chains on these three proteins, and thus downregulate NFκB signalling by preventing IκB phosphorylation [50–52]. Inhibition of CYLD activity (such as occurs in cylindromatosis through mutation) removes this dampening effect on the NFκB pathway, and increases resistance to apoptosis, which may be the mechanism of tumour formation in this disease. CYLD has also been shown to suppress JNK signalling activated by immune stimuli, but not stress-induced JNK activation, by negatively regulating the activation of MKK7, an upstream kinase known to mediate JNK activation by immune stimuli [53].

Other reports have identified a potentially large family of novel DUBs, the ovarian tumour protein (OTU) family. Balakirev et al. [54] described two OTU domain proteins, Otubain 1 and 2, to have DUB activity. Evans et al. [55] characterized a 100-kDa zinc finger protein, Cezanne (cellular zinc finger anti-NFκB), that is a negative regulator of NFκB signalling, and determined that the N-terminal OTU domain conferred its DUB activity. This domain is shared by some 80 OTU proteins [55], with 14 human proteins [17]. One human OTU protein, A20, can cleave both K-48 and K-63-linked ubiquitin chains *in vitro*, but *in vivo* appears to have specificity for K-63 chains. However, A20 also possesses a ubiquitin ligase (E3) domain, which can assemble K-48-linked ubiquitin chains. A20 also acts as an inhibitor of NFκB signalling, by removing the (activating) K-63 chains on the tumour necrosis factor receptor interacting protein RIP, and then assembling K-48 linked chains on RIP by virtue of its ubiquitin ligase domain, resulting in the degradation of RIP and the inability to activate NNFκB [56]. The exact mechanism whereby

Cezanne inhibits NFκB signalling has not been elucidated, but presumably it functions by a similar mechanism to A20, on different components of the NFκB pathway. Thus, the NFκB signalling pathway is regulated at many steps by deubiquitination, and the cylindromatosis disease provides a clear example where mutation of a DUB can lead to cancer. The role of the ubiquitin pathway in NFκB signalling has been extensively reviewed [57–59].

In an amazing twist on the regulation of NFκB signalling by deubiquitination, the bacterial pathogen *Yersinia* uses a bacterially-encoded DUB as a virulence factor (YopJ) to inject into host cells to block the host proinflammatory response [60, 61]. Although originally reported to cleave the ubiquitin-like protein SUMO and share sequence similarity with SUMO-peptidases [60], a recent report reveals that YopJ, when overexpressed, acts as a promiscuous DUB, removing K-63-linked chains from TRAF2, TRAF6, and IκBα in much the same way as CYLD, but also cleaving K-48-linked chains from IκB and thus preventing activation of the NFκB transcription factors [61].

USP7 (discussed in detail in the following section) has also been reported as binding to the TRAF (tumour necrosis factor–receptor associated factor) family of proteins by virtue of its own TRAF domain, and that its transient overexpression is sufficient for suppressing NFκB induction by TRAF2 and TRAF6 [62]. USP7's function has not been further studied in this respect, and it is not known if it is acting in a similar manner to CYLD or A20.

9.2.4 USP7/HAUSP and p53

In 1997, Everett and colleagues identified a novel DUB that bound to the herpes simplex virus protein regulatory protein Vmw110 (ICP0) and termed it herpesvirus-associated ubiquitin-specific protease (HAUSP) [63]. This USP-type DUB is also known as USP7 in systematic nomenclature [64]. The normal cellular role for USP7 remained unclear until 2002, when it was demonstrated that USP7 could bind to the p53 tumour-suppressor protein and remove a polyubiquitin chain from it, thus stabilizing p53 [65]. This appeared to be a "classical" role for DUBs, in controlling the degradation of a ubiquitinated substrate by regulating the extent of polyubiquitination, and thus efficiency of targeting to the proteasome. In this context, USP7 would be considered a tumour-suppressor protein itself, given that it can stabilize the p53 tumour suppressor. Consistent with this, overexpression of USP7 in HeLa cells leads to their death by apoptosis [66]. However, subsequent investigations revealed a more complex role for USP7. These stemmed initially from studies using RNA-interference to knockdown USP7 protein levels, which was predicted to destabilize p53 in the absence of its "guardian" USP7. However, the opposite effect was observed: p53 was unexpectedly stabilized [67, 68]. The explanation: USP7 (a DUB) also interacts with Mdm2 (a ubiquitin ligase for p53), and Mdm2 itself can be ubiquitinated (most likely autoubiquitinated) and degraded by the proteasome. Thus, downregulation of USP7 results in degradation of Mdm2, and subsequently less ubiquitination and degradation of p53 [67]. How are these

opposing roles of USP7 regulated? One answer may be phosphorylation: the ATM protein kinase phosphorylates Mdm2 (and Mdmx) in response to DNA damage, and this lowers their affinity for USP7, thus resulting in more rapid Mdm2/Mdmx degradation [69]. Thus less p53 is ubiquitinated and degraded, and USP7 is now available to stabilize p53, both of which result in higher p53 levels.

The p53 tumour suppressor has a critical role in regulating cell growth, and is mutated in many cancers. It thus seems obvious to predict that alterations in USP7/HAUSP may also be a contributing factor in cancer. A recent study that investigated USP7 expression and p53 gene status in non-small cell lung carcinomas found that, in 93 of the 131 patients examined, either mutant p53 or reduced HAUSP expression was observed [70]. Reduced USP7 levels were associated with reduced p53 protein expression at statistical significance in tumours with wild-type p53 and more dramatically in tumours with mutant p53. The authors concluded that the simultaneous evaluation of both USP7 expression and p53 gene status was a significant indicator of poor prognosis in adenocarcinoma patients [70]. Unfortunately Mdm2 expression was not studied, but reduced USP7 levels linked to reduced p53 levels is consistent with the "simple" tumour-suppressor role for USP7 in regulating p53 levels by directly deubiquitinating it. Other factors may be at play here, and the role of ubiquitination and deubiquitination in the regulation of p53 is somewhat more complicated than the simplified picture presented above, which is further explored in Ref. [71].

It is worth recalling that USP7 was first identified as interacting with the herpes simplex virus protein ICP0 [63]. Subsequent work has shown that ICP0 is a RING finger ubiquitin ligase/E3 that targets several cellular proteins for degradation, including p53 [72]. ICP0 can also induce its own ubiquitination and degradation, and USP7 can control this process by deubiquitinating it. Furthermore, ICP0 can target USP7 for multiubiquitination and degradation, bringing in to question how these two reciprocal activities are balanced [73]. These authors propose that USP7-mediated stabilization of ICP0 is dominant over ICP0-induced degradation of USP7 during productive HSV-1 infection, and that the biological significance of the ICP0–USP7 interaction may be most pronounced in natural infection situations, in which limited amounts of ICP0 are expressed [73].

At least one other viral protein binds to USP7. The Epstein–Barr nuclear antigen 1 (EBNA1) protein binds USP7 [74], and EBNA1 binds to the same region of USP7 as does p53 [75]. Recent structural studies reveal that p53 binds the same "pocket" on USP7 as EBNA1 but makes less extensive contacts with it [76]. The functional consequence of this is that the EBNA1–USP7 interaction prevents USP7 from deubiquitinating p53, thus allowing p53 degradation and preventing apoptosis of infected cells [76]. Whether the herpes virus ICP0–USP7 interaction functions in an analogous manner has not been directly addressed, but given that ICP0 can directly target p53 for ubiquitination and degradation, independently of its ability to bind USP7 [72], a different mechanism may be at work. However, the targeting of the p53 "guardian" USP7 by viral proteins is one way these viruses can subvert the normal apoptosis pathways and allow survival and proliferation of infected cells.

9.2.5
USP33/VDU1, USP20/VDU2, and von Hippel–Lindau Disease

VDU1 (USP33) and VDU2 (USP20) are 59% identical DUBs of the USP family. Both VDUs interact with a component of an E3 ubiquitin ligase, VHL, a tumour-suppressor protein in which mutations are associated with von Hippel–Lindau disease. Both proteins interact with the β-domain of pVHL, leading to their ubiquitination and degradation by the proteasome. Interestingly, the β-domain of pVHL is the region that harbors the naturally occurring mutations in von Hippel–Lindau disease. Some of these mutations have been shown to disrupt VDU1/2 interaction with pVHL, implying an important role for VDU1/2 in von Hippel–Lindau syndrome [77, 78]. One target of the VHL ubiquitin ligase is the α-subunit of the transcription factor hypoxia-inducible factor 1 (HIF-1), that regulates genes involved in angiogenesis, glucose metabolism, and cell proliferation, invasion, and metastasis [79]. The inability to degrade HIF-1α leads to overexpression of HIF-1 target genes and can lead to a variety of tumours (reviewed in Ref. [80]). Recent work has shown that USP20/VDU2, but interestingly not USP33/VDU1, interacts with HIF-1α, and can specifically deubiquitinate and stabilize it, thus antagonizing the VHL ubiquitin ligase activity against HIF-1α [81]. This situation is similar to that of Mdm2, p53 and USP7 discussed above, although the relative affinity of HIF-1α for either VHL or USP20/VDU2 has not yet been explored, and adds another layer of regulation to the control of HIF-1 levels and activity in the cell. Whether USP33/VDU1 regulates ubiquitination HIF-1α under different conditions or cell types not studied by Li et al. [81], or of a completely different target of the VHL ubiquitin ligase, remains to be determined.

USP20/VDU2 and USP33/VDU1 have both been reported to bind to, and deubiquitinate, the cytoplasmic portion of an integral membrane ER-resident selenoenzyme, type 2 iodothyronine deiodinase (D2), that activates the pro-hormone thyroxine and supplies most of the 3,5,3′-triiodothyronine that is essential for brain development [82]. Ubiquitination of D2 is required for its downregulation through the ER-associated degradation (ERAD) pathway, and both USP20 and USP33 can rescue it from this fate. Interestingly, this report demonstrates that both USP20 and USP33 associate with the ER, and are possibly integral ER membrane proteins, at least in transiently transfected cells. The ubiquitin ligase for D2 has not been identified [82], so it is unclear whether the VHL ubiquitin ligase also targets D2, or whether a different ligase is responsible. However, it is clear from this example that DUBs can regulate the ubiquitination status of multiple target proteins, whether through the same or different ubiquitin ligases.

9.2.6
USP1, Fanconi Anaemia, and DNA Repair

Fanconi anaemia (FA) is a rare autosomal recessive disease that predisposes patients to developing a variety of cancers. At the cellular level, FA patients exhibit

chromosome instability and hypersensitivity to DNA cross-linking agents, indicating a role in DNA damage repair. The genetic basis for FA is diverse, and several proteins have been identified with links to the disease. At least seven of these, including a ubiquitin ligase FANCL, form a complex that targets another FA protein, FANCD2, for monoubiquitination (for review see Refs [83, 84]). This monoubiquitination is essential for FANCD2 to relocate to the sites of DNA damage, where it binds to BRCA1 and RAD51, and also co-localizes with BRCA2 (also known as FANCD1). The exact function of FANCD2 is not well understood, although it is known that its monoubiquitination does not target it for proteolysis, but rather targets it to sites of DNA damage. FANCD2 is also monoubiquitinated during normal progression through S-phase, and deubiquitinated on exit from S-phase. Nijman et al. [85] identified the DUB USP1 as interacting with FANCD2 at sites of DNA damage within chromatin and deubiquitinating it, and suggested that this event is required to inhibit or switch off FANCD2-mediated DNA repair, based on their observations that reduction of USP1 levels by RNAi resulted in protection from chromosomal breaks induced by a DNA cross-linking agent. The ability of USP1 to regulate the FA DNA repair complex makes it a candidate cancer-susceptibility gene, although mutations in this gene have not yet been reported.

9.2.7 DUBs Associated with BRCA1 and BRCA2

BAP1 is a UCH-type DUB which possesses two putative nuclear localization signals, and localizes exclusively in the nucleus of rhabdomyosarcoma cells. BAP1 interacts *in vivo* and *in vitro* with the RING finger domain of the breast/ovarian cancer susceptibility protein, BRCA1. The BAP1/BRCA1 interaction enhances BRCA1-mediated inhibition of breast cancer cell growth, probably through BAP1-mediated stabilization of BRCA1 or BRCA1 interacting proteins. The latter implies that BAP1 may be a tumour suppressor functioning through BRCA1 [86]. Interestingly, BAP1 does not interact with naturally occurring mutants of the RING finger domain of BRCA1 [87]. BRCA1 can form a heterodimer with another RING finger protein, BARD1 (BRCA1-associated RING domain), and this heterodimer has been shown to be an E3 ubiquitin ligase, initially by using truncated proteins [88] and more recently with full-length recombinant proteins [89]. Notably, BRCA1/BARD1 has been found to form ubiquitin chains with the unusual Lys-6 linkage [90, 91], which have a poorly characterized, but nonproteolytic role [10]. While it is not known whether BAP1 can bind BRCA1 when the latter is bound to BARD1, this appears unlikely, given that both BAP1 and BARD1 bind to the RING finger of BRCA1 [86, 89]. Recent work has shown that the BRCA1/BARD1 E3 ligase can auto-ubiquitinate, which stimulates its ligase activity towards other proteins, such as nucleosome core histones at the site of DNA damage [89]. Addition of recombinant BAP1 to multiubiquitinated BRCA1/BARD1 did not result in removal of ubiquitin, suggesting either that this is not the primary function of BAP1 or that additional factors are required *in vivo* [89]. However, as mentioned above, BAP1

may not be able to bind the preformed BRCA1/BARD1 heterodimer, and it is possible that BAP1 may bind the BRCA1 monomer to protect it from erroneous ubiquitination, and is displaced by BARD1 when the active ubiquitin ligase is formed.

BAP1 has been proposed to be a candidate tumour-suppressor protein [86], although a recent study [92] did not find any mutations in BAP1 in a series of 47 French familial breast cancer cases that were negative for BRCA1 or BRCA2 mutations. While this study concluded that BAP1 was not a high-risk breast cancer predisposing gene, a common BAP1 polymorphic variant was identified that was associated with moderate risk in sporadic breast cancers [92].

BRCA2 is a second breast cancer susceptibility protein that functions in the repair of DNA double-strand breaks, and individuals carrying a germ-line mutation in its gene are predisposed to breast, ovarian, and other cancers. One report reveals that USP11 is associated with BRCA2, that BRCA2 is constitutively ubiquitinated in whole cells, and that overexpression of USP11 results in deubiquitination of BRCA2 [93]. Induction of DNA damage with mitomycin C (MMC) led to increased ubiquitination of BRCA2 and decreased its protein level in the cell, an effect that was blocked by proteasome inhibitors, suggestive of proteasome-mediated degradation. Downregulation of USP11 by RNA interference resulted in sensitivity of cells to MMC-induced DNA damage in a manner that depended on BRCA2, but not on deubiquitination of BRCA2 by USP11. Thus these authors concluded that the pro-survival function(s) of USP11, although shown to be BRCA2 dependent, appear to be mediated through a USP11 substrate other than BRCA2 [93]. At least one other substrate has been reported; USP11 has been shown to stabilize RanBPM, the RanGTPase-binding protein required for correct nucleation of microtubules [94]. As discussed by Schoenfeld et al. [93], the Ran pathway does have some functional links to processes that involve BRCA2, such as formation of Rad51 foci and centrosome regulation, and thus it is possible that RanBPM may be the critical molecule regulated by USP11. The ubiquitin ligase responsible for ubiquitinating BRCA2 has not been identified, but given that it can form a complex with BRCA1 [95, 96], it is also possible that the BRCA1/BARD1 ubiquitin ligase ubiquitinates BRCA2 [93].

There are several observations that suggest that the Fanconi anaemia DNA repair pathway discussed above and the BRCA1 and BRCA2 DNA repair pathways are intimately linked, although the mechanistic details are not yet clear. First, the DNA damage-induced monoubiquitination of the Fanconi anaemia D2 protein (FANCD2) is reduced in cells that are defective for BRCA1 [97], which implies cross-talk at the level of DNA damage sensing and/or subsequent ubiquitination. Second, ubiquitinated FANCD2 co-localizes with BRCA1 at the sites of DNA breakage [97]. Third, the FANCD1 protein is actually a specific allele of BRCA2 [84], which would imply that USP11 may be part of the FA complex, as well as USP1. Notably, downregulation of USP11 and USP1 by RNA interference gives opposite responses with respect to sensitivity to DNA damage caused by MMC, suggesting that USP11 may be involved in the initiating phase of DNA damage repair, while USP1 is proposed to be at the terminating phase [85].

9.2.8
The Cytokine-inducible DUB-1/DUB-2/USP17 Family and Regulation of Cell Growth

Studies since the mid-1990s have identified a family of small USP-type DUBs in mice that are immediate–early cytokine-induced genes that have a role in regulating cell growth. DUB-1 is expressed mainly in B-lymphocytes in response to interlukin-3 (IL-3), peaking in early G1 phase and then rapidly declining. Interestingly, the continuous expression of DUB-1 arrests cells in G1 phase [98]. DUB-2 is induced rapidly by IL-2 in T lymphocytes and then rapidly downregulated [99]. Forced DUB-2 expression prolongs IL-2-induced gene activation by enhancing signalling through the JAK/STAT pathway, and suppresses apoptosis induced by cytokine withdrawal, thus allowing cells to survive [100]. In mice, DUB-1 and DUB-2 form part of a gene cluster of closely-related USP genes that also includes DUB-1A and DUB-2A [101, 102]. Similar clusters of orthologous cytokine-inducible genes, termed USP17, have been identified in humans [103, 104]. It has been shown that at least one of the human genes, originally termed DUB-3, is induced by IL-4 and IL-6, and also that its constitutive expression blocks cell proliferation and can initiate apoptosis [105]. This family has potentially huge diversity in humans, with a cluster of between 17 and 95 USP17 repeats on chromosome 4p, (USP17, RS447), and a cluster of some five genes on chromosome 8p [103]. How many of these genes are active remains to be determined [104].

The exact mechanism of action of the USP17-type DUBs was initially unknown, but they were assumed to modulate either the ubiquitin-dependent proteolysis or the ubiquitination state of an unknown growth regulatory factor(s) [98]. More recent work has revealed a likely substrate: the common cytokine receptor subunit γ(c) that is shared by the IL-2, -4, -7, -9, -15, and -21 receptors, and is essential for signalling by these cytokines. The γ(c) receptor is constitutively ubiquitinated by the ubiquitin ligase c-Cbl, which induces its internalization and downregulation [106]. DUB-2 has now been shown to deubiquitinate this receptor, and increasing DUB-2 expression correlates with an increased γ(c) half-life, resulting in the upregulation of the receptor [106]. Thus the effect of upregulating DUB-2 as an IL-2 immediate–early gene would be to increase receptor expression at the cell surface, thus prolonging IL-2-mediated signalling whilst DUB-2 is present. It is worth noting that while DUB-2 is induced by IL-2, mediated through the γ(c) receptor, DUB-1 is induced by IL-3, IL-5, and GMCSF, whose receptors share the common β(c) subunit [107]. It is thus tempting to speculate that DUB-1 may act in an analogous manner to DUB-2, but towards the β(c) subunit of the IL-3 receptor, thus prolonging IL-3 signalling. Other USP17-type DUBs may regulate other cytokine receptors, explaining in part the multiplicity of these DUBs.

9.3
Conclusions and Perspectives

This chapter has attempted to summarize some of the best understood examples of pathways where DUBs have a demonstrated role in regulating cell proliferation

and/or have been linked to cancer. Given the well-established role of the ubiquitin–proteasome pathway in regulating the degradation of many short-lived tumour suppressors, oncoproteins, transcription factors, and other regulatory proteins, and also the emerging roles of ubiquitination in regulating protein trafficking, it is not unexpected that defects in the ubiquitin pathway are linked to different cancers (see Refs [2, 108, 109] for review). Furthermore, a clear role for DUBs in regulating ubiquitination status, and thus degradation rate and/or trafficking of these substrates, is emerging. Thus it is no surprise that DUBs have emerged in recent years as oncoproteins or tumour-suppressor proteins themselves.

Many similarities are apparent from the different examples discussed above: in most cases, a DUB associates directly with an E3 ubiquitin ligase. This was not envisaged in early models of DUB function, where it was assumed that they would deubiquitinate substrates after, and separately from, the action of the ubiquitin ligase. In extreme cases, such as A20, the ubiquitin ligase and the DUB are located within the same polypeptide. Also in several cases, the ubiquitin ligase can ubiquitinate itself; its substrate; and its associated DUB. Furthermore, the DUB can deubiquitinate the substrate, and also often the E3 ligase. This complicated interplay is summarized in Table 9.1, for some of the examples discussed above. Of course, the functional consequence of this interplay is to enable the fine-tuning of the level or localization of a ubiquitinatable protein in the cell. This will ultimately depend

Table 9.1. Interplay between ubiquitin ligases, substrates, and DUBS.

Ubiquitin ligase; auto-Ub?*	Associated DUB	Substrate	Does ligase Ub DUB?*	Can DUB de-Ub ligase?*	Can DUB de-Ub substrate?*
Mdm2; yes	USP7/HAUSP	p53	Yes	Yes	Yes
ICP0 (viral); yes	USP7/HAUSP	p53	Yes	Yes	Yes
BRCA1/BARD1; yes	BAP1	histones	n.d.	No	n.d.
n.d.	USP11	BRCA2	n.d.	n.d.	Yes
VHL; nd	USP33 + USP20	HIF-1α	Yes	n.d.	Yes (USP20 only)
TRAF2/TRAF6; yes	CYLD	IKB	n.d.	Yes	Yes
A20 (Zn-finger); n.d.	A20 (OTU)	RIP	n.d.	n.d.	Yes
FANCL complex; n.d.	USP1	FANCD2	n.d.	n.d.	Yes
c-Cbl; yes	DUB-2/USP17	IL-2R γ(c)	n.d. (but DUB is Ubd)	n.d.	Yes

* Ub: ubiquitinate
n.d.: not determined.

on: the level of active ubiquitin ligase (which can be stabilized by the DUB), the level of active DUB (which can be destabilized by the ligase), the level of the target substrate in the cell when the ligase is activated, and which of the proteins are able to interact with each other at a given time. The latter will depend on their localization within the cell, and also the relative affinities of the three components for each other. Of course, these affinities will be influenced by other post-translational modifications of any of the components, most notably phosphorylation, but also other modifications such as SUMOylation. Presumably future efforts on these sets of DUBs, ligases, and substrates will focus on this interplay.

Does every ubiquitin ligase have a DUB associated with it? Purely by the numbers, this may be feasible if some sharing of DUBs is allowed. If there are at least 80 active DUB genes in humans, and alternate splicing may lead to at least twice this number of DUBs produced, that is still not quite enough to go around the approximately 230 RING finger, HECT, and cullin-based ubiquitin ligases in the mouse transcriptome [110], unless some sharing of DUBs is allowed. Still, it is conceivable that many ubiquitin ligases may have an associated DUB, and it will be the interplay between their opposing activities, as well as their affinities for their substrates, that will determine the final fate of a ubiquitinatable protein.

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Protein Degradation

Vol. 4: The Ubiquitin-Proteasome System and Disease

Edited by
R. John Mayer, Aaron J. Ciechanover, and Martin Rechsteiner

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Preface

There is an incredible amount of current global research activity devoted to understanding the chemistry of life. The genomic revolution means that we now have the basic genetic information in order to understand in full the molecular basis of the life process. However, we are still in the early stages of trying to understand the specific mechanisms and pathways that regulate cellular activities. Occasionally discoveries are made that radically change the way in which we view cellular activities. One of the best examples would be the finding that reversible phosphorylation of proteins is a key regulatory mechanism with a plethora of downstream consequences. Now the seminal discovery of another post-translational modification, protein ubiquitylation, is leading to a radical revision of our understanding of cell physiology. It is becoming ever more clear that protein ubiquitylation is as important as protein phosphorylation in regulating cellular activities. One consequence of protein ubiquitylation is protein degradation by the 26S proteasome. However, we are just beginning to understand the full physiological consequences of covalent modification of proteins, not only by ubiquitin, but also by ubiquitin-related proteins.

Because the Ubiquitin Proteasome System (UPS) is a relatively young field of study, there is ample room to speculate on possible future developments. Today a handful of diseases, particularly neurodegenerative ones, are known to be caused by malfunction of the UPS. With perhaps as many as 1000 human genes encoding components of ubiquitin and ubiquitin-related modification pathways, it is almost certain that many more diseases will be found to arise from genetic errors in the UPS or by pathogen subversion of the system. This opens several avenues for the development of new therapies. Already the proteasome inhibitor Velcade is producing clinical success in the fight against multiple myeloma. Other therapies based on the inhibition or activation of specific ubiquitin ligases, the substrate recognition components of the UPS, are likely to be forthcoming. At the fundamental research level there are a number of possible discoveries especially given the surprising range of biochemical reactions involving ubiquitin and its cousins. Who would have guessed that the small highly conserved protein would be involved in endocytosis or that its relative Atg8 would form covalent bonds to a phospholipid during autophagy? We suspect that few students of ubiquitin will be surprised if it or a ubiquitin-like protein is one day found to be covalently attached to a nucleic acid for some biological purpose.

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We are regularly informed by the ubiquitin community that the initiation of this series of books on the UPS is extremely timely. Even though the field is young, it has now reached the point at which the biomedical scientific community at large needs reference works in which contributing authors indicate the fundamental roles of the ubiquitin proteasome system in all cellular processes. We have attempted to draw together contributions from experts in the field to illustrate the comprehensive manner in which the ubiquitin proteasome system regulates cell physiology. There is no doubt then when the full implications of protein modification by ubiquitin and ubiquitin-like molecules are fully understood we will have gained fundamental new insights into the life process. We will also have come to understand those pathological processes resulting from UPS malfunction. The medical implications should have considerable impact on the pharmaceutical industry and should open new avenues for therapeutic intervention in human and animal diseases. The extensive physiological ramifications of the ubiquitin proteasome system warrant a series of books of which this is the forth one. The focus of this book is on the role of the UPS in disease.

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1
Ubiquitin Signaling and Cancer Pathogenesis

Kaisa Haglund and Ivan Dikic

1.1
Introduction

Post-translational modifications of proteins allow cells to respond dynamically to intra- and extracellular stimuli to control cellular processes [1]. A modification that has been given special attention among all possible modifications is protein ubiquitination, due to the frequency of its occurrence and the key role it plays in the inducible and reversible control of signaling pathways which regulate cellular homeostasis [2–4]. Tagging of proteins with ubiquitin occurs in a three-step process through the sequential action of the ubiquitin activating (E1), conjugating (E2) and ligase (E3) enzymes [5, 6]. Ubiquitination is a dynamic and reversible modification, and the rapid removal of ubiquitin from substrates and the processing of ubiquitin chains is catalyzed by de-ubiquitinating enzymes (DUBs) [7]. The regulation of DUBs is attracting increasing interest, since they serve to switch off the ubiquitin signal or to initiate a shift between different modifications of the same lysine residue. Moreover, there seems to be an interesting interplay between E3 ubiquitin ligases and DUBs. Interactions between E3s and DUBs have been shown to regulate the stability of E3s which undergo autoubiquitination. This type of interaction also leads the DUB to its substrate and regulates the target stability [7].

Ubiquitin modification can occur in multiple ways, making it a very diverse modification with distinct cellular functions (Figure 1.1). In its simplest form, a single ubiquitin molecule is attached to a single lysine residue in a substrate, which is defined as monoubiquitination [8, 9]. Alternatively, several single ubiquitin molecules can be attached to several different lysines, which is referred to as multiple monoubiquitination or multiubiquitination [10, 11]. Moreover, ubiquitin contains seven lysines itself that can be used to form various types of ubiquitin chain in an iterative process known as polyubiquitination [5, 12]. Interestingly, all seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) have the potential to be used in chain formation, giving rise to chains with different linkages or branches [13].

Monoubiquitination is involved in endocytosis of plasma membrane proteins, the sorting of proteins into the multivesicular body (MVB), budding of

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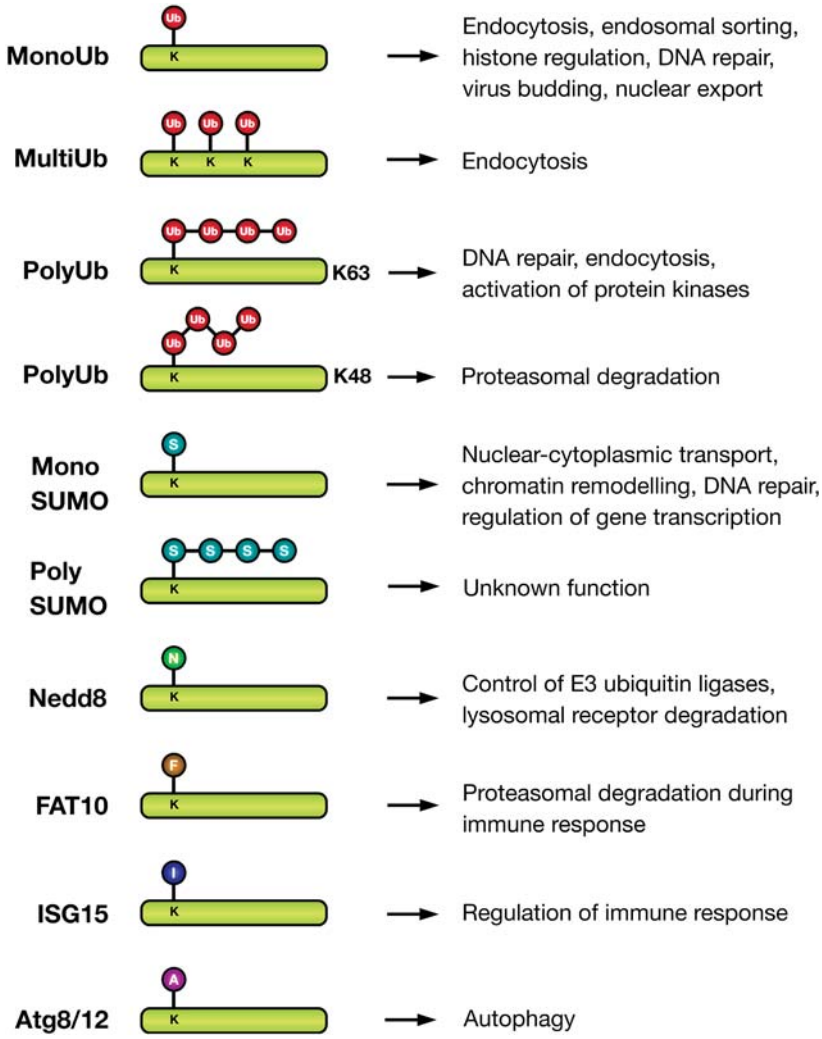

Fig. 1.1. Ubiquitin and ubiquitin-like protein (Ubl) modifications regulate a wide variety of cellular processes. Ubiquitin and Ubls share the same ubiquitin superfold and are collectively referred to as ubiquitons. All ubiquitons are attached via their C-terminal glycine residue to lysine residues in target proteins via a covalent isopeptide bond. Monoubiquitination (MonoUb) is essential for endocytosis and/or endosomal sorting of a variety of receptors, regulation of histones, DNA repair, virus budding and nuclear export. Tagging of several lysines with single ubiquitin molecules (MultiUb), is involved in endocytosis of certain RTKs and regulation of p53 localization. Polyubiquitination (PolyUb), the formation of ubiquitin chains via different lysines of ubiquitin, targets proteins for degradation in the 26S proteasome when linked via lysine 48, and has non-proteolytic functions, including control of DNA repair, endocytosis and activation of protein kinases when linked via lysine 63. Sumolyation controls several processes in the cell nucleus, including DNA repair, protein localization, chromatin remodeling and gene transcription. Neddylation regulates the activity of several E3 ubiquitin ligases, including Cbl, Mdm2 and cullins, and cooperates with ubiquitin to target EGFRs for lysosomal degradation [33, 34]. ISG15 and FAT10 are dimeric ubiquitons implicated in immune response [33, 34]. Atg8 and Atg12 play important roles in autophagy, the degradation of bulk cytoplasmic components, by contributing to the formation of autophagosomes during nutrient starvation of cells [33, 34]. Ub, ubiquitin; K, lysine; S, SUMO; N, Nedd8; F, FAT10; I, ISG15; A, Atg8/12.

retroviruses, DNA repair, histone activity and transcriptional regulation [8, 9, 14–16]. Multiple monoubiquitination is also involved in endocytosis of receptor tyrosine kinases (RTKs) and in nuclear export of p53 [10, 11]. In the case of polyubiquitination, the functions of polyubiquitin chains linked via lysines 48 and 63 have been best characterized. Proteins that are polyubiquitinated with Lys48-linked chains are recognized by ubiquitin-binding subunits of the 26S proteasome and are targeted for proteasomal degradation [5, 17]. Chains linked via Lys63, on the other hand, are involved in regulating endocytosis, DNA repair and activation of NF-κB [2, 14, 18–20]. Thus, whereas Lys48-linked polybiquitination was the first proteolytic signal described, it is becoming clear that monoubiquitination and Lys63-linked polyubiquitination function in several non-proteolytic cellular processes to regulate signaling networks.

1.1.1 Ubiquitin Signaling Networks

Ubiquitination is similar to phosphorylation and functions as a signaling device in cellular signaling networks. First, ubiquitination is an inducible event, which can be triggered by signals such as extracellular stimuli, phosphorylation and DNA damage [2]. This is associated with the fact that E3 ubiquitin ligases are tightly regulated by signal-induced mechanisms, such as post-translational modifications, compartmentalization, degradation and oligomerization [21, 22]. A prominent example is the ubiquitin ligase Cbl, which is recruited to a particular phosphotyrosine residue in the epidermal growth factor receptor (EGFR) following its ligand-induced activation, and subsequently tyrosine phosphorylation of Cbl itself promotes its ubiquitin ligase activity and consequently ubiquitination of the EGFR [23–25].

Second, ubiquitination is a reversible signal that is modulated by the action of DUBs, which is critical for the dynamic regulation of ubiquitin networks in the cell. The regulation of DUB activity is only beginning to be understood, and structural data indicate that these enzymes are in an active conformation only when bound to ubiquitin. Some DUBs require formation of complexes with other proteins in order to become active, and it has been reported that some are inhibited by phosphorylation or degradation [7]. For example, CYLD, an important DUB in the NF-κB pathway, undergoes inhibitory phosphorylation after TNF-α stimulation, leading to the accumulation of one of its substrates, Lys63-ubiquitinated TRAF2 [26].

Ubiquitin mediates many of its functions by interacting with highly specialized ubiquitin-binding domains (UBDs) in downstream effector proteins. More than 15 UBDs (UBA, UIM, IUIM, UEV, GAT, CUE, PAZ, NZF, GLUE, UBM, UBZ, VHS etc.) have been discovered so far [13, 27–31]. The structures of most of these domains have been elucidated when they are complexed with ubiquitin and it appears that they have many different tertiary structures and bind ubiquitin with relatively low affinity (50–100 μM) [13, 30]. The low affinity of UBD–ubiquitin interactions allows rapid assembly and disassembly of interaction networks, which

facilitates dynamic biochemical processes [9, 13]. Moreover, it is thought that a local increase in the concentration of UBD-containing proteins and UBDs, for example by the formation of multimeric complexes or the presence of several UBDs within the same protein, might increase the rate at which UBD–ubiquitin interactions occur [9, 13, 30]. Furthermore, some UBDs can bind several ubiquitin molecules simultaneously, as has been reported for the UIM of the endocytic sorting protein Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) [32]. Due to its versatility, the numerous substrates that can be tagged with ubiquitin and the various proteins containing UBDs, ubiquitination is thus involved in complex networks of interactions in time and space that regulate key cellular functions, such as signaling, endocytosis, cell cycle and DNA repair.

1.1.2 Ubiquitin-like Proteins

The complexity of cellular signaling networks is further increased by modifications with ubiquitin-like (Ubl) proteins, including the small ubiquitin-related modifier (SUMO), Neural precursor cell-expressed developmentally downregulated 8 (Nedd8), interferon-stimulated gene 15 (ISG15), FAT10, Atg8 and Atg12 [33, 34], all of which regulate a variety of physiological processes (Figure 1.1). All Ubls share a similar three-dimensional structure, the ubiquitin superfold which is a β-grasp fold. Despite the varying degrees of sequence similarity, all proteins containing this fold are collectively known as ubiquitons [34].

In a manner similar to that involved in the tagging of proteins with ubiquitin, Ubls are covalently attached to their target proteins via a cascade of three enzymes (E1, E2, E3) which are partially specific for each of the Ubls [33]. As with ubiquitin, Ubls most frequently attach to lysines, although the free N-terminus can be an attachment site for both for ubiquitin and Ubls. In contrast to the ubiquitin system, Ubls generally form mono-conjugates with the substrates and not polymeric chains (Figure 1.1). SUMO conjugates have been observed, however, but their function is not yet known [35]. It is very likely that there are specialized interaction domains for all the Ubls, although they have only been described for a subset. SUMO-interacting motifs (SIMs) have been assigned [36–39], and some known UBDs interact not only with ubiquitin, but also with Nedd8 [40]. Moreover, it is interesting to note that UBDs and SIMs bind at distinct surface locations on ubiquitin and SUMO, respectively, resulting in highly specific interactions which provide some insights into the different cellular functions of these two proteins [1].

In many cases, there is an active interplay between ubiquitin and Ubls in the regulation of individual proteins and/or cellular pathways. For example, the same lysine residue can be modified with either ubiquitin or SUMO, leading to the activation of completely different downstream pathways. The modification of PCNA (proliferating cell nuclear antigen), that forms a clamp that recruits DNA polymerases to the replication fork, with either ubiquitin or SUMO induces error-prone DNA repair or DNA synthesis, respectively [14]. Moreover, there is apparent cooperation between ubiquitin and Nedd8 during downregulation of the epider-

mal growth factor receptor (EGFR). EGF stimulation triggers Cbl-mediated neddylation of the EGFR, which in turn promotes the subsequent Cbl-mediated ubiquitination of the receptor and its degradation [40].

Further complexity in Ubl signaling networks results from the fact that Ubl domains can be found within the genetically-encoded sequence of proteins. Many proteins containing Ubl domains interact with the proteasome, but there are also several examples in which the ubiquitin fold is involved in mediating protein–protein interactions in signal transduction cascades, consistent with the important role of ubiquitin and Ubls in both degradation and signaling pathways [34].

1.2 Ubiquitin in Cancer Pathogenesis

The development of cancer is a multi-step process which results from mutations in the cellular pathways that control signaling, endocytosis, cell-cycle and cell-death and interactions between the tumor and its surrounding tissue [41]. Deregulation of components of the ubiquitination machinery appears to be a common theme in the development of cancers [4, 42–44]. Mutations or overexpression of numerous E3 ubiquitin ligases can convert them to potent oncogenes and some E3s and DUBs act as tumor suppressors (Table 1.1). Several substrates that are affected by alterations in E3 and DUB activity play key roles in the cell cycle, DNA repair, NF-κB signaling, RTK signaling and angiogenesis and their levels or activity are precisely regulated by ubiquitination (Table 1.1; Figure 1.2). In the following sections we will highlight the nature of role that the ubiquitin system plays in maintaining the homeostatic balance of these processes and why its deregulation promotes the development of different types of tumors.

1.2.1 Ubiquitin in Cell Cycle Control

Deregulation of cell-cycle control is a fundamental characteristic of cancer. Uncontrolled proliferation of cancer cells occurs because the precise regulation of the cell cycle has been disrupted [41]. Progression through the cell cycle is mediated by cyclin-dependent kinases (CDKs) whose activity is regulated by cyclins and CDK inhibitors (CDKIs) [43]. These undergo ubiquitin-mediated proteolysis which results in their periodic expression, ensuring that the cell cycle proceeds at normal speed. Cyclins act as accelerators of the cell cycle, whereas CDKIs function as brakes. Therefore, cyclins (D1 and E) are frequently overexpressed in human cancers and the CDKI p27 is a prominent tumor suppressor [43, 45, 46].

Three structurally-related cullin-dependent E3 ubiquitin ligases, SKP1-CUL1-F-box-protein (SCF)/Skp2, SCF/Fbw1 and anaphase-promoting complex/cyclosome (APC/C), are involved in regulating the levels of cyclins and CDK inhibitors by promoting their polyubiquitination and degradation in the proteasome [43].

Table 1.1. Summary of pathways and proteins regulated by ubiquitination and whose deregulation leads to the development of cancer.

Pathway	Deregulated protein	Function	Type of deregulation	Substrate	Ubiquitin modification	Cancer type	References
Cell cycle	SCF/Skp2	E3 ubiquitin ligase subunit, oncogene	Overexpression	p27, cyclin E	Lys48-linked polyubiquitination	Lung cancer, malignant melanoma, lymphoma	43
	SCF/Fbw7	E3 ubiquitin ligase subunit, tumor suppressor	Mutation	Cyclin E	Lys48-linked polyubiquitination	Ovarian cancer, breast cancer, endometrial cancer	43
	APC/C	E3 ubiquitin ligase, tumor suppressor	Mutation	Cyclin B, securin	Lys48-linked polyubiquitination	Colorectal cancer	46
DNA repair	Mdm2	E3 ubiquitin ligase, oncogene	Overexpression	p53	Lys48-linked polyubiquitination	Non-small cell lung cancer, soft-tissue carcinoma, colorectal cancer	59
	HAUSP	DUB, tumor suppressor	Mutation	p53, Mdm2	Deubiquitination	Non-small-cell lung cancer	67
	BRCA1	E3 ubiquitin ligase, tumor suppressor	Germline mutation	γ-tubulin	Polyubiquitination	Breast cancer, ovarian cancer	68, 69
	FANCL	E3 ubiquitin ligase, tumor suppressor	Mutation	FANCD2	Monoubiquitination	Fanconi anemia-related cancers	68, 74
NF-κB signaling	CYLD	DUB, tumor suppressor	Mutation	NEMO, TRAF2, TRAF6, Bcl-3	Deubiquitination of Lys63-linked ubiquitin chains	Cylindromatosis	53
RTK signaling	Cbl	E3 ubiquitin ligase, proto-oncogene	Mutation	RTKs	Multiple monoubiquitination	Breast cancer, glioblastoma, head and neck cancer, lymphoma	81–84, 86, 87
Angiogenesis	SCF/VHL	E3 ubiquitin ligase subunit, tumor suppressor	Mutation	HIF1α	Lys48-linked polyubiquitination	Kidney cancer, blood vessel tumors in the CNS	78

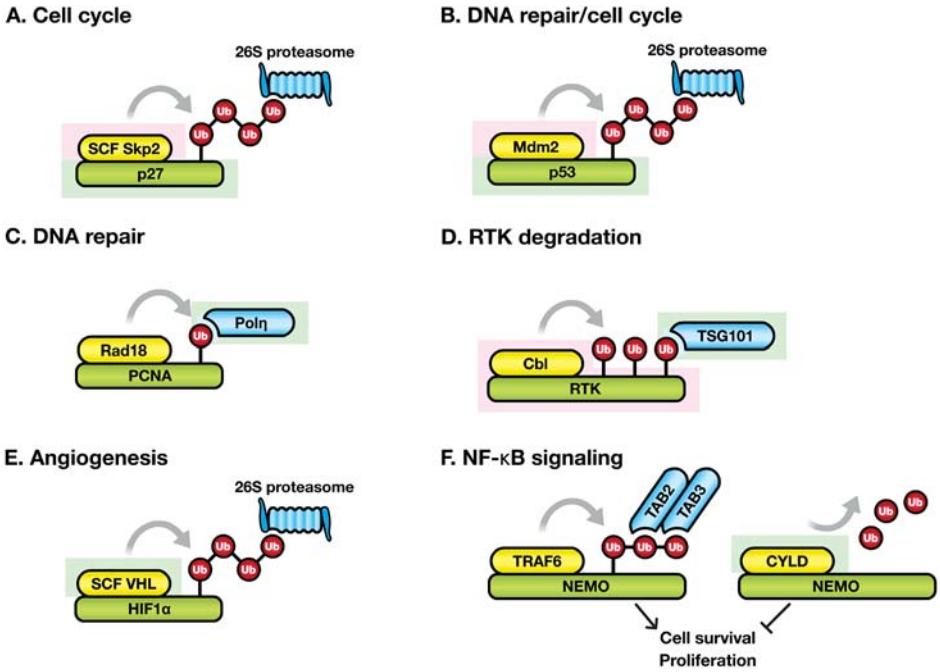

Fig. 1.2. Overview of cancer-relevant ubiquitin-dependent pathways. SCF/Skp2, Mdm2, Rad18, Cbl, SCF/VHL and TRAF6 all are E3 ubiquitin ligases (yellow) that mediate specific types of ubiquitination of their respective substrates which are indicated in the figure (p27, p53, PCNA, RTKs, HIF1α and NEMO) (green). The proteasome, which has UBD-containing subunits, and UBD-containing proteins (Polη, TSG101, TAB2/3) are shown in blue. (A, B, E) Lys48-linked polyubiquitination of p27, p53 and HIF1α leads to their proteasomal degradation, promoting cell cycle progression (p27, p53) or block of production of pro-angiogenic factors (HIF1α). SCF/Skp2 and Mdm2 act as oncogenes, because their overexpression leads to increased proliferation and the development of cancer. SCF/VHL, on the other hand, acts as a tumor suppressor, since its mutation leads to the accumulation of HIF1α, aberrant angiogenesis and tumorigenesis. (C) Rad18 mediates monoubiquitination of PCNA, a modification responsible for recruiting ubiquitin binding domain (UBD)-containing TLS polymerases to the site of DNA damage. Mutation of TLS polymerase Polη leads to a variant of a skin tumor syndrome called Xeroderma pigmentosum. (D) Cbl mediates multiple monoubiquitination of RTKs, which is recognized by ubiquitin-binding domains in proteins of the endocytic sorting machinery, including TSG101. Mutation of the Cbl binding site in RTKs, mutations of Cbl that abolish its ubiquitin ligase activity, or mutation in TSG101 all lead to defective receptor sorting and degradation, causing constitutive signaling and tumorigenesis. (F) TRAF6 mediates Lys63-linked polyubiquitination of NEMO, which recruits the UBD-containing proteins TAB2/3, leading to activation of the protein kinase TAK1 that is required for NF-κB activation. CYLD, the DUB that removes Lys63-linked chains from NEMO, is mutated in a cancer syndrome called cylindromatosis. Tumor suppressors are indicated in turquoise and oncogenes or proto-oncogenes in pink.

SCF/Skp2 targets among others p27 and cyclin E, and SCF/Fbw1 targets cyclin E for polyubiquitination and proteasomal degradation, events that regulate the G1–S transition (Figure 1.2) [47]. APC/C, on the other hand, promotes polyubiquitination and degradation of mitotic cyclins and securin, which are required for termination of the mitotic cycle and separation of the sister chromatids, respectively [46]. In this way APC/C maintains the normal chromosome number, alterations of which are a prevalent form of genetic instability in human cancers. These E3 ubiquitin ligases thus act at different time points during the cell cycle and importantly they appear to interplay in a regulatory loop [43].

Due to their central function in cell cycle progression, aberrant expression or mutations of SCF/Skp2, Fbw1 or APC/C have been found in several human cancers (Table 1) [43, 45, 46]. Skp2 has oncogenic properties in transgenic mouse models, is frequently overexpressed in lung cancers and its overexpression is correlated with poor prognosis in a wide range of cancer types [43]. Fbw1, on the other hand, acts as a tumor suppressor. Mutations in the *FBW1* gene have been reported in ovarian, breast and endometrial cancer, often correlated with increased cyclin E levels [43]. APC/C also functions as a tumor suppressor and is mutated in more than 70% of colorectal carcinomas [46]. Thus, cumulative evidence indicates that deregulation of the ubiquitin system in cell-cycle control is closely linked to the development of cancer.

1.2.2 Ubiquitin in the NF-κB Pathway

The NF-κB family of transcription factors triggers the expression of genes that are central mediators of cell survival, proliferation, and innate and adaptive immune responses. The role of NF-κB in cancer is connected to its constitutive activation of anti-apoptotic signals in both pre-neoplastic and malignant cells, and its emerging role in regulating tumor angiogenesis and invasion [48]. NF-κB activation is controlled by ubiquitination of several of the components of the NF-κB pathway [2, 18, 49]. A key step in the activation of NF-κB is its release from the inhibitor IκB and its subsequent translocation from the cytoplasm to the nucleus where it triggers the expression of its target genes. A central regulator of this process is the IκB kinase (IKK) complex, which consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO). IKK promotes IκB phosphorylation which recruits the E3 ubiquitin ligase SCF-βTRCP to IκB which in turn promotes Lys48-linked ubiquitination and proteasomal degradation, thereby releasing NF-κB [18, 49].

Another type of ubiquitin modification is exemplified by Lys63-linked polyubiquitination which also plays a central role in NF-κB activation by activating protein kinases. Both IKK and the kinase that activates IKK, TGFβ-activated kinase (TAK1), require Lys63-linked chains synthesized by the E3 ubiquitin ligase TNF receptor associated factor 6 (TRAF6) for their activation [18]. IKK activation requires the modification of the regulatory subunit NEMO with Lys63-linked chains [50]. TAK1 activation depends on the interaction between the UBDs of the TAK1-binding

proteins TAB1, 2 and 3 with substrates modified with Lys63-linked polyubiquitin chains, and ubiquitinated NEMO is a likely interaction partner (Figure 1.2) [51].

Since ubiquitination plays a central role in NF-κB activation, its removal by DUBs is critical to the downregulation of the NF-κB signal. To date, two DUBs have been identified to have important roles in regulating the NF-κB pathway, A20 and cylindromatosis (CYLD). A20 has a dual role in downregulating NF-κB signaling. First, A20 specifically removes Lys63-linked ubiquitin chains from the receptor-interacting protein (RIP), an essential mediator of TNF receptor 1 (TNFR1) signaling, and subsequently it attaches Lys48-linked ubiquitin chains to promote its proteasomal degradation [52]. Whether there is a genetic link between A20 and the risk of cancer still needs to be established [4].

CYLD was originally identified as a tumor suppressor gene that is mutated in familial cylindromatosis, an autosomal dominant disease characterized by multiple tumors of the skin appendages [53]. CYLD contains a ubiquitin C-terminal hydrolase (UCH) domain and acts as a DUB that removes Lys63-linked chains from several NF-κB pathway members, including the ubiquitin ligases TRAF2 and TRAF6, the IKK subunit NEMO and the transcriptional co-activator Bcl-3 (Figure 1.2) [54–58]. In this way CYLD regulates the duration of NF-κB activation and its loss thus correlates with tumorigenesis.

These examples illustrate that modification of pathway components containing Lys63-linked ubiquitin chains (NEMO, TRAFs, RIP, Bcl-3) triggers the activation of NF-κB, whereas ubiquitin removal is a common theme in its inactivation, thereby preventing excessive cell proliferation and tumor development.

1.2.3 Ubiquitin as a Signal in DNA Repair

The maintenance of DNA integrity is pivotal to the prevention of cancer-promoting mutations in the genome. Cells have therefore developed elaborate DNA repair systems to respond to DNA damage. Emerging data show that ubiquitin modification plays a major role in DNA repair response both by regulating cell cycle arrest (p53, Mdm2, HAUSP, BRCA1 and FANCD2) and by controlling trans-lesion DNA synthesis (PCNA and TLS polymerases).

1.2.3.1 p53 Pathway

The *p53* gene is mutated in more than 50% of human cancers. p53 is a transcription factor with an essential role in promoting cell-cycle arrest, apoptosis and DNA repair when cells encounter DNA damage. In this way, p53 hinders proliferation of damaged cells and acts as a tumor suppressor [59, 60]. In order to maintain cellular homeostasis, the levels of p53 are highly regulated in cells. In unstressed cells, the levels of p53 are kept low and this is mediated by ubiquitin-dependent proteasomal degradation. Mdm2 is a RING-type E3 ubiquitin ligase responsible for promoting both monoubiquitination and Lys48-linked polyubiquitination of p53 in a dose-dependent manner (Figure 1.2) [11]. Monoubiquitination of p53 promotes its nuclear export and polyubiquitination, its degradation by nuclear

proteasomes. Normally, the interaction between Mdm2 and p53 is disrupted when cells encounter DNA damage or other stresses, promoting an accumulation of p53 in the nucleus, cell-cycle arrest and DNA repair [61]. Overexpression of Mdm2, on the other hand, leads to aberrant deactivation of p53, which is observed in many types of tumors (Table 1.1) [59, 62, 63].

Herpes simplex-associated ubiquitin-specific protease (HAUSP) is involved in p53 deubiquitination and stabilization [64, 65]. Importantly, its overexpression is sufficient to promote cell-cycle arrest and apoptosis, suggesting that it could act as a tumor suppressor [65]. On the other hand, disruption of the *HAUSP* gene in human cancer cell lines by targeted homologous recombination, also leads to p53 stabilization and activation [66]. These contradictory results could be explained by the presence of other targets of HAUSP, such as Mdm2, which determine p53 levels [64]. Nevertheless, mutations of the *HAUSP* gene are associated with an increased risk for non-small-cell lung cancer [67].

1.2.3.2 **BRCA1 and FANCD2**

The breast cancer susceptibility genes *BRCA1* and *BRCA2* and products of the Fanconi anemia (FA) gene act as tumor suppressors. They function in a network of interconnected biological processes and have important roles in cell-cycle checkpoint control and DNA repair of double strand breaks by mediating homologous recombination [60]. Germline mutations in one allele of either *BRCA1* or *BRCA2* cause hereditary breast and ovarian cancer syndrome and mutations in FA genes (*FANCA, FANCB, FANCC, FANCD1, FANCD2* etc.) can cause FA, a genetic disorder associated with increased susceptibility to cancer [68].

BRCA1 acts as a RING-type E3 ubiquitin ligase and its activity is increased when it is complexed with the structurally and functionally related BRCA1-associated RING domain 1 (BARD1) ubiquitin ligase [69]. Specific mutations in the RING domain of BRCA1 abolish its ubiquitin ligase activity and tumor suppression capabilities. Interestingly, BRCA1 and BARD1 preferentially promote formation of Lys6-linked chains, a chain type that seems to be primarily involved in substrate stabilization [69].

BRCA1- and *BARD1*-deficient mice show centrosome amplification, defective G2–M checkpoint control and genetic instability [69]. Among the ubiquitinated targets of BRCA1/BARD1 is the centrosome component γ-tubulin [70]. Following their duplication during cell division, centrosomes help to form the spindle apparatus that segregates the duplicated chromosomes into daughter cells. Mutation of the ubiquitination site in γ-tubulin leads to amplification of centrosome numbers, a defect associated with chromosome missegregation and the development of cancer [70].

When DNA is damaged, BRCA1 binds to FANCD2 in nuclear foci that are required for cell-cycle checkpoint control and DNA repair [71]. The localization of FANCD2 to these foci is promoted by its monoubiquitination, suggesting that monoubiquitin-binding proteins might be involved in its recruitment [72]. FANCD2 undergoes monoubiquitination in BRCA1–/– cells, indicating that another E3 ubiquitin ligase promotes this modification [73]. Indeed, a component

of the nuclear FA–protein complex, FANCL, possesses E3 ubiquitin ligase activity against FANCD2 via its RING-finger-like plant domain (PHD) [74]. Deubiquitination of FANCD2 by ubiquitin-specific protease 1 (USP1), on the other hand, may play an important role when cells restart the cell cycle after DNA damage [75].

1.2.3.3 PCNA and TLS Polymerases

DNA damage blocks the progression of the replication fork and in order to avoid stalling the replication process and circumventing the damaged sites, cells replace the high-fidelity replicative polymerase Polδ with one of the five specialized low stringency DNA polymerases which are able to perform trans-lesion DNA synthesis (TLS) across different types of damage [76]. That TLS is crucial for cells is emphasized by the fact that defects in TLS polymerases can cause disease. Mutations in TLS polymerase Polη are found in patients suffering from a variant of Xeroderma pigmentosum, a UV-induced skin tumor syndrome [77].

Proliferating cell nuclear antigen (PCNA) functions to recruit different polymerases to the site of DNA replication or repair, and its ubiquitination and deubiquitination plays a major role in the polymerase switch. Non-ubiquitinated PCNA recruits the replicative polymerase Polδ during DNA replication. Certain types of DNA damage, on the other hand, induce Rad18-mediated monoubiquitination of PCNA. This modification triggers the recruitment of TLS polymerases, all of which contain UBDs, the so-called ubiquitin-binding motif (UBM) or ubiquitin-binding zinc finger (UBZ) (Figure 1.2) [14, 27]. After trans-lesion synthesis has taken place, the low fidelity TLS polymerases are exchanged for Polδ to ensure accurate continued replication. Therefore, the DUB USP1 removes ubiquitin from PCNA during normal replication to allow recruitment of Polδ and is degraded once the DNA becomes damaged, again allowing monoubiquitination of PCNA and recruitment of the TLS polymerases [20].

1.2.4 Ubiquitin Networks in Angiogenesis

Rapidly growing tumors require efficient blood and nutrient supply and therefore secrete growth factors, such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), to promote angiogenesis, the formation of new capillaries. Therefore, it is not surprising that an anti-angiogenic protein, such as VHL (von Hippel-Lindau), would be a tumor suppressor [78]. The *VHL* gene encodes a component of an SCF-like ubiquitin ligase and is mutated in patients suffering from the familial cancer susceptibility, von Hippel-Lindau syndrome, that is associated with cancer of the kidney and tumors in the blood vessels of the central nervous system [78, 79]. Under normoxic conditions, VHL binds to the hydroxylated α-subunits of the hypoxia-inducible factor (HIF) heterodimeric transcription factors and targets them for polyubiquitination and proteasomal degradation (Figure 1.2) [80]. During hypoxic conditions, HIF1α is not hydroxylated and can thus not be bound by VHL, leading to its stabilization. HIF1α then triggers

the transcription of several genes encoding pro-angiogenic growth factors, including VEGF, PDGFβ and transforming growth factor α (TGFα) [78]. Mutation of VHL is thought to lead to constantly increased levels of HIF1α and its target growth factors even under normoxic conditions [78], thus stimulating the formation of new blood vessels and tumors.

1.2.5 Ubiquitin Networks in Receptor Endocytosis

Constitutive receptor tyrosine kinase (RTK) signaling, resulting from receptor overexpression, autocrine growth factor loops and activating mutations, can cause cell transformation and cancer [81]. Moreover, loss of negative regulation of RTKs is an important factor contributing to enhanced receptor signaling [82–84]. RTKs are downregulated by endocytosis and lysosomal degradation, which requires ligand-induced Cbl-mediated receptor multiple monoubiquitination, Lys63-linked polyubiquitination and neddylation (Figure 1.2) [9, 10, 40, 85]. Ubiquitin attached to RTKs serves as a sorting tag that is recognized by UBD-containing endocytic proteins along the endocytic pathway, ensuring that they targeted into the inner vesicles of the multivesicular body (MVB), which destines them for lysosomal degradation [9, 15, 16]. Therefore, RTK mutations that lead to the loss of the binding site for the ubiquitin ligase Cbl in addition to Cbl mutants lacking ubiquitin ligase activity, cause defective downregulation of the receptor [83, 84]. Prominent examples of RTKs that have been found mutated in tumors and have escaped Cbl-mediated ubiquitination and degradation include EGFR (EGFRvV, v-erbB and EGFRvIII), MET (TRP-MET) and c-Kit (v-Kit) [83, 86, 87]. Oncogenic forms of Cbl (v-Cbl, Cbl-70Z, ΔY368-Cbl, ΔY371-Cbl) all lack ubiquitin ligase activity and are thought to act as dominant negative proteins and to compete with endogenous Cbl for binding to activated RTKs [24, 25]. Deletions of the extracellular area of the EGFR (EGFRvIII) are found in approximately 40% of glioblastomas and the EGFR family member ErbB2 is frequently overexpressed in breast cancer [87]. Overexpression of ErbB2 favors the formation of EGFR/ErbB2 heterodimers which recruit Cbl less efficiently, and are thus not degraded, but rather recycled back to the cell surface [88–90].

Interestingly, components of the endosomal sorting complex required for transport (ESCRT) machinery that sorts ubiquitinated cargo into the MVB [15, 91], are also linked to the development of tumors. Mutations in the components of the ESCRT-I tumor susceptibility gene 101 (TSG101) and hepatocellular carcinoma-related protein 1 (HCRP1) have been implicated in tumor development [84, 92, 93]. TSG101 contains a ubiquitin-binding UEV domain that binds to ubiquitinated cargo and is required for effective receptor sorting into the MVB (Figure 1.2) [15]. Moreover, mutations of *erupted* (TSG101) and *Vps25* (an ESCRT-II component) have been shown to cause neoplastic tumor growth in the fruit fly [94–98]. Thus, proper ubiquitin-dependent lysosomal degradation of activated RTKs prevents constitutive receptor signaling and carcinogenesis.

1.3
Targeting Ubiquitin Networks in Cancers

Due to its common deregulation in the development of cancers, targeting the ubiquitin system in cancer therapeutics emerges as a promising approach. The major challenge is to develop drugs that specifically act on the desired ubiquitin system component or substrate without affecting other pathways. Possible strategies involve inhibiting ubiquitin activation or conjugation, ubiquitin ligase activity of oncogenic E3s, by blocking either E2 or substrate binding, or inhibiting the degradation of cancer-preventing tumor suppressors [99]. Since the ubiquitin activation and proteasomal degradation steps involve ATP-dependent and proteolytic enzymes, respectively, which are classical drug targets, they represent therapeutically attractive points of intervention [99]. The major concern with these strategies, however, is their wide action on numerous substrates and pathways within the cell which may produce severe side effects. Intervening in the E3–substrate interaction therefore represents a more selective approach which could lead to more effective treatment and fewer nonspecific effects (Figure 1.3).

1.3.1
Targeting Interactions between E3s and their Substrates

This strategy has been successfully applied when targeting the interaction between the oncogenic E3 ubiquitin ligase Mdm2 and the tumor suppressor p53 with two

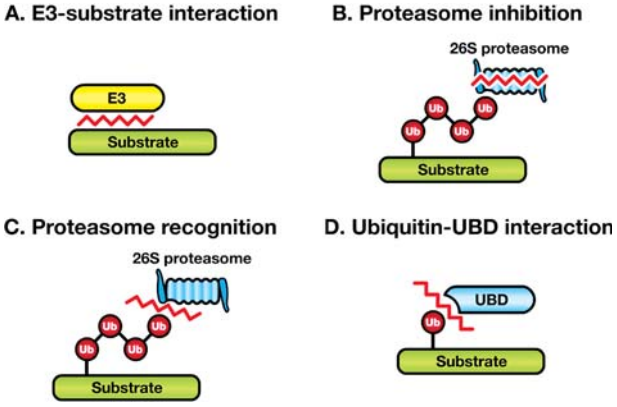

Fig. 1.3. Different approaches for targeting the ubiquitin system in cancer therapy. (A) Interference with the interaction between the E3 ubiquitin ligase and the substrate. Examples: Nutlin and RITA. (B) Inhibition of the catalytic activity of the proteasome. Example: Bortezomib. (C) Interference with the interaction between Lys48-polyubiquitinated substrates and the proteasome. Example: Ubistatins. (D) Interference with the interaction between ubiquitinated substrates and the corresponding ubiquitin-binding domain (UBD)-containing proteins. The main difficulty with all these strategies is in achieving optimum specificity and selectivity. See the main text for more details.

types of small molecule inhibitors that were identified in anti-cancer drug screens, Nutlins (cis-imidazole derivatives) and RITA (2,5-bis(5-hydroxymethyl-2-thienyl)furan). Nutlins occupy the p53 binding pocket of Mdm2 and RITA binds p53 and in this way they both prevent the p53–Mdm2 interaction [99]. Consequently, both compounds stabilize p53, leading to p53-dependent cell-cycle arrest in cancer cell lines and the inhibition of tumor growth in nude mice [99]. Although the initial studies on these inhibitors seem promising, their bioavailability and usefulness in the treatment of human cancer need to be thoroughly studied. Despite higher specificity, major concerns still remain: do these interventions yield unwanted effects such as affecting other substrates of Mdm2, other interaction partners of p53 or p53-related proteins such as p63 and p73? These basic concerns apply in each of the cases where the interaction surface between the E3 ubiquitin ligase and the substrate is targeted.

1.3.2
Targeting the Proteasome

Surprisingly, the biggest success so far in targeting the ubiquitin system in cancer therapy has been the development of Bortezomib, a small molecule proteasome inhibitor that binds reversibly to the active site of the 20S proteasome subunit [100]. Despite concerns regarding the lack of specificity due to the inhibition of the entire proteasomal protein degradation system, this inhibitor is being successfully used clinically in the treatment of relapsed, refractory multiple myeloma, and is being studied in a variety of hematological cancers and solid tumors, including non-Hodgkin's lymphoma, prostate, breast and non-small-cell lung cancers. Bortezomib is thought to inhibit cell proliferation by blocking the degradation of proteins involved in cell-cycle control and apoptosis (including p53, cyclins and IκB) [101]. Interestingly, Bortezomib shows selective cytotoxicity against cancer cells compared to normal cells both *in vitro* and *in vivo* [99]. Therefore, development of strategies involving proteasome inhibitors may be useful in the therapy of certain types of tumors (Figure 1.3).

1.3.3
Other Approaches

Apart from targeting the ubiquitin system itself, some clinically-effective monoclonal antibodies and small molecule inhibitors have been shown to promote ubiquitin-dependent degradation of oncogenic proteins. Such an example is Herceptin, a monoclonal antibody used for the treatment of breast cancer tumors overexpressing ErbB2 which increases Cbl-mediated ErbB2 ubiquitination and degradation [102].

Another promising and challenging approach to targeting the ubiquitin system in cancer therapy is to alter the ubiquitin-induced protein–protein interactions in cells [4]. Although this approach offers more specificity than any of the above-mentioned strategies due to the fact that there are more ubiquitin-induced

interactions than existing enzymes, there are still many difficulties related to this strategy. The main obstacles include targeting the flat and hydrophobic interaction surface between ubiquitin and UBDs and dealing with the low affinities of such interactions. Modulators of polyubiquitin chain recognition, the ubistatins, have been shown to bind specifically to the interfaces between Lys48-linked ubiquitin molecules, and to inhibit ubiquitin-dependent proteasomal degradation of certain substrates (Figure 1.3) [103]. The ubistatins are however not cell-permeable, but it may be possible to use them to target interaction surfaces between ubiquitin and UBDs after further developing them and increasing their bioavailability. Other types of inhibitor of ubiquitin–UBD interactions could target either the hydrophobic surface of ubiquitin containing Ile44, with which most UBDs interact, or specific UBDs (Figure 1.3). However, these approaches are also associated with issues of specificity, since ubiquitin and ubiquitin chains are attached to numerous proteins and UBDs are found in a vast variety of proteins. Despite these drawbacks, the increasing interest and knowledge gained in this field will ensure that several novel strategies for targeting the ubiquitin system with higher specificity will be developed in the near future.

1.4 Conclusions and Future Perspectives

In conclusion, we note that there is a strong link between alterations in ubiquitin signaling networks and the hallmarks of cancer, including uncontrolled proliferation and cell cycle divisions (SCF ligases, NF-κB), increased cellular signaling (RTKs, NF-κB), defective endocytosis (Cbl, RTKs, TSG101), increased cell survival (NF-κB), defective DNA repair (p53, BRCA1, TLS polymerases) and increased angiogenesis (VHL). A complete understanding of the interplay between ubiquitination and other post-translational modifications such as phosphorylation and acetylation, between ubiquitin ligases and DUBs, as well as ubiquitin and Ubls in cellular networks will have a great impact on our insight into cancer-promoting mechanisms and our ability to design smart drugs for the treatment of cancer.

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2
Regulation of the p53 Tumor-suppressor Protein by Ubiquitin and Ubiquitin-like Molecules

Dimitris P. Xirodimas

2.1
Functional Domains of p53

The p53 protein is a sequence-specific transcription factor, which either induces or represses expression of a variety of genes. This change in gene expression leads to either cell cycle arrest or apoptosis depending on cellular conditions. The p53 pathway is activated by a variety of genotoxic agents such as ultraviolet (UV) light [1], ionizing radiation [2], chemotherapeutic drugs [3] as well as by non-genotoxic treatments such as withdrawal of growth factors, hypoxia [4], heat shock, depletion of ribonucleoside triphosphates [5]. All these stimuli induce a nuclear accumulation of p53, whereas in normal unstressed cells the protein is present in immunologically undetectable levels. The kinetics of this response may vary depending on the stimulus applied, for example ionizing radiation results in a fast and transient p53 accumulation, while UV radiation induces more prolonged protein stabilization.

The p53 protein can be divided into three independent functional domains which coordinate and regulate the activity of each other in the complete protein. The N-terminus, which includes the first 100 amino acid residues, has been shown to mediate the transcriptional transactivation function of p53 and to be crucial for p53-mediated apoptosis. Components of the transcriptional machinery such as the TATA-associated factors TAFII70 and TAFII31 (subunits of TFIID) [6, 7], the p62 subunit of the transcription/repair factor TFIIH [8] or the co-activators CBP/p300 [9, 10] have been shown to interact with this region of p53. The N-terminus of p53 also contains a proline-rich domain (amino acids 62–91), which is important for the induction of p53-mediated apoptosis [11–13]. Amino acid residues 100–290 of human p53 form an independently folded protease-resistant domain, which binds to DNA in a sequence-specific manner [14, 15]. The DNA binding domain has been selected as a target in the process of tumor progression, as 90% of the missense point mutations in p53 identified in tumors are located in this domain and are responsible for the loss of the biological activity of wild type p53 [16, 17]. The C-terminus of human p53 contains the nuclear localization signal (amino acids 315–320) and the oligomerization domain (amino acids 324–355), which allows

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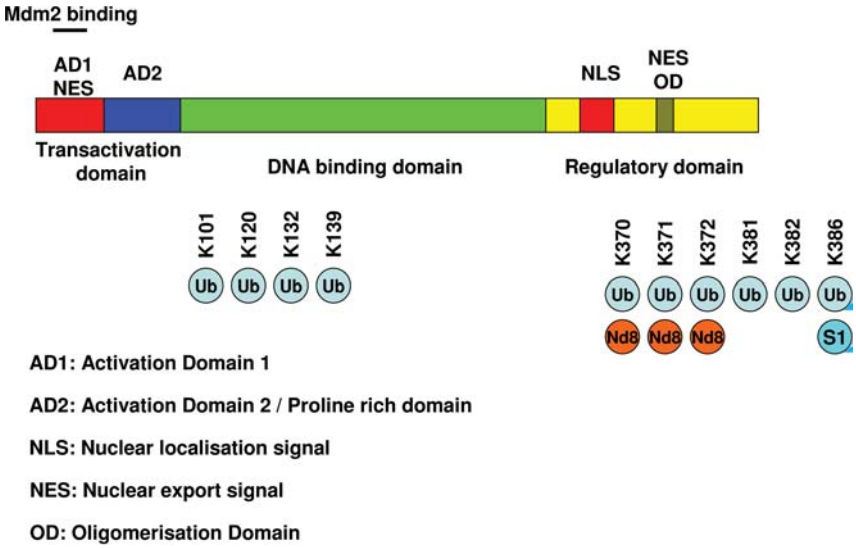

Fig. 2.1. Functional domains of p53. [6, 7, 11–13, 14–15, 18, 19, 20, 41–44, 72–74]

the formation of tetrameric p53 structures [18, 19]. The last 30 C-terminal p53 amino acid residues (363–393) are involved in the non-specific DNA and RNA binding activity of p53 as well as negatively regulating the sequence-specific DNA binding activity of the core of p53 [20]. Up until recently, the structure of the p53 gene was considered to be simple with the use of a single promoter and the production of two in mouse and three in human splice variants. It appears that the structure of the p53 gene is more complex than at first thought and through the use of an internal promoter in intron 4 and alternative splicing, six new p53 isoforms can be synthesized. Biologically, some of the p53 isoforms can differentially bind to p53 promoters, modulating the expression of p53 target genes [21].

2.2 The Family of Ubiquitin-like Molecules

Ubiquitin is the senior member of a growing family of small polypeptides, the ubiquitin-like family. These molecules are related to ubiquitin but are standing as unique pathways in controlling diverse biological processes. This family includes SUMO/sentrin/Smpt3 (SUMO-1, -2 and -3), NEDD8/Rub1, ISG15/UCRP, Fub, Fat 10 and Apg12 [22, 23]. Mechanistically, their conjugation is very similar to that of ubiquitin. The immature molecule is initially processed by a specific protease to expose at the C-terminus a di-glycine motif. Conjugation of the mature molecule on the ε amino group of a substrate lysine residue involves three main enzymatic activities. An E1 activating enzyme through an ATP-dependent step forms a high

energy thiolester bond between an internal cysteine residue and the C-terminal glycine of the ubiquitin-like modifier. In the second step through transesterification, a thiolester bond is formed between the ubiquitin-like molecule and the E2 conjugating enzyme. Finally, through the action of an E3-ligase the lysine of the substrate is covalently modified [24–26]. However, at least for ubiquitin there is evidence that a fourth activity may also be involved (E4-ligase) which stimulates the formation of poly-ubiquitin chains [27]. Biochemical studies have demonstrated that at least for SUMO-1, -2 and -3 covalent modification of the substrate requires the activity of only the first two enzymes mentioned above. It is believed that an E3 ligase increases the efficiency of the transfer of the modifier from the E2 enzyme onto the substrate. For SUMO-1, -2, -3 the E1 enzyme is a heterodimer of SAE1/AOS1 and SAE2/Uba2, whereas the E2 conjugating enzyme is Ubc9 [28–31]. Modification of substrates with SUMO typically occurs on a lysine within a consensus sequence ψKXD/E (where ψ is a hydrophobic residue) [32]. The conjugating enzyme Ubc9 interacts with this motif, possibly explaining the conjugation of substrates in the absence of an E3-ligase activity [33–35]. SUMO-2 and -3 but not -1 have internal consensus sequences and can therefore form poly-SUMO chains [36]. For NEDD8 the E1 is also a heterodimer of APPBP1 (or Ula1 in yeast) and Uba3 proteins and the E2 conjugating enzyme is Ubc12 [37, 38]. These set of enzymes are unique for each pathway and they cannot use ubiquitin or other members of the family.

2.3
E3 Ligases for p53

There are two distinct families of E3-ligases. The HECT (Homologous to E6-AP Carboxyl Terminus, see below) family of E3s which make a thiolester bond with ubiquitin and the RING Finger E3s which instead facilitate the transfer of ubiquitin from the E2 to the substrate. A common phenotype for the RING ligases is their autocatalytic activities which enables them to control their own modification and stability [26]. A number of E3 ligases have been identified as regulators of p53 modification with ubiquitin/SUMO/NEDD8 (see Table 2.1). It was during the early 1990s that the first ligase was identified as a p53 regulator by the fact that the human papillomavirus protein E6 directly interacts with p53 and recruits the E6AP (E6 associated protein) E3-ligase. This results in p53 ubiquitination and proteasomal degradation [39]. Adenoviral proteins E1B55K and E4orf6, which also interact with p53, cooperate in a cullin-based E3-ligase complex to stimulate the degradation of p53 [40]. This is one of the mechanisms by which viral infection neutralizes p53 function and promotes cell proliferation. It was not until 1997 that the first cellular E3-ligase was identified which directly interacts with p53 and controls p53 levels through the ubiquitin/proteasome pathway. The Mdm2 oncogene product was known to interact with the N-terminus of p53 inhibiting p53 transcriptional activity by competing for p53 binding with factors of the basal

Table 2.1. E3-ligases which control the function of p53. [39, 40, 50–52, 53, 54, 59, 60–62, 63–67]

Ligase	Type
E6AP	HECT
E1B55k/E4orf6	Cullin based ligase
Mdm2	RING
COP1	RING
Pihr2	RING
CHIP	U-Box
ARFBP1	HECT
Topors	RING
WWR1	HECT
CARPs	RING
PIAS family	RING

transcriptional machinery [7, 41–44]. However, the significance of the p53–Mdm2 interaction was clearly demonstrated from gene "knock out" experiments in mice [45, 46]. Deletion of Mdm2 appeared to be embryonic lethal, but in contrast mice deficient for both Mdm2 and p53 were viable and developed normally, suggesting that a key activity of Mdm2 is to downregulate the growth-suppressing effects of p53. This motivated different groups to look more carefully at the effects of Mdm2 binding on p53. One approach was to create p53 mutants that were unable to interact with Mdm2 or Mdm2 mutants that were deficient for p53 binding and to use them in co-transfection experiments in cells. Interaction of p53 with Mdm2 dramatically reduced the p53 steady state levels, demonstrating that p53 is degraded through its interaction with Mdm2 [47, 48]. In another approach, peptides which were selected using phage-displayed peptide libraries, and which could disrupt the p53–Mdm2 interaction, were shown to stabilize p53 and activate p53-dependent transcription without the administration of any genotoxic stimuli [49]. Further biochemical studies showed that Mdm2 plays a direct role in the process by acting as an RING finger E3-ligase promoting the modification of p53 with ubiquitin [50–52]. As mentioned above p53 as a transcription factor induces the expression of different genes, one of them being the mdm2 gene itself, thus creating a negative feedback loop. Since then, two other RING finger ligases have been identified which also participate in a negative feedback loop with p53. Pihr2 and Cop1 E3-ligases were shown to stimulate p53 ubiquitination and proteasomal degradation and p53 induces transcription of the pihr2 and cop1 genes [53, 54]. Therefore, during the p53 response to stress stimuli, a variety of ubiquitin ligases can be induced which can then suppress the function of p53. Mdm2 is overexpressed in sarcomas, and increased protein levels of Pirh2 and Cop1 were recently detected in lung and breast tumors respectively [55–58]. These tumors contain wild-type p53, suggesting that the overexpression of these ligases could account for an over-silenced p53 function. ARF-BP1/Mule/HectH9 was recently identified as a HECT-type E3 ligase, which also negatively controls p53 levels. It was found to interact

with the p14ARF tumor suppressor (see below) and to control p53 independently of Mdm2. However, ARF-BP1 is not a p53-induced gene and it was also shown to have p53-independent functions [59]. The chaperone-associated ubiquitin ligase CHIP can also suppress the levels of p53. CHIP through its interaction with Hsc70 and Hsp90 facilitates ubiquitination and degradation of chaperone-associated proteins [60]. Given the conformational flexibility of p53, its stability could be regulated through a transient association with molecular chaperones. TOPORS ligases were shown to stimulate modification of p53 both with SUMO and ubiquitin but the physiological implications are not known [61, 62]. A more specific role for the PIAS (Protein Inhibitor of Activated STAT) family of RING ligases in the SUMO conjugation of p53 has been demonstrated [63–65]. Recently, the WWP1 (WW domain-containing protein 1) ligase was shown to ubiquitinate p53 but this interaction seems to increase the levels of p53 [66]. In this case the increase was associated with a cytoplasmic accumulation of p53 and reduction in its transcriptional activity. Also, the CARP (caspase-8 and -10 associated RING proteins) family of apoptotic inhibitors were demonstrated to specifically suppress the levels of p53 phosphorylated at Ser15/20 [67].

2.4
Modification of p53 with Ubiquitin

Covalent modification of substrates with ubiquitin can occur in different formats. Ubiquitin can be conjugated as a single moiety or can form ubiquitin chains through modification of a pre-existing ubiquitin via an internal lysine residue. There are seven lysine residues in ubiquitin which can be used as acceptor sites (K6, K11, K27, K29, K33, K48 and K63). Formation of chains through different lysines creates a unique functional signal. For example chain formation through K48 is regarded as a signal for targeting the substrate for proteasomal degradation, whereas K63 linkage is involved in post replicative DNA repair, translation and endocytosis. One of the most extensively studied roles of p53 modification with ubiquitin is targeting p53 for 26S proteasomal degradation [68–71]. Initial studies identified the regulatory C-terminus of p53 as the domain required for p53 modification with ubiquitin. Specifically, mutation of six lysine residues (K370, K372, K373, K381, K382, K386) located in the last 30 amino acids of p53 dramatically reduced the levels of Mdm2-mediated ubiquitinated p53 [72, 73]. This p53 mutant is expressed at higher protein levels compared to wild-type p53 and has increased transcriptional activity. Additional lysines in the DNA binding domain of p53 (K101, K120, K132, K139) were recently identified [74]. The role of the C-terminal lysines in regulating p53 function was further addressed in a mouse model system where the six or seven C-terminal lysines in p53 where mutated to arginine. Interestingly, the mice were viable, developed normally and the stability of the p53 mutants was very similar to that of the wild-type p53. However, the p537KR mutant showed more rapid DNA damage in the thymus compared to the wild type [75] whereas deficiencies in the activation of p53 target genes in the p536KR

mutant mice were observed [76]. It has to be noted however, that lysines are target residues for multiple post-translational modifications and therefore, phenotypes observed in *in-vivo* or *in-vitro* model systems are difficult to attribute to a particular modification. Furthermore, since different modifications may have different biological outcomes the overall functional effect of lysine mutants may represent an average of the individual responses. Initial biological studies suggested that the Mdm2–p53 interaction leads to nuclear export and degradation of p53 in the cytoplasm. This model was based on the identification of nuclear export sequences in Mdm2 and p53 and on the observation that inhibitors of the Crm1-dependent nuclear export such as leptomycin B, resulted in nuclear accumulation of p53 and activation of the response. By using heterokaryon assays, where two different types of cells are fused to create a cell with two discrete nuclei and a common cytoplasm, it was shown that p53 and Mdm2 can shuttle from one nuclei to the other through the common cytoplasm [77–79]. However, the interaction of p53 with Mdm2 is not sufficient to mediate export to the cytoplasm. Mutation of a critical cysteine residue in the RING finger of Mdm2 (C464A), which renders Mdm2 inactive as an E3-ligase but still able to interact with p53, severely impaired the ability of Mdm2 to facilitate nuclear export of p53 [80]. These data suggested that ubiquitin may act as the signal for this translocation event, leading to degradation of p53 in the cytoplasm. This step however, does not appear to be the only mechanism by which p53 is targeted to the proteasome as subsequent studies have shown that Mdm2-mediated ubiquitination and degradation of p53 may occur in the nucleus in the absence of any nuclear to cytoplasmic transport [81–83]. Further analysis suggested that the extent of p53 ubiquitination may control this process. Low levels of Mdm2 promote mono-ubiquitination of p53 facilitating p53 nuclear export, whereas high Mdm2 levels were able to promote multi-ubiquitination of p53 and proteasomal degradation in the nucleus. This model was supported by the observation that a p53 protein fused at its C-terminus with ubiquitin (mimicking mono-ubiquitination) was localized in the cytoplasm [84]. This appears to be a specific signal for ubiquitin as fusion of NEDD8 and SUMO-1 failed to change the localization of p53 [85]. This means of regulation could represent physiological conditions where in unstressed cells with low levels of Mdm2, p53 is exported to the cytoplasm for degradation or for transcription-independent p53 functions, such as the induction of apoptosis through interaction with mitochondria. However, at later stages of the p53 response or in malignancies where the levels of Mdm2 are high, p53 is poly-ubiquitinated and degraded in the nucleus.

2.5 Requirements for Mdm2-mediated Ubiquitination of p53

Ubiquitin modification of a substrate requires the direct interaction of the E3-ligase. Clearly, Mdm2 is the most intensively studied p53 E3-ligase and much information has now been amassed with regard to its requirements for the promotion of p53 ubiquitination and proteasomal degradation. Mdm2 is a 90-kDa protein

divided into four major conserved domains: an N-terminal domain (amino acids 23–108), a central domain which contains a highly acidic region (amino acids 209–275), a zinc finger domain (amino acids 289–333) and a RING finger domain (amino acids 460–490) [86, 87]. The studies in which the role of Mdm2 as a regulator of p53 stability was discovered, also showed that direct interaction with p53 is necessary for this process [47, 48]. Studies using peptide mimetics, peptide phage display libraries approaches, crystallographic or mutational analysis showed that amino acids 14–27 in p53 interact with a hydrophobic pocket in the N-terminus of Mdm2. More specifically, Phe 19, Try 23 and Leu 26 are crucial for this interaction as their side chains are buried within the Mdm2 hydrophobic pocket and are the main binding contacts [43, 49, 88–90]. The RING finger domain is required for the E3-ligase activity of Mdm2 and for its suppressive role towards the transcriptional activity of p53. Mutations of potential zinc-coordinating residues in this domain showed that an intact RING finger is required for Mdm2 to promote p53 and its own ubiquitination [50, 51, 91]. Furthermore, this domain of Mdm2 is important for its interaction with its homolog, Mdmx and it is believed that heterodimer formation is mediated via interaction of their RING finger domains [92]. Much interest has been focussed on the central domain of Mdm2 and its role in the regulation of p53 and Mdm2 stability. This domain is responsible for many of the Mdm2 interactions with regulatory proteins such as p14ARF, p300, YY1, Rb, ribosomal proteins, Kap1 and TAFII250 (see below). In particular, deletion of the acidic domain inhibits the ability of Mdm2 to promote p53 degradation. Experiments with hybrid mutants containing Mdm2 and Mdmx domains also showed the importance of the acidic domain in targeting p53 for degradation [93, 94]. Further mutational analysis of potential phosphorylation of serine residues in mouse Mdm2 (Ser238, 240, 244, 251, 254, 258, 260) showed that these mutants were deficient in promoting p53 degradation. However, their ability to ubiquitinate p53 was not affected [95]. Lack of p53 degradation despite efficient ubiquitination was previously shown with an in-frame Mdm2 deletion mutant (amino acids 217–246) deficient for p300 binding and with p53 mutants [82, 96]. This highlights that proteasomal degradation of p53 involves a post-ubiqutination step, which could be regulated through phosphorylation of Mdm2 and/or interaction with additional proteins. However, apart from the main binding area for p53 and Mdm2, additional areas of the proteins interact as secondary binding motifs. From NMR studies on Mdm2 fragments bound to N-terminal p53 peptides, it was suggested that Mdm2 is conformationally flexible, and subject to allosteric regulation upon substrate binding [89]. Consistent with this, is the fact that *in-vitro* interaction of Mdm2 with RNA renders Mdm2 capable of binding to p53 lacking the N-terminus [97]. Further biochemical and biological studies have shown that the acidic domain of Mdm2 can interact with a flexible region in p53 within the DNA binding domain, which is frequently found unfolded in human tumors [98, 99]. While interaction of p53 with Mdm2 through their N-termini is important for p53 degradation, the secondary binding interface may control efficient p53 ubiquitination and proteasomal degradation [100]. On the other hand, p53 needs to have the correct oligomerization status for proper association and processing by

NLS: Nuclear localisation signal

NES: Nuclear export signal

NoLS: Nucleolar localisation signal

Fig. 2.2. Functional domains of Mdm2. [86, 87, 116]

Mdm2. p53 mutants unable to form tetramers are deficient in Mdm2 binding and degradation [101]. These conformational/structural requirements for Mdm2 and p53 to assemble a complex which can be processed by the proteasome, underlie the complexity and precise regulation of these processes.

2.6 Regulation of p53 Ubiquitination

2.6.1 E2 Conjugating Enzymes

As mentioned above, modification of a substrate with ubiquitin or ubiquitin-like molecules involves three well-characterised enzymatic activities. For ubiquitin, there is one E1-activating enzyme and multiple E2-conjugating enzymes; each of the E2 enzymes is capable of cooperating with different E3-ligases to promote modification of the substrates. Different mechanisms exist to modulate the modification status of p53 with regard to ubiquitin. Characterization of the specificity of Mdm2 in the selection of E2-conjugating enzymes for p53 ubiquitination showed that *in vivo* the UbcH5B/C are physiological E2s for Mdm2. These enzymes are involved in the formation of K48-linked ubiquitin chains. Downregulation of these E2s by siRNA led to the accumulation of p53 and Mdm2 proteins in cells. However, the accumulated p53 was transcriptionally inactive and this was due, at least in part, to the concomitant accumulation of the Mdm2 protein. Interestingly, this study also showed that known activators of the p53 response, such as doxorubicin and actinomycin D reduced the levels of expression of UbcH5B/C. This raises the possibility that the levels of E2-conjugating enzymes could be affected by signaling pathways that control the levels of p53 [102]. Ubc13 an E2-conjugating enzyme, which is involved in the formation of complexes with ubiquitin variant proteins (Uevs) to stimulate the formation of K63-dependent ubiquitin chains, was reported to promote the cytoplasmic localization of p53. As with UbcH5B/C the levels of Ubc13 were modulated by stress stimuli, in this case ionizing radiation

caused reduction of Ubc13 levels. Ubc13 was found to directly interact with p53 and it still not known whether an E3-ligase complex is required to produce this effect [103].

2.6.2 Interacting Proteins

The p53 pathway is also controlled through the interaction of p53 and Mdm2 with other cellular proteins. The $p14^{ARF}$ tumor suppressor is now well established as a regulator of the Mdm2 protein. ARF (p14 in human, p19 in mice) was identified as an alternative transcript of the Ink4α/ARF locus, which also expresses the $p16^{Ink4\alpha}$ cyclin dependent kinases inhibitor [104, 105]. ARF has been shown to directly interact with Mdm2 and to protect p53 from Mdm2-mediated proteasomal degradation [106–108]. Mechanistically, initial studies proposed that ARF, which itself is predominantly localized in the nucleolus, sequesters Mdm2 to the same compartment. This disrupts the interaction with p53 allowing it to accumulate in the nucleoplasm [109, 110]. Other studies proposed that ARF was able to inhibit the nuclear export of the p53–Mdm2 complex via a step involving the nucleolus [111]. However, protection of p53 from Mdm2-mediated degradation by ARF through the nucleolus does not seem to be the only mechanism of action. ARF mutants which were localized in the nucleoplasm were reported to stabilize p53 and activate the p53 response [112].

Biochemical studies showed that ARF was able to inhibit the E3-ligase activity of Mdm2 [113, 114]. Further *in vivo* studies showed that ARF has a differential role in controlling the ligase activity of Mdm2. Blockade of the proteasomal degradation of p53 and Mdm2 by ARF was accompanied by the inhibition of p53 ubiqutination but not Mdm2 auto-ubiquitination [115]. Mdm2 contains a cryptic nucleolar sequence at the C-terminus (amino acids 466–473) which becomes exposed on interaction with ARF. Mutation of this signal makes Mdm2 resistant to nucleolar localization when ARF is bound [116]. This Mdm2 mutant (Mdm2NoLS), despite its inability to degrade p53, is still auto-ubiquitinated. Expression of ARF as with wild-type Mdm2, blocked proteasomal degradation of Mdm2NoLS without affecting its auto-ubiquitination [115]. This suggests that Mdm2 can be ubiquitinated and degraded outside the nucleolus and ARF can block this step in the absence of nucleolar sequestration. This is consistent with the idea that the "trans" and "auto" ubiquitination of Mdm2 are differentially modulated. ARF also has p53- and Mdm2-independent functions and recently the ARF-BP1 ligase, which controls p53 stability was also shown to be inhibited by ARF [59].

The role of the nucleolus in controlling the function of p53 was recently expanded. In an elegant study, DNA damage of the nucleolus was shown to be necessary and sufficient to stabilize p53 and activate its response [117]. Furthermore, ribosomal proteins such as L11, L5, L23 whose nucleolar localization is part of the ribosome assembly process, were also shown to protect p53 from proteasomal degradation [118–122]. These studies identified a link between the p53

pathway and ribosomal biogenesis, with p53 sensing any nucleolar stress possibly through Mdm2. Interestingly, ribosomal protein L26 was shown to interact with the 5′ untranslated region of p53 mRNA and increase p53 translation during DNA damage [123]. Therefore, by increasing protein synthesis and decreasing proteasomal degradation of p53 an optimal response is achieved. This is further supported by an elegant study showing that Mdm2 protein is rapidly degraded as a result of DNA damage, which then leads to the accumulation of p53 [124]. The involvement of the translational process in regulating p53 stability had been previously suggested with the demonstration that Mdm2 was able to increase protein synthesis of the full length p53 and a shorter form through a second initiation site at residue 47. This form of p53 (p53/47) lacks the N-terminal Mdm2 binding site and by oligomerizing with the full length p53 regulates the stability and function of the full-length p53 [125]. The Mdm2 homolog Mdmx has proven to be an important regulator of p53 [87]. Knockout of the Mdmx gene in mice caused embryonic lethality which was rescued by the concomitant deletion of p53 [126–128]. Mdmx can inhibit p53 transcriptional activity through its interaction with p53 at its N-terminus [129, 130]. However, despite the presence of a C-terminal RING domain which is structurally similar to Mdm2, the ability of Mdmx to promote p53 ubiquitination is very low compared to that of Mdm2 [131]. When overexpressed, Mdmx was shown to block proteasomal degradation of p53 and Mdm2. Mdmx was shown to localize predominantly in the cytoplasm but Mdm2 was able to recruit Mdmx into the nucleus, which led to the inhibition of proteasomal degradation of p53 and Mdm2. However, in contrast to ARF expression, Mdmx preferentially inhibited the auto-ubiquitination activity of Mdm2 but not the ubiquitination of p53 [132–134]. On the other hand, Mdm2 as an E3-ligase can control the levels of Mdmx and it appears that these two proteins cooperate to control each other's stability and the function of p53 [135–137].

A similar autoregulatory loop was identified between Mdm2 and the tumor susceptibility gene 101 (tsg101). Deletion of tsg101 in mice caused embryonic lethality and the accumulation of p53 and this lethality was delayed by the concomitant deletion of p53. TSG101 was found to interact with both Mdm2 and p53 to control their stability. Expression of TSG101 stabilized Mdm2 and decreased the levels of p53, presumably through the increase in Mdm2 levels. This effect was dependent on the ubiquitin conjugating-like domain (Ubc) found in TSG101, possibly affecting Mdm2 ubiquitination. On the other hand Mdm2 is capable of destabilizing TSG101, creating an autoregulatory loop which controls the stability of p53 [138, 139]. These data suggest that apart from the regulation of Mdm2 ligase activity either towards itself or the substrate, there is control of a post-ubiquitination step, essential for the proteasomal processing of p53 and Mdm2.

Biochemical studies suggested that Mdm2 as an E3-ligase promotes the initial modification of p53 with ubiquitin as multiple single moieties (multi mono-ubiquitination), suggesting that additional factors may be involved in the formation of poly-ubiquitinated p53 [140]. Indeed, the transcriptional co-activator p300 and the transcription factors YY1 (Yin Yang 1) were shown to directly interact with Mdm2 and cooperate in the generation of polyubiquitin chains. Both factors interact with the central domain of Mdm2 which plays an important role in p53 degradation

[141, 142]. Mdm2 showed similar cooperation in stimulating the ubiquitination of p53 through the central acidic domain, with the transcriptional co-repressor Kap1, the transcription factor TAFII250, the Mdm2 binding protein MTBP and gankyrin a protein which is commonly overexpressed in hepatocellular carcinomas [143–146]. Transfer of ubiquitinated substrates to the proteasome is typically mediated through carrier proteins, which can interact with both the ubiquitin chain (through a UBA domain) and the proteasome (through a UBL domain) [23, 147]. Rad23 (*S. cerevisiae*) or the human homolog, hHR23A and hHR23B, proteins are involved in the global nucleotide excision repair (NER) pathway and they contain both UBA and UBL domains. A post-ubiquitination role for hHR23 was proposed in the regulation of p53 degradation. An Mdm2–hHR23 complex was identified and the presence of hHR23 was found to be required for genotoxic activation of the p53 response [148–150]. In addition, the retinoblastoma susceptibility protein (pRb) protects p53 from proteasomal degradation and inhibits the anti-apoptotic effects of Mdm2 by its interaction with Mdm2 (amino acids 273–321). However, pRb did not inhibit the suppressing effects of Mdm2 on p53 transactivation activity but rather on p53-mediated transrepression [151].

2.6.3
By Other Post-translational Modifications

Additional post-translational modifications can regulate the ability of Mdm2 to promote p53 ubiquitination and proteasomal degradation. In response to DNA damage both p53 and Mdm2 are targets for phosphorylation events which control the suppressive function of Mdm2 on p53. Phosphorylation/dephosphorylation events involving Ser15, Thr18 and Ser20 are thought to regulate the interaction of Mdm2 with p53 and therefore its capacity to modulate p53 stability. The ATM/ATR family of kinases activated after DNA damage are thought to be involved in these events either by directly phosphorylating p53 (Ser15) or indirectly through the activation of other kinases such as Chk1, Chk2 and Plk3 (Ser20). These phosphorylation events can also "prime" p53 for additional modifications, such as the recruitment of histone acetyltransferases, which acetylate p53 at Lys382 [152–155]. Acetylation of p53 is implicated in the control of p53 stability and its transcriptional activity [156]. This interplay between different modification events was highlighted by the finding that Mdm2 associated with HDAC1 histone deacetylase, resulting in the deacetylation of p53 lysines known to be present in their acetylated form [157]. Deacetylation of p53 is further facilitated by the action of the Kap1 co-repressor. Kap1 was shown to directly interact with Mdm2 and recruit HDAC1 to p53, resulting in deacetylation and Mdm2-dependent increase in the ubiquitination of p53 [146]. It is conceivable, that these lysines become accessible to other modification pathways that use the same residues in p53, such as ubiquitin and ubiquitin-like molecules. This highlights that different signaling pathways can alter or finely modulate the function of p53 by synergizing or competing in its modification. Not surprisingly, the role of Mdm2 as a regulator of p53 stability is controlled by its degree of phosphorylation. When the DNA is damaged, Ser395 and Tyr394 in Mdm2 are phosphorylated by ATM and its downstream tyrosine

kinase c-Abl respectively, which then inhibits the ability of Mdm2 to degrade p53 [158–160]. In the case of Ser395, phosphorylation blocks Mdm2-mediated nuclear export and degradation of p53 [161]. The subcellular localization of Mdm2 is also controlled by other induced phosphorylation events. Survival signaling pathways such as AKT activation leads to the phosphorylation of Mdm2 at Ser166 and Ser186 [162, 163] which stimulates the nuclear import of Mdm2 and the decrease in p53 levels. Activation of c-Abl also leads to the phosphorylation of Tyr276 in Mdm2, which then blocks the Mdm2-mediated degradation of p53. This phosphorylation event also facilitates the interaction between Mdm2 and ARF, which leads to nucleolar localization of Mdm2 [164].

2.7 De-ubiquitination of p53

Modification of substrates with ubiquitin and ubiquitin-like molecules is a very dynamic process and is brought about by the action of de-conjugating enzymes which specifically remove the modified molecule from the substrate. Various de-ubiquitinating enzymes which control the p53 degradation process have been identified. HAUSP/USP7 was first identified as a p53 interacting protein, which could block/reverse Mdm2-mediated ubiquitination of p53 and activate the p53 response [165]. However, this effect appears to be more complex as it depends on the relative amounts of HAUSP in cells. As mentioned previously, Mdm2 as a RING ligase is able to promote its own ubiquitination and proteasomal degradation. HAUSP was also shown to interact with Mdm2 to prevent its auto-ubiqutination and degradation. Therefore, in the total absence of HAUSP (gene knockout in cells) the levels of Mdm2 dramatically decrease thus causing an increase in p53 levels [166]. However, a partial reduction in HAUSP levels (siRNA knockdown in cells) caused the levels of p53 protein to decline, suggesting that under these conditions the ubiquitination of p53 but not of Mdm2, was affected [167]. The role of HAUSP in the p53–Mdm2 pathway was shown to be regulated by the death-associated protein Daxx. By simultaneously interacting with HAUSP and Mdm2, Daxx facilitates the de-ubiquitination and stabilization of Mdm2. On the other hand, Daxx promotes Mdm2-mediated ubiquitination and degradation of p53. In the presence of DNA damage Daxx dissociates from Mdm2, leading to Mdm2 destabilization and the activation of the p53 response [168]. Furthermore, HAUSP was shown to control the levels of Mdmx. The de-ubiquitinating activity of HAUSP is impaired after DNA damage, providing a possible mechanism for the rapid and transient destabilization of Mdm2/Mdmx which has been observed under conditions of stress [124, 169, 170]. Two more de-ubiquitinating enzymes were recently reported to control the p53–Mdm2 pathway. USP2a was identified in a bacterial two-hybrid screen for novel Mdm2 interactors and was shown to specifically de-ubiquitinate Mdm2 but not p53. Overexpression of USP2a caused an accumulation of Mdm2 and a decrease in p53 levels through the proteasome pathway. This may be relevant to 50% of prostate tumors where the USP2

gene (encodes for USP2a and USP2b) is found to be amplified. As many of these tumors contain wild-type p53, overexpression of USP2a could explain the suppression of the p53 pathway [171]. USP28 a de-ubiquitinating enzyme found in complex with one of the DNA damage mediator proteins, 53BP1, was reported to be a critical regulator of the apoptotic response induced by p53 after exposure to ionizing radiation (IR) [172].

2.8 SUMO-1/sentrin/smpt3

In contrast to ubiquitin, SUMO modification cannot act as a degradation signal for the 26S proteasome. In fact, in a few reported cases SUMO has been found to block proteasomal degradation by competing with ubiquitin. One of the emerging roles for SUMO conjugation is to control transcriptional activity. With regard to p53, initial reports suggested that conjugation with SUMO-1 increases its transcriptional activity. Lysine 386 was shown to be the acceptor residue for SUMO-1 and is one of the lysines required for the conjugation of ubiquitin to p53; it is also present in the identified SUMO consensus sequence ψKXD/E [173, 174]. Two-hybrid screens originally identified members of the PIAS family of ligases (see above) as interactors with p53 and soon after biochemical and biological studies demonstrated the role of PIAS as regulators of p53–SUMO conjugation [64]. Different groups have assessed the role of these ligases in p53 function with contradictory data [175]. Based on the initial studies it appears that expression of ubc9 and SUMO-1 increased p53 transcriptional activity [173, 174, 176]. However, expression of the PIAS ligases (PIAS1 and PIASx) enhanced p53 SUMO conjugation but resulted in suppression of p53 transcriptional activity [65]. This effect was dependent on the intact E3-ligase activity, as RING finger point mutants were unable to stimulate p53–SUMO conjugation and suppress p53 function. In a more recent study, PIASy was also shown to stimulate p53–SUMO conjugation but the effect of this PIAS member was to activate p53 transcriptional activity [63]. The effect of the SUMO pathway on p53 function becomes more complex, as the p53 regulators Mdm2 and Mdmx are also SUMO conjugated. Removal of SUMO-1 from Mdm2 through the action of the SUMO protease, SUSP4, resulted in increased auto-ubiquitination of Mdm2 and an increase in p53 stability. The levels of SUSP4 were increased after UV-induced DNA damage, providing another example of cooperation between different conjugation pathways in the activation of the p53 response [177].

2.9 NEDD8/Rub1

NEDD8 shares the greatest homology with ubiquitin amongst the family of ubiquitin-like molecules. However, a distinct set of enzymatic activities are involved in

NEDD8 modification of substrate proteins. Genetic experiments in yeast, plants, *C. elegans*, Drosophila and mice have shown an important role for NEDD8 in cell cycle and cell viability [178–181]. However, up until recently the only well-described substrate for NEDDylation was the cullin family of proteins. Cullins are scaffold proteins for the SCF–ubiquitin ligase complex (Skip-1, Cullin, F-box). The role of cullin NEDDylation is to increase the ubiquitin ligase activity of the complex towards its substrates [182]. Two of the first identified non-cullin substrates for NEDD8 were the p53 and Mdm2 proteins. Mdm2 as a RING finger ligase promoted *in vivo* modification of itself and p53 with NEDD8. This modification required the C-terminus of p53 but in contrast to ubiquitin a p53 mutant with three lysines in that domain (K370, K372, K373) mutated into arginine, was deficient in Mdm2-mediated NEDDylation. The role of NEDD8 in the control of p53 function was assessed in the TS41 system. These Chinese Hamster Ovary (CHO) cells carry a temperature sensitive mutation in the APP-BP1 gene (one of the components of the NEDD8 E1-activating enzyme; see above) [183]. When these cells were grown at restrictive temperatures (where the pathway is switched off) the transcriptional activity of p53 was increased. Furthermore, in the absence of the NEDDylation pathway Mdm2 was more potent in inhibiting p53 activity. These data suggest a suppressive role for the NEDD8 conjugation pathway in the transcriptional activity of p53 [184]. The FBX011 protein, a member of the F-box protein family and a component of the SCF complex, was reported to promote the modification of p53 with NEDD8 but not with ubiquitin. Expression of FBX011 did not affect the protein levels of p53 but inhibited p53 transcriptional activity, consistent with a suppressive role for NEDD8 in p53 function [185].

2.10
Therapeutic Intervention through the Ubiquitin Pathway

As previously mentioned, in many tumors, despite the presence of a wild-type p53 gene the pathway is not functional and in some cases this may result from the overexpression of E3-ligases which over-suppress the function of p53. There is evidence to suggest that tumor cells may be more sensitive to the activation of p53 compared to healthy cells, making the activation of p53 an attractive target for potential anti-cancer drugs [186]. The studies, which identified small peptides that disrupt the p53–Mdm2 interaction, showed that this was sufficient to increase the levels of p53 in the absence of any genotoxic stimuli [49]. This provides a proof of principle for targeting the interaction between p53 and Mdm2 or the function of the E3-ligase Mdm2. In support of the former strategy, small molecules called nutlins were isolated as potent and specific inhibitors of the p53–Mdm2 interaction. Nutlins bind in the hydrophobic pocket of Mdm2, in which the N-terminus of p53 is buried. These drug-like molecules were shown to stabilize and activate p53 only in tumor cells that contained wild-type p53 [187]. The ligase activity of Mdm2 has also been used as a target for small molecule inhibitors. Using an *in vitro* Mdm2–p53 ubiquitination assay, compounds were isolated that inhibited

p53 ubiquitination. Interestingly, these molecules did not affect Mdm2 auto-ubiquitination and had a similar effect to ARF expression in cells [188]. In other studies, compounds which inhibited the auto-ubiquitination activity of Mdm2 were isolated *in vitro*. At first glance, this may seem paradoxical as use of inhibitors of this class will lead to an accumulation of the Mdm2 protein, which in principal could still interact with p53 and inhibit its function. However, these compounds were shown to stabilize p53 and Mdm2 in cells, and to activate p53 transcriptional activity [189]. Inhibitors of proteasome function such as PS-341 are now being used as treatment for multiple myeloma. The exact mechanism of the anti-tumor action of these compounds is not known, but is characterized by an accumulation of p53 and the induction of phosphorylation at Ser15 [190]. As previously mentioned, over 50% of human tumors contain mutant p53 with the majority of mutations located in the core DNA-binding domain of the protein. More specifically, these mutations either involve residues which make direct contact with DNA ("contact mutants"), or residues that provide structural stability, and proper positioning of the DNA contact residues ("structural mutants"). Restoring the wild-type activity of p53 mutants could be a valuable approach to suppress the uncontrolled growth of these tumors. This strategy is being tested with small molecules such as PRIMA-1, MIRA-1 and CP-31398, which can restore wild type conformation to some p53 mutants [191]. The role of the ubiquitin pathway in the transcriptional silencing of p53 mutants has not yet been addressed. There is evidence to suggest that structural mutants of p53 are thermodynamically unstable leading to an increased association with the chaperone machinery. Chaperone-associated ligases, such as CHIP can promote proteasomal degradation of both mutant and wild-type p53, but this process is more efficient in the case of the mutant protein. Since wild-type p53 is conformationally flexible, it is possible that it takes part in a dynamic interaction with the chaperone machinery, but this event is more profound for the more thermodynamically unstable p53 mutants [60, 192]. Furthermore, mutations in the DNA-binding domain caused an increase in p53 ubiquitination [98]. Clearly, investigation of the role of the ubiquitin pathway in the regulation of mutant p53 function could provide a new approach to restoring the wild type function of mutant p53 in tumor cells.

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3
The Ubiquitin–Proteasome System in Epstein-Barr Virus Infection and Oncogenesis

Maria G. Masucci

3.1
Introduction

The modification of intracellular proteins by covalent attachment of ubiquitin or ubiquitin-like polypeptides and the degradation of some of these conjugates by the proteasomes are critical events in the regulation of cellular metabolism, proliferation, differentiation and death (reviewed in [1]). These processes are therefore major targets for pathogens that often devote a significant part of their genomes to genes whose products modify the cellular environment or protect the infected cells from the host immune attack. The involvement of the ubiquitin–proteasome system in the life cycle of viruses includes a role in virus entry, virus exit and maturation, regulation of viral and cellular gene expression and modulation of cellular functions including cell cycle, apoptosis and antiviral responses such as interferon production and antigen processing. This chapter will focus on the first identified human tumor virus Epstein-Barr Virus (EBV), a gamma-herpesvirus that is involved in the pathogenesis of a broad spectrum of malignancies of lymphoid and epithelial cell origin (reviewed in [2]). As a result of its relatively large coding capacity, EBV has evolved unique strategies for persistence in the infected host by parasitizing the complex life cycle of its primary target, the B-lymphocyte. Many of these strategies involve modulation of the ubiquitin–proteasome system.

3.2
Viral Interference with the Ubiquitin–Proteasome System

The ubiquitin–proteasome system plays a pivotal role in viral infection and pathogenesis. As extensively discussed elsewhere in this book and in a number of recent reviews, ubiquitin-dependent proteolysis is achieved through two successive steps: the covalent attachment of ubiquitin to the target protein, and the degradation of the ubiquitinated protein by the 26S proteasome with the release of peptide fragments and reusable ubiquitin [1]. The ubiquitination step involves

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three sequential enzymatic reactions that entail the ATP-dependent activation of the C-terminal glycine of ubiquitin by a ubiquitin-activating enzyme (E1), the transfer of the activated ubiquitin to a ubiquitin-conjugating enzyme (E2) and the subsequent formation of a covalent isopeptide bond between the activate C-terminus of ubiquitin and ε-amino group of a lysine residue in the substrate catalyzed by a ubiquitin–protein ligase (E3). After at least four rounds of ubiquitination involving the Lys48 or, less frequently, Lys29 residue of the previously conjugated ubiquitin, the substrate is recognized and subsequently degraded by the 26S proteasome (reviewed in [3]). The specificity of proteolysis appears to be achieved primarily at the step of ubiquitination, mainly due to the capacity of the E3 enzymes to recognize only one or a few specific substrates. The E3s can be divided into three groups: the homologous to E6-associated protein carboxyl terminus (HECT)-domain subfamily E3s that are themselves ubiquitinated before transferring ubiquitin to the substrate; the single-subunit really interesting new gene (RING)-finger subfamily, where the substrate recognition site and the RING domain involved in E2 binding reside in the same protein; the multi-subunit RING-finger subfamily where the substrate recognition and RING domains are found in separate subunits of the ligase, which allows the construction of a huge variety of enzymes with different substrate specificity and finely tuned activity (reviewed in [4]). The efficiency and stability of ubiquitination is also regulated by the activity of deubiquitinating enzymes (DUBs) that hydrolyze the isopeptide bonds between two adjacent ubiquitins, or between ubiquitin and the substrate. More than 90 DUBs have been identified in the human genome (reviewed in [5]). Based on their structure and function, these can be classified into at least five distinct families: ubiquitin-specific proteases (USP), ubiquitin carboxyl-terminal hydrolases (UCH), ovarian tumor (OTU) domain-containing proteases, Josefines, and the Jab1/MPN domain-associated metalloisopeptidase (JAMM) group of hydrolases. In addition to the recycling of damaged or misfolded proteins, the ubiquitin–proteasome system is responsible for the constitutive and induced turnover of regulatory proteins that control a wide variety of cellular functions, including the cell cycle, transcription, translation, signal transduction, antigen processing and apoptosis [1]. Furthermore, modification of proteins by polyubiquitin chains linked through Lys63, by single ubiquitin, or by a growing family of ubiquitin-like molecules including SUMO, Nedd8, ISG15, FAT10, LC3 and several more, each requiring a dedicated set of specific E1, E2 and E3 enzymes, does not result in proteasomal degradation but regulates essential functions such as DNA repair, endo- and exocytosis, protein trafficking between different cellular compartments, and autophagy [6].

Viruses exploit and manipulate this complex system of protein modification and degradation in many different ways. Viral entry and exit from the infected cell follow physiological routes for uptake and export of macromolecules that are controlled by mono or polyubiquitination of receptors and transport proteins. Likewise, the trafficking of viral regulatory and structural proteins in and out of the nucleus and other cellular compartments requires the same type of ubiquitin and

ubiquitin-like modification that guide these processes in uninfected cells. In addition, viruses are obligate intracellular parasites that must exploit cellular metabolic processes for energy production and synthesis of proteins and nucleic acids whose regulators are controlled by the ubiquitin–proteasome system. Interference with these regulatory pathways is achieved through the production of multifunctional viral proteins that either mimic the activity of the cellular enzymes, often E3 ligases, or act as chaperones that redirect the activity of the cellular enzymes to new targets whose modification or destruction is required for successful virus infection. In addition, viral or cellular proteins may be rescued from proteasomal degradation by interfering with ubiquitination or with the degradation of ubiquitinated proteins by the proteasome. Finally, modulation of the efficiency and specificity of proteasomal processing is exploited by many viruses as a means of interfering with antigen presentation to protect the infected cells from immune attack. Examples of these viral strategies have been found in the life cycle of EBV (Figure 3.1).

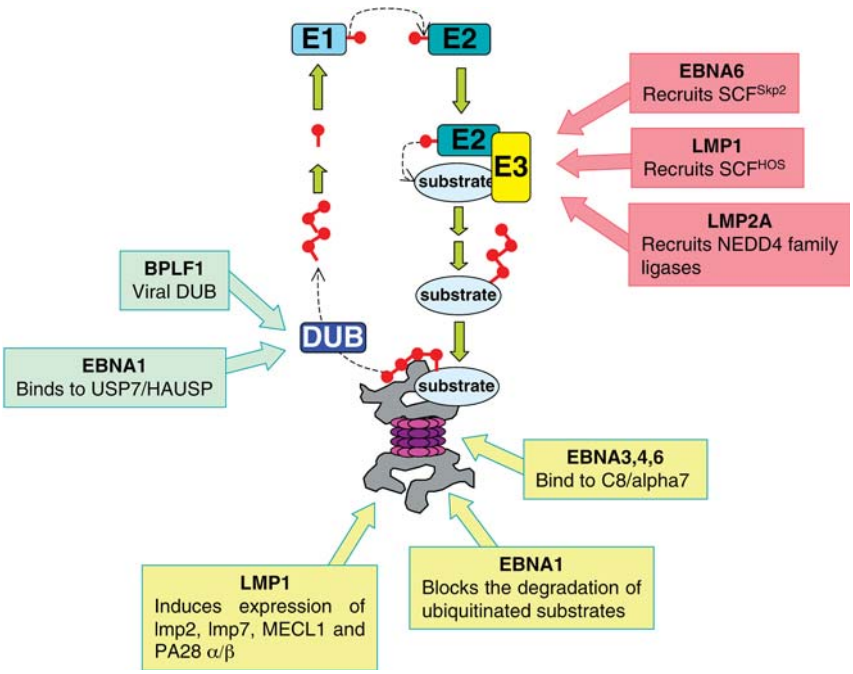

Fig. 3.1. EBV proteins that interfere with the ubiquitin–proteasome system. Proteins are targeted for proteasomal degradation by conjugation with ubiquitin, which requires the activity of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). The polyubiquitinated substrate is then bound to the proteasome which unfolds the substrate and cleaves the protein into small peptides. Ubiquitin is recycled through the activity of deubiquitinating enzymes (DUB). Different components of the ubiquitin–proteasome system are targeted by EBV proteins.

3.3 The EBV Life Cycle

EBV is a largely non-pathogenic virus that establishes persistent infections in over 90% of the adults worldwide (reviewed in [7]). Primary infection usually occurs during early childhood and is generally asymptomatic while delay until adolescence or adulthood is often associated with a benign self-limiting lymphoproliferative disease known as glandular fever, or infectious mononucleosis (IM). Like other herpesviruses EBV establishes productive or latent infections in different cell types. Epithelial cells of the oropharynx are believed to be the major site of virus replication while B-lymphocytes sustain mainly non-productive infections and are the site of persistence in healthy carriers. Infection of B-lymphocytes *in vitro* results in the expression of a restricted set of viral genes that are collectively known as the "latent" gene to distinguish them from the "lytic" genes that characterize the productive virus cycle. The latent genes encode six nuclear and three membrane-associated proteins known as EBV nuclear antigens (EBNA)-1, -2, -3 (or 3A), -4 (or 3B), -5 (or LP) and -6 (or 3C) and latent membrane proteins (LMP)-1, -2A and -2B, respectively (reviewed in [2]). In addition, two untranslated RNAs, the EBER-1 and -2 involved in the regulation of interferon production [8], and other RNAs of unknown function have been detected in all the infected cells [8, 9]. Expression of the latent EBV proteins is associated with growth transformation *in vitro* and *in vivo*, which underscores their capacity to interfere with cellular pathways that regulate B-cell proliferation and differentiation.

Healthy EBV-infected individuals carry between 1 to 100 latently EBV-infected B lymphocytes per million cells in the peripheral blood [10–12]. The EBV genome is not integrated in the host-cell DNA and the infected B-cells carry multiple autonomously replicating virus episomes. This, and the establishment of latency in B-lymphocytes which cycle between resting and activated proliferative states, pose a special challenge to the life-long maintenance of the virus reservoir. In the case of EBV this problem has been solved by the development of multiple programs of viral gene expression that are adapted to different stages of B-cell activation/differentiation (Table 3.1). In the absence of effective immune surveillance, as observed *in vitro* or *in vivo* during primary infection and in severely immunosuppressed individuals, EBV-infected B-cells express a latency program, called Latency III, which includes all nine latent viral proteins. This program is associated with autonomous B-cell proliferation and is exemplified by the lymphoblastoid cell lines (LCLs) that can be established by *in vitro* EBV infection of B-cells from virtually all individuals. LCL cells resemble B-blasts which have been activated by encounter with the cognate antigen; they secrete immunoglobulins and express several activation markers and adhesion molecules. It is believed that this growth-inducing latency program is required to expand the pool of infected cells before the establishment of effective immunity, increasing thereby, the likelihood of access to the memory B-cell compartment. Indeed, proliferating EBV-infected cells are highly sensitive to innate and specific immune responses and are only found in the blood of IM patients before the establishment of specific immunity.

Table 3.1. EBV-latency programs and their expression in normal B-lymphocytes and EBV-associated malignancies.

Viral program	Expressed viral genes		B cell type	Disease
	Proteins	RNAs		
Latency I	(LMP2A EBNA1)	(EBERs; BARF0)	Memory cells[a]	Burkitt's lymphoma[b]
Latency II	EBNA1; LMP1; LMP2A; -2B	EBERs; BARF0; miRNAs	Centroblasts (germinal centers)	Hodgkin's disease, Peripheral T cell lymphoma, Nasal T/NK cell lymphoma, Nasopharyngeal carcinoma, Lympho-epithelioma (stomach, salivary glands)
Latency III	EBNA1-6; LMP1; LMP2A; -2B	EBERs; BARF0; miRNAs	Lymphoblasts	Infectious Mononucleosis; AIDS-related immunoblastic lymphoma; Post-transplant lymphoproliferative disease

a LMP-2A mRNA may be the only viral transcript detected in circulating memory cells. Complete silencing of the viral genome is likely to occur in some memory cells.
b LMP-2A is usually not expressed in BL cells while some tumors express EBNA-3, -4 and -6.

The virus-infected cells that circulate in the blood of healthy EBV carriers are non-proliferating memory B-lymphocytes where the viral genome is completely silenced or viral gene expression is restricted to the LMP2 membrane proteins either alone, or together with the nuclear antigen EBNA1. This viral program is called Latency I [10, 13]. Memory B-cells are both long-lived and poorly immunogenic and are therefore an ideal viral reservoir but, due to their continuous circulation though different body milieus, they are also exposed to new encounters with their cognate antigens. This could reactivate the latent virus and trigger the productive cycle, which yields infectious virus but is regularly accompanied by cell death. While continuous low levels of virus production and infection of new B-lymphocytes could potentially assure the persistence of the virus for the entire life of the infected host, the demonstration that the same virus strain can be isolated from healthy carriers over decades suggests that most if not all latently-infected cells never undergo lytic replication. As discussed below, this is achieved through the capacity of LMP2A to interfere with signaling through the B-cell receptor. An intermediate form of latency characterized by the expression of EBNA1, LMP1 and LMP2 (Latency II), has been identified in germinal center B-lymphocytes [14, 15]. LMP1 regulates both B-cell activation and apoptosis, which allows the survival, expansion and further differentiation into memory cells of infected B-lymphocytes which will reach the lymphoid follicles (reviewed in [16]).

Since very few EBV-infected cells are found in blood or lymphoid tissues of healthy carriers, our knowledge of viral gene expression in these cells rests exclusively on the detection of viral transcripts by highly sensitive PCRs as there is no

evidence for protein expression. However, the expression of different combinations of latent viral proteins in different stages of B-cells activation/differentiation is strongly supported by studies of EBV-associated malignancies (Table 3.1). Thus, Latency III is expressed in the immunoblast-like cells of EBV carrying lymphoproliferative disorders that arise in severely immunosuppressed individuals, such as transplant recipients and AIDS patients [17], while Latency I is found in EBV carrying Burkitt's lymphoma (BL) whose cells are phenotypically similar to memory B lymphocytes [18]. In line with the germinal center cell origin of Hodgkin's Disease (HD) lymphomas, the EBV-positive forms of this tumor express Latency II (reviewed in [19]). This type of latency is also expressed in non-B-cell tumors of both hematopoietic and epithelial cell origin, including T cell lymphomas, NK cell lymphomas and hemophagocytic syndrome lymphomas, nasopharyngeal carcinoma (NPC) and lymphoepitheliomas originating from stomach, thymus and salivary glands (reviewed in [20, 21]). Each of the latency programs is likely to contribute in specific ways to the biology of the tumor in which it is expressed.

While studies on EBV-carrying cells of normal and tumor cell origin have yielded a wealth of information with regard to the mechanisms by which the virus manipulates the cellular environment during latency, our knowledge of other stages of the virus cycle is lagging behind due to the lack of an easily accessible *in vitro* model of lytic infection and difficulty in obtaining adequate amounts of infectious virus. This handicap is now being overcome with the help of recombinant DNA technologies, but major gaps still exist in our understanding of the early and late events of virus infection such as virus entry, uncoating and delivery of the viral DNA to the nucleus of the infected cells, the assembly of new virus and the release of infectious virus particles.

3.4 EBV and the Ubiquitin–Proteasome System

3.4.1 EBNA1

Because of its specific role in the virus life cycle, EBNA1 is the only viral protein expressed in all types of EBV-infected cells (Figure 3.2). Dimers of EBNA1 bind to the dyad symmetry and family of repeat sequences in the origin of latent plasmid replication (oriP) and coordinate the replication of the viral episomes with cellular DNA and their partitioning during cell division. EBNA1 is also a transcriptional regulator that acts on the two major latent promoters for EBNA transcription, Wp and Cp, and on its own latent promoter Qp (reviewed in [22]). Most of the identified functional domains of EBNA1, including a nuclear localization signal, dimerization and DNA binding domains, reside in the C-terminal half of the protein, while most of the N-terminal half is occupied by a Gly-Ala repeat (GAr) that varies in length between different EBV isolates [23].

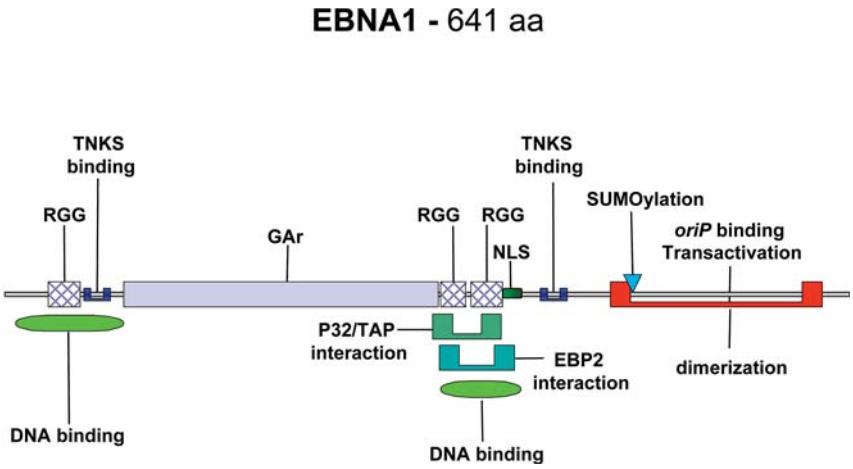

Fig. 3.2. Schematic representation of functional domains in EBNA1. EBNA1 is required for *oriP*-dependent virus replication and partitioning of the viral episome to replicating latently infected cells. These activities are regulated by interaction with tankyrase (TNKS) that also mediates poly-ADP ribose (PAR)-modification of EBNA1. Interaction of EBNA1 with EBNA1-binding protein 2 (EBP2) is required for tethering the viral episomes to the cellular mitotic chromosomes. The arginine–glycine repeat (RGG) domains are involved in binding to RNA. EBNA1 contains one nuclear localization signal (NLS) and one putative SUMOylation site. Interaction with p32/TAP is associated with the translocation of EBNA1 to the cytoplasm.

The regular or even exclusive expression of EBNA1 in EBV-associated malignancies makes it an ideal target for tumor-specific immune intervention, which has prompted an intensive search for specific T-cell responses that could be selectively boosted in cancer patients. This search was initially frustrated by the failure to identify EBV-specific CTLs capable of recognizing cells that express EBNA1 through recombinant vaccinia or adenovirus vectors (reviewed in [24]). This peculiar feature of EBNA1 was shown to be due to the presence of the GAr since removal of this domain resulted in accelerated protein turnover and efficient presentation of endogenous epitopes [25, 26], and reconstituted the capacity of EBNA1 to trigger specific rejection responses in a mouse tumor model [27]. Later studies have demonstrated that EBNA1-specific effectors exist but are either MHC class II restricted CD4 positive T-cells (reviewed in [28]), or MHC class I restricted CD8 positive T-cells that recognize epitopes derived from recombinant EBNA1 exogenously fed to antigen-presenting cells through an as yet poorly characterized cross-talk process between the endocytic – MHC class II restricted – and exocytic – MHC class I restricted – pathways of antigen processing [26]. More recent studies using highly sensitive methods capable of detecting small amounts of cytokines produced by individual effector cells have finally confirmed that MHC class I restricted epitopes can be generated from endogenously expressed EBNA1 [29–31], although processing appears to be less efficient than that for regular endogenous antigens. Several possible explanations can be envisaged to explain this escape from the

GAr-induced blockade of proteasomal processing. The EBNA1 epitopes may be produced from Defective Ribosomal Products (DRiPs) that, unlike the intact EBNA1, are targeted for proteasomal degradation [32]. Although attractive, this explanation does not account for the observation that the majority of the identified MHC Class I restricted epitopes in EBNA1 are located in the C-terminus of the protein, downstream of the GAr, and should therefore also be protected in the context of DRiPs. A more challenging possibility has been suggested by the recent finding that endogenous EBNA1 may gain access to the exocytic pathway of antigen processing by autophagy [33]. Thus, EBNA1 accumulates slowly in cytosolic autophagosomes after the inhibition of lysosome acidification while inhibition of autophagy decreases the presentation of MHC class II restricted epitopes to CD4-positive T-cell clones. It remains to be seen whether this pathway might also be involved in the generation of MHC class I restricted epitopes.

While the biological significance of the GAr in the context of EBV-specific immune surveillance awaits further clarification, elucidation of its mechanism of action is particularly interesting since this is the first example of a protein domain that blocks antigen presentation and this feature could be exploited in immunological and gene therapy settings. Using an *in-vitro* processing assay based on *in-vitro* translated substrates it was shown that the GAr is a specific inhibitor of ubiquitin–proteasome-dependent proteolysis [34] and acts as modular transferable element that can abrogate or severely inhibit the degradation of a variety of viral and cellular proteasome substrates. Several characteristics of this stabilization signal were resolved by using a set of chimeric reporters involving IκB [35], p53 [36], and green fluorescent protein (GFP)-based proteasome substrates [37]. The activity of the GAr was shown to be largely independent of its location in the target protein [34–37] and was not restricted by the type of ubiquitin ligase involved in substrate modification [35–37]. Fusions of the GAr to green fluorescent protein (GFP)-based reporters that are targeted for degradation with varying efficiency showed that the GAr counteracts the degradation signal in a length-dependent manner [37]. EBNA1 itself could also be targeted for ubiquitin-dependent proteolysis using a strong degradation signal, which resulted in the efficient presentation of EBNA1-derived CTL epitopes [38]. The only requirement for activity appears to be the presence of a sufficiently long stretch of alanines or similar small hybrophobic residues, preferably interspersed by one, two or three glycine residues which may act by increasing solubility [39]. This, together with the demonstration that ubiquitinated GAr containing IκB cannot form stable complexes with the proteasome [35], and that the repeat does not affect the interaction of ubiquitinated substrates with the S5a ubiquitin-binding subunit of the 19S cap [36, 40], suggests that the hydrophobic domain produced by the GAr may directly affect the interaction of the substrate with the proteasome. This possibility is supported by the demonstration that a synthetic GAr peptide could inhibit the degradation of biotinylated lysozyme *in vitro* [40]. Interestingly, GAr-containing chimeras were also shown to be protected from proteasomal degradation in yeast [41, 42], suggesting that the viral repeat targets a conserved step in proteasomal processing. By embed-

ding the GAr within ornithine decarboxylase (ODC), a natural proteasome substrate that does not require ubiquitin conjugation, Zhang and Coffino demonstrated that the GAr acts as a stop signal for proteasome processing *in vitro*, resulting in partial proteolysis [42]. Introduction of the GAr into an ODC degradation domain-destabilized GFP, led to the accumulation of degradation products that still contain the repeat, suggesting that the GAr may interfere with the unfolding activity of the proteasome, which could halt degradation. This possibility was recently substantiated by the demonstration that the production of intermediates is influenced by the position of the GAr relative to a folded domain within the substrate. The spacing between the GAr and a downstream folded domain appears to be critical for intermediate production [43]. These findings support a model whereby positioning of the GAr domain within the ATPase ring reduces the efficiency of nucleotide hydrolysis and substrate unfolding. If this impairment takes place, insertion pauses and proteolysis are limited to the portion of the substrate that has already entered the catalytic chamber of the proteasome.

The finding that presentation of EBNA1 epitopes can occur in spite of the protective activity of the GAr, points to a non-immunological role for the effect of the repeat on EBNA1 stability. Indeed, using both affinity chromatography and TAP-tagging approaches it was recently shown that EBNA1 interacts with the ubiquitin-specific protease USP7 [44, 45], also known as herpes virus-associated ubiquitin-specific protease, HAUSP. This DUB was first identified by virtue of its interaction with the ICP0 protein of herpes simplex virus type 1 [46], a viral E3 ligase that is required for efficient initiation of the HSV-1 lytic cycle [47]. An EBNA1 mutant defective for USP7 binding exhibited the long half-life and lack of MHC class I presentation typical of wild-type EBNA1, indicating that USP7 is not directly involved in the regulation of EBNA1 turnover. However, disruption of USP7 binding enhanced the replication of an EBV *ori*P-containing plasmid. This may be due to a direct effect of USP7 on the ubiquitination of EBNA1 or of other cellular substrates that interact with, and regulate the activity of *oriP*. Indeed, tankirase-1 (TRF1), a negative regulator of telomere length that also interacts with *oriP* and binds to EBNA1 inhibiting *oriP*-dependent replication [48, 49], is a substrate for ubiquitin-dependent proteolysis [50]. Only the telomere-unbound form of TRF1 is ubiquitinated and degraded, suggesting that specific rescue of this protein bound to *oriP* could play an important role in the regulation of latent EBV replication. It is also possible that binding to EBNA1 may affect cellular functions that are normally regulated by USP7. Recent evidence indicates that USP7 is a key regulator of p53 and Mdm2 [51]. EBNA1 and p53 bind to the same pocket in USP7 but p53 makes less extensive contacts resulting in significantly lower binding affinity [45, 52]. Thus, EBNA1 could efficiently compete for p53 binding and prevent its deubiquitination, which would promote Mdm2-dependent degradation. Functional studies indicated that binding of EBNA1 to USP7 can protect cells from apoptosis by lowering the levels of p53 [52], providing a structural and conceptual framework for understanding how EBNA1 might contribute to the survival of EBV-infected cells.

3.4.2 EBNA6 (EBNA3C)

The progression of the cell cycle and stability of cell cycle checkpoint proteins is controlled by the ubiquitin–proteasome system. A critical regulator of cell cycle molecules is the Skp1/Cul1/F-box E3 ligase SCF^{Skp2} that mediates the polyubiquitination and degradation of E2F and several E2F transcriptional targets, including p27 and c-Myc. A link between the EBV nuclear antigen EBNA6 (EBNA3C) and SCF^{Skp2} was recently demonstrated, providing a new insight into the mechanism for cell cycle regulation by EBV (Figure 3.3). EBNA6 is one of three high molecular weight EBNAs encoded in the BamHI-E region of the EBV genome and was shown to be essential for B-cell immortalization [53]. Transfection of EBNA6 into EBV-negative cells of lymphoid and epithelial cell origin was shown to correlate with decreased Rb protein levels [54]. EBNA6 forms a stable complex with Rb in cells treated with inhibitors of the proteasome and interacts with Rb *in vitro* through a conserved motif within amino acids 140–149 that has been linked to the regulation of SCF^{Skp2}. Indeed, transfection of a dominant negative SCF^{Skp2} reduced the ability of EBNA6 to promote the degradation of Rb. SCF^{Skp2} has no detectable effect on Rb levels in the absence of EBNA6, suggesting that EBNA6 may specifically usurp this ligase to enhance Rb degradation. Capture of SCF^{Skp2} by EBNA6 may have additional effects on the regulation of cell cycle progression since EBNA6 also associates with the cyclin A/cdk2 complexes through a small region between amino acids 130 and 159 that shows high affinity for the conserved mammalian cyclin box in amino acids 206 to 226 of cyclin A [55]. Binding of EBNA6 to cyclin

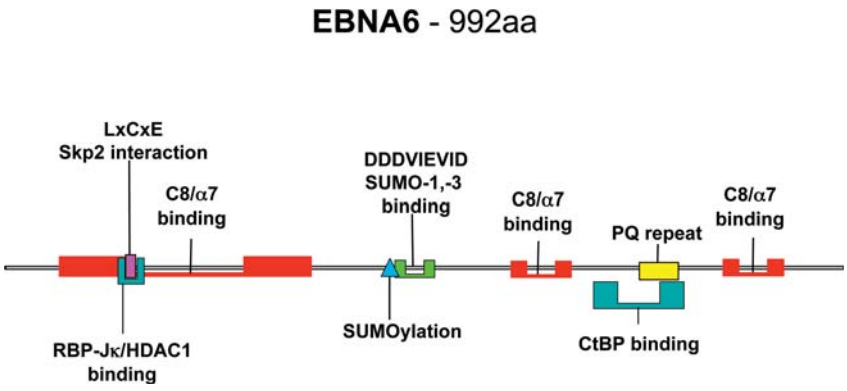

Fig. 3.3. Schematic representation of functional domains in EBNA6. EBNA6 (EBNA3C) is one of three nuclear antigens encoded in the BamHI E region of the viral genome and cooperates with EBNA2 in the regulation of viral and cellular genes. The RBP-Jκ, HDAC1 and CtBP binding sites, involved in transcription regulation, and the glutamine–proline repeats are indicated. EBNA6 interacts with SCF^{Skp2}, with SUMO-1 and SUMO-3 and contains one putative SUMOylation site. In addition, EBNA6 interacts with the C8/α7 subunit in the α-ring of the 20S proteasome.

A induces SCF^{Skp2}-dependent degradation of p27 and enhances the activity of the kinase [56].

In addition to its effect on cellular proteins, EBNA6 participates in the regulation of viral gene expression and cooperates with EBNA2 in transactivating the LMP1 promoter [57]. A region of the protein between amino acids 365 and 545, that was shown to be necessary and sufficient for LMP1 coactivation, interacts with SUMO-1 and SUMO-3 in yeast two-hybrid screens. This region is also required for localization of EBNA6 to PML bodies and for modification by SUMO-1, SUMO-2, and SUMO-3, but neither of these functions appears to be necessary for transcriptional coactivation [58]. Furthermore, coactivation was inhibited by mutation of a short sequence between amino acids 509 and 515 (DDDVIEVID) which bears close similarity to residues 84–90 of SUMO-1 (EEDVIV) and the resulting mutants lost the capacity to bind to SUMO-1 and SUMO-3. This conserved region is important for the binding of SUMO to UBC9 and ULP-1 [59] and a similar motif is found in the E1 enzymes of plants [60] and in SNF2 domain proteins that are involved in chromatin remodeling and transcriptional regulation [61], suggesting that it may mediate binding to SUMOylated substrates. It has been speculated that through this motif EBNA6 may bind to a repressor, such as HDAC-1, and inhibit its effects on another transcription factor(s) at EBNA2-regulated promoters.

A yeast two-hybrid screen using EBNA6 as bait revealed an interaction with the C8 (alpha7) subunit of the 20S proteasome [62]. The interaction was confirmed in glutathione S-transferase (GST) pull-down experiments that also revealed interaction between C8 and the two other members of the high molecular weight EBNA family: EBNA3 (EBNA3A) and EBNA4 (EBNA3B). Co-immunoprecipitation of the EBNA3 proteins with C8/alpha7 was also demonstrated after transfection of expression vectors into B cells. The interaction between these viral proteins and GST-C8 appears to be more robust than the interaction between C8 and the cyclin-dependent kinase inhibitor p21(WAF1/CIP1), which results in degradation of p21 by the 20S proteasome [63]. Consistent with their ability to bind directly to the 20S proteasome, the EBNAs were degraded *in vitro* using purified 20S proteasomes but the significance of this finding is unclear since the viral proteins have a relatively long half-life in EBV-infected cells. It remains to be seen whether these interactions serve any other purposes, such as for example, the targeting of specific cellular substrates to the proteasome.

3.4.3 LMP1

Expression of the Latency III program is potentially unfavorable for EBV since the uncontrolled proliferation of virus-infected cells could kill the host, as indeed happens in patients suffering from severe congenital or iatrogenic immune deficiencies. To counteract the hazard, viral proteins expressed in Latency III increase the immunogenicity of the infected cells by regulating the expression of adhesion and co-stimulatory molecules and enhance the activity of various components of

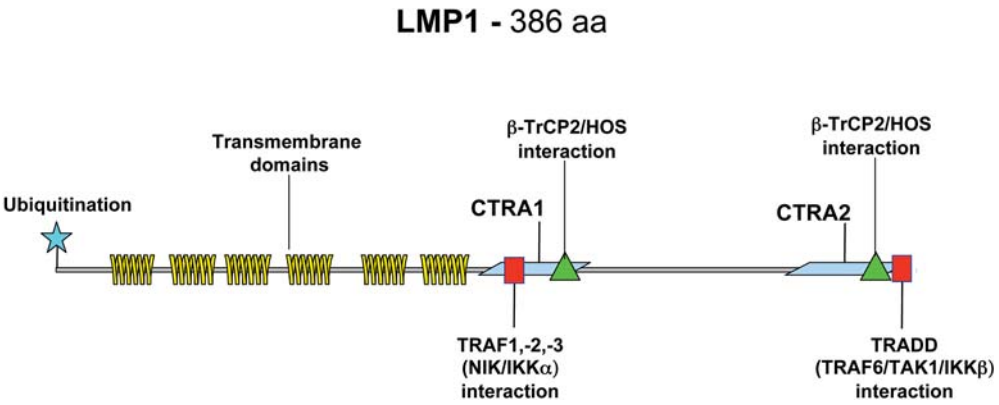

Fig. 3.4. Schematic representation of functional domains in LMP1. LMP1 is one of two EBV membrane proteins expressed in latently-infected cells and mediates NF-κB activation by acting as a constitutive receptor of the CD40/TNFR family. Two C-terminal activating regions (CTAR) are involved in NF-κB activation through the canonical (TRADD, TRAF6, TAK1, IKKβ) and alternative (TRAF2/NIK/IKKα) pathways. LMP1 is ubiquitinated at the N-terminus and contains two SCF^{HOS} binding sites.

the antigen presentation machinery, which renders the infected cells easily attacked by the immune responses. LMP1 plays a pivotal role in this regulation by interfering with a major effector of lymphoid cell activation and function, the NF-κB signaling pathway (Figure 3.4).

LMP1 is the only EBV protein with recognized oncogenic activity. Transfection in mouse or human fibroblasts and epithelial cells confers tumorigenicity in immunosuppressed animals [64–66] and LMP1 transgenic mice develop hyperproliferations and lymphomas [67, 68]. LMP1 contains a short N-terminal cytoplasmic domain followed by six membrane-spanning domains and a large cytoplasmic C-terminal domain that is involved in signaling (reviewed in [2]). Through the transmembrane domain LMP1 forms multimers that localize to lipid rafts together with LMP2A and a variety of cellular proteins involved in B-cell proliferation and function [69]. LMP1 acts as a constitutive receptor of the CD40/tumor necrosis factor-receptor (TNFR) family and induces expression of the NF-κB and JNK transcription factors [70, 71]. Similar to TNFR, LMP1 binds to TRAF1, TRAF2 and TRADD, and activates the NF-κB-inducing kinase (NIK) and the IkB kinases (IKKα and IKKβ) [72]. Activated IKK phosphorylates IκB, which leads to recognition by the WD40 domains of F-box proteins belonging to the β-TrCP family, including β-TrCP1 and β-TrCP2 (also termed FWD1/Fbw1a and HOS/Fbw1b). These F-box proteins interact with Skp1, Cullin1, and Roc1/Rbx1 to form the $SCF^{b\text{-}TrCP/HOS}$ E3 ubiquitin ligases (reviewed in [73]) that ubiquitinate IκB, which results in proteasomal degradation of the inhibitor and activation of NF-κB-dependent transcription [74]. Recent findings suggest that the regulation of NF-κB signaling by LMP1 may be more complex than originally thought. The LMP1

protein encoded by the prototype B95.8 strain of EBV was shown to interact with the HOS subunit of SCF^{HOS} via one canonical and one cryptic HOS recognition site [75]. Mutations of these sites abrogated HOS binding and increased the transforming activity of LMP1, which correlated with its increased ability to induce IκB degradation and NF-κB-mediated transcription without further activation of IKK. Furthermore, overexpression of HOS in cells expressing the B95.8 LMP1 enhanced the degradation of IκB and activation of NF-κB without significant effect on the stability of the viral protein. Thus, the B95.8 LMP1 appears to act as a pseudosubstrate for SCF^{HOS}, which by reducing the levels of endogenous HOS available to interact with phosphorylated IκB, may result in fine-tuning of LMP1-induced NF-κB signaling. Interestingly, the HOS interacting domains in LMP1 are mutated or deleted in the NPC-derived LMP1-Cao variant that exhibits enhanced tumorigenic properties in epithelial cells [64], providing an interesting clue to the possible role of LMP1 mutations in malignancies.

Several components of the antigen presentation pathway are also upregulated in LMP1-expressing cells including the transporters associated with antigen processing (TAPs, [76, 77] and some subunits of the proteasome [78], which results in enhanced enzymatic activity of the proteasome and altered cleavage specificity, thereby promoting the antigenicity of EBV-infected cells. In addition, LMP1 itself is a short-lived protein [79] and a substrate of ubiquitin-dependent proteolysis, which generates several epitopes for MHC class I restricted T-cell recognition of EBV-infected cells [80]. However, LMP1 also contains two epitopes with strong homology to an immunosuppressive domain found in a retroviral membrane protein, that strongly inhibits the activity of both cytotoxic T-lymphocytes and natural killer cells *in vitro* [81]. Thus, processing of LMP1 may suppress EBV-specific immune responses, as was shown to occur at the tumor site of EBV positive HD lymphomas [82]. The mechanism of ubiquitin-dependent processing of LMP1 is also quite puzzling since the single lysine residue in the C-terminal domain of the LMP1 encoded by the prototype B95.8 EBV strain, is often mutated in wild-type EBV isolates and, furthermore, is not required for ubiquitination. Minor modification of the N-terminus resulted in full stabilizations, confirming that this is the site for ubiquitin conjugation [83]. This unusual ubiquitination linkage has been described for only a few cellular substrates that either do not contain lysine residues or whose lysine residues may be inaccessible (reviewed in [84]). Interestingly, N-terminal ubiquitination is also involved in the degradation of the second EBV membrane protein, LMP2 [85]. The two EBV proteins co-localize in the cell membrane and it is therefore possible that the same ligase may be involved in their ubiquitination.

3.4.4 LMP2

Studies with EBV deletion mutants show that LMP2A and its N-terminal truncated variant LMP2B are not required for B-cell transformation [86] (Figure 3.5). Yet, the regular expression of these proteins in the most restricted forms of latency

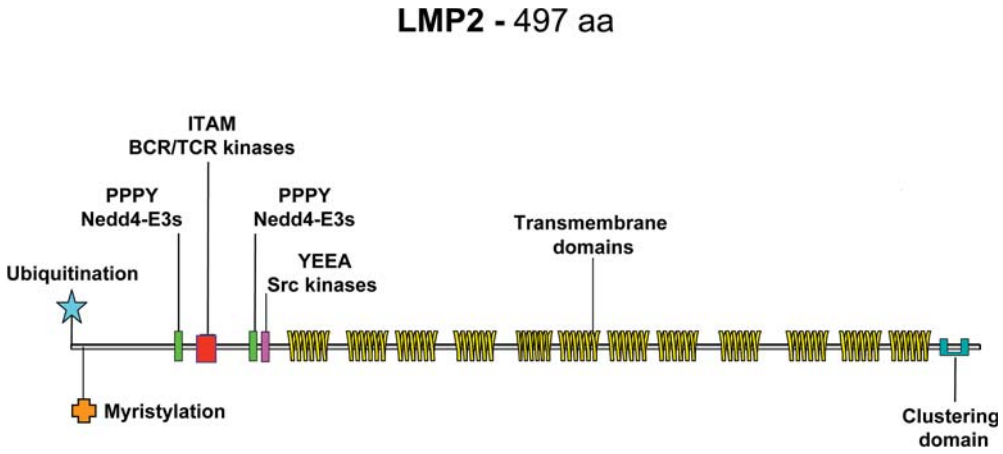

Fig. 3.5. Schematic representation of functional domains in LMP2. LMP2 is the second EBV-encoded membrane protein expressed in latently infected cells. It interferes with BCR/TCR signaling by acting as a scaffold for binding BCR/TCR-associated and Scr-family kinases and Nedd4-family ubiquitin ligases. LMP2 is ubiquitinated at the N-terminus.

suggests that they fulfill an important function in the biology of EBV infection. EBV persists in B-lymphocytes that are also the primary site of virus reactivation. The mechanisms that lead to the breakdown of latency are poorly understood but studies with tumor cell lines suggest that triggering the B-cell receptor (BCR) plays a critical role [87]. It has been proposed that LMP2A regulates this process through its ability to inhibit BRC signaling [88, 89] although recent studies question this scenario since LMP2A was shown to activate B-lymphocytes and induced antibody production and plasma cell differentiation in a transgenic mouse model [90]. The molecular mechanism by which LMP2 interferes with B-cell receptor signaling has been extensively studied. It has been shown that the N-terminus of LMP2A contains several ITAM motifs that mediate in the interaction with the SH2-domain of the BCR-associated tyrosine kinases Lyn and Syk, and two Pro-Pro-Pro-Pro-Tyr (PY) motifs that are known to interact with proteins containing WW modules [91]. In screening for interacting proteins two laboratories have independently identified members of the Nedd4 ubiquitin ligase family [92, 93]. These include AIP4/Itchy, WWP2/AIP2 and KIAA0439 which contain a C-terminal HECT domain. Binding of these E3s to LMP2A induces ubiquitination of Lyn and Syk and this correlates with the accelerated degradation of Lyn. Ubiquitination of Syk does not appear to affect its turnover although accelerated degradation of a small pool of activated Syk cannot be excluded. Other ligases are also involved in this signaling cascade. Phosphorylation of Syk causes constitutive activation of the Syk substrate SLP-65 (SH2 domain-containing leukocyte protein-65), which in turn induces the formation of a ternary complex composed of the ubiquitin ligase Cbl, C3G and

the protooncogene CrkL (CT10 regulator of kinase-like enzyme) [94]. Cbl-b ubiquitinates Syk, which is important for its function as a negative regulator of BCR signaling [95]. LMP2A itself is specifically ubiquitinated by AIP4 and WWP2 at the N-terminus and this is required for modulation of BCR signaling. While further studies are needed to clarify the significance of LMP2A-induced ubiquitination of Lyn and Syk, these data confirm that the expression of scaffolds that recruit components of the ubiquitin–proteasome system is a common viral strategy for selective inactivation of cellular substrates.

LMP2A is also part of the Latency II program expressed in non-B cell tumors and its expression was shown to enhance the metastatic potential of NPC. The effect in T lymphocytes appears to be analogous to that observed in B cells since LMP2A was shown to interact with the T-cell receptor-associated tyrosine kinases Lck, Fyn and ZAP-70/Syk and stable expression of LMP2A downregulated T-cell receptor levels and signaling in the T cell line Jurkat [96]. Recruitment of IAP4 via the PPPPY motifs was required for LMP2A ubiquitination and regulated the stability of LMP2A and LMP2A–kinase complexes. In epithelial cells, LMP2A activates the phosphatidylinositol 3 -OH kinase/Akt and β-catenin signaling pathways [97, 98]. The ITAM was essential for the activation of Akt by LMP2A in human foreskin keratinocyte (HFK), while both the ITAM and PPPPY motifs contributed to LMP2A-mediated accumulation and nuclear translocation of β-catenin [99]. Furthermore, the PPPPY motifs were critical for LMP2A-mediated inhibition of epithelial cell differentiation in semi-solid methylcellulose medium. LMP2A was also shown to influence the adhesion and migration and invasiveness of epithelial cells, possibly by regulating the expression of the epithelial cell integrin α6β4 [100]. An unexpected role of the Syk kinase in this phenotype was recently suggested by the finding that Tyr 74 and 85 in the ITAM of LMP2A are essential for both Syk activation and LMP2A-induced epithelial cell migration [101]. It remains to be seen whether IAP4, c-Cbl or other E3 ligases participate in this regulation.

3.4.5
BZLF1 (Zta) and BRLF1 (Rta)

The immediate early proteins of EBV, BZLF1 (Zta) and BRLF1 (Rta), mediate the switch from latent to productive infection by activating the transcription of early and late viral genes as well as a variety of cellular genes that are required for progression of the lytic virus cycle (reviewed in [102]). The molecular pathways leading to this switch are only partially understood but a common downstream event, for EBV as for other herpes viruses, is the dispersion of promyelocytic leukemia bodies (PML, ND10) in the nucleus of the host cell [103]. The PML protein is induced by interferon, involved in major histocompatibility complex class I presentation, and necessary for certain types of apoptosis [104]. Modification of PML by SUMO-1 is known to be required for the formation of PML bodies [105]. Both Zta [106] and Rta [107] are modified by SUMO-1 and Rta was shown to interact with the SUMO E2 conjugase, Ubc9 and E3 ligase, PIAS1, in yeast two-hybrid

screens, GST-pull-down assays, co-immunoprecipitation, and confocal microscopy. Expression of Zta and Rta induces PML dispersion in EBV-positive cells but Zta alone is sufficient to produce this effect in EBV-negative cell lines. Similar to the corresponding proteins in HSV-1 (ICP0) and CMV (IE1), Zta reduces the amount of SUMO-1-modified PML, probably by competing with PML for limiting amounts of SUMO-1. Furthermore, the capacity of Rta to transactivate a reporter plasmid containing a specific responsive element was greatly increased by SUMOylation of Lys19 [107]. Thus, SUMO-1 modification of these viral immediate early proteins appears to promote lytic EBV replication by enhancing their transactivating activity as well as by modulating the function of cellular proteins that are targeted by SUMO-1 modification.

3.4.6 BPLF1

Recent reports describing the identification of genes encoding for proteins with DUB activity in adenovirus [108], SARS corona virus [109–111] and human herpes viruses [112], demonstrated that this step of the of the ubiquitin-dependent proteolytic machinery is also specifically targeted during viral infection. DUB activity was demonstrated in the N-terminal 500 residues of the largest tegument protein of herpes simplex virus 1 (HSV-1), UL36, as well as in the UL36 homologs of other herpesviruses, including the EBV-encoded BPLF1 protein [112]. These proteins are expressed late in the replication cycle of herpes viruses and are essential for the production of infectious virus particles [113]. UL36 has been implicated in a sequence-specific interaction with the viral genome [114] and studies carried out using a temperature-sensitive mutant suggest that it plays a role in the release of viral DNA from incoming nucleocapsids, viral DNA synthesis, and late gene expression [115]. The role of its DUB activity in these processes is not understood. The ubiquitination machinery regulates the membrane protein trafficking systems involved in the formation of caveolae and multivesicular bodies (MVBs) that are required for the entry and exit of some viruses from infected cells [116, 117]. At least two DUBs, AMSH and UBPY, regulate the activity of MVBs. Both DUBs interact with ESCRT proteins via STAM (signal transducing adaptor molecule) and show specificity for Lys63- and Lys48-linked polyubiquitin chains *in vitro*. AMSH (associated molecule with the SH3 domain of STAM) binds to clathrin and to mVps24/CHMP3, a component of ESCRT III complex, and is markedly stimulated by STAM, indicating that activation is coupled to association with the MVB-sorting machinery [118]. RNA-mediated knockdown of UBPY results in increased global levels of ubiquitinated protein and accumulation of ubiquitin on endosomes, while UBPY-depleted cells have more and larger MVBs [119]. Perhaps herpes viruses exploit the deubiquitinating activity encoded by UL36, and its homologs that are produced in the final stages of virus replication, to manipulate ubiquitin modifications during viral egress. Interestingly, the viral enzymes do not show strong homology with eukaryotic DUBs, making this new family of ubiquitin-specific proteases an attractive target for selective inhibition.

3.5 EBV-associated Malignancies

Specific genetic alterations contribute to the pathogenesis of EBV-associated malignancies. This concept is clearly illustrated by BL, a highly malignant B-cell tumor that is characterized by the regular presence of chromosomal translocations involving *c-myc* on chromosome 8 and one of the immunoglobulin heavy or light chain genes on chromosomes 14, 2 or 22 [120]. EBV-carrying BL is the most frequent childhood malignancy in endemic malaria areas of subtropical Africa and Papua New Guinea and occurs at a 100-fold higher frequency in AIDS patients. The role of EBV as a co-factor in the pathogenesis of this malignancy is supported by the observation that a genetically and phenotypically similar but EBV-negative variant of the tumor occurs worldwide at a much lower frequency. The EBV-positive tumors express only EBNA1 [18] or, in some cases, EBNA-1 and the high molecular weight EBNA-3, -4 and -6 [121]. The B-cell proliferation and activation-associated viral proteins, EBNA2 and LMP1 are not expressed and, as a result, EBV-positive BL cells do not express B-cell activation markers, adhesion or co-stimulatory molecules and grow as single cell suspensions rather than in clumps [18]. Also characteristic of the tumor cells are the low levels of MHC class I and selective loss of certain class I alleles, HLA A11 in particular [122], which may contribute to the poor immunogenicity of these cells [123]. In addition BL cells are deficient in their ability to present endogenous antigens [77], which correlates with the downregulation of the interferon- -inducible subunits of the proteasome and the peptide transporters TAP1 and TAP2 [76, 78]. Studies on *in vitro* EBV-transformed cell lines that carry inducible EBNA2 and *c-myc* genes and recapitulate the viral and cellular gene expression program of BLs, have shown that overexpression of c-Myc in cells expressing Latency I drives the BL cell phenotype [124].

Surprisingly, in spite of their apparent defect of proteasome function, BL cells were shown to be resistant to treatment with doses of proteasome inhibitors that are readily toxic for LCL cells [125]. Since the turnover of short- and long-lived protein proceeded virtually undisturbed in BL cells treated with the proteasome inhibitors, other proteases likely contribute to the regulation of protein turnover. Indeed, DUB activity and the serine protease tripeptidyl peptidase (TPP)-II, were shown to be overexpressed in BL cells and were upregulated on induction of c-Myc. The upregulation of DUBs may offer a selective advantage in cells with poor proteasome activity since the free polyubiquitin chains that may accumulate as a consequence of slow proteasomal processing are highly toxic. Furthermore, DUBs may also be involved in regulating the turnover of specific substrates that control the survival of these cells. Analysis of DUB expression and activity using a set of functional probes followed by mass spectrometry revealed consistent differences between BL and LCL cells. In particular, USP7/HAUSP was highly expressed in LCLs while UCH-L1 was regularly overexpressed in the tumor-derived cell line [126]. UCH-L1, also known as PGP9.5, is an abundant neuronal protein and mutations at or around the putative catalytic site have been associated with familial

Parkinson's disease [127] and with other neurodegenerative disorders characterized by the formation of protein aggregates including spinocerebellar ataxia [128] and Huntington's disease [129]. In addition, high levels of UCH-L1 have been detected in a variety of human malignancies including neuroblastoma [130], colon carcinoma [131], non small-cell-lung carcinoma [132, 133], pancreatic carcinoma [134], prostate and breast carcinomas [135, 136] and renal carcinoma [137] and appear to be associated with the more malignant and invasive forms of these tumors. The physiological targets of UCH-L1 are unknown. *In vitro*, UCH-L1 acts as a C-terminal hydrolase for ubiquityl esters and amides but has no activity with regard to larger ubiquitin conjugates [138]. In addition, recent evidence suggests that, depending on its level of expression and capacity to form homodimers, UCH-L1 may act as an E3 ligase [139]. Interestingly, UCH-L1 was shown to interact with Ubc9, RanBPM and Jab1 in a yeast two-hybrid screen [140]. Ubc9 is the E2 for SUMO and RanBPM is a regulator of the small G protein Ran [141] while Jab1 is a Jun activation domain-binding protein that can bind to p27(Kip1) and is involved in the cytoplasmic transport of p27(Kip1) for its degradation [142]. Jab1 is also a component of the COP9 signalosome [143] and acts as a Cullin de-Neddylase [144]. UCH-L1 co-localizes with Jab1 in the perinuclear cytoplasm of contact-inhibited cells while, under serum-re-stimulation, nuclear translocation of both UCH-L1 and Jab1 coincides with a reduced level of p27(Kip1) in the nucleus. Therefore, UCH-L1 may contribute to p27(Kip1) degradation via its interaction and nuclear translocation with Jab1.

Recent findings suggest an unexpected role for TPPII in the phenotype of BL cells. TPPII is an evolutionarily conserved cytosolic serine protease of the subtilisin family that removes tripeptides from the free N-terminus of oligopeptides [145–149]. The 138-kDa subunit of TPPII forms large oligomeric complexes of 5–9 MDa with a twisted ribbon structure that are detected in the cytoplasm and associate with the plasma membrane of most cell types [145, 146, 150, 151]. It has been proposed that TPPII may act downstream of the proteasome to accelerate the production of free amino acids from longer precursors by generating tripeptide intermediates that are easily degraded by other cellular exopeptidases [152]. In addition, TPPII appears to play a more specific function in the processing of certain cellular substrates as it is the main cholecystokinin-inactivating enzyme in rat brain [151] and regulates apoptotic responses by promoting the maturation of procaspase-1 in macrophages infected with the enteropathogenic bacterium *Shigella flexneri* [153]. Recent evidence suggests that TPPII may have a role in antigen processing [154, 155], probably through its capacity to cleave long peptides fragments produced by the proteasome [154, 156]. The observations that TPPII is overexpressed in cells adapted to grow in the presence of lethal concentrations of proteasome inhibitors [150, 157, 158], rescues transfected cells from acute intoxication with these inhibitors [150, 152, 157] and increases the survival and tumorigenicity of proteasome inhibitor adapted cells in a mouse lymphoma model [159], suggest a possible role for this enzyme in tumor progression. Indeed, TPPII overexpression correlates with accelerated proliferation and accumulation of centrosome and chromosome aberrations in BL as well as in transfected 293HEK cells,

whereas functional knockdown of TPPII by shRNA resulted in growth retardation and the generation of polynucleated cells that failed to complete cell division [160]. Furthermore, TPPII overexpressing cells evaded mitotic arrest induced by spindle poisons and displayed high levels of polyploidy despite the constitutively high expression of major components of the spindle checkpoints [161]. This was accompanied by upregulation of inhibitors of apoptosis (IAPs) and resistance to p53-induced apoptosis, suggesting that TPPII may allow the transit through mitosis and the survival of cells with severe mitotic spindle damage. Collectively, these findings suggest that TPPII may participate in the regulation of critical events that control the homeostasis of cell division. In particular, the accumulation of centrosome abnormalities and multipolar spindles together with the capacity to overcome spindle checkpoints, point to a possible involvement of TPPII in the early phases of mitosis, at or around the time of centrosome duplication.

3.6 Concluding Remarks

The unraveling of the different strategies by which EBV exploits and manipulates ubiquitin/proteasome-dependent proteolysis, together with a better understanding of how these manipulations assist the virus during productive infection, latency and malignant transformation, will provide new strategies for meeting the challenge of this potentially oncogenic viral infection. Interference with the interaction between LMP2A and BCR/TCR-associated tyrosine kinases may release the blockade of productive infection and promote the elimination of virus-infected cells either directly or indirectly, through the exposure of numerous highly immunogenic epitopes that could target the infected cells for destruction by CTLs. Induction of EBNA1 processing may also sensitize the infected cells to CTL-mediated rejection since EBNA1-specific precursors are present in EBV carriers. It is likely that the endocytic, exocytic and autophagic pathways of antigen processing play different roles in different cell types or pathologic conditions and a better understanding of the interplay between these modes of protein processing will be required to develop effective strategies for enhancing the degradation of the Gar-containing protein. Our current detailed understanding of the interaction between EBNA1 and USP7/HAUSP based on structure determination, may also provide a conceptual basis for the rational design of inhibitors that will selectively affect the capacity of the viral protein to interfere with cellar functions that control proliferation and apoptosis. Finally, it seems reasonable to assume that, as clearly demonstrated for other virus infections, modulation of components of the ubiquitin–proteasome system will interfere with EBV assembly and maturation. Changes in the pattern of antibody responses to viral antigens associated with the productive cycle and increased virus load are observed in EBV-associated malignancies and play an important diagnostic and prognostic role in NPC, pointing to a possible role of virus production in the pathogenesis of this tumor [162]. Thus, interference with the late stages of virus replication by modulation of the

ubiquitin–proteasome system may provide a new means by which to influence the natural history of at least some EBV-associated malignancies.

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4
HECT Ubiquitin-protein Ligases in Human Disease

Martin Scheffner and Olivier Staub

4.1
Introduction

It is commonly accepted that the specific recognition of substrate proteins of the ubiquitin-conjugation system is mainly mediated by the action of E3 ubiquitin-protein ligases [1–3]. It is, therefore, not surprising that E3s constitute the largest class of enzymes known to be involved in ubiquitination, with the human genome encoding more than 500 putative E3s or E3 complexes. Deregulation of the activity of a still increasing number of E3s has been associated with the development of human disease including cancer, cardiovascular, immunological, and neurological disorders. For example, mutations in the *brca1* gene that interferes with the E3 activity of the respective protein product, have been linked to hereditary breast cancer in a certain percentage of cases [4, 5]. Similarly, amplification of the *hdm2* gene resulting in increased levels of Hdm2, which acts as an E3 ligase for the tumor suppressor p53, has been linked to a certain percentage of soft tissue sarcomas [6, 7]. Thus, both inappropriate inactivation ("loss of function", e.g. BRCA1) and inappropriate activation ("gain of function", e.g. Hdm2) of E3s can have pathophysiological consequences. Based on the presence of distinct amino acid sequence motifs, proteins with E3 activity can be roughly grouped into three classes: HECT E3s, RING-finger E3s, and U-box E3s [8, 9]. The basic mechanisms by which E3s facilitate ubiquitination of substrate proteins.

4.2
Definition of HECT E3s

Functional and structural studies indicate that in a simplified view, all known E3s have a modular structure consisting of at least two functional domains. The RING-finger domain, the U-box, or the HECT domain of the respective E3 mediates the interaction with its cognate ubiquitin-conjugating enzyme E2 [1–3]. The other domain is required for specific interaction with the respective target protein

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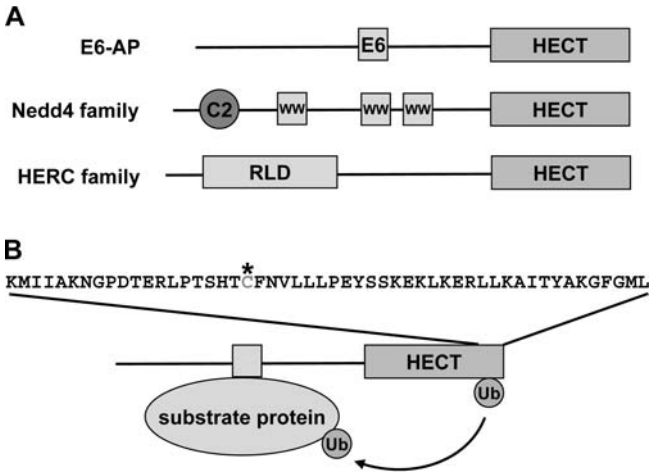

Fig. 4.1. The family of HECT ubiquitin-protein ligases. (A) Schematic representation of the three subfamilies of HECT E3s. All members of the HECT family of E3s are characterized by the C-terminal HECT domain. The HECT domain consists of approximately 350 amino acid residues and represents the catalytic domain of HECT E3s. Nedd4 family members are characterized by an N-terminal C2 domain and the presence of several WW domains (as representative the schematic structure of Smurf2 is shown; see also Figure 4.3). The HERC family comprises six members, which are characterized by the presence of one or several RLD domains (as representative the structure of HERC5 is shown schematically). The schematic structure of E6-AP, the founding member of the HECT family, is shown as representative of the third subfamily. Members of this subfamily are characterized by the notion that, apart from the HECT domain, they do not share any significant similarities in amino acid sequence. (B) HECT E3s have the ability to form thioester complexes with ubiquitin in the presence of E1 and their cognate E2s and are assumed to directly catalyze the covalent attachment of ubiquitin to lysine residues of substrate proteins. The sequence of the C-terminal 52 amino acid residues of E6-AP are shown with the cysteine residue involved in thioester complex formation and with ubiquitin marked with an asterisk.

and, thus, determines the substrate specificity of the respective E3. In the case of HECT E3s, both domains are generally displayed on a single polypeptide chain (Figure 4.1), although auxiliary factors may affect or alter the substrate specificity of a given HECT E3.

Members of the HECT E3 family are large proteins ranging in size from approximately 80 kDa to more than 500 kDa and have been found in all eukaryotic organisms examined. They are characterized by a C-terminal region of about 350 amino acids in length that shows significant similarity to the C terminus of E6-AP (see below) and, therefore, has been termed the "HECT domain" (*H*omologous to *E*6-AP *C T*erminus) [8, 9]. Functional characterization of E6-AP revealed that, in the presence of its cognate E2s, the HECT domain forms thioester complexes with ubiquitin. Furthermore, a conserved cysteine residue within the HECT domain

is required for both ubiquitin thioester complex formation and E3 ligase activity [8, 9]. This indicates that, in contrast to RING-finger and U-box E3s, the HECT domain does not only function as a binding site for E2s but, in addition, plays a catalytic role in the final attachment of ubiquitin to a substrate protein (Figure 4.1).

Similarly to RING-finger and U-box E3s, a common nomenclature that would unambiguously identify a given protein as member of the HECT E3 family has not been introduced. In the following, we will therefore refer to individual members of the HECT E3 family with their respective trivial names that are most commonly used in the literature.

4.3 Human HECT E3s and their Role in Disease

Database analyses indicate that the human genome encodes 28 different HECT proteins (K. Hoffmann and H. Scheel, personal communication). Based on the presence of distinct amino acid sequence motifs, human HECT E3s can be classified into three subfamilies: HECT E3s with RCC1-like domains (RLDs) termed HERC E3s (*HE*CT and *RC*C1-like domain), HECT E3s with WW domains (Nedd4/Nedd4-like proteins), and HECT E3s that neither contain RLDs nor WW domains (Figure 4.1). RLDs and WW domains represent known protein–protein interaction domains and, thus, provide some information about potential interaction partners of the respective E3s.

RLDs were originally described as interaction sites for small GTP binding proteins [10, 11]. Indeed, HERC1 was shown to bind to and act as guanine nucleotide exchange factor for ARF1 [12]. However, no evidence has been provided that ARF1 represents a ubiquitination substrate for HERC1. Thus, the interaction with ARF1 may not be related to the E3 function of HERC1 suggesting that, at least in some cases, HECT E3s are multifunctional proteins. Recent evidence has suggested that HERC1 interacts with TSC2, a GTPase-activating protein of the Rheb GTPase, and targets it for degradation [13]. Interestingly, TSC2 negatively affects the mTOR pathway and has been associated with tuberous sclerosis complex, an inherited disease characterized by hamartoma formation in various organs. However, if and how HERC1 is involved in the development of this disease remains to be elucidated.

Although the RLD may represent an interaction motif for small GTP-binding proteins, it is also involved in the interaction with other proteins. We have recently obtained evidence that HERC2 interacts with E6-AP via RLD2 (Figure 4.1) (U. Kogel, S. Glockzin and M. Scheffner, unpublished data) indicating that, similar to RING-finger E3s, HECT E3s have the potential to form hetero-oligomeric E3 complexes. Apart from HERC1, none of the members of the HERC subgroup of HECT E3s has been etiologically associated with human disease so far. However, mutations in the *herc2* gene have been linked to pathophysiologi-

cal phenotypes in mice [14, 15] and *herc2* expression may be affected in some Angelman syndrome patients (see below). Furthermore, HERC5 has recently been reported to act as an E3 ligase for ISG15, a ubiquitin-like protein that is expressed when cells are stimulated with interferon [16, 17]. However, the physiological significance of this observation is presently unclear and will not be discussed further.

The WW domain represents a highly conserved protein domain that binds to proline-rich regions of interacting proteins [18–20]. Based on the actual sequence of the proline-rich region bound, WW domains have been classified into four groups, with group I domains having a preference for PPXY motifs, group II/III domains for poly-P regions, and group IV for P motifs containing phosphorylated S or T [19, 20]. The best characterized members of the WW domain subgroup of HECT E3s are Nedd4, Itchy, and Smurf. Since these proteins have been associated with human disease or processes with pathophysiological potential, these proteins will be discussed below in more detail. As indicated above, the third subgroup of HECT E3s is characterized by the notion that its members do not share any known protein–protein interaction motifs with other HECT E3s. Members of this subgroup comprise E6-AP and MULE/ARF-BP1/HectH9, both of which have been associated with human cancer.

4.4 E6-AP

E6-AP was the first human E3 that was identified at the amino acid sequence level and represents the founding member of the HECT family of E3s. E6-AP (*E6-A*ssociated *P*rotein) was originally isolated as a protein that binds to the E6 oncoprotein of cancer-associated human papillomaviruses (HPVs) and, in complex with E6, targets p53 for ubiquitination and proteasome-mediated degradation [21, 22]. Subsequently, it was shown that loss of the E3 activity of E6-AP is associated with the development of a hereditary neurological disorder, the Angelman syndrome [23–25]. Thus, E6-AP represents a prime example of the hypothesis that deregulation of components of the ubiquitin-conjugation system contributes to human disease: inappropriate activation of E6-AP contributes to carcinogenesis ("gain of function"), while inactivation results in a neurological disease ("loss of function") (Figure 4.2).

4.4.1 E6-AP and Cervical Cancer (Cancer of the Uterine Cervix)

Cervical cancer represents the second most frequent cancer in women worldwide with approximately 400 000 new cases each year. It is commonly accepted that infection with certain HPV types represents the most significant risk factor for the development of cervical cancer (for review see [26]). Approximately 30 HPV types have been associated with lesions of the anogenital tract and these HPVs

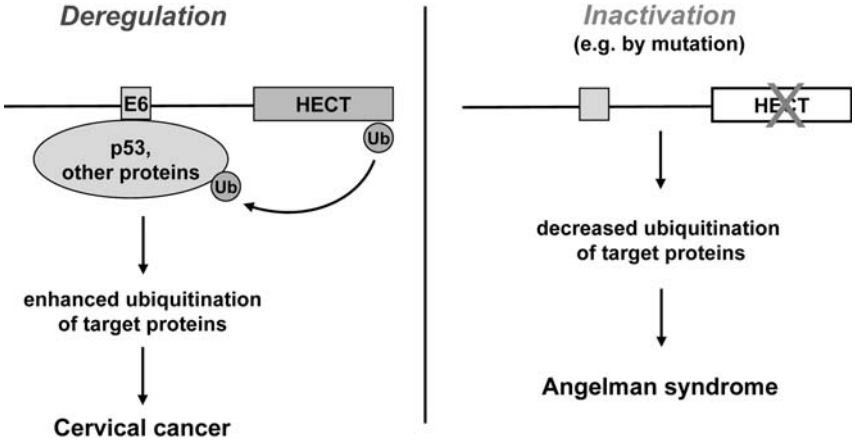

Fig. 4.2. Deregulation of E6-AP activity contributes to the development of human disease. For details, see text.

can be classified into "low risk" and "high risk" types based on their association with malignant lesions. While low risk HPVs are generally associated with benign lesions including condyloma accuminata, women that are infected with high risk HPVs have a significantly increased risk of developing cervical cancer. High risk HPVs encode two major oncoproteins termed E6 and E7 and the respective genes are the only viral genes that are generally retained and expressed in cervical cancer tissues. Furthermore, a number of studies have shown that continuous expression of both E6 and E7 is required for the viability of HPV-positive cancer cells.

As indicated above, it is well established that E6 utilizes E6-AP to target p53 for degradation and, thus, inactivation. Similarly, E7 targets the retinoblastoma susceptibility gene product pRB and the pRB-related proteins p107 and p130 for proteasome-mediated degradation [27–30]. A potential reason for inactivating p53 and pRB family members via degradation rather than by other means is provided by the notion that in HPV-positive cells, E6 and E7 are maintained at rather low levels. Thus, a catalytic mechanism (assuming that one E6 or E7 molecule can target more than one p53 or pRB molecule for degradation) would assure that p53 and pRB family members are efficiently inactivated even if E6 and E7 are expressed at lower amounts in infected cells than their respective target proteins [30].

What is the physiological significance of E6/E6-AP-induced degradation of p53? In contrast to many other tumor types (approximately 40% of all human tumors harbor a mutated p53 gene) the p53 gene is very rarely mutated in cervical carcinomas [26, 30]. Thus, E6/E6-AP-induced degradation of p53 can be considered as functionally equivalent to inactivation of p53 by mutation of the p53 gene, although the situation in HPV-positive cancers may be somewhat more complicated (for

detailed discussion of this issue, see [30–32]). Furthermore, there is good evidence to indicate that in HPV-negative cells, E6-AP plays no, or only a minor, role in p53 degradation [33–36]. In fact, a number of RING-finger E3s including Hdm2, Pirh2, COP1, and HECTH9/ARF-BP1/MULE (see below) have been reported to mediate p53 ubiquitination and degradation in HPV-negative cells [37–42]. Furthermore, genetic experiments in mice clearly indicate that Mdm2, the mouse homolog of Hdm2, is a major antagonist of p53 [43]. Taken together, the published data indicate, that the E6 oncoprotein utilizes E6-AP to target p53 for degradation under conditions where the normal pathways for p53 degradation are not functional. Indeed, it was reported that, for as yet unknown reasons, the Hdm2-dependent pathway of p53 degradation is inactive in cervical carcinoma cell lines [44]. In support of this notion, interference with E6-AP expression by antisense RNA-based approaches or by RNA interference results in the accumulation of p53 and activation of its transcriptional and growth-suppressive properties [36, 45].

It is commonly accepted that the ability of high risk E6s to target p53 for degradation contributes to virus-induced cellular transformation. However, it is also clear that the E6 protein has oncogenic activities that are independent of p53. Numerous cellular proteins including the PDZ domain-containing proteins hDlg, MAGI-1, MUPP-1, and hScrib have been reported to interact with high risk E6 proteins [46–50] and experiments with transgenic mice indicate that the ability of E6 to interact with PDZ domain-containing proteins contributes to its oncogenic potential [51, 52]. Furthermore, it has recently been reported that the E6/E6-AP complex targets NFX1-91 for ubiquitination and degradation [53]. NFX1-91 acts as a transcriptional repressor of the gene encoding hTERT, the rate-limiting and catalytic subunit of telomerase. Thus, this activity of the E6/E6-AP complex may critically contribute to the immortalizing activities of E6.

Unlike the interaction with p53, binding of E6 to PDZ domain proteins is independent of E6-AP [46]. Interestingly, E6 targets hScrib for degradation in an E6-AP-dependent manner, whereas hDlg, MAGI-1, and MUPP-1 have been reported to be targeted for degradation by E6 in an E6-AP-independent manner [54–56]. Thus, it was speculated that E6 may interact with E3 ligases other than E6-AP. However, by using E6-AP-deficient cells we have recently obtained strong evidence that, within cells, E6-mediated degradation of hDlg and MAGI-1 is dependent on the presence of E6-AP (P. Kuballa, K. Matentzoglu, and M. Scheffner, unpublished data). We are therefore proposing that all the known proteolytic properties of E6 depend on E6-AP. Finally, it should be mentioned that E6-AP may not only be utilized by E6 but may represent a direct target for E6, since binding of E6 targets E6-AP for self-ubiquitination and degradation [57]. Although the physiological significance of this observation is presently unclear, it can be speculated that an E6-induced decrease of intracellular E6-AP levels should have profound effects on the stability of E6-independent substrates of E6-AP. However, whether this is indeed the case, remains to be determined. Thus, to fully understand the role of E6-AP in cervical carcinogenesis, it is important to identify and characterize the cellular pathways that are affected by E6-AP in normal (i.e. HPV-negative) cells.

4.4.2 E6-AP and Angelman Syndrome

The "Angelman syndrome" (AS) was first described in 1965 by the pediatrician Harry Angelman [58]. AS is characterized by mental retardation, movement or balance disorder, characteristic abnormal behaviors, and severe limitations in speech and language. It is a genetic disorder with an incidence of approximately 1 in 10 000 to 1 in 40 000 and has been linked to chromosome 15q11-13 known as the Prader–Willi/Angelman region [59, 60]. This region is known to contain a bipartite imprinting center and, accordingly, contains maternally and paternally imprinted genes. The Prader-Willi syndrome and AS represent two clinically distinct disorders with Prader–Willi syndrome resulting from paternal genetic deficiency and AS from maternal genetic deficiency [61]. Interestingly, studies in mice have shown that the ube3a gene encoding E6-AP is biallelically expressed in all somatic cells with the exception of Purkinje cells (cerebellum), hippocampal neurons and mitral cells of the olfactory bulb, in which the paternally-derived ube3a gene is silenced [62]. Indeed, all of the genetic abnormalities associated with AS affect expression of the maternal ube3a gene and/or the ubiquitin ligase activity of E6-AP. Development of AS appears to be the result of several genetic mechanisms with deletion of the 15q11-13 region of the maternal chromosome accounting for approximately 70% of cases. The Prader–Willi/Angelman region is approximately 4 Mb in size and is bounded by duplicons of the *herc2* gene that may predispose to chromosomal rearrangements. Other mechanisms include uniparental paternal disomy, defects in imprinting, and single point mutations in the ube3a gene [59–61, 63]. In this context, it should be noted that E6-AP affects the activity of nuclear hormone receptors and this property of E6-AP does not appear to be related to its ubiquitin ligase function [64, 65]. However, since the relevance of this property of E6-AP for the development of AS or cervical cancer is unclear (e.g. this property is not affected in those E6-AP mutants that are derived from AS patients with point mutations in the ube3a gene), it will not be discussed further.

To understand why loss of the ubiquitin ligase activity of E6-AP results in the development of AS, it is essential to identify the cellular pathways that involve E6-AP. An obvious possibility in this regard is the identification and characterization of proteins that serve as ubiquitination substrates of E6-AP. Several E6-independent substrates of E6-AP have been reported, including HHR23A and HHR23B (the human homologs of *S. cerevisiae* RAD23), Blk (a member of the Src-family of non-receptor tyrosine kinases), Bak (a human pro-apoptotic protein), and Mcm7 (which is involved in DNA replication) [66–69]. However, whether deregulated degradation of these proteins is involved in the pathogenesis of AS patients is presently unclear. Similarly, it should be noted that in E6-AP null mice, cytoplasmic levels of p53 are significantly increased in postmitotic neurons [70]. Although it is possible that this suggests that E6-AP may play a more prominent role in p53 degradation in certain tissues or at certain stages of cellular differentiation, it seems likely that the observed increase in p53 levels is an indirect rather than a

direct effect of the loss of E6-AP expression. Taken together, although the function of E6-AP in cervical cancer is at least in part understood, the pathways that involve E6-AP in the absence of the HPV E6 oncoproteins and that are deregulated in AS patients remain to be identified.

4.5 HECTH9

In 1995, a HECT protein termed UREB1 (*U*pstream *R*egulatory *E*lement *B*inding protein *1*) was reported to interfere with p53-mediated transcriptional transactivation [71]. However, the physiological significance of this interaction remained unclear and, subsequently, it was found that the UREB1 protein studied represented a significantly N-terminally-truncated version of the actual full-length protein. Recently, several groups have reported on the identification of substrate proteins of full-length UREB1 including histones, p53, the anti-apoptotic protein Mcl-1, and the proto-oncoprotein c-Myc and the respective authors have referred to UREB1 using various names including E3histone, ARF-BP1 (ARF-Binding Protein 1), MULE (Mcl-1 Ubiquitin Ligase E3), and HECTH9 [42, 72–74] (in the following discussion we will refer to UREB1/E3histone/ARF-BP1/MULE/HectH9 simply as HectH9 [75]). HECTH9 consists of 4374 amino acid residues and, in addition to the HECT domain, contains three domains known to serve as protein–protein interaction sites, namely a BH3 domain, a WWE domain, and a UBA domain (UBA domains mediate interaction with ubiquitin). The BH3 domain is required for the interaction of HECTH9 with Mcl-1 [73], whereas the interactions sites for p53 and c-Myc have not as yet been mapped in detail [42, 74]. Interestingly, while HECTH9-mediated ubiquitination targets p53 and Mcl-1 for proteasome-mediated degradation, c-Myc is not targeted for degradation by HECTH9. HECTH9 modifies c-Myc with ubiquitin chains that are linked via lysine residue 63 (K63) of ubiquitin [42, 73, 74] and K63-linked ubiquitin chains are known to serve non-proteolytic roles [1–3]. Indeed, HECTH9-mediated ubiquitination of c-Myc appears to be required for transactivation of multiple target genes of c-Myc and induction of cell proliferation. The notion that HECTH9 acts as a positive effector of cell proliferation is supported by the observations that HECTH9 plays an important role in p53 degradation, that its activity is negatively regulated by the human tumor suppressor p14ARF (or p19ARF in mice), and that RNAi-mediated downregulation of HECTH9 expression interferes with the growth of p53 null cells. The latter observation indicates that HECTH9 has p53-independent pro-proliferative properties [74]. However, whether these p53-independent properties are related to HECTH9-mediated activation of c-Myc and whether p14ARF affects c-Myc activation, remains to be determined. Finally, it should be noted that the observation that HECTH9 targets the anti-apoptotic protein Mcl-1 for degradation cannot be readily associated with its pro-proliferative properties. Nonetheless, the findings that the HECTH9 gene is overexpressed in various human tumors and

that HECTH9 expression is required for proliferation of at least a subset of tumor cells, suggests that HECTH9 represents an attractive target for the development of molecular strategies in the treatment of cancer [76].

4.6 HECT E3s with WW Domains

HECT E3s with WW domains share a common structure including an N-terminal calcium-dependent phospholipid binding C2 domain [77], two to four WW domains [18, 20], and the HECT domain (for recent reviews on Nedd4/Nedd4-like proteins see [78–80]). There are nine members of Nedd4-like HECT E3s encoded in the human or mouse genome (Figure 4.3), and orthologs are found in all eukaryotic organisms including fly, worm, and yeast [78]. Extensive alternative splicing of some, possibly all family members, contributes to the diversity of the protein family [81–85]. Nedd4/Nedd4-like proteins are involved in a plethora of functions, including endocytosis, trafficking, degradation of membrane proteins, control of cell growth, and virus budding [78] and therefore play a role in a number of pathologies including hypertension, cancer, and immune diseases.

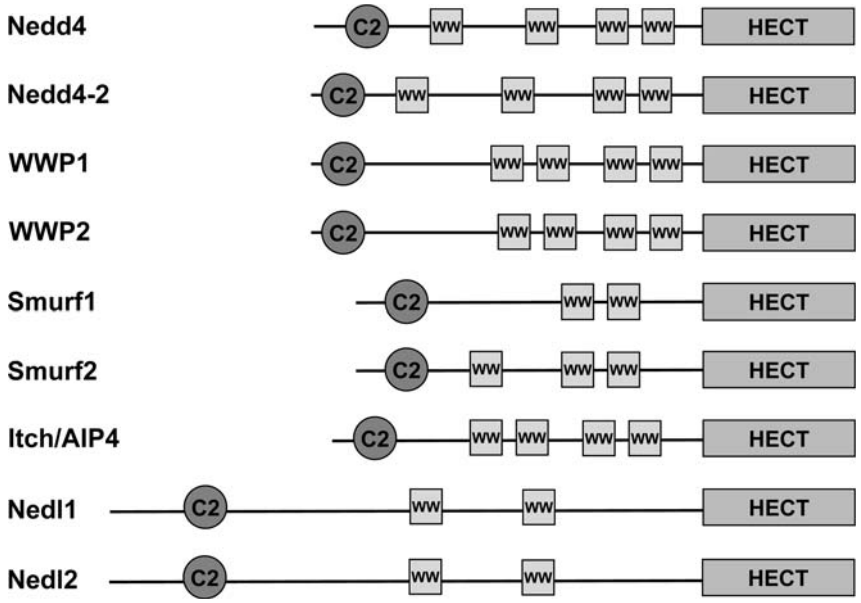

Fig. 4.3. The family of human Nedd4/Nedd4-like proteins. Schematic view of the family members showing the C2 domain, WW domains, and the HECT domain.

4.6.1 Nedd4/Nedd4-2

Nedd4 (Neuronal precursor cell Expressed Developmentally Downregulated 4) is the founding member of the Nedd4/Nedd4-like family of proteins and was originally identified in a subtractive screen using mRNAs derived from different stages during brain development [86]. The gene encoding Nedd4 (also referred to as Nedd4-1) is located on chromosome 15q22 and is ubiquitously expressed in all tissues [87–89]. It was originally described as a protein containing three WW domains and a C-terminal HECT domain [90], but it is now evident that there are multiple alternative transcripts encoding different forms of Nedd4 which either have or do not have the C2 domain and varying numbers of WW domains. Nedd4 has a close relative, Nedd4-2, encoded by a different gene at chromosomal location 18q21. As is the case for Nedd4, Nedd4-2 is ubiquitously expressed, with abundant expression in the kidney, heart, and liver [89, 91, 92]. It has been suggested that both proteins play a role in a hereditary form of hypertension, Liddle's syndrome.

4.6.1.1 Nedd4/Nedd4-2 and Liddle's Syndrome

Liddle's syndrome is a rare genetic form of hypertension first described in 1963 [93, 94]; it is characterized by early onset of severe hypertension, hypokalemia, metabolic alkalosis, and low circulating levels of renin and aldosterone. Patients are treated with a low Na^+ diet and administration of triamterenes, which are specific inhibitors of the epithelial Na^+ channel (ENaC). All the available data suggest that there is a defect in the Na^+-reabsorbing epithelia of the distal nephron. Such cells have on their apical side (facing the urinary compartment) ENaC, which allows entry of Na^+ into the cell, and on the basolateral side the Na^+,K^+-ATPase, which extrudes Na^+ out of the cell into the blood compartment. This Na^+ transport is highly regulated by the renin–angiotensin–aldosterone system. In support of a defect in this transport system, mutations in the genes encoding ENaC subunits were found to be at the origin of Liddle's syndrome [95, 96]. ENaC is a transmembrane protein complex that is composed of three homologous subunits which assemble in a tetrameric structure ($2\alpha1\beta1\gamma$); each subunit contains two transmembrane domains, one extracellular loop and two short cytosolic N and C termini. Of relevance are the short proline-rich regions in the C termini of each subunit, which are referred to as PY-motifs (consensus: L/P-P-X-Y-X-X-ø, where ø is a hydrophobic amino acid [97]). These PY-motifs serve as binding sites for the WW domains of Nedd4 and/or Nedd4-2, which ubiquitinate ENaC on the N termini of the ENaC subunits [88, 89, 91, 92, 97–101]. Such ubiquitination leads to the rapid internalization of ENaC and rapid degradation in the endosomal/lysosomal system, as described for many membrane proteins [102–106]. In Liddle's syndrome, a PY-motif is mutated or deleted either in the β- or in the γ-subunit [95, 96, 107], leading to impaired interaction with Nedd4 or Nedd4-2 and consequently reduced ubiquitination and internalization, resulting in the accumulation of channels at the plasma membrane (Figure 4.4).

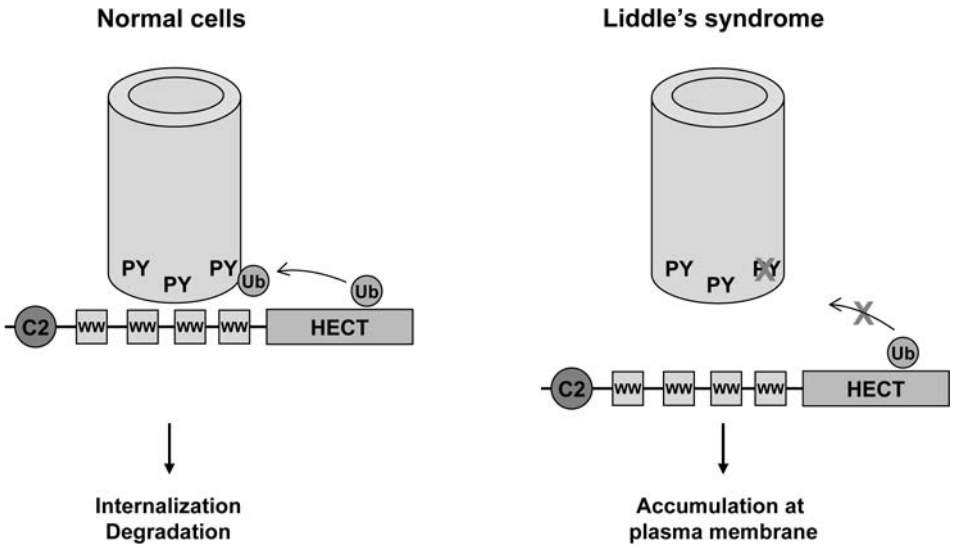

Fig. 4.4. Defective regulation of ENaC by Nedd4-2 in Liddle's syndrome. In normal cells, Nedd4 or Nedd4-2 interacts via the WW domain/PY-motif interaction with the ENaC complex. In Liddle's syndrome, this interaction is impaired, causing reduced ubiquitination, internalization of ENaC, and consequently accumulation of the channel at the plasma membrane.

Which Nedd4/Nedd4-like protein is physiologically controlling ENaC? The majority of evidence indicates Nedd4-2 to be the relevant regulator in the kidney: (1) functional and biochemical evidence from heterologous expression experiments indicates that Nedd4-2 is a more potent regulator of ENaC than Nedd4-1 and can be co-immunoprecipitated with ENaC from intact cells [89, 91, 98, 100]. (2) RNA interference experiments suggest that Nedd4-2, but not Nedd4-1, is involved in the negative control of transepithelial Na^+ transport in epithelial cells [108]. (3) Nedd4-2 is expressed together with ENaC in the principal cells of the distal nephron and its expression is controlled by dietary Na^+. A high-Na^+ diet leads to high Nedd4-2 expression (and inversely to low ENaC expression), whereas a low-Na^+ diet produces the opposite effect, suggesting that Nedd4-2 is itself regulated in the physiology of Na^+ reabsorption [109]. (4) Nedd4-2 interacts with and becomes phosphorylated by the aldosterone-induced "serum and glucocorticoid-induced kinase" Sgk1, a key regulator of Na^+ transport. Phosphorylation of Nedd4-2 creates binding sites for 14-3-3 proteins, which interfere with the ENaC/Nedd4-2 interaction, causing, as in Liddle's syndrome, an increase in the number of channels at the cell surface [98, 100, 104, 105, 110–112]. (5) Polymorphisms in the Nedd4-2 gene are linked to hypertension [113–116]. However, definite proof that Nedd4-2 controls Na^+ transport in the distal nephron will require its inactivation

and analysis in a transgenic mouse KO model, or the genetic linkage of the Nedd4-2 locus to Liddle's syndrome or other forms of hypertension.

4.6.1.2 Nedd4 and Retrovirus Budding

Nedd4/Nedd4-like proteins have attracted considerable attention following the finding that they play a role in budding of many viruses. Viral budding is a process that largely takes advantage of the cellular mechanisms that control targeting of proteins for lysosomal degradation via the multiple vesicle body (MVB) pathway (for reviews, see [102, 117]). Essentially, late domains on viral gag proteins recruit components of the MVB machinery (e.g. Tsg101) and promote the budding of the virus. Late domains may contain either a P(S/T)AP, a PY motif, or a tyrosine-based sorting motif $YP_x(n)L$. P(S/T)AP has been shown to interact with the UEV domain of Tsg101, allowing recruitment of ESCRT-I and, subsequently, ESCRT-II and ESCRT-III complexes. These complexes usually induce membrane invagination and vesicle formation towards the interior of the late endosome. In the case of viruses, this interaction allows the virus to bud into the lumen of late endosomes, and eventually to leave the cell via exocytosis, or alternatively, to assemble the ESCRT complexes at the plasma membrane and subsequently bud from there. The P(S/T)AP and the PY-motif have been demonstrated to be functionally exchangeable.

Although it has been shown that the viral PY-motifs bind to Nedd4 family members, it is not clear how this promotes particle release. Ubiquitylation of Gag may recruit the MVB machinery, for example via binding to the Tsg101 protein or other ubiquitin-binding proteins of the ESCRT pathway. Alternatively, it has been shown that Nedd4 and Tsg101 can interact with each other, which may be a way to recruit Tsg101 to the Gag protein and induce virus budding [118]. Furthermore, studies using HTLV-1 mutants, which have either the PY motif or the P(S/T)AP motif mutated, showed that mutation of the PY-motif leads to the accumulation of the virus at the plasma membrane, whereas mutations of the P(S/T)AP motif leads to accumulation in endosomes, suggesting that HTLV-1 first interacts at the plasma membrane with Nedd4 and later with Tsg101 in endosomes [119].

4.6.2 Itch and the Immune Response

Mouse Itch, the ortholog of human AIP4, is a member of the Nedd4/Nedd4-like HECT E3 family and was identified as the protein encoded by the itchy locus of chromosome 2 in non-agouti lethal 18H mice [120, 121]. In these mice, Itch is inactivated by gene inversion, simultaneously with promoter inactivation of the agouti gene, leading to a darker colored coat [120]. Depending on the genetic background, the mice present two different, but related phenotypes: with the JC/Ct background, they display an inflammatory disease of the large intestine, whereas with the C57L/6J background they present a fatal disease involving changes in the lung, spleen, lymph nodes, skin, ear, thymus, and stomach. In each organ, the phenotype suggests hyperactivation of processes typical of chronic inflammation.

Moreover, these mice, also referred to as *itchy* mice, are characterized by skin and ear scarring, caused by constant scratching when older than 16 weeks. Larger spleens and lymph nodes, likely due to hyperproliferation of lymphocytes, are also observed. This phenotype points to a critical role for Itch in the negative control of the immune system. Indeed, defects in T-cell development and function are characteristic in Itch null mice. When pathogens invade the host, an adaptive immune response is elicited through the generation of specific T cells. Antigenic stimulation drives naïve $CD4^+$ T cells into Th1 and Th2 cells which can be distinguished by their cytokine profiles and functions [122, 123]. Th1 cells produce IL-2 and IFN-γ, which are effective in counteracting viral infections and other intracellular pathogens, whereas Th2 cells produce IL-4 and IL-5, which are involved in the elimination of extracellular helminthic pathogens. Itch plays a role in Th2 differentiation as evidenced in older Itch−/− mice. In this context, T cells can be chronically activated and display increased proliferation in response to stimulation with anti-CD3 or anti-CD3 plus anti-CD28. This stimulation is accompanied by increased production of IL-4 and IL-5 and a biased differentiation of $CD4^+$ cells into Th2 cells [124]. Consistently, the Th2-dependent serum concentrations of IgG1 and IgE in itchy mice are increased. During Itch-mediated T-cell differentiation, Itch binds to either JunB or c-jun, transcription factors which are involved in the gene regulation of Th2 cytokines such as IL-4. Itch binds via its WW domains to the PPXY motif in Jun-B/c-jun and promotes Jun-B/c-jun ubiquitination and consequently their proteasomal degradation (Figure 4.5) [124].

Itch may also play a role in immune self tolerance. Although an efficient immune response is important for protection of the host, the immune system also has mechanisms to prevent excessive damage to normal cells and tissues, a process known as self tolerance. Defects in Th1 responses can result in autoimmune diseases such as type 1 diabetes or rheumatoid arthritis, whereas excessive Th2 cell activation can lead to asthmatic or allergic symptoms. There are a number of

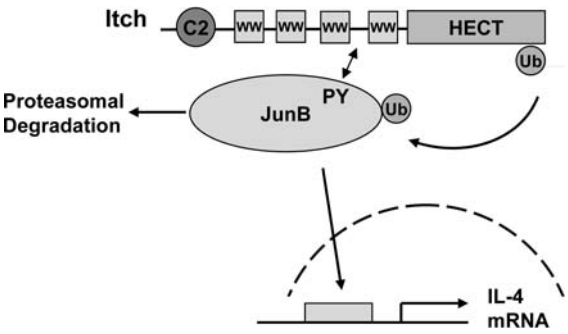

Fig. 4.5. Itch-dependent regulation of c-jun/JunB. Itch interacts via the WW domain/PY-motif with c-jun or JunB, thereby ubiquitinating/targeting c-jun for proteasomal degradation. In Itch null mice, increased c-jun activity leads to increased expression of IL-4.

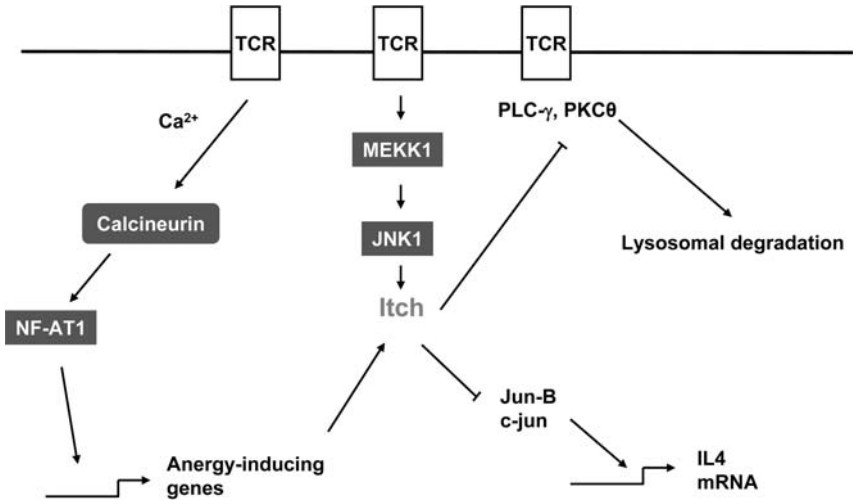

Fig. 4.6. Role of Itch in T-cell anergy. Engagement of the T-cell receptor without co-stimulation leads to mobilization of intracellular Ca^{2+} and activation of calcineurin. Calcineurin dephosphorylates and activates NF-AT1, promoting the transcription of the genes involved in anergy, including Itch. Itch becomes phosphorylated via the MEKK1/JNK1 pathway and ubiquitinates c-jun/JunB, PLC-γ, and PKCθ, which are then targeted for proteasomal (c-jun/Junb) and lysosomal (PLC-γ, PKCθ) degradation, respectively.

mechanisms involved in the induction of peripheral immune tolerance, including peripheral deletion of autoreactive T cells, generation of Tregs, and T-cell anergy. [125, 126]. Of particular interest is T-cell anergy, which is a state of unresponsiveness in T cells that is achieved when the T-cell receptor (TCR) is engaged without co-stimulation of accessory molecules such as the CD86 receptor [127]. There are multiple mechanisms that seem to be relevant in T-cell anergy and some of them involve Itch (Figure 4.6). In the absence of co-factors, TCR engagement is sufficient to stimulate mobilization of intracellular free calcium ions, causing activation of calcineurin, a calcium-sensitive phosphatase. Calcineurin dephosphorylates the NF-AT1 transcription factor which becomes activated and stimulates the transcription of putative "anergy genes", whose products are responsible for keeping the T cell in an anergic state [128]. Among a number of signaling proteins and other ubiquitin-protein ligases (Grail, Cbl-B), Itch is induced under anergic conditions [129]. Upon stimulation, Itch and its close relative Nedd4 become membrane associated, most likely via their C2 domains, resulting in binding and subsequent mono-ubiquitination of PKC- and PLC-γ1, proteins which are important in TCR signaling. The mono-ubiquitinated PKC- and PLC-γ1 are channeled via the ESCRT pathway (including Tsg101, which is also upregulated in calcium induced T-cell anergy) into lysosomes, where they are degraded [129].

The activity of Itch can be regulated by phosphorylation, either negatively or positively. Tyrosine phosphorylation by the Fyn kinase causes a negative effect on

Itch activity [130], whereas TCR-mediated signaling via the mitogen and extracellular kinase 1 (MEKK1) and JNK1 [131] has a positive effect. Consistent with this, phosphorylation of Itch by MEKK1 and JNK1 stimulates JunB and c-jun turnover. T cells derived from MEKK1 *KD* mice that express a catalytically inactive form of MEKK1, show reduced JNK activation following engagement of the TCR and the CD28 auxiliary receptor. Moreover, both peripheral T cells and thymocytes hyperproliferate in response to stimulation with antibodies to CD3 and CD28, and within 4 h of receptor engagement express larger amounts of IL-4, consistent with the requirement of phosphorylated Itch to negatively control Th2 activation. The MEKK1–JNK–Itch pathway is important in T-cell anergy and Th2 tolerance and airway inflammation [132]. Both *in-vitro* and *in-vivo* assays show that Th2 cells that either express an inactive MEKK1 mutant or are devoid of JNK1 or Itch cannot become immune tolerized, whereas Th1 tolerance is not affected. This breakdown of Th2 tolerization leads to airway inflammation [132].

4.6.3 Smurfs

Smurf1 and Smurf2 are two other, closely related members of the Nedd4/Nedd4-like proteins family that have been extensively studied in recent years and are particularly important in a number of pathophysiological conditions, including cancer and bone homeostasis.

Smurf1 (*Sm*ad *u*biquitination *r*egulatory *f*actor 1) was originally identified as an interacting partner of Smad1, a protein involved in TGF-β signaling [133]. The TGF-β superfamily of cytokines is involved in a plethora of biological functions, including embryonic development, regulation of cell growth and differentiation, and apoptosis. Moreover, mutations in TGF-β pathway components are associated with a number of human diseases, including cancer and osteoporosis. There are roughly 40 members of the TGF-β ligands and they can be divided into two groups, the TGF/activin/nodals and the bone morphogenetic proteins (BMPs), each group stimulating different signaling pathways. TGF-β ligands bind to Ser/Thr kinase receptors. These receptors are composed of type I and type II class receptors, which heterodimerize on ligand binding. The type II receptor phosphorylates the type I receptors, which become activated and stimulate Smads, which are involved in the TGF-β or the BMP pathways. The type I receptor phosphorylates serine residues on the receptor-regulated Smads (or R-Smads), Smad-1, -2, -3, -5, and -8. Smad-1, -5, and -8 are involved in the BMP pathway, whereas Smad-2 and -3 have a signaling function in the TGF-β pathway. Once phosphorylated, R-Smads complex with the common co-SMAD, Smad-4, and translocate to the nucleus, where they interact with DNA-binding proteins and regulate transcription. In addition to R-Smads and co-Smads, there are so-called inhibitory or I-Smads (Smad-6 and -7) that are important for the negative regulation of TGF-β and BMP pathways. They inhibit TGF-β and BMP signaling by competing with R-Smads for association with type I receptors or by targeting receptors for ubiquitin-mediated degradation. Ubiquitination, via Smurf1 or -2, is an alternative negative

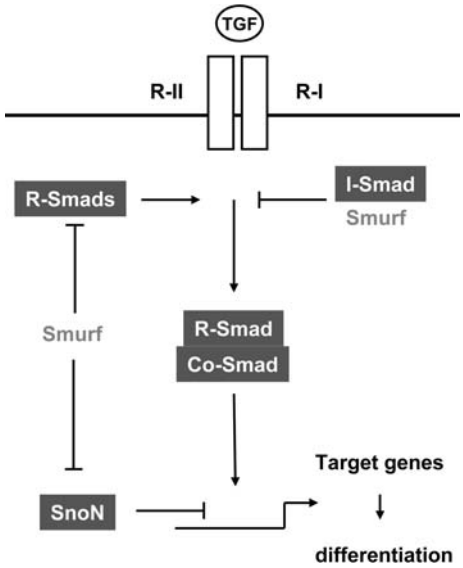

Fig. 4.7. Role of Smurf in the TGF-β pathway. TGF-β ligand stimulates heterodimerization of type I and type II Ser/Thr kinase receptors, leading to the phosphorylation of the type I receptor by the type II receptor. This recruits receptor-regulated Smads (R-Smads), which become phosphorylated. Upon phosphorylation, R-Smads interact with the common Smad, Smad4, and the complex translocates to the nucleus, where it interacts with co-factors and stimulates transcription of the genes involved in differentiation. The pathway is negatively regulated by inhibitory Smads (I-Smads), by SnoN, and by Smurfs. Smurfs can interact with and ubiquitinate R-Smads and can be recruited by I-Smads to the receptor where they induce receptor ubiquitination and internalization. Moreover, Smurfs are also involved in SnoN ubiquitination.

mechanism by which TGF-β and BMP signaling is regulated. Thus Smurf1/2 can act at different levels in the signaling pathway (Figure 4.7).

As mentioned above, Smurf1 has been identified as a ubiquitin-protein ligase that interacts with Smad1 via WW domain/PY-motif interaction. By doing so, it ubiquitinates and suppresses the steady state levels of the BMP signaling proteins Smad-1 and Smad-5 (but not Smad-2 or -4) [133]. Smurf2 associates with Smad-1, -2, and -3, giving rise to a decrease in the steady state level of Smad1 and, to a lesser extent, of Smad2 [134–136]. The interaction between Smurf2 and Smad2 is dramatically increased when Smad2 becomes activated by the TGF-β pathway, indicating that Smurf2 plays a particular role in the ligand-dependent regulation of the pathway. Destruction of activated Smad2 occurs in the nucleus via proteasomal degradation [137]. Similarly, activated Smad3 is also targeted for proteasomal degradation but in this case, it is ubiquitinated by the SCF/Roc1 E3 ligase complex [138].

Smurfs can also use adapters for the regulation of the TGF-β pathway. Smad2/Smurf2 complexes are formed after TGF-β stimulation and Smad2 then recruits Smurf2 to the transcriptional co-repressor SnoN [136]. As SnoN is an inhibitor

of the TGF-β transcriptional response, its degradation may thereby facilitate TGFβ signaling. A different mechanism involves Smad7, which is able to interact with Smurf2 in the nucleus. This interaction causes export of Smurf2 from the nucleus and interaction of the complex with the TGF-β receptor. In concert with the E2 enzyme UbcH7 [139], Smad7 becomes ubiquitinated and triggers the internalization and degradation of itself and the receptor [140]. Interestingly, this action involves the lipid raft/caveolar internalization pathway; alternatively the receptor can also internalize via the classical clathrin-dependent pathway, thereby maintaining TGF-β signaling [141]. In a similar manner to Smurf2, Smurf1 is also recruited by Smad7 to the TGF-β receptor [142]. For nuclear export, it uses the protein CRM-1 [143], which is an importin β-related nuclear transport receptor and physically interacts with a nuclear export signal in the HECT domain of Smurf1 [142, 144]. Furthermore, both Smad6 and Smad7 can also translocate Smurf1 to the BMP receptors and induce ubiquitination of theses receptors [145].

4.6.3.1 Smurfs and Cancer

The TGF-β pathway generally has an inhibitory effect on growth, both *in vitro* and *in vivo*. As described above, steady-state expression levels of the proteins in this pathway are tightly regulated to ensure proper function. It can therefore be expected that improper steady state levels are frequently associated with cancer [146]. Although the majority of Smad mutations associated with cancer are loss of function mutations [147, 148], there are a number of others that affect the steady state level [146]. Oncogenic missense mutations in Smad4 (L43S, G65V, R100T, or P130S) or in Smad 2 (R133C, or a nonsense mutation at position 515, Q407R, L369R) lead to increased ubiquitination and proteasomal degradation as compared to the normal proteins [149–154]. Hence, certain oncogenic Smad mutations destabilize the protein and thereby inactivate TGF-β regulation. It is not yet known which ubiquitin-protein ligase(s) is/are involved, but they may very well be different from Smurf1 or Smurf2. It seems conceivable that the above-mentioned mutations may cause misfolding of the proteins and, thus, the misfolded proteins may be recognized by E3 ligases involved in quality control. However, there is also evidence for the involvement of Smurf ligases in certain types of cancer. It has been found that Smurf2 is highly overexpressed in esophageal squamous carcinoma and is associated with a poor prognosis of this disease [155].

In addition to its tumor suppressive effect, TGF-β can also display, under certain circumstances such as in late-stage tumors, a tumor-promoting effect [156]. This can be attributed to its ability to promote malignant progress, invasiveness, and metastasis. Hence a decrease in Smurf levels causing an increase in TGF-β signaling may stimulate late-stage tumors. RNF11, a Ring-H2 protein that is highly expressed in invasive breast cancer [157], was recently shown to interact with Smurf2 and to target Smurf2 for ubiquitin–proteasome-mediated degradation. Furthermore, it has been found that RNF11 can interfere with Smurf2-mediated ubiquitination of the TGFβ receptor. By blocking Smurf2 activity, RNF11 may thus enhance TGF-β signaling and its tumor-promoting activity [157].

4.6.3.2 Smurfs and Bone Homeostasis

The bone mass is balanced between constant resorption and new formation. These properties are assured by specialized cells, the osteoclasts which are responsible for resorption, and the osteoblasts which produce bone [158]. Osteoblasts develop from mesenchymal progenitors and require the osteoblast-specific transcription factors RunX2 and Osterix. These proteins are essential for the differentiated osteoblasts to synthesize alkaline phosphatases, type 1 collagen, osteocalcin, and bone sialoprotein and to deposit them in the bone extracellular matrix. Osteoblast differentiation and their bone-forming activities are subject to control by members of the TGF-β/BMP superfamily (Figure 4.8). BMPs promote osteoblast differentiation and bone ECM deposition, whereas TGF-β interferes with this process by inhibiting Runx2 and osteocalcin activity. Because both TGF-β and BMPs take advantage of the Smads, it is not surprising that the Smurfs, especially Smurf1, also play a role in osteoblast function. Overexpressing Smurf1 in osteoblast precursor cells interferes with BMP-induced osteoblast differentiation [159, 160], whereas RNA interference-mediated downregulation of Smurf1 expression or expression of catalytically inactive Smurf1 enhances osteoblast differentiation [159, 161]. Thus, overexpression or suppression of Smurf1 controls the expression of Smad proteins, particularly of Smad1 and/or Smad5, thereby enhancing or interfering with the BMP signaling pathway [159–161]. Alternatively, Smurf1 is also capable of binding to Runx2, partly via interaction with the WW domain/PY motifs and additionally via Smad6, this interaction then destabilizes Runx2 and inhibits osteo-

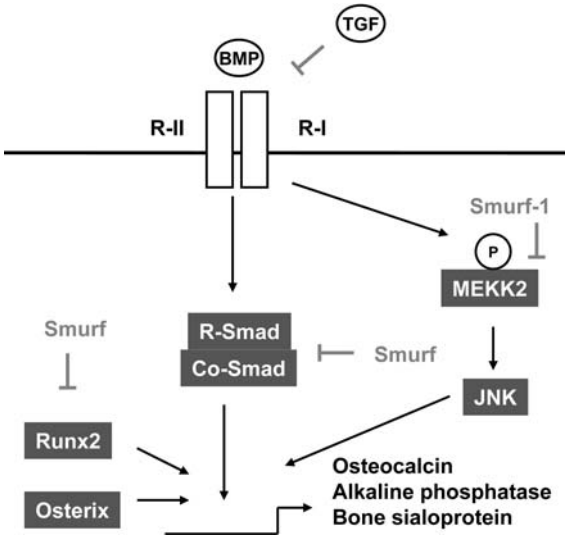

Fig. 4.8. Smurf and bone homeostasis. Whereas TGF-β has a negative effect on Runx2 and osteocalcin activity, BMP stimulates these processes via the Smad pathway. Smurfs can interfere with BMP signaling by ubiquitinating R-Smads, Runx2, and phosphorylated MEKK2.

blast differentiation [160–162]. Transgenic mice which overexpress Smurf1 display significantly reduced bone formation and this provides further support for the suggestion that Smurf1 plays a role in bone homeostasis [160].

The strongest argument for a role in bone homeostasis comes from Smurf1–/– mice [163]. These mice are viable and develop normally, become fertile and have a similar life expectancy as wild-type mice. However, starting from approximately 4 months of age, they show an increase in bone mass caused by enhanced osteoblast activity [163]. Because there is no developmental defect in bone formation, it is likely that Smurf1 does not play an essential role in early osteoblast formation, but rather is important in bone-forming activities in mature osteoblasts. Surprisingly, neither BMP signaling nor Runx2 activity seem to be affected by the inactivation of Smurf1. Rather it is JNK, which has previously been shown to play a role in osteoblast function, which is constantly phosphorylated in Smurf–/– mice, whereas phosphorylation of JNK in normal mice requires stimulation by BMP. Consequential to this phosphorylation, downstream transcription activity is enhanced in Smurf–/– cells. Smurf1 does not interact with JNK, but with MEKK2, an upstream activator of JNK. Autophosphorylation of MEKK2 appears to be indispensable for its interaction with Smurf1 and for its ubiquitin-dependent degradation. Expression of constitutively active MEKK2 or JNK, or inactive MEKK2 in osteoblasts demonstrates that these kinases regulate osteoblast activity.

The lack of effect of Smurf1 inactivation on the BMP pathway may be explained by the compensatory action of Smurf2. Indeed, Smurf1–/– mice show an increase in Smurf2 expression, and, double KO mice for Smurf1 and Smurf2 are embryonic lethal, supporting the idea that Smurf1 and 2 have overlapping functions. It remains to be shown how MEKK2 becomes activated in the control of osteoblast activity. The involvement of Smurf1 in human pathologies with dysregulated bone homeostasis (such as osteoporosis) remains to be demonstrated. However, the fact that Smurf1 inactivation has no effect on the maintenance of skeletal integrity may be useful for developing therapeutic strategies for treating age-related bone loss such as osteoporosis.

4.7 Concluding Remarks

As described above, deregulation of the activity of certain HECT E3s is intrinsically involved in the development of distinct human diseases. Thus, an important question is whether HECT E3s or – in those cases where E3 activity is lost during disease development via mutation – their respective substrate proteins or proteins that regulate the interaction of HECT E3s with their substrate proteins (e.g. Sgk1 kinase which negatively affects Nedd4-2/substrate interaction) represent potential targets in the development of treatments for the respective disease. Unfortunately, the physiologically relevant substrate proteins of most HECT E3s are not yet known and their identification may be hampered by the notion that a given protein is not only recognized as a substrate by a single E3 but by several

E3s. Nonetheless, identification of the substrate proteins of E6-AP, for example, should provide significant insight into the mechanisms by which loss of the E3 activity of E6-AP results in the development of the Angelman syndrome. In cases in which inappropriate activation of HECT E3s contributes to disease (e.g. overexpression of the respective HECT E3 or of auxiliary factors affecting the substrate specificity of the E3), there may be direct interference with the E3 activity of the respective HECT E3 or interference with the interaction between the E3 and particular substrate proteins, for example by small molecules. Indeed, downregulation of E6-AP expression has been reported to selectively activate the p53 tumor suppressor pathway in HPV-positive cells. Although this (inactivation of E6-AP) may not appear to be an attractive approach for the treatment of cervical cancer (since the viability of HPV-positive cells depends on the continuous presence of E6 and E7, molecular strategies to target these viral oncoproteins represent the approaches of choice), this observation indicates that HECT E3s can serve as potential targets for the development of molecular therapeutic approaches. Finally, it should be noted, that ubiquitination is a reversible process by the action of deubiquitinating enzymes. Since deubiquitinating enzymes are proteolytic enzymes with a spatially-defined catalytic center, they may more easily be made tractable by small molecules than by HECT E3s. It will, therefore, be particularly important to understand which of the E3s/deubiquitinating enzymes are involved in regulating the stability of distinct target proteins.

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5
Ubiquitin-independent Mechanisms of Substrate Recognition and Degradation by the Proteasome

Martin A. Hoyt and Philip Coffino

5.1
Introduction

As its name implies, the marking of proteasome substrates by ubiquitin conjugation is a central feature of the ubiquitin–proteasome system (UPS), and a topic which has been the subject of much investigation. There are, however, examples of proteins whose degradation by the proteasome has been divorced (or at least partially separated) from the process of ubiquitination. Given the predominance of ubiquitin-mediated targeting in proteasome function, and the central role many of these targets play in both regulation and maintenance of cellular homeostasis, it is fair to question the significance of a few exceptional cases. While one may be tempted to ascribe special import to such cases, and such perceptions may hold true, here we advocate a more modest view: that by examining the mechanisms of ubiquitin-dependent and -independent targeting systems, and identifying their point of convergence on the proteasome, we may illuminate something of general interest about proteasome biology.

What constitutes evidence for ubiquitin-independent degradation via the proteasome? A preliminary and obvious criterion is the lack of detectable ubiquitin conjugates of a particular proteasome substrate. However, since rapidly degraded proteins often exist at extremely low steady-state levels in the cell, these conjugates can be difficult to detect, even for proteasome substrates whose degradation has been demonstrated to require ubiquitination. Ubiquitin conjugates commonly represent only a small fraction of the steady-state population, and proteasome inhibition is often required for such conjugates to accumulate to detectable levels. Furthermore, existing polyubiquitin chains can be rapidly removed from substrate proteins by the action of cellular deubiquitinating enzymes. Given the difficulties of detection, the lack of detectable ubiquitinated species, is by itself insufficient to establish the ubiquitin-independence of protein degradation.

In addition to the problem of detection of ubiquitin conjugates, it is worth noting that ubiquitination is not a sufficient criterion to establish that a particular protein

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is a substrate for proteasomal degradation. As an example, ubiquitination is required for the function of the Met4 transcription factor of the yeast *Saccharomyces cerevisiae*, but this modification does not necessarily lead to its degradation [1–4]. This is true in spite of the observation that the Met4 ubiquitin chain is extended through lysine 48 of ubiquitin [1], a canonical linkage involved in the formation of the ubiquitin proteasomal recognition signal [5]. Additionally, as we will discuss below, some proteasome substrates are degraded by both ubiquitin-dependent and -independent pathways. The coincidence of ubiquitination and proteasome-mediated degradation is therefore not necessarily a causal relationship, as others have noted [6].

A second experimental approach used to establish that degradation of a particular proteasome substrate is independent of its ubiquitination, involves the perturbation of the ubiquitination machinery itself. It is anticipated that lesions which prevent or greatly impair ubiquitination should have little effect on the turnover of substrates not dependent on this modification. Implicit in this assumption is that ubiquitination is directly involved in the degradation of the substrate protein, and that secondary effects of the global inhibition of ubiquitination will be minor. This approach is limited by the lack of drugs that target the ubiquitination process specifically. Instead genetic approaches are most often used. Temperature-sensitive mutants of the ubiquitin-activating enzyme (E1) are available in animal cells [7, 8], and in *S. cerevisiae* a hypomorphic allele of the E1 gene (*UBA1*) was generated by a mutagenic insertion of a minitransposon in its promoter region [9]. Mutants of any particular ubiquitin-conjugating enzyme (E2) or ubiquitin ligase (E3) are also available in yeast.

As an alternative to global perturbations of the ubiquitination machinery, the ubiquitination of a particular substrate can be prevented by the removal of the target lysine residues within that substrate via mutation. When utilizing this methodology, consideration must be given to the effects such potentially extensive mutation could have on the structure and function of the protein in question. In the case of p21 (see Section 5.2.2), Sheaff and co-workers [10] demonstrated that when all six lysines residues were mutated to arginines, the mutant protein could bind, inhibit, and be phosphorylated by cyclin–Cdk complexes, and thus its normal function and structure were not severely affected. In addition to the structural consequences of lysine mutation, attention must also be given to sites of potential ubiquitin modification other than lysine residues. A lysine-less mutant of the muscle-specific transcription factor MyoD is ubiquitinated and targeted for proteasomal degradation through its N-terminus when no other modification site is available [11]. Human $p14^{Arf}$, which lacks any lysine residues, and its mouse homolog ($p19^{Arf}$) that contains a single lysine, were also shown to be ubiquitinated at their N-termini [12]. Other naturally occurring lysine-less proteins also appear to be ubiquitinated at their N-termini [13]. Ubiquitination can occur even when internal lysines or an accessible N-terminus are unavailable. Cadwell and Coscoy [14] recently demonstrated that MIR1, an E3 ubquitin ligase encoded by the Kaposi's sarcoma-associated herpesvirus, can mediate the ubiquitination of major

histocompatibility complex class I (MHC I) molecules at an available cysteine residue via a thiol-ester bond.

Lastly, the ubiquitin-independence of protein degradation can be demonstrated by reconstitution using purified components in an *in-vitro* assay system. While this approach is more rigorous in that the presence of ubiquitin can be completely excluded by using recombinant proteins produced in prokaryotes, it also requires that all the necessary components have been identified beforehand, insuring that each can be purified in the active form and that they can be combined effectively. *In-vitro* assays are also useful in distinguishing whether the 20S or 26S form of the proteasome is the relevant protease, since this distinction is difficult to ascertain *in vivo*.

5.2 Ubiquitin-independent Proteasome Substrates

In discussing ubiquitin-independent substrates, we will first describe various substrates and the evidence that their turnover occurs independently of ubiquitin modification, followed by a discussion of these potential alternate mechanisms of proteasome recognition, and what can be learned through using such substrates.

5.2.1 Ornithine Decarboxylase

Ornithine decarboxylase (ODC) is a homodimeric enzyme that catalyzes an initial and key regulatory step in the biosynthesis of polyamines. ODC is a remarkably labile enzyme. Its activity undergoes marked and rapid changes in response to various biological perturbations, most prominently brought about by signals that promote cell growth [15]. Although subject to regulation by transcriptional and translational means, most of the dynamic changes in ODC protein levels depend on changes in stability [16]. A clue to the source of ODC lability came from comparing the protein sequences of vertebrate ODCs to those of an African trypanosome [17, 18]. Although vertebrate and parasite ODCs exhibit similar specific activities and a sequence homology of approximately 70%, the vertebrate proteins uniformly include a C-terminal extension of 35–40 amino acids, here termed cODC, that is absent in the corresponding parasite enzyme. Transfection of mammalian cells with DNAs encoding mouse and trypanosomal ODCs revealed that the former enzyme turned over with a short half-live, but that the latter is stable. Revealingly, truncated mouse ODC lacking cODC proved to be stable [17]. Conversely, appending cODC to the trypanosomal enzyme made it labile [18]. When tested within a common cellular context, cultured mammalian cells, the presence of cODC proved to be the necessary and sufficient determinant of rapid turnover. Degradation was found to be mediated by the proteasome, as shown by both biochemical studies using purified components and by genetics [19, 20]. The conclusion that cODC

mediates degradation by the proteasome proved true in broader structural and biological contexts. Appending cODC to a variety of stable proteins is sufficient to destabilize them [21, 22]. The cODC degradation signal is recognized in eukaryotic organisms ranging from man to fungi and plants [23, 24].

The accumulation of excess cellular polyamines leads to a rapid reduction in ODC activity mediated by its regulatory protein antizyme (AZ) [25]. AZ inhibits ODC activity by dissociating the ODC homodimer to form catalytically inactive ODC-AZ heterodimers, and by facilitating the degradation of ODC by the 26S proteasome through the increased exposure of its C-terminal degradation signal. Although AZ augments the degradation of ODC, it is worth emphasizing that the cODC signal is both autonomous and portable as it destabilizes proteins with no capacity to associate with AZ and in biochemical and cellular contexts devoid of AZ.

An alternate pathway for the ubiquitin-independent degradation of ODC by the 20S proteasome, which appears to be elicited under conditions of oxidative stress, has recently been described [26]. This process is inhibited by NAD(P)H quinone oxidoreductase 1 (NQO1), an enzyme involved in the reduction of various quinones that can use NADH or NADPH as electron donors, which was also shown to inhibit the ubiquitin-independent degradation of p53 (see Section 5.2.4). This NQO1-mediated inhibition is alleviated by dicoumarol, a specific inhibitor of NQO1. The dicoumarol-induced degradation of ODC is accelerated by AZ, but apparently does not require cODC. The relevant effect of AZ in this case appears to be its ability to dissociate ODC homodimers: the degradation of a double mutant form of ODC, unable to bind AZ or to form homodimers, was accelerated by dicoumarol treatment, while degradation of a homodimeric ODC that could not interact with AZ was unaffected. The degradation of the monomeric ODC mutant, *in vitro*, was carried out by 20S (but not 26S) proteasomes, and inhibited by the interaction of NQO1 with ODC.

5.2.2
p21$^{Waf1/Cip1}$

p21 is a member of the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors, which block progression through the G_1/S transition of the cell cycle [27]. Accordingly, the amount of p21 is often elevated in cells that are undergoing exit from the cell cycle, such as during terminal differentiation. Cellular levels of p21 are regulated both by its rate of synthesis and degradation. Treatment of cultured mammalian cells with proteasome inhibitors leads to the accumulation of ubiquitinated forms of p21 and a concurrent stabilization of the protein, without changes in its rate of synthesis. However, mutation of all six lysine residues, which could serve as sites of ubiquitin conjugation, prevented the formation of ubiquitinated p21 species, but had no effect on the half-life of the mutant protein [10]. The N-terminus was ruled out as a possible alternate site of ubiquitin attachment due to acetylation of the terminal residue [10, 28]. Furthermore, turnover of p21 was unaffected in ts20TG^R and tsBN75 cell lines harboring temperature-sensitive alleles of the ubiquitin-activating enzyme E1, at both the permissive and restrictive temperatures [28].

In contrast proteins whose turnover was known to be dependent on ubiquitination, such as the cyclins D1 and E, were stabilized in the mutant cell line.

The ubiquitin-independent degradation of p21 appears to occur through a direct interaction of its C-terminus with the C8 (α7) subunit of the 20S proteasome [29]. Deletion mutants of p21 lacking this C-terminal domain stabilize p21 *in vivo*, and prevent its degradation by purified 20S proteasomes *in vitro*. The E3 ubiquitin ligase MDM2 targets both itself and p53 for ubiquitin-dependent degradation via the UPS [30]. Recently, MDM2 was found to regulate p21 activity in cells by mediating its proteasomal turnover independently of p21 ubiquitination [31]. Ectopic expression of MDM2 in cultured mammalian cells resulted in the accelerated degradation of p21 even when MDM2 mutants lacking ubiquitin ligase activity, or the lysine-less version of p21 were used [31]. It has not been determined whether the MDM2-mediated degradation of p21 depends on the interaction of p21 with the C8 subunit of the proteasome, however.

5.2.3
Retinoblastoma Protein

The human retinoblastoma protein (Rb) is a nuclear protein that inhibits the expression of genes dependent on the E2F family of transcription factors [32]. Rb can be targeted for proteasomal degradation by the human papillomavirus E7 oncoprotein [33], and by the pp71 protein of human cytomegalovirus [34]. Several observations imply that the latter process is ubiquitin independent: the absence of high molecular weight ubiquitinated forms of Rb, even following treatment with proteasome inhibitors; the lack of an effect of the dominant negative ubiquitin K48R mutant on Rb turnover; and the capacity of pp71 to direct the degradation of Rb in a cell line bearing a temperature-sensitive E1 (ts20) [35].

Recently MDM2 overexpression was shown to lead to a reduction in the half-life of the Rb protein, by a mechanism strikingly similar to that of MDM2-mediated turnover of p21 [36]. The MDM2-mediated turnover of Rb was shown to be ubiquitin-independent *in vivo*, as judged by criteria similar to those used by Kalejta and Shenk [35]. The degradation of Rb *in vitro* was accomplished by the 20S proteasome, and Rb co-fractionated with the 20S proteasome in cell lysates during gel filtration chromatography. Both Rb and MDM2 were shown to interact with the C8 subunit of the 20S proteasome, and MDM2 was shown to facilitate the Rb–C8 interaction [36]. The results of this study and those regarding p21 suggest a model whereby MDM2 functions to tether substrates to the 20S proteasome to facilitate their degradation. This model is supported by the observation that MDM2 mutants that bind C8, but not Rb, fail to degrade Rb [36].

5.2.4
p53 and p73

The tumor suppressor p53 is a labile protein that is targeted for destruction by the action of the MDM2 ubiquitin ligase. Like ODC, the stability of p53, and the related

p73 protein, can be negatively regulated by NQO1 and dicoumarol treatment reversed this effect [37]. NQO1 does not inhibit MDM2 ubiquitination-mediated degradation of p53 [38], rather it blocks a ubiquitin-independent degradation pathway for p53 [37]. The ubiquitin-independence was demonstrated using a ubiquitin-depleted reticulocyte lysate-derived degradation system following dicoumarol treatment, and in a cell line bearing a temperature-sensitive E1 mutant.

As with ODC, the 20S proteasome was shown to be responsible for the ubiquitin-independent degradation of p53 and p73 [39]. The p73 gene is translated into p73α and p73β isoforms by alternate splicing of its transcript. While the degradation of p53 and the p73α isoform was inhibited by NQO1, the degradation of p73β, which lacks the C-terminal SAM domain, present in p73α, was not inhibited by NQO1. Both p53 and p73α were able to bind NQO1, while p73β was not. Because NQO1 was shown to co-fractionate with 20S proteasomes in this study, the authors proposed a model whereby NQO1 prevents unrestrained proteolysis by the 20S proteasome [39].

5.2.5
Human Thymidylate Synthase

Thymidylate synthase (TS) is an enzyme responsible for the formation of dTMP by the reductive methylation of dUMP, and is an essential source of nucleotides used in DNA synthesis. Ligand binding to TS, following treatment with inhibitors, results in both a change from an "open" to a "closed" conformation, and stabilization of the enzyme [40]. When compared to prokaryote TS, the human enzyme contains a 29-amino acid N-terminal extension that is structurally disordered and is dispensable for normal catalytic activity. Truncations of this N-terminal extension were found to have varying effects on the stability of the enzyme depending on the extent of the deletion [41]. Deletion of amino acids 2–7 from the N-terminus resulted in almost complete stabilization of the thymidylate synthase protein, and further analysis identified Pro2 as a critical residue [42]. Degradation of TS was mediated by the 26S proteasome, but ubiquitin-conjugated forms of the enzyme were undetectable in cells [41]. Experiments using either a temperature-sensitive E1 allele, or a "lysine-less" mutant of the enzyme, supported the conclusion that ubiquitination was not required for TS degradation.

5.2.6
Rpn4

Rpn4 is a transcriptional activator of genes encoding the proteasome subunits of the yeast *Saccharomyces cerevisiae* [43, 44]. It is itself a target for rapid degradation by the proteasome [45], and thus forms a feedback loop in which decreased proteasome activity leads to Rpn4 accumulation and increased expression of proteasomal genes. Degradation of Rpn4 by the proteasome occurs by both ubiquitin-dependent and -independent mechanisms, and the degradation signals for both pathways map to the N-terminal region of Rpn4 [46]. Ubiquitination

occurs on lysines in the N-terminal 229 amino acids of Rpn4, and is mediated by the Ubr2 ubiquitin ligase [47]. However, mutating all 11 lysines in an N-terminal fragment of Rpn4 only partially stabilized the truncated protein [46]. Similarly, the turnover of the full-length protein was largely unaffected in a yeast *uba1-2* mutant defective in the activity of the ubiquitin-activating enzyme, E1 [45], pointing to the existence of a ubiquitin-independent degradation pathway. The ubiquitin-independent degradation signal resides at or proximal to the Rpn4 N-terminus, since either extension by the addition of an epitope tag, or deletion of the first 10 amino acids of the native N-terminus leads to partial stabilization, and these modifications combined with lysine mutations in the truncated protein lead to complete stabilization [46].

5.2.7
NF-κB and IκBα

The vertebrate transcription factor NF-κB functions in a number of signaling pathways including those involved in immune and inflammatory responses. Ubiquitination plays a central role in the regulation of the NF-κB pathway in mammalian cells (reviewed in [48]). However, ubiquitin-independent proteasome-mediated proteolysis also plays a role in this pathway. The IκBα inhibitory protein binds to and sequesters the NF-κB dimer in the cytoplasm by masking its nuclear localization signal. In response to appropriate stimuli, IκBα is phosphorylated and subsequently targeted for proteasomal degradation by ubiquitination, allowing NF-κB to transit to the nucleus. Apart from the ubiquitin-dependent degradation of IκBα in stimulated cells, basal turnover of this protein occurs when it is in its monomeric form, not associated with p65. The rapid turnover of monomeric IκBα by the proteasome in unstimulated cells was shown to be ubiquitin-independent by the following criteria: (1) No high molecular weight ubiquitin conjugates of IκBα were detected in unstimulated cells. (2) Mutants lacking a C-terminal domain required for signal-dependent ubiquitination and degradation of IκBα retained their basal instability. (3) A mutant form of the IκBα protein in which all the lysine residues had been removed or mutated to arginines, also did not affect the basal instability of the protein. (4) The constitutive turnover of IκBα, like that following signal-dependent stimulation, was sensitive to proteasome inhibition [49].

NF-κB is synthesized as a p105 precursor protein which is processed to the mature p50 form. Limited proteasome-mediated proteolysis of the C-terminal domain of p105 appears to be the mechanism by which p105 is processed [50, 51]. Remarkably, a recent study [52] provides evidence that p105 processing can be carried out by the 20S proteasome in the absence of ubiquitination. In this study processing of NF-κB to the p50 form was accomplished in a purified *in-vitro* system using only the 20S core particle of the proteasome. The processing *in vivo* also appears to occur independently of prior ubiquitination. Processing was unaffected in a temperature-sensitive E1 mutant cell line, and following mutation of "critical" lysine residues around the region required for processing. It should be noted that

in this study, not all lysine residues were mutated, thus the possible role of the unmutated lysines as sites of ubiquitination cannot be dismissed. This model of p105 degradation is supported by the results of an earlier study which showed that the viral Tax protein facilitates interactions between NF-κB and an α and β subunit of the 20S proteasome [53].

The conclusion that the processing of the p105 form of NF-κB is ubiquitin independent must be reconciled with earlier findings which strongly implicated ubiquitination in this process [50]. Using cell-free systems to study p105 processing, it was shown that the process was ATP dependent; that p105 could be polyubiquitinated in the *in-vitro* system; that methylated ubiquitin (lacking free amine groups) inhibited both p105 polyubiquitination and processing; and that the activity of a specific ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) was required.

5.2.8
Steroid Receptor Co-activator-3

The steroid co-activator receptor-3 (SRC-3 or AIB1) is a transcriptional co-activator that is encoded by an oncogene which is frequently amplified in breast cancer cell lines [54]. SRC-3 interacts with steroid hormone receptors and plays a role in proliferation, specifically in response to estrogen [55]. SRC-3 is degraded, in a ubiquitin-independent manner, by the REGγ (PA28γ) regulatory complex of the 20S proteasome [56]. REGγ is a member of a family of related 11S proteasome activators. In contrast to the related REGα and REGβ proteins that form a heteroheptameric regulatory complex, REGγ forms a homoheptameric complex and confers different peptide substrate specificities than the REGαβ complex [57]. Li and coworkers [56] found that SRC-3 could be immunoprecipitated in complexes containing REGγ and that SRC-3 interacted with REGγ through its histone acetyltransferase (HAT) domain (residues 1081–1417). Reducing REGγ expression in cells by means of RNA interference reduced SRC-3 turnover, and REGγ overexpression enhanced SRC-3 degradation. Degradation required the interaction of REGγ with SRC-3, since deletion of the SRC-3 HAT domain prevented SRC-3 degradation. Furthermore, a REGγ mutant incapable of activating the 20S proteasome also prevented SRC-3 degradation. The degradation of SRC-3, but not the related SRC-1 protein, by REGγ and the 20S proteasome could be reconstituted *in vitro* using purified components in the absence of either ATP or prior ubiquitination.

5.2.9
c-Jun

c-Jun is a member of the AP-1 family of transcription factors that must homodimerize or heterodimerize with other factors in order to recognize specific DNA-binding sites. c-Jun is a short-lived protein that is ubiquitinated *in vivo* [58]. Jariel-Encontre and co-workers [59] demonstrated that c-Jun could be degraded *in vitro* using fractionated rat liver lysates depleted of ubiquitin or the ubiquitin-

activating enzyme E1. These extracts were shown to be incapable of modifying their endogenous proteins with radiolabeled ubiquitin, or of directing the degradation of recombinant p53, which depends on ubiquitination. This degradation of c-Jun in the absence of ubiquitin was mediated by the proteasome, since it was both ATP dependent and inhibited by the immunodepletion of proteasomes. Furthermore, c-Jun degradation could be accomplished using purified 26S proteasomes alone, but not by the 20S form, indicating that no other factors in the lysate were required for ubiquitin-independent degradation.

5.3
Mechanisms of Ubiquitin-independent Degradation

One question is central to the discussion of both ubiquitin-dependent and -independent pathways of proteasome-mediated proteolysis: what are the minimal structural elements a protein must have to be recognized and processed by the proteasome? For the majority of substrates, the simple conventional answer is that conjugation to a polyubiquitin chain containing Lys48 linkages is sufficient. However, as we described above, a wide variety of proteasome substrates can potentially be degraded in the absence of such ubiquitin modification. This leads to the fundamental question regarding ubiquitin-independent proteolysis: are ubiquitin-independent substrates inherently "ubiquitin-like" (that is recognized by same receptor), or is their mechanism of recognition by the proteasome completely divorced from the ubiquitin pathway? Given the evidence at hand, it seems that either alternative is possible, depending on the substrate in question.

The ubiquitin-independent substrates we have described can be roughly divided into two categories: those recognized and degraded by the 20S core particle alone, and those that require the intact 26S holoenzyme. Some fall into both categories. The requirements for degradation of a substrate by the 20S proteasome are likely to differ greatly from those processed by the 26S proteasome.

For those substrates that depend on the proteasome holoenzyme (or more specifically the 19S regulatory particle), one simple explanation of their lability is that they contain ubiquitin-like domains that mediate their interaction with the proteasome. Recent biochemical evidence suggests the cODC domain, in spite of lacking any obvious homology to ubiquitin, is such a domain [60]. The capacity of cODC to direct degradation depends on its mimicry of a polyubiquitin chain. Using purified rat 26S proteasomes, protein substrates carrying cODC and those with a ubiquitin oligomer attached were found to act as mutual competitive inhibitors, these experiments demonstrated that cODC and the ubiquitin conjugate co-occupy a common proteasome element required for substrate recognition. Alternately, the ubiquitin and cODC receptors might occur at distinct, but proximal, sites within the proteasome such that occupancy at one site prevents access to the other. Whether polyubiquitin chains compete with other ubiquitin-independent substrates, such as c-Jun, for the 26S proteasome has not yet been determined.

What sequences in ubiquitin-independent proteasome substrates act as signals for degradation by the 20S proteasome? In the case of ODC, the cODC signal required for degradation by the 26S proteasome is wholly dispensable for degradation by the 20S form under conditions of oxidative stress [26]. The observation that monomerization of ODC is required for degradation under these conditions suggests that the relevant alternative signal resides at, or proximal to, the dimer interface.

Both p21 and Rb interact with the C8/α7 subunit of the 20S proteasome and these interactions appear to be facilitated by the MDM2 ubiquitin ligase [29, 36]. The 31 C-terminal residues (134–164) of p21 are required for its interaction with C8, and this sequence was sufficient to confer this binding ability on p27, which normally does not interact with C8 when attached to its C-terminus [29]. The residues required for C8 interaction mapped to the C-terminal RbC pocket of Rb (residues 772–928) and this same region is required for binding MDM2 [61]. MDM2 itself appears to interact with C8 through its RING-finger domain, since a point mutation in this domain (C464A) greatly impairs MDM2–C8 interaction [36]. Whether, the NQO1-inhibited degradation of ODC and p53 by the 20S proteasome also involves such interactions with the C8 subunit remains to be determined.

How do substrates of the 20S proteasome gain access to catalytic sites sequestered within the core particle? The axial channel of the 20S core particle is normally occluded by interactions of the N-termini of its α subunits when the 20S core is not associated with regulatory complexes [62]. This gating impairs the degradation of both peptide and protein substrates by the 20S proteasome. An associated substrate must therefore be able to disrupt these interactions in order to gain access to the axial channel and the internal active sites. In the proteasome holoenzyme, association of the 19S or 11S/PA28 regulatory complexes reorganizes the N-terminal residues of the α subunits and leads to an "open-gate" conformation [62, 63]. It was found that purified 20S proteasomes progressively lose the auto-inhibition of peptidase activity by the N-termini of the α subunits unless they are maintained in the presence of potassium ions [64]. It therefore seems possible that the degradation of some substrates by the 20S proteasome might be non-specific and due to the spontaneous activation of the α subunits if these proteasome are not purified and stored in buffers containing potassium ions.

In addition to activating the 20S core particle to an open-gate conformation, substrate degradation also requires the unfolding of substrates to accommodate their insertion through the narrow (~13 Å) axial channel. For substrates degraded by the 26S proteasome, this unfolding is thought to be accomplished by the ATPase subunits of the 19S regulatory particle. For 20S substrates two possibilities seem obvious: first, a substrate may be unstructured, thus obviating the need for unfolding prior to internalization. A protein may either be inherently unstructured, such as p21, which lacks an ordered conformation unless bound by Cdk2 [65], or alternatively, a substrate may be rendered unstructured by modification prior to proteolysis [66, 67]. Second, unfolding a structured 20S substrate could be accomplished by associated proteins not intrinsic to the 19S regulatory

particle. For example, the degradation of oxidized calmodulin requiring the presence of the chaperone Hsp90, is stimulated by ATP, and is sensitive to Hsp90 inhibitors [68]. The proteolysis of peptide substrates was unaffected by Hsp90 in these experiments, indicating that the chaperone is not acting to alter the gating conformation of the 20S proteasome, but more likely assisting in proteasome association and unfolding prior to entry. In contrast, the processing of the NF-κB p105 precursor by the 20S proteasome occurs in the absence of associated chaperones [52]. In this case, the absence of other proteasome-associated factors was established rigorously by mass spectrometric analysis. Apparently p105 is sufficient to enable processing by highly purified 20S complexes. This is likely a property of the C-terminal half of the precursor, since this region in isolation is degraded by purified 20S proteasomes following endoproteolytic cleavage from NF-κB p50 [52].

Is proteasome association itself sufficient to render a protein a substrate for proteolysis? There is an entire class of proteasome-associated "adaptor" proteins, such as the Rad23 and Dsk2 proteins of *S. cerevisiae*, which interact with the proteasome through ubiquitin-like domains but are not themselves subjected to degradation [69, 70]. This suggests that proteasome interaction by itself is insufficient to initiate destruction (see below). In the case of ubiquitinated substrates, native substrates cannot be used to resolve this question without further experimental manipulation. When subjected to such tests, a Lys48-linked polyubiquitin chain *per se* has proven insufficient (for examples, see [71, 72]). These data imply that the position of substrate conjugation matters, as may local sequence contiguous to the site of conjugation. Is that because of an effect of localization on the capacity of the ubiquitin chain to act as an association element with the proteasome? More specifically, is proteasome association but one attribute conferred by polyubiquitin, or is it sufficient on its own?

In principle, the question can be answered by providing an alternative method for delivering a protein to the proteasome, one that bypasses a need for ubiquitin conjugates. If any stable protein can be delivered to proteasomes without using ubiquitin and thereby undergo degradation, proteasome association must be the only requirement for degradation. This strategy was tested by conditionally tethering the non-substrate protein His3 to a non-essential intrinsic proteasome protein, Rpn10 [73]. Localizing His3 to the proteasome in this way converted His3 into a substrate. This result implies that localization promotes degradation, but it may nonetheless be insufficient: other components of the delivery system used in these experiments (or His3 itself) may provide further signals or structures required for degradation. Tethering provides a very high local concentration, which, in the example cited, was sufficient, but generality of this conclusion requires additional experimental testing. On the face of it, this cannot be a full description of substrate specification, or else the proteasome would be continually digesting bits of itself. And, as mentioned above, there exists numerous examples of proteasome-associated proteins that are not subjected to proteolysis [69, 70]. This conclusion is further supported by the recent work of Matouschek and colleagues that suggests that proteasome association cannot be the whole story [74]. They provided evidence

instead for a two-element model, whereby an association tag, usually polyubiquitin, collaborates with an unstructured region that is required as a proteasome entry site. The question of whether localization is sufficient remains unresolved.

The degradation tag of ODC, cODC, provides a favorable test bed to answer these questions. A cysteine thiol of cODC, at position 441 of native mouse ODC, proved critical in determining the molecular basis for cODC action[75]. Removing that thiol or replacing it with a hydroxyl abolished both degradation and the ability of cODC to act as a competitive inhibitor of native ODC degradation. C441 is therefore essential for localization to the proteasome of proteins containing cODC. To assess whether association is sufficient, we performed experiments using constructs containing GFP, a compact single-domain globular protein lacking prominent unstructured protrusions, to dissect cODC function [76]. A fusion of GFP to cODC (GFP-cODC) is degraded by proteasomes, but the identical molecule with C441 thiol deleted (GFP-cODC^{C441A}) is not degraded, nor is it recognized by proteasomes. Fusing the non-essential proteasome protein Rpn10 to GFP results in the association of this fusion protein (Rpn10–GFP) with the proteasome. Despite its association with proteasomes, Rpn10–GFP is stable and not rapidly degraded. Adding cODC to Rpn10–GFP, to make Rpn10–GFP–cODC, produces a protein that is degraded, as expected; this protein has two potential interaction elements for the proteasome, Rpn10 and cODC. If the thiol of C441 within cODC is removed by mutation to make Rpn10–GFP–cODC^{C441A}, degradation persists. Because cODC^{C441A} cannot mediate docking, it must provide some other essential function, a function that accounts for the different properties of Rpn10–GFP (stable) versus Rpn10–GFP–cODC^{C441A} (unstable). The cODC^{C441A} element provides no specific structural information necessary for degradation in the context of Rpn10–GFP–cODC^{C441A}; a variety of alternate carboxyl termini inserted in place of cODC^{C441A} can support degradation. We infer that interaction alone does not make a substrate; association must be supplemented by the presence of an unstructured region. This finding is consistent with the two-element model of Matouschek [74].

5.4 Conclusion

An increasing interest in the role of proteolysis in the regulation of gene expression has led not only to a better understanding of the ubiquitin–proteasome system, but also to an increasing awareness of those substrates that are (in whole or in part) not completely dependent on the canonical degradation pathway. Our understanding of substrate–proteasome interactions has been broadened and deepened by extending investigations beyond ubiquitin conjugation and the 26S proteasome. A consideration of the variety of mechanisms utilized by the proteasome not only provides an appreciation of the complexities of the proteolytic machine, but potentially provides novel tools with which to dissect those mechanisms.

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6
Endoplasmic Reticulum Protein Quality Control and Degradation

Antje Schäfer, Zlatka Kostova and Dieter H. Wolf

6.1
Introduction

Life is full of risks. This holds true even for the most important molecules of a cell, i.e. its proteins. Many steps that can go wrong lie ahead of a protein, starting from its birth or synthesis, all through its development into a mature, biologically active entity, [1, 2]. Problems include premature inhibition of synthesis, improper folding, incorrect maturation and, in the case of oligomeric proteins, lack of interacting partners resulting in orphan proteins. The cell has to make sure that these abnormal or orphan proteins are rapidly eliminated. In mammalian cells, failure to do so may lead to severe protein folding diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Creutzfeldt Jacob disease, bovine spongiform encephalophathy (BSE, cattle) and many others [3–8]. In eukaryotic cells, aside from a small portion of protein synthesis within the mitochondria, the majority of proteins are synthesized on cytoplasmic ribosomes. Secretory proteins, proteins of the cell membrane, the lysosome (vacuole), the Golgi apparatus and the endoplasmic reticulum (ER) have to be imported from the cytoplasm into the ER in an unfolded state, folded within this organelle and subsequently delivered to predetermined cellular sites in their biologically-active forms [9, 10]. About one-quarter of the proteome traverses the secretory pathway [11]. The ER provides the cell with an optimized environment that deals with the heavy load of folding work with its high concentration of chaperones. Nevertheless, folding of secretory proteins often fails. The ER fights this problem with two distinct, but interconnected mechanisms. The first is an ER-dedicated stress response that remodels the ER in such a way that its folding capacity increases and this is known as the unfolded protein response (UPR) [12, 13]. The second is a strict ER protein quality control system (ERQC) followed by the elimination of improperly folded proteins and protein complexes unable to assemble into higher order structures (ERAD) [14–22]. Both events are components of what has been called the ER quality control and degradation (ERQD) process [23]. UPR and ERQD are tightly interconnected: UPR induction increases the capacity of ERQD and failure of ERQD leads to UPR induction. ERQD requires an initial

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recognition step, which retains and/or retrieves improperly folded proteins in the ER and decides whether "misfolding" has occurred. In a second step, the misfolded protein is handed over to an elimination system. Due to the prevailing dogma of the time that secretory proteins, once imported into the ER, were trapped in the secretory pathway, unable to return into the cytoplasm [24], the idea of an ER-localized proteolytic system for degradation of the misfolded proteins was proposed, but was difficult to conceive [25]. Shortly thereafter it was shown that misfolded proteins of the ER membrane can become targets of the cytosolic ubiquitin–proteasome system [26–29]. The delivery mechanism of misfolded proteins of the ER to the ubiquitin–proteasome system, however, remained an enigma. In a study on yeast, using a misfolded mutant of the vacuolar enzyme Carboxypeptidase yscY (CPY*) Hiller et al. showed that after translocation into the ER, the mutant protein was fully glycosylated, then retro-translocated out of the ER into the cytoplasm where it was ubiquitinated and degraded by the proteasome [30, 31]. At nearly the same time, a virus-induced "dislocation" and proteasomal degradation of the major histocompatibility complex (MHC) class I molecule from the ER was reported [32]. In addition, a report concerning the retro-translocation of a mutated yeast pheromone peptide, α-factor, from the ER to the cytoplasm and its degradation by the proteasome, was published [33]. These studies overturned the existing dogma and completely reshaped our thinking with regard to the mechanism of elimination of misfolded proteins from the ER. The model eukaryote *Saccharomyces cerevisiae,* easily amenable to genetic, molecular biological and biochemical experimentation, has been a pacemaker in the study of ER protein quality control and degradation (ERQD). Since ERQD is a "housekeeping" mechanism common to all eukaryotic cells, research in yeast is likely to uncover the basic principles conserved in all mammalian cells and will continue to pave the way for the elucidation of the ERQD mechanism and for our understanding of this central cellular process.

6.2
ER-import, Folding and the Unfolded Protein Response

Proteins destined for secretion or for residence within the compartments of the secretory pathway enter the ER via a translocation channel known as the Sec61 translocon. The translocon is composed of three integral membrane proteins, Sec61, Sbh1 and Sss1 in yeast and Sec61α, Sec61β and Sec61γ in mammalian cells [9, 34]. Nascent polypeptides enter the ER in an unfolded state. Their folding in the ER lumen, accompanied by chemical modifications such as disulfide bond formation and addition of carbohydrates, requires a multitude of enzymes and chaperones. Among those are peptidyl-prolyl isomerases, disulfide bond modifying enzymes (Ero1p, PDI and others), classical chaperones (BiP, Kar2 in yeast) and co-chaperones, the oligosaccharyltransferase complex, N-glycan-modifying enzymes such as glucosidases I and II, and α-1,2-mannosidase and, in mammalian cells, UDP-Glc: glycoprotein glucosyltransferase as well as lectin-like

chaperones (calnexin, calreticulin) [8, 35]. Properly modified and folded proteins are packed into vesicles and transported to the Golgi apparatus from where they proceed towards their final destination [8, 11, 14]. A highly sophisticated protein quality control system residing in the ER scans proteins for correct folding. If anything goes wrong with folding or membrane insertion, the ER reacts with the two interconnected mechanisms: the unfolded protein response (UPR) and, if all measures fail, the ER-associated protein quality control and degradation (ERQD) pathway. These mechanisms work together: the unfolded protein response (UPR) provides the ER with an increased capacity to fold proteins and control their folding [12, 13, 36] and upregulates the components of the machinery capable of degrading misfolded proteins [37–39]. Loss of ERAD leads to constitutive induction of UPR. Loss of UPR and ERAD results in dramatically decreased cell viability [36, 38, 39].

In yeast, a highly conserved transmembrane kinase of the ER, Ire1, monitors the folding capacity of the ER. Ire1 is composed of an ER-lumenal domain that senses the presence of misfolded proteins and a cytoplasmic tail consisting of a kinase and an endoribonuclease domain. ER stress, resulting from the accumulation of misfolded proteins, leads to oligomerization and autophosphorylation of Ire1, which activates the kinase function. Ire1 is targeted to the inner nuclear membrane via a nuclear targeting sequence [40]. Here, the endoribonuclease domain of Ire1 participates in the nonconventional splicing of the b-ZiP transcription factor HAC1 (XBP-1 in metazoans) mRNA. Splicing of the HAC1 mRNA leads to the synthesis of an active transcription factor that triggers the synthesis of UPRE-controlled genes, including genes encoding components of the ERQD machinery. It was initially proposed that Ire1 is activated when the major Hsp70 of the ER, BiP (Kar2 in yeast), usually bound to the ER-lumenal domain of Ire1, is titrated out by increasing amounts of misfolded proteins [36]. The finding that the conserved core region of the lumenal domain of dimerized Ire1 forms a deep hydrophobic groove has led to the proposal that Ire1 binds misfolded proteins directly [41]. The discussion continues as recent data indicate that this groove is too narrow for peptide binding and that the dimerization of Ire1 does not require the direct binding of unfolded proteins [42]. However, regardless of whether Ire1 senses the presence of misfolded proteins directly or indirectly, it is certain that UPR responds to subtle changes in the misfolded protein content of the ER. Mammalian cells express two other sensors in addition to IRE1: (i) the ER transmembrane kinase PERK containing a cytoplasmic elF2α kinase domain and (ii) the ER transmembrane transcription factor ATF6. Binding of misfolded proteins to PERK activates the kinase domain. This leads to inhibition of translation and upregulation of the transcription factor ATF4 that initiates the transcription of some UPR target genes. Transmembrane ATF6, on the other hand, reaches the Golgi apparatus when there is an accumulation of misfolded proteins, where a proteolytic cleavage releases the cytoplasmic transcription factor domain. The transcription factor enters the nucleus to initiate gene transcription. Equipped with a set of three distinct sensors for misfolded proteins, IRE1, PERK and ATF6, mammalian cells respond to the accumulation of misfolded proteins in the ER in a more nuanced fashion than yeast. These

responses range from increased ERQD, in everyday life, to induction of apoptosis under conditions of permanent stress [13].

6.3 General Principles and Components of ERQD (Endoplasmic Reticulum Quality Control and Protein Degradation)

The initial components of ER-associated protein degradation were, to a large extent, discovered via yeast genetics studies. The first indication that the cytoplasmic ubiquitin–proteasome system participated in the degradation of a misfolded ER protein came from studies on a yeast *sec61-2* mutant harboring a defective ER-translocation channel that, at restrictive temperatures, was degraded following ubiquitination: a mutation in the ubiquitin-conjugating enzyme Ubc6 restored the growth of *sec61-2* cells at the restrictive temperature leading to the conclusion that the Sec61-2 protein was not degraded in this mutant under these conditions [29]. The role of the cytoplasmic proteasome in the degradation of an ER membrane protein was discovered when the fate of a mutated ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) was studied in human cells. The ΔF508 mutation renders the CFTR protein unable to leave the ER to reach the plasma membrane. Trapped in the ER, mutant CFTR undergoes rapid degradation by the proteasome which results in the manifestation of the disease state, cystic fibrosis [27, 28]. Up till then, the ubiquitin–proteasome system was known to be an essential and selective protein degradation machinery required for signal-induced protein elimination as well as for removal of misfolded cytoplasmic proteins [43–46]. Proteins destined for degradation by the proteasome were known to be polyubiquitinated via a cascade of three enzymes. First, an ubiquitin-activating enzyme utilizing ATP to form an anhydride bond between the carboxyl of the C-terminal glycine of ubiquitin and the phosphate group of AMP, transfers this activated ubiquitin onto the active site cysteine of the enzyme to form a thioester bond. Second, the thioester-bonded ubiquitin is transferred to a ubiquitin-conjugation enzyme (Ubc, E2) retaining the active thioester bond. Finally a ubiquitin protein ligase (E3) links the ubiquitin in an isopeptide bond to the -amino group of a lysine side chain of the protein to be degraded, either directly or via the E2. When internal lysines are not available, ubiquitin can be linked with a peptide bond to the N-terminus of a protein [47]. For degradation by the proteasome, a polyubiquitin chain has to be formed on the lysine 48 residue of each preceding ubiquitin. Ubiquitin chains of four and more units are recognized by the proteasome, initiating protein degradation [44, 48]. The finding that mutated ER membrane proteins are degraded by the ubiquitin–proteasome system [26–29] did not give any information about the elimination mechanism of these proteins. A mutational analysis of yeast expressing a mutant and, therefore, misfolded vacuolar Carboxypeptidase yscY (CPY*) disclosed the mechanistic steps of ERAD. It was discovered that mutant CPY* is imported into the ER completely, it is fully N-glycosylated, recognized as unable to fold properly, retrograde transported back into the cytoplasm

in its glycosylated form, ubiquitinated and, finally, degraded by the proteasome [19–21, 30, 31, 49]. The ubiquitin-conjugating enzymes Ubc6 and Ubc7 [31] and, later, Ubc1 [39] were found to catalyze the ubiquitination of CPY*. Genetic and biochemical analyses of yeast strains exhibiting defective CPY* degradation resulted in the discovery of the following additional ERQD components, listed in chronological order: Der1 (degradation of the ER), a protein spanning the ER membrane four times with its N- and C-termini located in the cytosol [37, 50]; soluble ER α-mannosidase I [51]; Cue1, a type I protein of the ER [52]; Kar2p, the major Hsp70 chaperone of the ER lumen [53] and its J-domain co-chaperones Jem1 and Scj1 [54]; Der3, a RING-finger ubiquitin-protein ligase (E3) spanning the ER membrane six times with the N- and C-termini facing the cytosol [55–57]; Der5/Pmr1, an ER/Golgi-located Ca^{2+} pump [58]; Png1, a cytoplasmic peptide N-glycanase [59]; Htm1/Mnl1, an ER lectin [60, 61]; the trimeric AAA-ATPase complex of the cytoplasm consisting of Cdc48, Ufd1 and Npl4 [62–64]; Dsk2 and Rad23, two UBA-UBL domain proteins [65]; Der7/α-glucosidase I involved in N-glycan trimming [66]; Yos9, a lectin-like protein of the ER lumen [67–70]; Ubx2 a protein containing an UBX domain and spanning the ER membrane twice, the N- and C-termini reaching into the cytosol [71, 72] and Usa1, another double-pass ER membrane protein [73, 74]. Studies on the regulated degradation of the HMG-CoA-reductase isozyme 2 in yeast [75] revealed that its downregulation is dependent on several *HRD* gene products (HMG-CoA reductase degradation) and the proteasome. *HRD1* was found to be identical to *DER3,* encoding the ER ubiquitin protein ligase involved in CPY* degradation [57, 75]. *HRD3* encodes a single-pass transmembrane protein with a long N-terminal portion in the ER lumen and a short C-terminal tail in the cytoplasm. Hrd3 is also required for the degradation of misfolded CPY* [76]. Degradation of HMG-CoA reductase 2 was also dependent on the Golgi P-type ATPase, Cod1/Spf1 [77]. Like Pmr1, Cod1, is also required for CPY* degradation [78]. The overlap of the "*DER*" genes with the "*HRD*" genes and the subsequent finding that most of the components required for ERAD are also required for the downregulation of HMG-CoA-reductase 2 shows that the cell uses the degradation machinery of the ER not only for the elimination of misfolded secretory proteins but also for the regulation of central metabolic enzymes located in the ER. A screen for mutants defective in the degradation of fusion proteins carrying the Deg1 degradation signal of the MATα2 repressor yielded Doa10, a novel ubiquitin-protein-ligase of the ER [79]. Like Hrd1/Der3, Doa10 is an ER membrane E3 containing a RING-finger domain. The protein spans the membrane 14 times with both termini facing the cytoplasm [80].

6.4 Mechanism of ERQD

The involvement of carbohydrate chains in the recognition of misfolded proteins was revealed by the finding that a mutation in α-mannosidase I resulted in considerable retardation of CPY* degradation [51]. Upon entry into the ER,

pre-assembled oligosaccharide chains of the structure $Glc_3Man_9GlcNAc_2$ are transferred onto asparagine residues within the consensus Asn-X-Ser(Thr) sequence of the nascent polypeptide chain. While the polypeptide chain folds, the Glc_3-Man_9-$GlcNAc_2$ structure undergoes trimming. Sequential action of glucosidases I and II leads to the removal of the two terminal glucose residues, resulting in a Glc_1Man_9-$GlcNAc_2$ structure. In mammalian cells, the chaperones calnexin and calreticulin specifically recognize and bind this mono-glucosylated oligosaccharide. These lectin chaperones and foldases such as peptidyl-proline isomerases and disulfide isomerases bind and facilitate folding of the protein into its correct native structure. During this process the last and innermost glucose residue is also removed by glucosidase II. If the protein has not folded properly by the end of the time allocated for glucose-trimming, the de-glucosylated oligosaccharide is re-glucosylated by the folding sensor UDP: glucose glycoprotein glucosyltransferase (UGGT) to restore the $Glc_1Man_9GlcNAc_2$ structure and allow time for a new round of folding. If folding remains unsuccessful, α-mannosidase I removes the outermost mannose residue from the de-glucosylated oligosaccharide, thus preventing secretion of the protein from the ER and committing it to ERAD [14, 81]. In yeast, the re-glucosylation mechanism by UGGT does not exist. Instead, following removal of the three glucose residues by glucosidases I and II [66, 82], the slow acting α-mannosidase I [51] seems to be the only timer for the decision process of secretion or degradation. Terminally misfolded proteins containing the $Man_8GlcNAc_2$ structure are retained in the ER to undergo ERAD.

The first protein assembly required for the degradation of soluble, ER lumenal misfolded CPY* and ER membrane HMG-CoA reductase was described in yeast as a complex between the E3 ubiquitin protein ligase Hrd1/Der3 and its partner Hrd3 [55, 83, 84]. The ubiquitin-conjugating enzyme Ubc7, recruited to the ER membrane by Cue1p [52] was found to interact with the RING-finger domain of Hrd1/Der3 [55], thus expanding the membrane complex to a Ubc7–Cue1–Hrd1/Der3-Hrd3 structure. Later, the membrane protein Usa1 and the first identified ERAD component Der1 [51] were shown to be part of the ERAD complex [73, 74, 85, 86]. More recently, transmembrane Ubx2 was identified as the link between the Ubc7–Cue1–Hrd1/Der3–Hrd3–Usa1–Der1 complex of the ER membrane and the trimeric Cdc48–Ufd1–Npl4 complex of the cytosol [71, 72] (Figure 6.1). Similarly, in the lumen of the ER, interactions of the lectin-like protein Yos9 and the major Hsp70 of the ER, Kar2 (BiP in mammalian cells) with Hrd3 were uncovered [73, 74, 85]. A detailed picture of the degradation pathway of N-glycosylated misfolded ER-lumenal and lumenal lesion-containing ER membrane proteins is slowly emerging. After synthesis in the cytoplasm secretory proteins are translocated into the ER where, during/after N-glycosylation, they undergo folding. An important component of the ER folding machinery is the Hsp70 chaperone Kar2, which on one hand assists folding, and on the other, keeps the protein that has not reached its native structure, in a soluble form [54, 87]. During the folding process, glucosidases I and II cleave the three terminal glucose residues of the $Glc_3Man_9GlcNAc_2$ glycan. Then, misfolded proteins become substrates of α-mannosidase I which trims the glycans further to create a $Man_8GlcNAc_2$ structure;

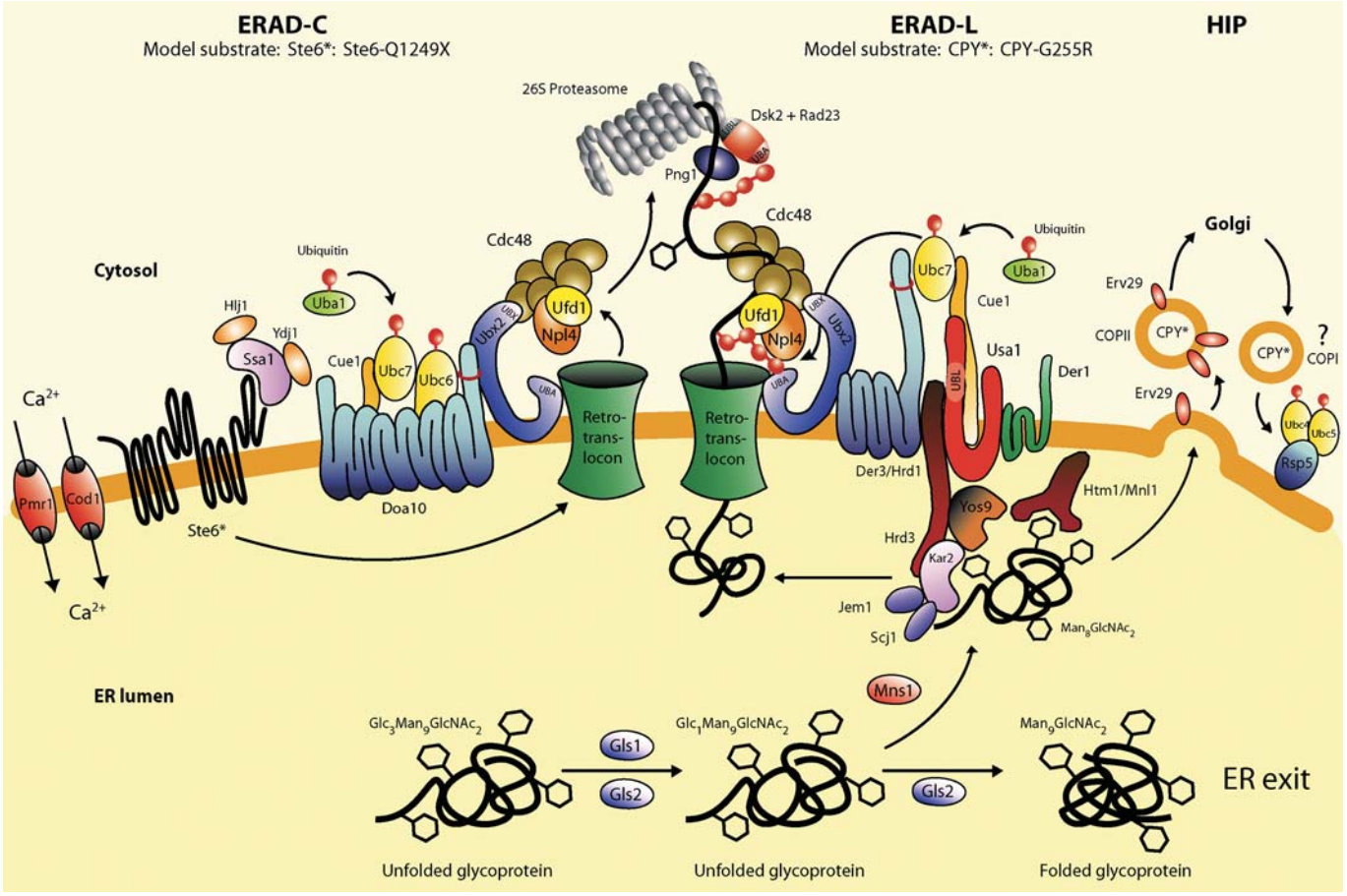

Fig. 6.1. ER protein quality control and degradation (ERQD). ER-associated degradation pathways: ERAD-C, ERAD-L and HiP.

the misfolded proteins are then recognized by and interact with the lectin-like proteins Yos9 and Htm1. Kar2 and Yos9 interact with the N-terminal lumenal tail of Hrd3 [73, 85] (Figure 6.1). One may speculate that Kar2 and Yos9 bind hydrophobic patches of proteins undergoing folding and deliver these to the lumenal tail of Hrd3, which acts as a receptor for misfolded proteins exposing hydrophobic amino acid stretches. So far, Yos9 is known to affect only the degradation of glycosylated proteins [67–70] even though the initial step of Yos9 binding to misfolded proteins is independent of their glycosylation status and the interaction of Yos9 with Hrd3 [73]. It is conceivable that the lectin domain of Yos9 scans the bound substrate, which is also interacting with Kar2 and Hrd3, for the presence of the $Man_8GlcNAc_2$ structure indicative of improper folding, for delivery to the ubiquitin-protein ligase Hrd1/Der3. Such a bipartite control mechanism, inspecting both the hydrophobicity and N-glycan structure, would ensure that only misfolded proteins are polyubiquitinated and degraded. It is interesting to note that the location of carbohydrates on a misfolded protein seems to be important for its degradation [88, 89]. The relationship between the N-glycan position and degradation is presently not understood. Similarly, the function of the second lectin-like protein involved in ERAD, Htm1/Mnl1p, [60, 61] is unclear. One may speculate that it also has a N-glycan screening function. While the role of most components of the Hrd/Der surveillance complex is emerging, the function of Der1 is still not understood. In yeast ERQD, Der1 is required only for the degradation of soluble substrates like CPY* [37, 50, 87]. Der1 orthologs (known as Derlins) have been found in mammalian cells and also shown to be involved in ERAD [90–92]. Derlin-1 is required for the degradation of MHC class I heavy chains induced by the human cytomegalovirus (HCMV) protein US11. Interestingly, MHC class I heavy chain degradation induced by the HCMV protein US2 is independent of Derlin-1 [92, 93]. The formation of homo-oligomers of Derlin-1 that span the ER membrane several times has given grounds for the speculation that Derlin-1 might be part of the retro-translocation channel [91, 92]. The link between the ER-membrane Hrd/Der ubiquitination assembly and the proteasome is a trimeric chaperone complex consisting of the AAA-ATPase Cdc48 (p97 in mammalian cells), Ufd1 and Npl4. It is believed that this complex pulls the polyubiquitinated proteins out of the ER or away from the ER membrane [62–64]. Delivery of ER-released polyubiquitinated substrates to the proteasome requires the UBA-UBL domain proteins Dsk2 and Rad23 to function, presumably, as adaptors preventing the dissociation and accumulation of free, unfolded protein chains in the cytosol [65, 94] (Figure 6.1). The ERQD mechanism, as described, leaves out Doa10, the second ERAD ubiquitin protein ligase. Doa10 has 14 transmembrane domains and resides both in the ER and the inner nuclear membrane [79, 80, 95]. The enzyme is not involved in the degradation of ER luminal-soluble CPY* [79]. While CPY* and membrane proteins carrying a misfolded domain in the ER lumen are degraded via the Hrd1/Der3 ubiquitin ligase pathway known as the ERAD-L (luminal) pathway [87, 96, 97], degradation of proteins carrying lesions on the cytoplasmic side of the ER membrane (such as Ste6*, a truncated version of the yeast a-factor transporter Ste6) were shown to be substrates of the Doa10 ubiquitin ligase [96, 98]. Accord-

ingly, this degradation pathway was called the ERAD-C (cytosolic) pathway [96]. As for the Hrd1/-dependent ERAD-L pathway, the main ubiquitin-conjugating enzymes required for polyubiquitination of ERAD-C substrates are Ubc6 and Ubc7 bound to Cue1 [96, 98]. The two pathways, ERAD-L and ERAD-C, converge at the Cdc48–Ufd1–Npl4 complex and the proteasome [74, 96, 98] (Figure 6.1). The degradation of Ste6*, with the truncated protein domain in the cytosol, requires the action of the cytoplasmic Hsp70 chaperone Ssa1 as well as the Hsp40 co-chaperones Ydj1 and Hdj1 (Figure 6.1). This requirement is similar to that of some misfolded cytoplasmic proteins, as discovered recently [99, 100]. The cytoplasmic Hsp70 chaperone machinery is, most likely, involved in the discovery of the misfolded, cytoplasmic protein domain. It is unclear if this machinery is also active in the unfolding of misfolded membrane-embedded proteins. Also recently, the existence of a third "pathway", ERAD-M, responsible for the degradation of membrane proteins with destabilized transmembrane domains has been postulated [74]. For such substrates degradation depends on the Hrd1/Der3 ubiquitin ligase and Hrd3 of the ERAD-L pathway, but it does not always require Der1. ERAD-M seems to converge with ERAD-L and ERAD-C at the Cdc48–Ufd1–Npl4 complex. Der1 is also not required for the degradation of CTG*, a chimeric ERAD substrate containing misfolded CPY* tethered to the ER membrane by the last transmembrane domain of the multidrug resistance transporter Pdr5 and fused to GFP on the cytoplasmic side of the ER [87]. It is unclear how or whether ER-lumenal CPY* distorts the transmembrane domain to make CTG* into an ERAD-M substrate. On the other hand, a process requiring Der1 is the degradation of Hrd1/Der3, which occurs when Hrd3 is absent [76]. Given the heterogeneity of both substrates and components, a simple classification of misfolded protein degradation within the ER into distinct pathways, such as ERAD-L, ERAD-C and ERAD-M, may not provide all the answers.

6.5 "Overflow" Degradation Pathways: ER-to-Golgi Transport and Autophagocytosis

A portion of the ER-luminal ERAD substrate CPY* [49], was shown to escape into the Golgi apparatus from where it is retrieved back to the ER. The secretory competence of the ER is essential for efficient ER-associated degradation [101]. Deletion of Der1 induces the unfolded protein response [37] and significantly increases the escape of CPY* into the Golgi apparatus. A second, Hrd1/Der3-independent degradation pathway (HIP: HRD independent proteolysis) has also been described, which becomes operative when CPY* is considerably overexpressed. Overexpression of CPY* likely saturates the HRD/DER pathway and leads to the induction of the HIP pathway which, like ERAD, is also regulated by the UPR. HIP requires ER to Golgi transport, the HECT domain ubiquitin-protein ligase Rsp5 and the ubiquitin-conjugating enzymes Ubc4 and Ubc5 instead of Ubc7 and Ubc1. Whether misfolded proteins enter the cytosol from the Golgi apparatus or the ER is not clear [102]. Transport of overexpressed misfolded proteins to the vacuole has

been reported as an overflow pathway [103]. Recent experiments suggest that only proteins carrying an intact ER exit signal are prone to transport to the Golgi apparatus when misfolded. If, despite the misfolding of parts of the protein, the ER exit signal remains correctly folded, it can be recognized by the Erv29 cargo receptor and channeled into the secretory pathway. From here, the misfolded protein is directed to the HIP pathway or possibly to the vacuole for degradation. This model proposes competition between the ERQD machinery and ER exit facilitators for binding to misfolded proteins. The relative strength of the export signal on an aberrant protein against the affinity for the ERQD machinery would then determine what fraction, if any, of a misfolded protein exits the ER [104]. To completely block the degradation of CPY* both the HIP and the HRD/DER pathways have to be eliminated, emphasizing that both pathways contribute to the disposal of CPY*. Recently, autophagocytosis has been described as an additional overflow pathway for misfolded proteins that functions in coordination with ER-associated degradation. In yeast, expression of the Z-variant of the human α-1 proteinase inhibitor (A1PiZ) responsible for human liver disease, leads to saturation of ERQD and transport of the excess protein to the vacuole. A portion of A1PiZ reaches the vacuole via the secretory pathway. However, another portion, which forms aggregates, reaches the vacuole via autophagocytosis [105]. The mechanistic diversity in a cell which ensures the disposal of mutated and misfolded proteins when the ERQD machinery is overloaded reflects the importance given to the avoidance of the accumulation of disease-causing protein "garbage".

6.6 The Retrotranslocation Channel

One of the missing links in the current knowledge of ERQD is the nature of the retro translocation channel that delivers the misfolded proteins of the ER lumen or membrane back into the cytosol. The existence of a protein import channel in the ER membrane containing Sec61 as the channel-forming component drew attention to Sec61 as a possible component of the export channel. Indeed, genetic [53, 76, 106] and biochemical [107] experiments in yeast and mammalian cells [32, 108, 109] indicated the involvement of Sec61 in the retrotranslocation of the substrates studied. However, none of the studies undertaken for the isolation of ERAD-specific protein complexes containing Hrd1/Der3 or Doa10 have identified Sec61 as a component of the system [74, 86]. It is, therefore, unlikely that protein import and retrograde export use one and the same Sec61 channel to enable bidirectional transport. Rather, Sec61 import channels may associate either with the Hrd/Der or the Doa10 complexes to gain directionality for retrograde transport. Alternatively, Sec61 may form a hybrid channel with the Hrd/Der or Doa10 complexes which have the capacity to form channel-like entities due to their polytopic nature. One may also envisage that only certain misfolded proteins, i.e. the hydrophilic soluble proteins of the ER lumen, require the help of Sec61 for retrograde transport. The failure to find a biochemical connection between one of the ERAD

membrane complexes and Sec61 may be due to the high mobility of Sec61, shuttling between protein import and retrograde transport channels. Finally, Sec61 may not be involved in retro translocation at all and the export channels may be solely composed of the Hrd/Der and/or Doa10 complexes. Since genetic data involve Sec61 in retrotranslocation [53, 76] this would indicate an indirect effect of Sec61 mutations on the retrotranslocation process. This may be due to the fact that the Sec61-2 mutant used in the genetic studies is itself degraded via ERAD [26, 29]. Experiments carried out under conditions that would arrest the channel in the process of substrate retrotranslocation may ultimately provide us with the correct picture. The discussion still continues as a recent study has shown that the Sec61 protein import channel binds 26S proteasomes and suggested that it acts as a proteasome receptor on the ER membrane [110].

Some studies indicate that not all ERAD substrates exploit the canonical ERAD machinery for the retrotranslocation step. The yeast pro-α-factor carrying mutated glycosylation sites is retrograde transported out of the ER and degraded by the proteasome without ubiquitination [33] or involvement of the AAA-ATPase Cdc48 [111]. *In vitro* studies have shown that the 19S cap of the proteasome was able to retrotranslocate mutant α-factor. Addition of 20S proteasome core particles to this *in vitro* system led to degradation of the substrate, indicating that the two processes are uncoupled [111]. Interestingly, a recent *in vitro* study in mammalian cells provides evidence that the Cdc48 (p97) machinery is not essential but rather facilitates the degradation of one of the most studied mammalian ERAD substrates, the cystic fibrosis transmembrane conductance regulator (CFTR) [112]. This observation suggests that the AAA-ATPases of Cdc48 and the proteasome 19S cap cooperate in the unfolding and extraction of the polytopic membrane protein out of the ER membrane.

6.7 Metazoan ERQD

ERQD and UPR are "housekeeping" processes essential for cell survival that have evolved early in the history of eukaryotic life. It is, therefore, expected that mechanisms similar to those identified in yeast operate in higher eukaryotic cells. Indeed, many of the yeast proteins involved in ERQD and UPR have mammalian counterparts with similar functions. Table 6.1 lists the mammalian counterparts of the known yeast ERAD components. As might be expected, due to the specialized needs of mammalian cells, several components of ERQD have multiplied and diverged in substrate specificity. For instance, while only one Der1 protein seems to operate in yeast ERAD (a homolog, Dfm1, has not been found to operate in ERQD) [21, 37, 50], three Der1 orthologs, Derlin-1, Derlin-2 and Derlin-3, have been discovered in mammalian cells. While Derlin-1 is required for human cytomegalovirus-US11-triggered elimination of MHC class I heavy chains [92, 93], Derlin-2 and Derlin-3, but not Derlin-1, are involved in the degradation of the null Hong Kong (NHK) mutant of α1-proteinase inhibitor [90]. Two orthologs of the

Table 6.1. ERAD components in yeast and their mammalian counterparts.

Yeast	Mammalian	References
Kar2	BiP/GRP78	117–119
Yos9	OS-9 (?)	120–122
Htm1/Mnl1	EDEM1, EDEM2, EDEM3	123
Der1	Derlin-1, Derlin-2, Derlin-3	90, 124, 125
Usa1	HERP	126–128
Hrd3	SEL1L	129
Hrd1/Der3	HRD1 (Synoviolin), Gp78	115, 130–133, 113, 134–136
Doa10	TEB4 (MARCH-VI)	80, 137
Ubc6	Ube2g1	138–141
Ubc7	Ube2g2	113, 142, 143
Ubx2/Sel1	KIAA0887 (?)	74
Cdc48	p97/VCP	124, 144, 145
Ufd1	UFD1	146–148
Npl4	NPL4	146–149
Dsk2	PLIC-1 and 2 (Ubiquilin1,2)	150, 151
Rad23	hHR23A and B	152, 153

yeast ubiquitin ligase Hrd1/Der3 were identified in mammalian cells, gp78 [113, 114] and Hrd1 [115]. The two enzymes show different substrate specificity: gp78, but not Hrd1, is involved in the regulation of mammalian HMG-CoA reductase degradation [116]. There is strong evidence that mammalian ERQD pathways merge at the Cdc48–(p97)–Ufd1–Npl4 machinery [91] prior to degradation of the selected misfolded proteins via the proteasome. Further studies will gradually complete the present mosaic of ERQD and finally provide us with the whole picture of this life-saving process.

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7
Interactions between Viruses and the Ubiquitin–Proteasome System

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7.1
Introduction

Ubiquitination plays an increasingly important role in the regulation of many essential cellular processes. Like phosphorylation, glycosylation, methylation and acetylation, ubiquitination is a post-translational modification that tags a protein allowing recognition by host cellular machinery that will in turn, direct its subcellular localization and fate. The cellular pathways regulated by ubiquitination are diverse and include proteolysis, membrane protein trafficking, transcription, cell cycle control and cell signaling [1–3]. In addition, ubiquitination is also involved in important viral processes such as entry and egress.

A productive viral infection requires the effective manipulation of host functions by different viral genes. As obligate intracellular parasites, viruses have co-evolved with their hosts and adapted many cellular pathways for their own requirements. Dissecting the role of viral proteins in disease pathogenesis has led to not only a greater understanding of the function of the viral proteins, but also allowed identification of cellular homologs and an improved understanding of normal cell physiology. The ubiquitin–proteasome system (UPS) is no exception to this. Indeed the UPS provides many fine examples of how different viruses manipulate the host ubiquitin machinery resulting in altered protein function. Since the best recognized function of the UPS is protein degradation, it is not surprising that viruses are particularly adept at exploiting this pathway, leading to the accelerated degradation of cellular proteins which may interfere with viral fitness – examples include the virally-induced degradation of p53, MHC class I and APOBEC3G. Viral proteins may also manipulate the UPS to affect membrane trafficking, control of cell cycle, DNA repair, alterations of the immune system and may disrupt virtually every pathway involved in the UPS. A detailed review of all these pathways is beyond the scope of this chapter; however a brief overview of the viral proteins known to interact with the UPS will be followed by a discussion of viral E3 ligases and viral proteins that recruit cellular E3 ligases.

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7.2 Overview of Viruses and the Ubiquitin–Proteasome System

7.2.1 Proteolysis

Proteolysis within the cell is closely linked to the UPS, and viruses can interfere with many stages of protein degradation including the proteasome, the ER-associated degradation pathway (ERAD) and lysosomal degradation pathways. The proteasome is the major non-lysosomal site of protein degradation within the cell. Lysine-48-linked polyubiquitinated proteins are normally targeted for degradation by the proteasome, but during certain viral infections, the normal proteasome-mediated degradation process is perturbed. To avoid presentation to cytotoxic T lymphocytes (CTL), the Epstein-Barr virus (EBV) encoded EBNA-1 gene product contains an internal glycine–alanine repeat motif that not only prevents degradation by the proteasome but also reduces its rate of translation, blocking the formation of "Defective Ribosomal Products" (DRiPs) and therefore prevents the subsequent release of peptides for binding MHC class I molecules [4–6]. Similarly, latency-associated nuclear antigen-1 (LANA-1) of Kaposi's sarcoma-associated virus (KSHV or HHV-8) contains a strongly acidic string of amino acids that will also block the presentation of *cis* encoded peptides to CTL [7]. The matrix phosphoprotein pp65 of human cytomegalovirus (HCMV) is a major virion component that also blocks proteasome-mediated peptide generation. As an abundant component of the virion, pp65 is already present in high concentrations when the virus enters the cell, and does not require *de novo* synthesis. pp65 decreases the presentation of peptides *in trans* derived from the major immediate early transactivator (IE1) of HCMV [8]. Hepatitis B virus protein X (HBX) binds the proteasome and inhibits its protease and chymotryptic peptidase functions, leading to enhanced virus replication [9–11]. Tat, the transcriptional activator encoded by human immunodeficiency virus (HIV), inhibits the peptidase activity of the 20S proteasome and interferes with formation of the 20S proteasome–11S regulator complex by interfering with levels of LMP2, LMP7 and MECL1 transcripts – the components of the immunoproteasome [12, 13].

7.2.2 Viruses and the ERAD Pathway

Endoplasmic Reticulum Associated Degradation (ERAD) is a quality control step that involves the dislocation or retrograde translocation of misfolded proteins from the lumen of the ER to the cytosol where they are destined for proteasome-mediated degradation [14]. Misfolded proteins must be selected by ER-resident chaperones and brought to sites of retro-translocation, where they are then transferred back across the ER membrane via a protein-conducting channel. This retro-translocation requires polyubiquitination and the cytosolic ATPase p97/Cdc48. Insight into this pathway was initially achieved through study of the US2 and US11

gene products of human cytomegalovirus (HCMV). US2 and US11 were originally identified as proteins that downregulate major histocompatibility (MHC) class I molecules from the surface of infected cells by catalyzing the rapid retro-translocation of MHC class I heavy chains from the lumen of the ER back to the cytosol [15–17]. By using these viral proteins as a model system, many details of ERAD have been elucidated. Polyubiquitination is required for US2- and US11-dependent degradation of class I heavy chains [18, 19]. However, while ubiquitination of the class I molecules themselves is required for US2-mediated dislocation, a "lysineless" class I molecule can be dislocated from the ER in the presence of US11. While there is still a requirement for ubiquitination in the dislocation process, the target of this ubiquitination has not been identified [20].

Derlin-1 was identified as a binding partner of US11, but not of US2, required for dislocation [21, 22]. It is a tetraspanning ER membrane protein, proposed to act as a channel for the dislocation of misfolded proteins from the ER [23]. Another protein associated with ERAD is p97, a cytosolic AAA-ATPase required to provide energy for driving proteins through the dislocation channel or for releasing dislocated proteins from the cytosolic face of the ER membrane [24]. Although Sec61 was initially identified as a component of the retro-translocation machinery involved in US2-dependent class I degradation [17], more stringent conditions of analysis have not confirmed this. Further analysis of US2-associated proteins required for ER dislocation identified a role for the signal peptide peptidase (SPP), an intramembrane-cleaving aspartic protease of the presenilin family [25, 26]. While depletion of SPP by RNA interference blocks US2-mediated class I heavy chain degradation, it remains unclear whether it is the protease activity of SPP, or an additional function that is required for class I dislocation.

7.2.3
Membrane Protein Trafficking and Endosomal Sorting

Ubiquitination plays a role in the regulation of both the endocytic and exocytic pathways. The direct ubiquitination of a cell surface receptor, as well as its adaptor protein, is used as a mechanism for receptor internalization [27]. The direct effect of surface receptor ubiquitination is cargo recognition, internalization and sorting by the cellular trafficking machinery. Cargo sorting based on ubiquitination may result in either recycling to the plasma membrane or lysosomal degradation. Fine regulation occurs by a combination of ubiquitination and deubiquitination of components of the endocytic pathway and the cargo itself, although the details remain to be defined [28, 29]. For some receptors monoubiquitination is a sufficient stimulus for internalization [30, 31]. However, an increasingly important role for lysine-63-linked polyubiquitination is being currently recognized [32–34].

The highly conserved Endosomal Sorting Complex Required for Transport (ESCRT) machinery is recruited from the cytosol for the sorting of predominantly ubiquitinated proteins to multivesicular bodies (MVB) formed from membranes of the late endosomal compartments [35]. Tumor Susceptibility gene 101 (TSG101), a component of ESCRT-I, is essential for the sorting of ubiquitinated proteins

to the MVB [36]. Depletion of TSG101 rescues the downregulation of MHC class I molecules caused by the K3 gene product of Kaposi's sarcoma-associated herpesvirus (KSHV), by allowing recycling rather than lysosomal degradation of the polyubiquitinated class I molecules [37]. Ubiquitin-mediated receptor downregulation by related viral E3 ligases will be discussed in more detail below. Murine cytomegalovirus (MCMV) encodes several proteins known to downregulate NKG2D ligands on the surface of infected cells in order to avoid activating natural killer (NK) cells – the first line of defence against MCMV infection. Glycoprotein 40 (gp40) is expressed from the m152 gene of MCMV and is known to target RAE-1 [38], while the related gene, m155, encodes a protein shown to downregulate H60 in a UPS-dependent manner [39]. The adenovirus gene products 10.4 K and 14.5 K form a heterotrimeric complex known as receptor internalization and degradation (RID). RID expression results in the endocytosis and degradation of several important death receptors including tumor necrosis factor receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (TR1, TR2) and FAS, thereby protecting adenovirus-infected cells from apoptosis. Although not much is known about the mechanism of action of RID, it is known that targeting of TNFR1 involves the mu2 subunit of adaptor protein 2 and that clathrin-dependent internalization proceeds at a normal rate. However, increased degradation probably results from RID-promoted sorting into the endosomal/lysosomal compartment [40, 41].

7.2.4 Viral Entry and Egress

The UPS is involved in many stages of viral pathogenesis. Two key aspects of viral biology are entry and egress from the cells in which they replicate. Proteasome inhibition blocks the transfer of mouse hepatitis virus particles from endosomes into the cytosol suggesting a role for the UPS in the entry of this virus into cells [42]. Nuclear penetration of certain parvoviruses, including the minute virus of mice and canine parvovirus was also shown to involve the UPS. Inhibition of the proteasome by MG132 resulted in an accumulation of virus particles in the perinuclear region of the cell [43]. Sumoylation of the capsid protein of murine leukemia virus (MLV) by Ubc9 and PIASy was required for an early stage of virus replication; in the absence of capsid sumoylation, reverse-transcribed viral genomes were unable to circularize and enter the nucleus for integration into the host genome [44].

Many viruses recruit the ubiquitin-dependent ESCRT machinery to their site of release at the plasma membrane to promote viral budding and their egress from the cell. Smaller enveloped viruses including retroviruses (HIV-1, HTLV-1, MLV, Rous sarcoma virus, equine infectious anemia virus), filoviruses (Ebola), rhabdoviruses (vesicular stomatitis virus, rabies virus), arenaviruses (Lassa fever virus, lymphocytic choriomeningitis virus) and paramyxoviruses (simian virus 5, human parainfluenza virus) all require virally encoded sequences called late assembly or L-domains for separation of virus from host cells. Various L-domain motifs have been documented including PTAP, PPxY, FPIV, LxxLF and YPxL [45]. For example

the PTAP motif within the p6 domain of HIV-1 Gag binds to TSG101 [46] and indeed the complete ESCRT-1 is required for PTAP-mediated virus budding [47]. Tal, the TSG101-associated ligase, is necessary for the multiple monoubiquitination of TSG101 and regulates retrovirus budding [48]. Thus virus budding is akin to MVB formation with membrane invagination and pinching off of vesicles into a non-cytoplasmic compartment. A second L-domain motif, PPxY recruits the cellular E3 ligase Nedd4 through interactions with this HECT E3 ligase's WW domain [49]. Both the PTAP and PPxY motifs are involved in Ebola virus and human T lymphotropic virus type 1 (HTLV-1) budding [50, 49]. Two HBV proteins, core and the envelope protein L, interact with gamma2-adaptin, while the core protein also interacts with the E3 Nedd4 through its L-domain-like PPAY motif. Mature virus production was also decreased when a catalytically inactive form of Nedd4 was expressed [51]. YPxL L-domains recruit the E3 ligase AIP1 to facilitate budding of viruses including equine infectious anemia virus [52], Sendai virus [53] and HIV-1 [54]. Ubiquitination of foamy virus glycoprotein LP leads to an increase in subviral particle release [55].

7.2.5
Transcriptional Regulation

The UPS is required for transcriptional regulation, and many viruses disrupt cellular transcription to favor virus replication and counter host anti-viral strategies. The herpesvirus transactivators are a case in point. ICP0 of herpes simplex virus type-1 (HSV-1) is a virally encoded E3 ligase that transactivates both viral and cellular genes and is discussed in more detail below. Ubiquitination of viral transactivators is also important as exemplified by a requirement for ubiquitination of the HSV-1 virion transactivator VP16 by the E3 ligase Met30 [56]. Ubiquitination of the HIV-1 transactivator Tat has been shown to increase its activity [57].

7.2.6
Cell Cycle Control

The cell cycle is controlled by the activity of cyclin-dependent kinases (cdks) which either drive or inhibit crucial events in cell division. Another key regulator of cell cycle progression is the anaphase-promoting complex (APC). Through its E3 ligase activity the APC targets cdks and other regulators of the cell cycle for degradation by the UPS [58]. Viruses often disrupt the cell cycle in order to upregulate cellular proteins required for virus replication. This may lead to dysregulation of the cell cycle and transformation. Indeed the tumor suppressor properties of two important cell regulatory proteins, p53 and retinoblastoma sensitivity protein (Rb), were recognized in part by their ability to suppress tumor formation caused by the expression of "oncogenic" proteins of certain viruses [59, 60].

The specific targeting of p53 for proteasomal degradation is a common theme for viruses. E6 of human papilloma virus (HPV) has long been known to target p53 for ubiquitin-dependent degradation [61]. E1B55K and E4orf6 of adenovirus recruit a cellular E3 to ubiquitinate p53 while the E3 ligase ICP0 of HSV-1 can

ubiquitinate p53 [62, 63]. Similarly, E7 of HPV and EBV nuclear antigen 3C (EBNA3C) induce ubiquitin-dependent degradation of Rb [64, 65]. The viral transactivator Tax of HTLV-1 prematurely activates the APC and disrupts mitosis by promoting the polyubiquitination of cyclin B1 [66]. ICP0 of HSV-1 also impacts on cell cycle progression, although reports differ as to whether or not ICP0 induces the degradation of the ubiquitin-conjugating enzyme (E2) cdc34 thereby stabilizing cyclins D1 and D3 [67, 68].

Programmed cell death or apoptosis is often a consequence of viral infection. The term "inhibitors of apoptosis" (IAP) was first used to describe a baculovirus protein that blocked host insect cell apoptosis during viral infection. This protein contained a zinc finger motif that was also found in other proteins known to regulate apoptosis [69, 70]. Now several virus families have been shown to encode IAP proteins with E3 activity as noted later in this chapter.

Gamma herpesviruses establish latency in cells of lymphoid origin, and must insure that their episomal genome is replicated during cell division. EBNA-1, a viral protein important for viral DNA replication and EBV genome segregation, binds a cellular deubiquitinating enzyme (DUB) – the ubiquitin-specific protease 7 (USP7) also known as the herpesvirus-associated USP (HAUSP) – although the functional significance of this is still under investigation [71].

7.2.7 Cell Signaling

During the course of infection viruses downregulate surface receptors in a variety of ways that often mimic normal cellular processes. Receptor down-modulation in response to ligand binding shows some similarities with viral down-modulation of class I MHC [72]. The process of viral interference with the class I presentation pathway has been extensively studied [73, 74] and there are many examples of ubiquitin-mediated regulation induced by viruses to circumvent cellular regulation. The RTA protein of KSHV induces ubiquitination and proteasome-mediated degradation of interferon regulatory factor 7 (IRF7) [75].

The Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway are important in regulating cytokine signal transduction, cell growth and cell survival. In HTLV-1-transformed T cells, induction of the deubiquitinating enzyme DUB-2 results in prolonged activation of the Jak/STAT pathway. This in turn suppresses apoptosis of the virally transformed cell [76]. As will be described below, many paramyxoviruses encode V proteins that control degradation of STAT proteins via the UPS system [77–79].

The Toll Like Receptors (TLRs) as extracellular pattern recognition receptors, together with the intracellular recognition receptors such as nucleotide-binding oligomerization domain (NOD) and protein kinase R (PKR), detect pathogen-associated molecular patterns (PAMPS) and initiate a cascade of cellular signals, culminating in the activation of NFκB and the production of proinflammatory cytokines, such as TNF-α, IFN-γ, and the release of interleukins. Activation of the NFκB signaling pathway therefore represents an important line of defence against

virus infection. Regulation of this signal cascade by the UPS occurs at three points – degradation of the inhibitor of NFκB (IκB), processing of NFκB precursors and the activation of the IκB kinase (IKK) [80]. The HBV E5 protein disrupts the interaction of the E3 ligase cCbl with the epidermal growth factor receptor (EGFR). This leads to a decrease in EGFR ubiquitination and degradation, and thus to an increase in EGFR signaling [81]. There is also a report that ICP0, the E3 ligase encoded by HSV-1, complexes with cCbl and its adaptor protein CIN85 to increase the degradation of the EGFR in cells transfected with ICP0 alone, and in cells infected with HSV-1 [82]. ICP0 has also been reported to activate NFκB by catalyzing the polyubiquitination of IκB [83].

The Wnt/β-catenin signaling pathway is important during development and differentiation, and is often dysregulated in cancer. The polyubiquitination and degradation of β-catenin by the E3 ligase "seven in absentia homolog 1" (Siah-1) is important for turning off signal-transduction through growth factor receptors. Expression of latent membrane protein 1 (LMP1) of EBV decreases the levels of Siah-1. Thus β-catenin levels are stabilized to activate signaling pathways [84]. Another protein expressed in cells latently infected with EBV is LMP2A. LMP2A is important for maintaining latency in B cells through its interaction with Nedd4 family E3 ligases [85]. In epithelial cells, LMP2A inhibits differentiation and activates β-catenin signaling, perhaps contributing to carcinogenesis in these cells [86]. β-Catenin also accumulated when an additional E3 ligase complex, beta-transducin repeat-containing protein (βTrCP), was sequestered in the cytoplasm by Vpu of HIV-1 [87].

7.3
Viruses and E3 Ubiquitin-Protein Ligases

The ubiquitin reaction involves (i) ubiquitin activation via an E1 enzyme, (ii) transfer of the ubiquitin via a cysteine residue to an E2 ubiquitin-conjugating enzyme and (iii) targeting of the charged ubiquitin from the E2 to the lysine residue of the target protein. This latter reaction is catalyzed by the ubiquitin E3 ligase that associates with the substrate and thereby confers specificity to the ubiquitination reaction. Ubiquitin E3 ligases come in three different forms, containing a HECT (homologous to E6AP carboxyl terminus), a RING (really interesting new gene) or a U-box (UFD2 homology) domain [88]. Although the E3 ligase activity of APC and SCF complexes was well known, it was not until an analysis of the subunits of APC revealed homology with a subunit of SCF that the idea that the RING-finger motif might represent a superfamily of E3 ubiquitin ligases was appreciated [89, 90] whereas the concept of a HECT family of E3 ligases was recognized much earlier [91]. The E6 protein encoded by HPV associates with a cellular protein E6AP and, in oncogenic strains of the virus, the E6/E6AP complex binds and targets the p53 tumor-suppressor protein for ubiquitin-mediated proteolysis [61, 92]. It was found that the E6AP ubiquitin E3 ligase directly accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and

transfers the ubiquitin to targeted substrates. Subsequently the HECT family of related proteins with a C-terminus sequence motif similar to that of E6AP was identified and shown to have E3 ligase activity [91]. The HECT family has 28 family members.

In contrast to the HECT family, other E3 ligases do not directly bind ubiquitin, but recruit a ubiquitin-charged E2 conjugating enzyme and promote transfer of ubiquitin to the target protein after substrate binding. These are the RING family of E3 ligases [93]. RING domains contain a variable number of interspersed cysteine and histidine residues which bind the two zinc ions required for E3 ligase activity and fold to form a cross-braced structure [88]. The arrangement of cysteines and histidines can be C_3HC_4 (RING-HC) or $C_3H_2C_3$ (RING-H2) for the canonical RING-finger-containing E3s or C_4HC_3 (RING-CH) for the more recently described RING-variant structure [94, 95]. The SCF E3 ligases containing multiple subunits including a Skp-Cullin-F-box are a subset of the RING domain-containing E3s. The U-box-containing family is based on an E2 binding domain first described in the yeast Ufd2 protein [96]. U-box-containing proteins adopt a structure that is very similar to a RING domain without the zinc coordination, but rather than ubiquitinating substrates directly, they polyubiquitinate substrates of other E3s and are thus also referred to as E4s [88]. While viruses encode a number of RING-containing E3 ligases, as yet no virally encoded HECT or U-box domain-containing proteins have been described. Viruses do, however, recruit both RING- and HECT-containing cellular E3 protein-ubiquitin ligases.

7.3.1 ICP0 – A Viral RING E3 Ligase in HSV Activation

The most widely studied viral RING-containing protein is ICP0 of HSV-1. ICP0 is an immediate early protein required for the activation of most viral and many cellular genes and is critical for the reactivation of HSV-1 from latency. ICP0 has a RING domain near its N-terminus, encoded in exon 2, which confers E3 ligase activity and induces the proteasome-dependent degradation of substrates including components of centromeres and PML bodies [97–99], although its exact protein substrates have yet to be defined. The RING domain is required for the accumulation of conjugated ubiquitin species, as is the RING finger of ICP0-related proteins from other alphaherpesviruses [100, 101]. Controversy exists about the ability of ICP0 to affect cellular levels of cyclins D1 and D3 through the degradation of cdc34 (also known as UbcH3), the cellular E2 component of the SCF complexes that target these cyclins for degradation. It remains controversial whether additional ICP0 E3 ligase activity, associated with exon 3 of ICP0 rather than the RING domain encoded within exon 2, promotes the autoubiquitination of cdc34 [102, 103]. When conditions were adjusted to allow a similar rate of progression of virus infection in the presence and absence of ICP0, no evidence was found for ICP0-mediated protection of cyclins D1 and D3, nor the degradation of cdc34 [67]. *In vitro* E3 ligase activity of the RING domain encoded within exon 2 has been demonstrated using the E2 enzymes UbcH5a and Ubc6 [104, 105]. Subsequently, the degradation of PML and Sp100 *in vivo*, activities associated with the dispersal

of ND10 and reactivation and lytic replication of HSV-1, was shown to depend on the RING-finger domain of ICP0 and the E2 ubiquitin-conjugating enzyme UbcH5a [106, 107]. The question of whether PML is a direct target of ICP0 and the relationship with SUMO-1-modified PML and the SUMO-specific protease SENP1 is complex and remains unresolved [108, 106].

Other targets of ICP0 include p53 [62], the translational repressor 4E-BP1 [109] and the cellular ubiquitin-specific protease USP7 [110]. Analysis of the interaction between ICP0 and USP7 is an elegant example of a viral gene product exploiting the cellular machinery to full use. USP7 binds ICP0 and protects ICP0 from autoubiquitination and subsequent degradation. This is important as autoubiquitination is a key feature of RING-containing E3 ligases and leads to the short half-life of these proteins. The interaction between ICP0 and USP7 is finely balanced. By binding USP7, ICP0 is stabilized and escapes autoubiquitination, but inevitably the interaction of these two protein leads to ubiquitination and degradation of USP7 itself. The stabilization of ICP0 by USP7 appears to be dominant over the ubiquitination and degradation of USP7 [111, 110], allowing the virus to synthesize only small amounts of ICP0 protein.

7.3.2
Preventing the Release of Interferon

All interferons (IFNs) induce an antiviral state in target cells causing impaired virus replication. Due to the effectiveness of the IFN response, many viruses encode gene products that utilize different mechanisms to counteract the release of IFN and development of the IFN-mediated antiviral state. IRF7, a key mediator of type I IFN induction, is targeted for degradation by binding RTA, the immediate-early nuclear transcription factor of KSHV. RTA has E3 ligase activity and blocks IRF7-mediated IFN-α and IFN-β mRNA production by promoting ubiquitination and degradation of the IRF7 protein in a proteasome-dependent fashion *in vivo* and *in vitro*. Like ICP0, RTA also autoregulates its own polyubiquitination and stability, and both activities require the N-terminal cysteine/histidine-rich domain of RTA [75]. Therefore, RTA manipulates the stability and function of IRF7 and provides a regulatory strategy for circumventing the innate immune defence system. Interestingly, RTA from the related gammaherpesvirus EBV is sumoylated by PIAS1 and Ubc9, and this enhances the transactivation function of RTA, although there have been no reports of E3 ligase activity for this protein [112].

Many other RING-containing proteins are encoded by viruses, including some that have been shown to have E3 ligase activity, such as those encoded by the baculoviruses. The Bombyx mori nucleopolyhedrovirus (BmNPV) has six predicted RING-finger proteins, four of these, IAP2, IE2, PE38 and CG30 can induce polyubiquitin chain formation *in vitro*, three in conjunction with the Ubc4/5 E2 conjugating enzymes [113]. The inhibitor of apoptosis Op-AIP3 from the related baculovirus Orgyia pseudotsugata M nucleopolyhedrovirus, was shown to have RING-dependent autoubiquitination activity and was able to ubiquitinate the host pro-apoptotic protein HID [114]. The white spot syndrome virus (WSSV) of shrimp encodes four proteins with RING-finger motifs one of which, WSSV249, has been shown to

interact with and sequester the shrimp E2 PvUbc. It shows little specificity in that it will also ubiquitinate in the presence of UbcH1, UbcH2, UbcH5a, UbcH5b, UbcH5c, UbcH6 and UbcH10 [115]. A second WWSV RING-finger protein, WSSV222, functions to block apoptosis by causing the degradation of the shrimp turnover suppressor-like protein TSL [116].

Poxviruses encode RING-finger proteins with both the classical C_3HC_4 and the RING-CH sequence motif. Variola (the causative agent of smallpox) and ectromelia virus (mousepox) both encode a RING protein p28 that *in vitro* functions with Ubc4 and UbcH5c, and forms lysine-63 linked polyubiquitin chains in the presence of Ubc13 [34]. Although host cellular targets have yet to be identified, p28 is known to be an important virulence determinant for ectromelia virus [34, 117].

7.3.3
Viral E3 Ligases Ubiquitinate and Dispose of Critical Immune Receptors

The first E3 protein-ubiquitin ligase activity described for a RING-CH protein was that of the mK3 protein of the murine gammaherpesvirus 68 (MHV-68). MK3 is unusual in that as well as its RING-CH domain it is a type III transmembrane protein including two transmembrane domains with both the N and C termini in the cytosol. Encoded within the N-terminal domain of the protein is a C_4HC_3 zinc finger similar to that of a plant homeodomain (PHD) and thus different from the canonical zinc fingers of E3 ubiquitin-protein ligases – RING-HC (C_3HC_4) or RING-H2 ($C_3H_2C_3$). However, the NMR solution structure of the highly related zinc-finger from the K3 protein of KSHV was determined to be a RING-CH or RING-variant similar to those of E3 ligases rather than a PHD zinc finger [94]. This RING-variant domain in membrane-associated proteins has since been identified in other viruses and indeed in the human genome where a family of proteins (dubbed MARCH for membrane associated RING-CH) has been shown to have E3 ligase activity [118].

7.3.4
Degradation of MHC Class I Molecules by the mK3 Protein of MHV-68 Virus

CTL recognize and kill a virally-infected cell through identification of viral peptides bound to MHC class I molecules at the surface of the infected cell. The importance of this viral defence pathway is emphasized by the number of different viruses that encode many different gene products to prevent MHC class I-mediated antigen presentation [73]. Indeed, many viruses encode several unrelated gene products whose main function appears to be subversion of the antigen presentation pathway. As described earlier, involvement of the UPS is best characterized by the US2 and US11 gene products from HCMV which subvert the ER quality control pathway to dislocate MHC class I heavy chains from the ER to the cytosol where proteasome-mediated degradation ensues [24].

MK3 uses a related mechanism in that it directly ubiquitinates MHC class I molecules and other components of the antigen presentation pathway. MK3 was

initially identified by screening a plasmid library of the MHV-68 genome for activities that downregulate cell surface MHC class I molecules. Overexpression of mK3 in cell culture resulted in a rapid proteasome-dependent degradation of MHC class I molecules and an inhibition of class I antigen presentation [119]. This proteasome-dependent degradation of conformational class I molecules was shown to require the N-terminal RING domain and immunoprecipitation of class I associated with mK3 in the presence of proteasome inhibitors yielded class I and a higher molecular weight "ladder" of ubiquitinated class I species [120]. Further experiments showed that mK3 associates with the ER-resident MHC class I peptide loading complex, and binding to the peptide loading complex was required for mK3 stabilization, thus preventing its rapid degradation [121, 122]. In addition to ubiquitination of MHC class I heavy chains, mK3 activity also results in degradation of the TAP peptide transporter and tapasin, both important components of the peptide loading complex [123, 124]. By targeting additional components of the peptide loading complex, as well as class I, mK3 is also able to degrade other immunoreceptors which are dependent on peptide loading, but lack cytoplasmic lysine residues and might otherwise escape ubiquitination. For example, the GPI-linked MHC class I like molecule Qa2 is effectively downregulated by mK3 but contains no relevant lysine residues.

The increased stability offered to mK3 by binding the peptide loading complex also allows mK3 to "buffer" cytokines such as IFN-γ which increase class I surface expression through upregulation of class I and components of the peptide loading complex – TAP and tapasin. The high levels of class I normally induced by virus infection through IFN-γ activity are balanced by an increase in mK3 stability on the peptide loading complex and therefore activity in degrading these components.

How does mK3-mediated ubiquitination of class I heavy chains from the ER lead to degradation by the proteasome? Like US11, the link between mK3-mediated MHC class I ubiquitination and subsequent proteasome-mediated degradation appears to be the ERAD complex containing Derlin1 and p97. In addition to the peptide loading complex, mK3 associates with Derlin1 and requires the AAA-ATPase activity of p97 for degradation of ubiquitinated class I molecules [125].

7.3.5
Degradation of Immunoreceptors by Kaposi's Sarcoma-associated Herpesvirus

Given the high degree of homology between mK3 and its homologs in KSHV, K3 and K5 [126, 127, 119], it was not surprising that these proteins were also found to function as E3 ubiquitin-protein ligases and to target specific immunoreceptors for degradation. However, a fundamental difference between these related viral ligases is that unlike mK3, K3 and K5 do not predominantly function in the ER but ubiquitinate their targets at the cell surface where they are internalized and in some cases degraded [128, 37]. More is known about how K3 downregulates its target, MHC class I, than K5 and its multiple targets. K3-dependent ubiquitination of class I molecules occurs in the late secretory pathway and is followed by inter-

nalization and sorting via the endosomal machinery leading to lysosomal degradation [37]. This endolysosomal sorting is clathrin dependent with a requirement for epsin – a clathrin adaptor [32]. Endolysosomal sorting is dependent on components of the ESCRT-I machinery [37], but independent of ESCRT-II [72]. For members of the tyrosine kinase growth factor receptor family, monoubiquitination provides a sufficient signal for internalization and degradation [129, 31]. While K3 recruitment of UbcH5 is necessary for monoubiquitination of surface MHC class I molecules this is not sufficient to signal class I degradation. Indeed K3 recruits the Ubc13 E2 conjugating enzyme which catalyzes the lysine-63-linked polyubiquitination of class I, leading to class I internalization and degradation [32]. K3 (but not K5) is also the first ubiquitin E3 ligase shown to ubiquitinate mutant class I molecules via a single cysteine residue in a thiol-ester linkage, as opposed to via a lysine residue [130].

K5 ubiquitinates and downregulates MHC class I molecules as well as other immunoreceptors including B7.2, ICAM-1, CD31 and CD1d [126, 127, 131, 132, 119]. The specificity of K5 activity and how it effectively targets so many different immunoreceptors is unclear. The functional consequences are a decreased activation of T cells [132], reduced endothelial cell migration [131] and inhibition of YTS (NK cell line) killing in an MHC class I unrestricted fashion [133]. Immunoprecipitation of MHC class I and B7.2 followed by western blotting with ubiquitin-specific antibody demonstrated K5-mediated ubiquitination *in vivo*, while *in vitro* ubiquitination was demonstrated with GST or GST-K5 plus ubiquitin, ATP, E1 and UbcH5a [128]. Endocytosis was implicated in the downregulation of ICAM-1 and B7.2 in B cells by the use of dominant negative dynamin [134]. While K5 expression results in the downregulation and lysosomal degradation of class I and B7.2, this is not the case for all targets; CD1d is downregulated but not degraded in cells expressing K5 [132], while CD31/PECAM is ubiquitinated and degraded by both the proteasome and lysosomes [131]. Further details about K5-mediated ubiquitination, including the E2 conjugating enzymes recruited, mono-versus-poly ubiquitination, and mechanism of target acquisition remain to be elucidated.

Other viral RING-CH E3 ligases have also been described. They all share a similar organization to the K3 family (a RING-CH domain followed by two transmembrane regions). The K3-related protein of myxoma virus (MV), M153R or MV-LAP, contains an N-terminal RING-CH domain and targets MHC class I, Fas and CD4 for internalization and lysosomal degradation [135–137]. Interestingly, optimal activity of M153R requires myxoma virus infection [138], suggesting the involvement of additional viral proteins. Some viral RING proteins function as part of larger SCF E3 protein-ubiquitin ligases as detailed below.

7.3.6 Viral SCF E3 Ligases

Some viral proteins function as part of the larger subunit type SCF ubiquitin ligases. The Cullin E3 ubiquitin ligases are a family of modular RING E3 ligases that consist of four main components: a Cullin (Cul1, 2, 3, 4a, 4b, 5, or 7), a RING-finger protein, an adaptor protein, and a substrate receptor. Cul1 serves as a scaf-

fold for the assembly of the catalytic components consisting at its C-terminus of the RING-finger protein Rbx1, which binds and activates the E2 conjugating enzyme. At the N-terminus Cul1 binds Skp1 and an F-box family member that serves as a specificity factor for substrate binding [139]. This organization allows the F-box protein to bring the substrate protein into the proximity of the ubiquitination machinery. Like papillomaviruses, other DNA tumor viruses target p53 for inactivation or degradation. Adenovirus expresses two proteins, E1B-55K and E4orf6 that form a complex with Cul5, Rbx-1 and elongins B and C to target p53 for ubiquitination and degradation. Both viral proteins act independently to bind p53 and prevent gene expression. In combination they induce ubiquitin-dependent degradation [140, 141].

The V proteins of paramyxoviruses all interfere with the Jak/STAT signal transduction pathway. Type II human parainfluenza virus V protein forms a complex with the damaged DNA binding protein (DDB1) and Cul4a to polyubiquitinate STAT2 *in vivo*, while the V protein of simian virus 5 (SV5) functions as an adaptor to bind STAT2 and DBB1 in complex with Cul4a facilitating the polyubiquitination and degradation of STAT1. The loss of STAT1 in turn leads to a decrease in affinity of V for STAT2 and presumably a dissociation of the complex [77, 142]. The structure of the DDBA–Cul4a–V protein complex shows that the viral protein is inserted into the double propeller pocket of the DDB1 protein while the third propeller of DDB1 binds to Cul4a allowing the V protein to recruit alternative substrates to the E3 ligase complex [143].

7.3.7
HIV Vif and APOBEC Function

Cytidine deamination by host cell apolipoprotein B editing complex (APOBEC) proteins is a potent anti-retroviral strategy. DeoxyC to dU deamination of the non-coding (minus) strand of the genome results in G to A hypermutations in the coding strand that are lethal to the virus [144]. Elucidation of how the HIV-1 virion infectivity factor (Vif) overcomes APOBEC3G has revealed a link with the UPS. HIV-1 Vif co-opts the Cul5 E3 ubiquitin ligase, acting in effect as the substrate receptor for its target APOBEC3G. The new SCF E3 ligase complex containing Cul5, elongin B and C and Vif induce lysine-48 linked polyubiquitination and proteasome-mediated degradation of APOBEC3G [145–147]. Vif binds to elongin C through a novel "suppressor of cytokine signaling" (SOCS)-box motif. Two highly conserved cysteine residues in Vif, outside the SOCS-box, are required for Cul5 interaction [148, 149]. APOBEC3F molecules are also targeted by Vif for ubiquitination and degradation through a Cul5-dependent SCF E3 ligase complex [150]. If the activity of Vif fails to neutralize all APOBEC3F/G activity within the cell, an additional HIV-1 accessory protein Vpr, forms a complex with Cul1 and Cul4 to target cellular uracil DNA glycosylase (UNG) for ubiquitination and proteasomal degradation [151]. UNG removal of the uracil base leaves the viral reverse transcripts open to error-prone translesion repair. By inducing the degradation of UNG, the number of abasic sites in viral reverse transcripts may be decreased, increasing the viability of progeny virus. Indeed, Vpr^+ viruses in a Vif^- background

replicated better than Vpr⁻ viruses highlighting the importance of cytidine deamination in the anti-viral arsenal of the host [151]. Given that the SCF type E3 ligases are a protein complex, it is sometimes difficult to distinguish a component of such a complex from a protein that recruits a complex. A discussion of viral proteins that recruit E3 ligases follows.

7.3.8 Viral Recruitment of E3 Ligases

Simian virus 40 large T antigen (SV40T) binds and inactivates the tumor suppressors p53 and Rb contributing to the transforming activity of this viral protein. SV40T associates with an SCF-like complex composed of Cul7, Rbx1, and the F box protein Fbw6 [152] suggesting that the UPS is involved in this activity.

EBV is an oncogenic virus that encodes several proteins which recruit cellular E3 ligases to disrupt the cell cycle and interfere with normal signaling pathways. EBV nuclear antigen EBNA3C associates with cyclinA/cdk2 complexes and recruits the SCF/Skp2 E3 ligase complex resulting in the ubiquitination and degradation of the kinase inhibitor p27 and dysregulation of the cell cycle [153]. EBV-encoded latent membrane protein 1 (LMP1) and latent membrane protein 2 (LMP2) are important oncogenic proteins that interfere with cell signaling in latently infected cells. LMP1 interaction with the SCF E3 ligase complex containing the "homolog of Slimb" (HOS) helps regulate NFκB activation. LMP1 interacts directly with HOS but is not a substrate of this E3 ligase complex. Mutations that abrogate HOS binding, increase LMP1-induced transformation by increasing IκB degradation and therefore NFκB transcriptional activity. Therefore, LMP1 sequestration of HOS may restrict NFκB activation in EBV immortalized cells to help minimize transformation [154]. LMP1 also enhances the stability of the E3 ubiquitin ligase Siah1 that leads to the proteasomal degradation of prolyl hydroxylases 1 and 3 that in turn protects hypoxia inducible factor 1 subunit alpha (HIF1α) from ubiquitination and degradation [155]. The net result is an increase in levels of HIF1α in EBV-infected cells.

EBV encoded proteins can also regulate B- and T-lymphocyte receptor signaling. LMP2A interferes with normal B cell receptor (BCR) signaling and provides a constitutively active survival signal in latently infected B cells [156]. The N-terminal cytoplasmic domain of LMP2A binds to the BCR-associated kinases Syk and Lyn and recruits the cellular E3 ligases AIP4 and KIAA0439, resulting in the ubiquitination and degradation of Syk and Lyn and the inhibition of BCR signaling [85, 157]. Similarly, LMP2A in association with AIP4 regulates T-cell receptor (TCR) levels providing a survival signal to T-cell tumors harbouring latent EBV [158].

HIV-1 and related retroviruses encode many proteins that interact with cellular E3 protein-ubiquitin ligases during the course of infection. HIV-1 integrase associates with the E3 ligase Rad18, involved in cellular DNA repair. Rad18 expression stabilizes the integrase and may play a role in integration of the HIV-1 genome [159]. HIV-tat stimulates the transcriptional elongation of the HIV-1 genome by

recruiting the positive transcriptional elongation factor b (P-Tefb) to pause RNA polymerase II. Tat recruits the SCF E3 ligase Skp2 to ubiquitinate the catalytic subunit of P-Tefb, cdk9, which in turn allows optimal transactivation of the HIV-1 long terminal repeat [160]. Another HIV-1 accessory protein, Vpu binds to newly synthesized CD4 in the ER. Phosphorylated Vpu recruits the SCF βTrCP E3 ligase complex resulting in the ubiquitination, retro-translocation and proteasome-mediated degradation of CD4. In addition, Vpu sequesters the SCF βTrCP E3 ligase from its normal substrates which include β-catenin, IκBα and ATF4 [87, 161–163].

7.4 Conclusions

Viruses interact with and exploit the UPS at many points during their life cycle. Modification by ubiquitin plays a crucial role from initial entry of virus particles into the cell via ubiquitin-mediated endocytosis, through to assembly, egress and protection from immune surveillance. To replicate and avoid host anti-viral mechanisms viruses alternately mimic and interfere with host cellular processes, many of which are regulated by ubiquitination. Homology between viral and host gene products has and will continue to promote the use of viral proteins as tools and model systems to unlock the secrets of host protein function. This will both improve our understanding of viral pathogenesis and allow the development of superior anti-viral strategies.

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8
The Ubiquitin–Proteasome System in Parkinson's Disease

Kevin St. P. McNaught

8.1
Introduction

Parkinson's disease (PD) is a neurological disorder and is characterized clinically by bradykinesia (slowness of movement), rigidity, tremor, postural instability and gait dysfunction [1, 2]. However, non-motor features (e.g. autonomic dysfunction and dementia) often develop in these patients, especially during the advanced stages of the illness [1, 2]. PD affects both males and females, occurs in all racial/ethnic groups, and is found worldwide. Reports of incidence and prevalence rates of the illness vary, but most studies show that the occurrence of the disorder increases with aging [3, 4]. For example, Van Den Eeden and colleagues found an overall population incidence rate of PD annually to be 13.4 per 100 000 individuals, but this rate increases to 38.8 and 107.2 per 100 000 individuals in the age range of 60–69 and 70–99 years, respectively [4].

PD is defined pathologically by degeneration of the dopaminergic neurons in the substantia nigra pars compacta (SNc), leading to destruction of the nigrostriatal pathway, and consequently reduction of dopamine levels in the striatum [5]. Neuronal death with depletion of respective neurotransmitters also occur in other areas of the central nervous system (CNS), in particular the noradrenergic neurons in the locus coeruleus (LC), dorsal motor nucleus of the vagus (DMN), cholinergic neurons in the nucleus basalis of Meynert (NBM), and cells in the olfactory system [5–7]. Further, pathology can occur in some regions of the peripheral nervous system (PNS), such as autonomic ganglia (e.g. superior cervical ganglion) and the mesenteric plexus in the wall of the gut [5]. Pathology in the extranigral regions likely plays a role in the development of both motor and non-motor dysfunction in PD patients. Characteristically, neurodegeneration at the various pathological sites is accompanied by protein accumulation, aggregation and the formation of Lewy body inclusions in PD (Figure 8.1) [5, 6].

PD can occur through inheritance or may develop sporadically. Approximately 10–15% of cases are thought to be genetic in origin and specific linkages and gene mutations have been identified in small numbers of familial cases [8]. Most cases

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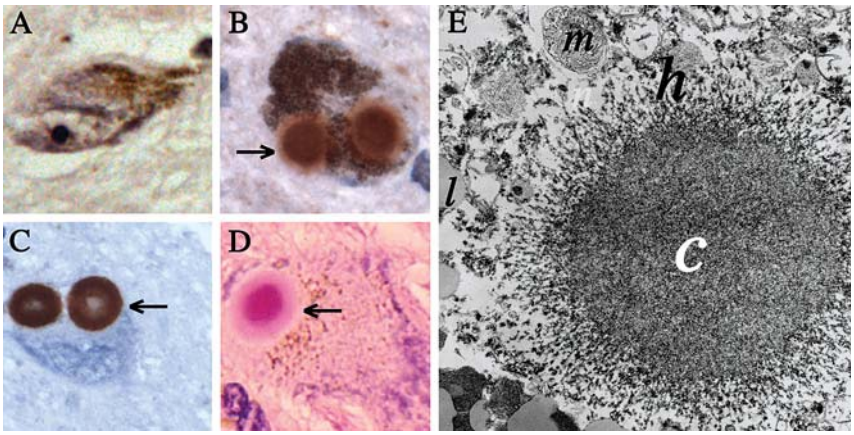

Fig. 8.1. Lewy bodies in the SNc in sporadic PD. (A–C) A standard immunohistochemical protocol; 3,3′-diaminobenzidine was used as a brown chromogen to stain sections of the SNc from normal control (A) and PD subjects (B, C) subjects. This procedure shows the presences of two Lewy bodies (arrows) containing ubiquitin (B) and -synuclein (B) in dopaminergic neurons in PD, but no inclusion body is present in normal controls (A). The dark granular substance in the neurons is neuromelanin. (D) Conventional Hematoxylin (blue) & Eosin (pink) histological staining reveals (arrow) a spherical Lewy body in SNc dopamine neurons with a distinct central core and a peripheral halo. (E) Electron micrograph of a Lewy body reveals that the core (*c*) contains granular material and the outer halo (*h*) is composed of radiating filaments. In panel E, (*l*) represents lipofuscin deposits, and (*m*) indicates a mitochondrion.

(≈90%) of PD are sporadic and of unknown cause. A widely held hypothesis relating to the cause of sporadic PD suggests that exposure to environmental toxins leads to the development of the illness in individuals who are rendered susceptible due to their genetic profile, poor ability to metabolize toxins and/or advancing age [9]. The pathogenic process is unknown, but has been linked to a variety of factors, including oxidative stress [10], mitochondrial dysfunction [11], inflammation [12], excitotoxicity [13] and apoptosis [14]. However, it is not clear as to how these cellular, biochemical and molecular changes relate to each other and to neuronal degeneration in PD.

In recent years, a growing body of genetic, postmortem and experimental evidence have converged to suggest that failure of the ubiquitin–proteasome system (UPS) and altered protein handling play a major role in the etiopathogenesis of sporadic and the various familial forms of PD (Figure 8.2) [15–17]. In this chapter, we will examine the range of defects that occur in the various hereditary and sporadic forms of PD, and consider how they might be linked to the UPS and lead to pathogenesis.

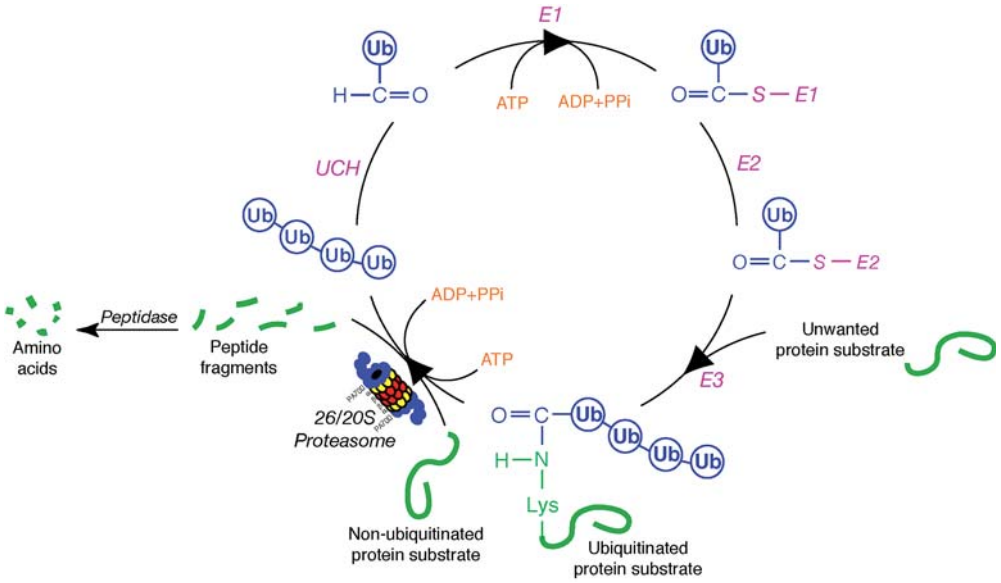

Fig. 8.2. The UPS and Parkinson's disease. Recent genetic, postmortem and experimental evidence suggest that failure of the UPS plays a role in the etiopathogenesis of familial and sporadic PD [15–17]. The UPS comprises two processes that occur consecutively to degrade unwanted proteins that are either abnormal (i.e. incomplete, mutant, misfolded, denatured, oxidized and otherwise damaged proteins) or normal (e.g. turnover of short-lived regulatory proteins). In the first step, a ubiquitin molecule (a 76-amino acid, 8.5-kDa polypeptide) is conjugated to unwanted proteins via a covalent isopeptide bond between the C-terminal Gly residue of ubiquitin and an internal Lys residue of the substrate protein. Thereafter, additional ubiquitin molecules are attached to the previously conjugated ubiquitin (at a Lys residue) in a sequential manner to form a polyubiquitin chain. Ubiquitination is ATP dependent and is mediated by three different enzymes acting in sequence, namely a ubiquitin-activating enzyme (E1) which activates ubiquitin by forming a thioester, followed by a ubiquitin-conjugating enzyme (E2) that carries activated ubiquitin as a thioester, and finally an ubiquitin ligase (E3) which transfers activated ubiquitin to the substrate protein. In mammalian cells, it appears that only one E1 enzyme exists, while 20–40 E2 enzymes have been identified and there are 500–1000 E3 enzymes which can be grouped into distinct families (e.g. HECT domain and RING-finger domain E3s). The selectivity of protein ubiquitination is assured by the fact that each E3 enzyme is specific for one or a limited number of different proteins. Additionally, some proteins require post-translational modification (e.g. phosphorylation of I B) before they can undergo ubiquitination and this provides a further degree of selectivity in the process [132]. In the second step of the UPS, unwanted proteins previously tagged by ubiquitin are unfolded by PA700 to permit entry into the inner chamber of the 26S proteasome complex where they are degraded in an ATP-dependent manner. The degradation products of 26S proteasomes are 2–25-residue peptide fragments that are further hydrolyzed by peptidases to produce their constituent amino acids which are re-used in protein synthesis [203]. Following recognition but before entry into the proteasome, polyubiquitin chains are separated from protein conjugates then disassembled by de-ubiquitination enzymes (ubiquitin C-terminal hydrolases) into monomeric ubiquitin which is re-used in the ubiquitination cycle. It is noteworthy that short peptides and some proteins (e.g. oxidatively damaged proteins) can be degraded by the 20S proteasome (the catalytic core of the 26S proteasome) without prior ubiquitination [24]. A variety of studies indicate that the failure of the UPS at various points as indicated, plays a role in protein accumulation, Lewy body formation and neurodegeneration in familial and sporadic forms of PD [15–17].

8.2 Protein Handling in the CNS

Neuronal activity is associated with the generation of proteins that are abnormal, such as incomplete, mutant, misfolded, denatured, oxidized and otherwise damaged proteins [18, 19]. This is prominent in the CNS due to the relatively high utilization of oxygen and elevated rate of metabolism, and the enzymatic- and auto-oxidation of neurotransmitters such as dopamine, all of which facilitate the production of reactive oxygen species and other free radicals that can induce protein damage [20, 21]. Abnormal proteins have a tendency to misfold, aggregate, interfere with intracellular processes and induce cytotoxicity [22–25]. As such, their production must be limited or they must be rapidly cleared so as to maintain the integrity and viability of cells [18, 19]. Indeed, in the CNS, a tight balance between the production of abnormal proteins and their clearance is crucial since these neurons have a limited ability for repair/regeneration and so are crucially dependent on existing neuronal populations. This equilibrium might be difficult to maintain in the CNS since the long lifespan of neurons is associated with alterations in a variety of intracellular process, such as oxidative stress and mitochondrial dysfunction which cause protein damage and accumulation [20]. Also, in the aging CNS, there is a marked increase in oxidative damage [20, 26, 27]. Finally, proteasomal function declines with aging in various regions of the CNS [20, 26, 28]. Thus, the CNS appears to be crucially dependent on protein clearance systems to maintain its structural and functional integrity.

There are two systems that mediate the majority of protein degradation and clearance within cells [29]. An autophagic process involving cathepsins (cysteine proteases) is responsible for the degradation of membrane and extracellular components following endocytosis into the lysosome [29]. The UPS is primarily responsible for the clearance of abnormal and cytoplasmic proteins and this process occurs in the cytoplasm, nucleus and endoplasmic reticulum (ER) of cells including neurons in the CNS (Figure 8.2) [18, 29–33]. This pathway also plays a significant role in the turnover of short-lived regulatory/functional proteins and is therefore intimately linked with a variety of inter-/intra-cellular processes [18, 29–31].

Molecular chaperones or heat shock proteins (HSPs), such as HSP70 and HSP90, are a highly conserved class of proteins that contribute to protein handling within cells [34, 35]. HSPs act to facilitate the proper folding and localization of proteins, and serve to prevent inappropriate interactions within and between proteins that can otherwise lead to misfolding and aggregation. Additionally, HSPs promote the refolding of proteins that become abnormal. Importantly, HSPs function synergistically with the UPS in several ways, notably in their ability to alter the folding pattern of abnormal proteins to facilitate their recognition and entry into 26/20S proteasomes for degradation [35–38]. HSPs also play a role in the assembly of the 26S proteasome complex [39].

Cells normally maintain a dynamic balance between the generation of abnormal proteins and their clearance by the UPS, HSPs and other proteolytic systems.

Disturbance of this equilibrium, either by the excess production of abnormal proteins or reduced degradation, leads to an adverse state called proteolytic stress [17, 40]. During proteolytic stress, poorly degraded or undegraded proteins tend to accumulate and aggregate with each other and with normal proteins [18, 24, 41]. Such protein aggregates can disrupt intracellular processes and induce cytotoxicity [22–25]. In recent years, several studies have shown that when UPS-mediated degradation fails, cells can activate a secondary and possibly cytoprotective response. This is the transport, sequestration and compartmentalization of poorly degraded/undegraded proteins and aggregates to form aggresomes and inclusion bodies [23, 42–45]. In many neurodegenerative disorders, where proteolytic stress is a key factor, protein aggregates and inclusion bodies can be seen within different compartments of the cell [46]. In PD, these proteinaceous inclusions are referred to as Lewy bodies and they are typically seen in the cytoplasm of neurons at the various pathological sites (Figure 8.1) [40, 47].

8.3 The UPS and Protein Mishandling in PD

During the past 10 years, there have been several discoveries of gene mutations that cause rare familial forms of PD [15, 17, 48]. Although the clinical spectrum and pathology of these illnesses often differ significantly from each other and from typical sporadic PD, it appears that they share similar pathogenic mechanisms, namely protein mishandling and aggregation [15, 17, 48]. This concept relates to the observation that these mutations can affect proteins that have a high propensity to misfold and aggregate, or impair the activity of UPS enzymes and related proteolytic systems [15, 17, 48]. Several studies have demonstrated that in patients with sporadic PD there is a reduction in proteasomal subunits and enzymatic activity in the SNc which degenerates, but an upregulation of proteasomal function in regions of the brain that have been spared [49]. Taken together, these findings suggest that failure of the UPS could be a central and common defect that underlies the pathogenesis of the various familial and sporadic forms of PD (Figure 8.2).

8.4 Parkin

An hereditary form of PD, autosomal recessive juvenile parkinsonism (AR-JP) first described in Japanese families, was linked to chromosome 6q25.2–q27 (PARK 2) in 1997 [50, 51]. This locus was found to host the gene that codes for a 465-amino acid/52-kDa protein called parkin [52, 53]. It is now appreciated that many deletions, point mutations and mutations spanning the entire parkin gene cause familial PD [15]. Estimates suggest that parkin mutations account for approximately 50% of early-onset (<45 years) familial cases of PD [54]. It is noteworthy

that parkin mutations have also been associated with late-onset (≥60 years old) hereditary PD [55].

Clinically, AR-JP is similar to common sporadic PD, but there are notable differences. AR-JP can have a very early age of onset, ranging from 7–72 years (average, 30 years), and demonstrates a rather slow rate of progression [54]. Similarly, the neuropathology of AR-JP differs from sporadic PD in that neurodegeneration in the familial disorder is restricted to the SNc and LC, whereas cell death is more widespread in sporadic PD [56]. Further, Lewy bodies or other protein aggregates are largely absent in patients with AR-JP [56]. It is also noteworthy that Lewy bodies have been found in a patient with parkin-linked autosomal dominant parkinsonism and clinical features more typical of sporadic PD [57].

Parkin is expressed in the cytoplasm, nucleus, golgi apparatus and processes of neurons throughout the CNS [58]. Several studies have shown that parkin is an E3 ubiquitin ligase [59–63]. Similar to other E3 ubiquitin ligases, parkin contains a RING-finger domain (comprising two RING-finger motifs separated by an in-between-RING domain) at the C-terminal, a central linker region, and a ubiquitin-like (UBL) domain at the N-terminal. Parkin acts in conjunction with several E2 enzymes, Ubc6, UbcH7 and UbcH8, to ubiquitinate a variety of substrates. These include synphilin-1, CDCrel-1, parkin-associated endothelin receptor-like receptor (Pael-R), an *O*-glycosylated isoform of α-synuclein (αSp22), cyclin E α/β-tubulin, p38 subunit of the aminoacyl-tRNA synthetase complex, and synaptotagmin X1 [15, 59, 61, 62]. Interestingly, parkin may polyubiquitinate proteins with linkages at lysine 48 (K48) or lysine (K63) [64]. Parkin has been shown to interact through its UBL domain with the 26S proteasome Rpn10/S5a subunit that, along with Rpt5/S6′, plays a role in the recognition of ubiquitinated substrates by the PA700 proteasome activator [31, 65]. It was shown that parkin also interacts with a protein complex containing CHIP/HSP70 and which promotes parkin's activity [66, 67]. Parkin also interacts with proteasomal subunits [68].

Precisely how parkin induces pathology in familial PD is not known, but could relate to a loss of E3 ubiquitin ligase and hence impairment of protein ubiquitination. The levels of parkin and its enzymatic activity are decreased in the SNc and LC which degenerate in AR-JP [56, 59, 60, 69]. This defect may thus underlie the accumulation of undegraded parkin substrates, including Pael-R and αSp22, in these brain areas [60, 62]. It has been shown that normal parkin prevents ER dysfunction and unfolded protein-induced cell death following overexpression of Pael-R in cultured cells and *Drosophila* [61, 62, 70]. So, it is reasonable to consider that accumulation of undegraded substrate proteins disrupts intracellular processes leading to neurodegeneration in familial PD.

Mutations of parkin in transgenic mice do not cause nigrostriatal degeneration as seen in AR-JP [71–74]. The frequency of point mutations, deletions and duplications of parkin is similar in AR-JP (3.8%) and normal control (3.1%) subjects [75]. Taken together, these observations raise the possibility that additional factors, for example exposure to environmental substances or other gene alterations, might be necessary to trigger the development of parkinsonism in some individuals carrying mutations in parkin.

8.5 UCH-L1

It was discovered in 1998 that an I93M missense mutation in the gene (4p14; PARK 5) encoding ubiquitin C-terminal L1 (UCH-L1), a 230-amino acid/26-kDa de-ubiquitinating enzyme, caused autosomal dominant PD in two siblings of a European family [76]. The parents were asymptomatic, suggesting that the gene defect causes disease with incomplete penetrance. The affected individuals had clinical features that resemble sporadic PD, including a good response to levodopa, but the age (49 and 51 years) of onset was relatively early. Postmortem analyses on one of the siblings revealed Lewy bodies in the brain [77]. More recent genetic screening studies have failed to detect UCH-L1 mutations in families with PD, suggesting that this mutations is a very rare cause of the illness [78]. Several studies have found that the UCH-L1 gene is a susceptibility locus in sporadic PD and that polymorphisms, such as the S18Y substitution, confer some degree of protection against developing the illness [79]. However, a recent study has failed to find any association between UCH-L1 polymorphisms and PD [80].

UCH-L1 is expressed exclusively in neurons in many areas of the CNS [81], and constitutes 1–2% of the soluble proteins in the brain [81–83]. It is not known how alterations in the UCH-L1 gene alters the UPS, proteolysis and protein levels in PD. Mutations in UCH-L1 causes a reduction in de-ubiquitinating activity *in vitro* and in the brain of transgenic mice with the neurological disorder gracile axonal dystrophy (GAD) [76, 84, 85]. Toxin- or mutation-induced inhibition of UCH-L1's activity leads to a marked decrease in the levels of ubiquitin in cultured cells and in the brain of GAD mice [85, 86]. Impairment of ubiquitin C-terminal hydrolases leads to degeneration of dopaminergic neurons with protein accumulation and formation of Lewy body-like inclusions in rat ventral midbrain cell cultures [86]. A recent study showed that UCH-L1 has E3 ubiquitin ligase activity, but it remains unclear if and how the PD-related mutation alters this function of the protein [87].

Therefore, it is possible that a mutation in UCH-L1 alters UPS function leading to altered proteolysis and ultimately cell death. However, more studies are required to decipher the mechanism by which mutations in UCH-L1 leads to pathogenesis in familial PD.

8.6 α-Synuclein

The first gene, at chromosome 4q21–q23 (PARK 1&4), to be associated with PD was reported in several European families during the 1990s [88–90]. Genetic analyses showed that the defects were A53T and A30P point mutations in the gene that encodes for a previously discovered 140-amino acid/14-kDa protein known as α-synuclein [89, 90]. Subsequently, an E46K mutation in α-synuclein was reported in another European family with autosomal dominant PD (plus features

of dementia with Lewy bodies) [91], but no other point mutation has been found. In recent years, duplication and triplication of the normal α-synuclein gene have been found to cause autosomal dominant PD in European and American families [92–97].

Familial PD caused by α-synuclein shares features of common sporadic PD, but there are also significant differences, in particular the relatively early age of onset (mean, between 40 and 50 years) and high occurrence of dementia in patients with α-synuclein mutations. Also, patients with duplication/triplication of the α-synuclein gene tend to have the neurodegenerative disorder, dementia with Lewy bodies, in addition to or instead of parkinsonism [92–97]. Pathological studies have shown that patients with the A53T mutation or multiplication mutation of the α-synuclein gene show a marked increase in α-synuclein levels with protein aggregation in various regions of the brain [95, 98, 99]. However, in patients with the A53T mutation, Lewy bodies are rarely present and there is a marked accumulation of α-synuclein and tau in the cerebral cortex and striatum [98, 99]. Also, patients with triplication of the normal α-synuclein gene have vacuoles in the cortex, neuronal death in the hippocampus and the inclusions bodies in glial cells [95]. These findings show that there are significant differences between the pathology in α-synuclein-linked familial and common sporadic PD.

α-Synuclein is a member of a family of related proteins that also include β- and γ-synucleins [100]. α-Synuclein, so called because of its intracellular localization to synapses and nuclear envelope when first discovered [101, 102], is expressed throughout the CNS [81]. The protein is enriched in presynaptic nerve terminals and associates with lipid membranes and vesicles [100]. The normal function of α-synuclein is unknown, but their is some evidence that it plays a role in synaptic neurotransmission [100, 103]. Since the discovery of α-synuclein-linked familial PD, there has been a great deal of effort aimed at deciphering how mutations in this protein induce neurodegeneration. The dominant mode of inheritance suggests a gain of function. Wild type α-synuclein is monomeric and intrinsically unstructured/natively unfolded at low concentrations, but in high concentration it has a propensity to oligomerize and aggregate into β-pleated sheets [104, 105]. Mutations in the protein increase this potential for misfolding, oligomerization and aggregation [104, 106–110]. Oligomerization of α-synuclein produces intermediary species (protofibrils) that form annular structures with pore-like properties that permeabilize synthetic vesicular membranes *in vitro* [106–108]. It has been suggested that protofibrils are the toxic α-synuclein species that are responsible for cell death [109], but this concept is largely based on studies of the biophysical and conformational properties of α-synuclein *in vitro*. With the discovery that Lewy bodies in patients with sporadic PD stained positively for α-synuclein, it has been considered that α-synuclein might also play an important role in the development of sporadic PD.

It is possible that the cytotoxicity of mutant/excess α-synuclein involves interference with proteolysis. Wild type α-synuclein is a substrate for both the 26S and 20S proteasome and is preferentially degraded in a ubiquitin-independent manner [111–113]. *In vitro* and *in vivo* studies have demonstrated that mutant α-synuclein,

which misfolds, oligomerizes and aggregates, is resistant to UPS-mediated degradation and also inhibits this pathway [114–116]. As a result, there is accumulation of a wide range of proteins in addition to α-synuclein in cells expressing mutant α-synuclein [114–116]. As previously discussed, high levels of undegraded or poorly degraded proteins have a tendency to aggregate with each other and other proteins, form inclusion bodies, disrupt intracellular processes, and cause cell death [22]. Recent studies indicate that α-synuclein can also be broken down by the 20S proteasome through endoproteolytic degradation that does not involve the N- or C-terminus [111–113]. This type of degradation yields truncated α-synuclein fragments which are particularly prone to aggregate, promote aggregation of the full length protein as well as other proteins, and cause cytotoxicity [117]. Thus, it is reasonable to consider that alterations in the α-synuclein gene can cause the UPS to fail and this defect may underlie protein aggregation, Lewy body formation and neurodegeneration in hereditary PD.

Numerous studies, employing a variety of approaches, have examined the effects of expressing familial PD-related mutant (and wild-type) α-synuclein in transgenic animals [118]. Expression of mutant (A53T, A30P) or wild-type α-synuclein in transgenic *Drosophila* [119], or the adenoviral-mediated expression of A53T mutant or wild type α-synuclein in the SNc of adult non-human primates (common marmosets) [120], causes dopamine cell degeneration. However, overexpression of A53T, A30P or wild-type α-synuclein causes inclusion body formation but does not cause neurodegeneration in transgenic mice [118]. Interestingly, the non-human forms of α-synuclein normally have a threonine in the alanine position and do not show aggregation as is found with the human mutation [89], possibly because α-synuclein is degraded differently in these species. α-Synuclein can also be degraded by the lysosomal system and there is evidence of impaired chaperone-mediated clearance by autophagy of the mutant form of the protein [121, 122].

The relative roles of the UPS and lysosomal systems in the degradation of wild-type and mutant α-synuclein has not been clearly defined, and it is possible that defects in the lysosomal systems could contribute to the protein accumulation and aggregation found in α-synuclein-linked familial PD. Additionally, the observation that not all carriers of point mutations in α-synuclein develop PD, suggests that additional factors, such as environmental toxins, might be required to trigger the development of PD in these individuals.

8.7 Dardarin/LRRK2

In 2002, a large Japanese family having autosomal dominant PD with incomplete penetrance was linked to a mutation on chromosome 12p11.2–q13.1 (PARK 8) [123]. This linkage has since been found in several families from different countries, and it is estimated that the mutation could account for 5% of familial cases of PD [8]. The gene defects in these patients are several missense mutations in

the gene encoding a 2527-amino acid/≈250-kDa protein called dardarin or LRRK2 (leucine-rich repeat kinase 2) [124, 125]. Notably, not all individuals with these mutations develop parkinsonism, suggesting the possible requirement of other etiological factors to act as a trigger for the illness [126].

The clinical spectrum of dardarin/LRRK2-linked PD is similar to sporadic PD and has an age of onset ranging from 32 to 79 years. There are, however, important pathological differences between these two forms of PD [123, 125, 127]. In dardarin/LRRK2-linked familial PD, all subjects have nigrostriatal degeneration, some have Lewy bodies and some do not have these inclusions; others have extensive cortical Lewy bodies consistent with "dementia with Lewy bodies", and some have tau-immunoreactive glial and neuronal inclusions consistent with tauopathies such as progressive supranuclear palsy. Interestingly, some patients with this mutation have a late onset form of PD with no family history and pathology changes characteristic of sporadic PD. It has been estimated that the LRRK2 mutation might account for as many as 7% of familial cases and 1.5–3% of cases of sporadic PD [126, 128, 129].

Dardarin/LRRK2 is expressed throughout the brain [124, 130], but its normal function is unknown. Based on its similarity with other proteins, it has been suggested that dardarin/LRRK2 might be a cytoplasmic kinase [124, 125]. It remains to be determined how mutations in dardarin/LRRK2 alter the structure and function of the protein, but a recent study indicated that PD-related mutations cause an increase in phosphorylation activity [131]. Some proteins, such as I B, require phosphorylation as a prerequisite to their ubiquitination and proteasomal degradation [132]. Indeed, the phopsphorylation status of serine 129 of α-synuclein appears to play a significant role in its ability to induce toxicity in *Drosophila* [133]. It will therefore be interesting to determine whether mutations in dardarin/LRRK2 lead to altered phosphorylation with increased aggregation of target proteins and impairment of the UPS.

8.8 PINK1

Autosomal recessive early-onset PD in several European families was found to result from missense and exon-deletion mutations in a gene located at chromosome 1p35–p36 (PARK 6) which codes for a 581-amino acid/62.8-kDa protein designated PINK1 (PTEN (phosphatase and tensin homolog deleted on chromosome 10)-induced kinase 1) [134–136]. This mutation has since been found in additional families, and may account for 1–2% of early-onset cases of PD [137, 138]. Clinically, this form of PD is characterized by early onset (20–40 years) of symptoms, slow progression and good response to levodopa [134, 138].

PINK1 is localized to mitochondria but additional studies are required to determine its cellular and anatomical distribution [136]. The normal function of PINK1 is unknown. It appears to be a serine/threonine protein kinase which phosphorylates proteins involved in signal transduction pathways [136]. In cell culture studies,

wild-type PINK1 prevents proteasome inhibitor-induced mitochondrial dysfunction and apoptosis, but this protection is lost with familial PD-related mutations [136]. These findings raise the possibility that mutations in PINK1 could render neurons susceptible to agents, such as abnormal proteins and toxins, that act on proteasomes to induce cell death.

Thus, the pathogenic process in PINK1-related familial PD might involve alterations in protein handling. It is interesting that familial PD-related mutations in PINK1 have been found in normal control subjects who do not have clinical features of parkinsonism [139], again raising the possibility that multiple factors may be necessary for the development of PD [140].

8.9 DJ-1

Genetic studies in 2001 of several European families with autosomal recessive early-onset parkinsonism found linkage to chromosome 1p36 (PARK 7) [141]. Subsequently, these families were found to have missense and deletion mutations in the gene that encodes a previously known 189-amino acid/20-kDa protein called DJ-1 [142–144]. Since then, no additional mutation in DJ-1 has been reported and it is thought that this defect could account for only <1% of early-onset cases of the illness [145]. Clinically, DJ-1-linked PD is similar to parkin-related PD, namely early onset (20–40 years) of symptoms, slow progression, presence of dystonia, levodopa-responsiveness, and the occurrence of psychiatric disturbance [142, 143]. At present, the neuropathological features in DJ-1 patients are not known.

In the CNS, DJ-1 is more prominent in astrocytes compared to neurons, and is present in the cytosol and nucleus of cells [146, 147]. The normal function of DJ-1 is not known. There is evidence to suggest that it acts as an antioxidant or a sensor of oxidative stress [148, 149]. Additionally, the molecular structure and *in vitro* properties of DJ-1 indicate that it might act as a molecular chaperone and a protease [150–152]. Recently, DJ-1 was found to interact with parkin, CHIP and HSP70, suggesting a link to these proteolytic systems [153].

The mechanism by which mutations in DJ-1 induces pathogenesis is unknown. The recessive pattern of inheritance raises the possibility that the mutations induce a loss of function of the protein. The PD-related mutations (e.g. L166P) destabilize and inactivate the protein, impair its proteolytic activity, and promote its rapid degradation by the proteasome [150, 154]. In cell cultures, overexpression of DJ-1 protects cells from oxidative stress, but knockdown of DJ-1 increases susceptibility to oxidative stress, endoplasmic reticulum stress and proteasomal inhibition [148, 149]. Further, mutations in DJ-1 reduce its ability to inhibit the aggregation of α-synuclein both *in vitro* and *in vivo* [155]. Interestingly, a recent study has shown that deletion of DJ-1 in transgenic mice does not induce neurodegeneration [156], suggesting that other factors might be involved in the pathogenic process in PD.

Thus, it may be speculated that mutations in DJ-1 lead to a loss of its putative antioxidant, chaperone and proteolytic activity. Such defects, if proven to be the case in future studies, would indicate that altered protein handling also plays a role in the pathogenesis of this familial form of PD.

8.10 Proteasomal Dysfunction in Sporadic PD

The majority of PD cases occur sporadically with insidious onset and are of unknown cause. At present, there is no convincing evidence to suggest that a defect in either parkin, UCH-L1, α-synuclein, dardarin/LRRK2, PINK1 or DJ-1, is responsible for sporadic PD. However, these or other genes could be involved as a susceptibility factor in this form of the illness. It is widely believed that gene and/or aging-related-susceptibility coupled with exposure to environmental toxins underlies the etiology of sporadic PD [9]. Thus, it is interesting that variability in the genes encoding α-synuclein [157], parkin [158], UCH-L1 [79], but not PINK1 [159], dardarin/LRRK2 [160], or DJ-1 [161], have been associated with an increased risk of developing sporadic PD. Several etiopathogenic factors, including oxidative stress, mitochondrial dysfunction [11], inflammation [12], excitotoxicity [13] and apoptosis [14], have been linked with the neurodegenerative process. Most recently, failure of the UPS, specifically at the level of the 26/20S proteasome, has been implicated in the pathogenesis of sporadic PD [17].

8.10.1 Altered Proteasomal Function

Over many years of research in PD, there has been indirect, but nevertheless significant, findings which suggested that proteasomal dysfunction plays a role in the vulnerability and degeneration of the SNc and perhaps other regions in PD. The mRNA level and enzymatic activity of 26/20S proteasomes and proteasome activators decrease with advancing age in the midbrain and other areas of the CNS [28, 162, 163]. In comparison with other brain areas, the SNc has a higher level of basal oxidative stress and protein oxidation; these processes are elevated in parallel with aging [27]. Therefore, declining proteasomal activity coupled with increasing oxidative protein damage with advancing age could underlie the age-related increase in susceptibility of the SNc to proteolytic stress and degeneration. Indeed, mild neuronal loss with Lewy bodies are found in the SNc of 10–15% of individuals who die over the age of 65 years without clinical evidence of neurological disorder [6, 164]. This condition, referred to as incidental Lewy body disease (ILBD), occurs with 10 times the frequency of PD and is thought to represent the pre-symptomatic phase of sporadic PD [164].

There is a marked increase in the levels of oxidatively damaged, 4-hydroxynonenal-conjugated, nitrated, phosphorylated and ubiquitinated proteins in the SNc and other areas of the brain in PD [47]. Indeed, protein aggregates and Lewy body

inclusions containing a wide variety of proteins, including α-synuclein and ubiquitin, can be seen at the various pathological sites in patients with PD [47]. These observations suggest that the UPS may be inhibited and/or saturated, resulting in protein accumulation and aggregation in the disorder. The accumulation of both ubiquitinated and non-ubiquitinated proteins (e.g. oxidized proteins and α-synuclein) [24, 111–113] in the brain indicates that a defect in proteolysis at a central and common point, i.e. the 20S proteasome core, is likely since both groups of proteins accumulate in the illness.

Studies of proteasomal function have shown that the chymotrypsin-like, trypsin-like and PGPH enzymatic activities of the 26/20S proteasomes were reduced by approximately 44–55% in the SNc in PD compared to age-matched controls [165–168]. In contrast, the three proteolytic activities of the proteasome were unchanged in regions that do not degenerate in PD, namely the frontal cortex, striatum, hippocampus, pons and cerebellum [165–168]. Interestingly, Tofaris and colleagues showed proteasomal impairment in PD cases with relatively mild neuropathology, suggesting that altered proteasomal function occurs early in the pathogenic process [167]. There is a 40% reduction in the content of proteasome α-subunits, but not β-subunits, in the SNc in PD compared to age-matched controls [165]. In contrast, the levels of α-subunits were increased by 9% in the cerebral cortex and by 29% in the striatum in PD. Immunohistochemical staining demonstrated reduced levels of 26/20S proteasomal α-subunits, but not β-subunits, within dopaminergic neurons in the SNc of PD subjects compared to age-matched controls [165]. As discussed previously, the PA700 proteasome activator is a complex of over 20+ different subunits with varying molecular weights [31]. In PD, there was either no change (42, 46 and 95 kDa bands) or up to a 33% loss (52.5, 75 and 81 kDa bands) of PA700 subunits in the SNc [165]. In contrast, there was a marked increase in the levels of subunits at the 81, 75, 52.5 and 42 kDa bands in the frontal cortex and/or the striatum of PD subjects compared to controls. This observation is consistent with other studies showing a significant upregulation of proteasomal function in cortical areas in patients with PD [168]. In normal control subjects, the levels of the PA28 proteasome activator were very low in the SNc compared to the frontal cortex and striatum [165]. In PD brains, PA28 immunoreactivity was almost undetectable in the SNc and levels were reduced in the frontal cortex (24%) and striatum (16%) in comparison to controls [165]. These findings indicate that, in sporadic PD, there is inhibition of proteasomal function in regions that degenerate while there is upregulation, perhaps a compensatory and cytoprotective response, in areas that do not degenerate. Interestingly, a recent study showed that proteasomal function is impaired in lymphocytes of PD but not Alzheimer's disease patients, although this defect might be related to drug therapy [169].

8.10.2
Role of Proteasomal Dysfunction in the Neurodegenerative Process

The involvement of altered proteasomal function in the pathogenesis in PD is supported by several observations. Proteasomes not only play a critical role in

Table 8.1. Alterations in UPS-linked cellular processes in Parkinson's disease.

Cellular processes linked to the UPS	Alterations in Parkinson's disease
Degradation and clearance of abnormal proteins [18]	Yes: failure of the UPS and protein aggregation
Antioxidant defense mechanisms [171, 176]	Yes: oxidative stress [10]
Mitochondrial function [172, 188, 200, 204]	Yes: complex I activity impaired [11]
Inflammatory response [174, 177]	Yes: microglial activation and gliosis [12, 205]
Immune processes [206]	Yes: complement activation [12]
Apoptotic signaling [172, 175]	Yes: apoptotic cell death [14]
Synaptic function and neurotransmission [207]	Yes: altered basal ganglia function [208, 209]
Signal transduction [210]	Yes: altered neuronal activity [208, 209]
Protein transport/trafficking [211]	Yes: inclusion body formation [47]
Gene transcription [212]	Yes: altered expression of a variety of proteins [186]
Development and differentiation [207]	–
Regulation of cell cycle and division [213]	–

The ubiquitin–proteasome system (UPS) controls the levels of short-lived regulatory/functional proteins that mediate a wide variety of cellular processes. Thus, failure of the UPS to degrade proteins not only causes protein accumulation and aggregation, but it also alters cellular functions. Many of these cellular and biochemical defects occur in Parkinson's disease and likely play role in the neurodegenerative process.

the degradation and clearance of unwanted proteins, but they also play a major role in controlling the levels of short-lived regulatory/functional proteins and are intimately linked with a variety of cellular processes (Table 8.1). Indeed, proteasomes are linked with antioxidant defense mechanisms [170, 171], mitochondrial activity [172, 173], inflammatory responses [174], and anti-apoptotic pathways [175] (Table 8.1). Thus, inhibition of proteasomal function disrupts these processes and causes oxidative stress [176], mitochondrial dysfunction [176], pro-inflammatory reactions [177] and apoptotic cell death [172]. Most of these proteasome-linked cellular processed have been found to be altered in PD, further supporting the concept that proteasomal dysfunction plays a role in the pathogenic process (Table 8.1).

Typically, inhibition of proteasomal function causes protein accumulation and the formation of aggresomes which are intracytoplasmic proteinaceous inclusions formed at the centrosome in response to inadequate protein degradation [23, 43, 178]. The demonstration that Lewy bodies contain the centrosome-related marker γ-tubulin, UPS components and HSP, and share other compositional and organization features of aggresomes, suggests that they might form in an aggresome-related manner as a result of proteolytic stress PD [40, 47]. Indeed, this concept raises the possibility that Lewy bodies might represent a cytoprotective response aimed at promoting the survival of the neurons in which they are formed in PD [47, 179].

Impairment of proteasomal function typically induces cell death and this often occurs via an apoptotic mechanism. It has been shown that application of proteasome inhibitors to cultured cells, or injection of these agents into the brain of rats, induces preferential degeneration of dopaminergic neurons in the SNc [86, 180–184]. In these model systems, dopaminergic cell death is accompanied by an accumulation of protein and the formation of α-synuclein/ubiquitin-immunoreactive inclusions that resemble Lewy bodies [86, 180–184]. Recently, it was shown that systemic exposure of rats to PSI (Z-Ile-Glu(OtBu)-Ala-Leu-al, a peptide aldehyde) or epoxomicin (Ac(Me)-Ile-Ile-Thr-Leu-EX, a peptide α′,β′-epoxyketone), which are synthetic and bacterial proteasome inhibitors respectively, induces a model that closely recapitulated many features of PD [185]. Proteasome inhibitor-treated rats developed progressive, PD-like, motor dysfunctions that could be improved with the administration of dopaminergic agents (i.e. levodopa and apomorphine). Positron emission tomography imaging (PET) demonstrated a gradual loss of dopaminergic nerve terminals in the striatum, and postmortem analyses showed striatal dopamine depletion and progressive neurodegeneration with apoptosis and inflammation in the SNc. Also, neuronal death occurred in the LC, DMN and NMB. At the various pathological sites in the rats treated with proteasome inhibitors, there was a 43–82% inhibition of proteasomal function, accumulation of proteins and the formation of intraneuronal α-synuclein/ubiquitin-positive inclusions which resemble Lewy bodies. Thus, this model based on inhibition of proteasomal function more closely recapitulates the behavioral, imaging, pathological and biochemical features of sporadic PD than any other model of the disorder described to date.

Taken together, the above observations suggest that altered proteasomal function could play a key role in protein accumulation, Lewy body formation and neurodegeneration in the SNc and perhaps other brain regions in sporadic PD.

8.10.3
The Cause of Proteasomal Dysfunction

The question arises as to what causes proteasomal dysfunction in sporadic PD. This is not known, but there are several possibilities. It could result from undiscovered gene mutations. Interestingly, DNA microarray analyses were recently used to demonstrate a reduction in the mRNA levels of 20S proteasome α-subunits (PSAM2, PSMA3 and PSMA5) and a non-ATPase subunit (PSMD8/Rpn12), and an ATPase subunit (PSMC4/Rpt3) of PA700, in the SNc in PD [186]. Proteasomal dysfunction may develop secondary to the other biochemical defects that occur in PD, such as oxidative stress or mitochondrial dysfunction [49, 187–189]. Indeed, there could be a close relationship and interplay between proteasomal dysfunction and the various cellular, biochemical and molecular changes that have been detected in PD [49]. An alternative hypothesis is that proteasomal dysfunction in PD could be the result of exposure to toxic substances. Inhibitors of the proteasome are widely distributed in the environment [190]. They are produced by bacteria (e.g. actinomycetes which infect the below-ground portion of crops) [191,

192], fungi (e.g. *Apiospora montagne* which infests wheat/flour) [193], plants [194–196], and the chemical/pharmaceutical industry [190, 197]. Indeed, lactacystin and epoxomicin, which are among the most potent proteasome inhibitors known, are naturally produced by actinomycete (*Streptomyces*) bacteria [198, 199]. These microbes are found globally in the soil and aquatic habitats of gardens and farmland, and are well known for infecting root vegetables and potatoes (causing "scab" formation) [198, 199]. Also, structurally-related analogs and the active pharmacophore of natural and synthetic compounds known to potently inhibit the proteasome, such as PSI, are also present in the environment [190]. Notably, agrochemicals such as the fungicide maneb (specifically its active metabolite) [197] and pesticides including rotenone, have been shown to impair proteasomal function [172, 200, 201]. Thus, humans could be exposed to proteasome inhibitors via the food chain. Indeed, the distribution of proteasome inhibitors in agrarian environments could underlie the finding that rural living and drinking well water are both associated with a high risk of developing PD [202].

8.11 Conclusion

The etiology of PD has fascinated researchers since the illness was discovered. Over the years, a variety of factors have been considered and it remains unclear if or how they contribute to development of the disorder. In recent years, there has been increasing evidence to suggest that failure of the UPS and altered protein handling as key, and perhaps common, are defects that might underlie the various familial and sporadic forms of PD. The evidence for UPS dysfunction in some forms of PD is substantial and convincing. In other types of PD, however, the evidence implicating UPS failure is speculative but is nevertheless intriguing. A determination that the UPS plays a role in the etiopathogenesis of disorder is not merely academic as this could reveal novel targets that can be exploited to develop neuroprotective medicines and possibly a diagnostic biomarker that are currently not available to patients with PD.

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9
The Molecular Pathway to Neurodegeneration in Parkin-Related Parkinsonism

Ryosuke Takahashi

9.1
Introduction

Parkinson's disease (PD) is the most common neurodegenerative disease of the motor system amongst elderly people. The prevalence of PD is approximately 1% of people by the age of 70 years [1]. PD is characterized by a progressive loss of dopaminergic neurons in the pars compacta of the substantia nigra accompanied by the formation of Lewy bodies. Lewy bodies are intra-neuronal fibrillary inclusions mainly composed of α-synuclein [2]. They are regarded as the hallmark of idiopathic PD. Loss of neurons within the pars compacta of the substantia nigra causes progressive motor disturbances, classically tremor, rigidity, bradykinesia and postural instability. To date, there is no known effective therapy to prevent or retard neurodegeneration as a result of PD [1, 3].

Most cases of PD develop sporadically, however, fewer than 10% of cases are familial and presumably inherited [4]. Autosomal recessive juvenile parkinsonism (AR-JP) accounts for approximately 50% of cases of early-onset familial PD in affected European families [5]. It is characterized by several unique features, including young age of onset (usually under 40 years of age), dystonia, and a marked response to dopamine. The neuropathological hallmark of AR-JP is selective degeneration of dopaminergic neurons in the substantia nigra zona compacta, similar to that observed in the idiopathic form of PD. However, AR-JP is not usually associated with Lewy bodies [6, 7].

Mutations in the parkin gene are responsible for AR-JP [8]. In this chapter, the role of parkin in the ubiquitin–proteasome system will be focused and discussed in light of recent findings.

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9.2
Parkin is an E3 Ubiquitin Ligase

9.2.1
Parkin and the Ubiquitin-Proteasome System

Parkin is a 465-amino acid protein characterized by a ubiquitin-like domain at its NH_2-terminus, as well as two RING-finger motifs and an IBR (in-between RING fingers) motif at its COOH terminus (RING-IBR-RING or RBR domain) [9]. The RING domain has been shown to be a feature of ubiquitin ligase involved in the ubiquitination reaction [10]. Polyubiquitination involves a sequence of reactions performed by ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligating (E3) enzymes. E3 interacts with specific substrate(s) and facilitates the formation of covalent bonds between the COOH terminus of ubiquitin and ε-lysine, either on a target protein or on the last ubiquitin of a protein-bound polyubiquitin chain in concert with its partner E2s. Yeast protein UFD2 is a multi-ubiquitin chain elongation factor, also called E4, required for efficient multi-ubiquitination of a substrate [11]. Polyubiquitin chains are thought to be potent targeting signals for the degradation of proteins within 26S proteasomes.

Several groups have shown that wild-type parkin is an E3 ubiquitin ligase [12–14] (Figure 9.1). Parkin ubiquitinates substrate proteins or itself in concert with E2s, such as UbcH7, UbcH8, Ubc6 and Ubc7 [12–14]. Moreover, several AR-JP-related missense mutations have been identified in the ubiquitin-like

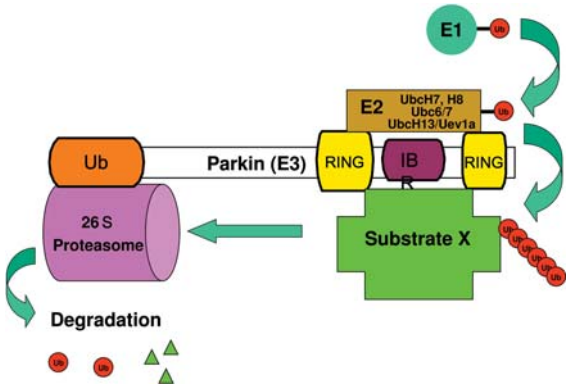

Fig. 9.1. Function of parkin in the ubiquitin proteasomal pathway. Parkin is an E3 ubiquitin ligase that recognizes substrate X and promotes ubiquitination in adjunct with two other ubiquitination enzymes, E1 and E2. Polyubiquitinated substrate X is recognized and degraded by the 26S proteasome. The N-terminal ubiquitin-like domain and the C-terminal RING-IBR-RING domain of parkin serve as recruitment domains for 26S proteasome and E2 enzymes, respectively. Some of the known substrates of parkin associate with its RING-IBR-RING domain.

domain of the parkin gene [15]. Furthermore, an NMR analysis has revealed binding between the ubiquitin-like domain of parkin and the Rpn 10 subunit of the 26S proteasome [16], strongly suggesting the link between Parkin and the UPS (Figure 9.1).

9.2.2 Proteasome-independent Role of Parkin

Polyubiquitin chains are formed through distinct types of linkages using one of the seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) within the previous ubiquitin molecule [17].

Recently, parkin was shown to be a dual function ubiqutin ligase that mediates both K48- and K63-linked polyubiquitination [18]. K48-linked polyubiquitin chain, the best characterized form of polyubiquitin, leads the proteins to degradation via 26S proteasomes, constituting the ubiquitin–proteasome system as mentioned before. On the other hand, K63-linked chains act as proteasome-independent signals in several different cellular pathways [17]. Dual specificity seems to be determined by the E2 enzymes that parkin recruits. In the assembly of a K63-linked polyubiquitin chain, parkin interacts with the UbcH13/Uev1a heterodimer [18]. Parkin mediates K63-linked, proteasome-independent ubiquitination of its substrate synphilin 1 [19]. Although K63-linked ubiqitination is implicated in inclusion body formation, further study is required to clarify its physiological relevance [20].

9.2.3 Multiple Monoubiquitination is Mediated by Parkin

Surprisingly, two recent reports have shown that Parkin mediates multiple monoubiquitination *in vitro* [21, 22]. Both reports have demonstrated that the second RING finger is responsible for E3 activity in an *in-vitro* ubiquitination assay, where bacterially-produced recombinant Parkin was used. Previous findings that mutations in regions other than the second RING finger showed reduced E3 activity *in vivo* might be ascribed to their insolubility and sequestration [23–26]. Parkin itself as well as maltose binding protein (MBP) connected to Parkin as a pseudosubstrate and p38 as a substrate, have been shown to be monoubiquitinated *in vitro* and *in vivo* respectively. In contrast to a previous report that Parkin accelerates polyubiquitin chain formation [18], Parkin has been shown to mediate monoubiquitination in concert with Ubc13 as well as Ubc7 or Ubc H7 under pure *in vitro* conditions [21, 22]. These results suggest that Parkin may mediate monoubiquitination regardless of its partner E2s.

A recent report showed that Parkin mediates monoubiquitination of an adaptor protein Eps15 with two ubiquitin-interacting motifs (UIMs) [27]. Eps15 interacts with and positively regulates the endocytosis of ubiquitinated epithelial growth factor receptor (EGFR). Parkin-mediated ubiquitination of Eps15 inhibits its ability to bind with and promote endocytosis of EGFR, resulting in suppression of EGFR

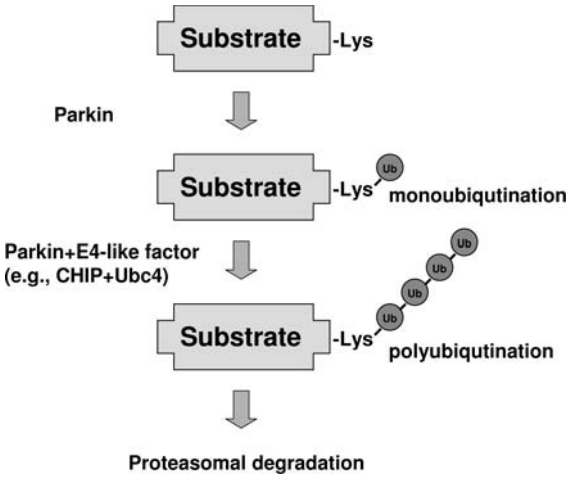

Fig. 9.2. Hypothetical two-step ubiquitination of Parkin substrates. At the first step, Parkin monoubiqutinates its substrate. Then, E4-like factors promote the elongation of polyubiquitin chain on the substrate molecules, thereby targeting the substrates to 26S proteasome. CHIP and its partner E2 Ubc4 are candidates for such E4-like molecules [28].

internalization and degradation, and promoting phosphoinositide 3-kinase (PI(3)K)-Akt signaling. Since Akt plays an important role in neuronal survival, this proteasome-independent function of Parkin may explain some aspects of neurodegeneration.

On the other hand, monoubiquitination may lead to proteasomal degradation. A previous report showed that the carboxy-terminus of Hsc70-interacting protein (CHIP), a U-box motif containing E3 protein, together with Ubc4, serves as an E4-like protein and cooperates with Parkin to form polyubiquitin chains [28]. Given the presence of E4-like factor, monoubiquitination catalyzed by Parkin may eventually target the proteins to degradation via the 26S proteasome (Figure 9.2). Whether Parkin can mediate both monoubiquitination and polyubiquitination should be re-examined and clarified in light of the recent findings.

9.2.4
Modulators of Parkin E3 Activity

Parkin is a component of a high molecular weight complex located in cells and the function of parkin seems to be modulated by its binding partners [28, 29]. Two parkin-associated proteins have been shown to promote the elimination of Pael-R by parkin: CHIP and Hsp70 [28].

CHIP contains a U-box motif, which is structurally similar to the RING-finger motif and exhibits U box-dependent E3 activity [30–32]. On the other hand, CHIP has been shown to downregulate chaperone ATPase activity [33]. Moreover, CHIP

has been shown to ubiquitinate improperly-folded protein in a chaperone-dependent manner [32]. When bound to parkin however, CHIP markedly enhances parkin-mediated ubiquitination of Pael-R *in vitro* [28]. Consistent with this observation, overexpression of CHIP accelerates Pael-R degradation in cultured cells, leading to a marked reduction in the steady-state level of Pael-R protein.

In contrast to CHIP, Hsp70 has been observed to inhibit ubiquitination of Pael-R *in vitro* and to increase levels of the soluble form of Pael-R *in vivo*, presumably by facilitating the proper folding of Pael-R. Moreover, Hsp70 inhibits CHIP-mediated degradation of soluble and probably functional Pael-R, so that only insoluble aggregates of the receptor are removed.

It has also been shown that bcl-2-associated athanogene 5 (BAG5), a BAG-family member, directly interacts with parkin and the chaperone Hsp70. BAG5, similar to CHIP, downregulates chaperone ATPase activity. Within this complex, BAG5 inhibits both parkin E3 ubiquitin ligase activity and Hsp70-mediated refolding of misfolded proteins. BAG5 enhances parkin sequestration within protein aggregates and attenuates parkin-dependent preservation of proteasome function [34].

Two binding partners of Parkin, 14-3-3 eta and Nrdp1/FLRF are also found to be negative regulators of Parkin E3 activity [35, 36].

9.3 Substrates of Parkin

9.3.1 Parkin Substrates and their Recognition Mechanisms

Although Parkin may mediate diverse forms of ubiquitination, it is likely that some, but not all, are involved in the UPS. Given that Parkin targets its ubiquitinated substrates to the 26S proteasome, Parkin dysfunction should lead to the accumulation of its substrate. Accumulation of toxic substrate(s) of Parkin (substrate-X) due to loss of parkin E3 activity or disruption of the parkin–proteasomal interaction in AR-JP patients with a genetic defect of parkin, should result in the development of dopaminergic neurodegeneration.

Based on this hypothesis, the identification of such toxic substrate(s) is the key to understanding the molecular mechanisms underlying AR-JP.

To date, 13 proteins have been identified as substrates of parkin [37]: CDCrel-1 , synaptotagmin XI [38], synphilin-1 [39], glycosylated α-synuclein [40], α/β-tubulin [41], the p38 subunit of an aminoacyl-tRNA synthetase (ARS) complex [42], *P*arkin-*a*ssociated *e*ndothelin receptor-like *r*eceptor (Pael-R) [43], the expanded form of polyglutamine [44], and cyclin E [45], SEPT5_v2/CDCrel-2 [46], misfolded dopamine transporter [47], far upstream element-binding protein 1 [48], RanBP2 [49] and Eps15 [27] (Table 9.1). It has been shown that the unmodified form of α-synuclein, a major component of Lewy body, is not a substrate for Parkin [39].

Although there are no apparent common properties among Parkin substrates, it has been noted that several different substrates are found within Lewy bodies.

Table 9.1. The reported substrates of parkin

Protein	Biological function	Lewy body
O-glycosylated α-synuclein	Septin family protein with unknown function	–
CDCrel-1	Isoform of α-synuclein with unknown function	N.D.
(Misfolded) Pae1 receptor	Orphan G-protein coupled receptor	+
p38 subunit of the aminoacyl-tRNA synthetase	Role in protein biosynthesis	+
Synaptotagmin XI	Regulation of exocytosis of neurotransmitters	+
Expanded polyglutamine(polyQ) proteins	Aberrant proteins responsible for polyQ diseases	–
α/β-Tubulins	Microtubule proteins	+
Synphilin-1	α-Synuclein-binding protein	+
Cyclin E	Cell cycle regulation of mitotic cells; unknown function in neurons	N.D.
SEPT5_v2/CDCrel-2	SEPT5_v2 is highly homologous with CDCrel-1	N.D.
Misfolded dopamine transporter	Regulation of dopamine uptake	N.D.
Far upstream element-binding protein-1	A binding partner of p38	N.D.
RanBP2	Small ubiquitin-related modifier (SUMO) E3 ligase family protein	N.D.
EPS15	Adaptor protein with ubiquitin-interacting motifs (UIMs)	N.D.

N.D., not detected.

Considering that the components of Lewy bodies consist of misfolded proteins, molecular chaperones and proteasome subunits, it is likely that Parkin ubiquitinates a subset of misfolded proteins. Consistent with this idea, an expanded form of polyglutamine, which is a causative agent of polyglutamine diseases such as Huntington's disease, has been identified as a parkin substrate [44]. Moreover, there is evidence to suggest that binding between parkin and polyglutamine is mediated by Hsp70, which is known to be a binding partner of Parkin. Hsp70-mediated substrate recognition explains the diversity of substrate specificity observed with parkin (Figure 9.3).

Among the various substrate molecules discussed above, the Pael receptor (Pael-R), CDC-rel1, cyclin E, synphilin-1, and the p38 subunit of aminoacyl tRNA synthetase have either been shown or suggested to promote cell death under certain conditions, and so represent the proteins which are most likely relevant to neurodegeneration in AR-JP.

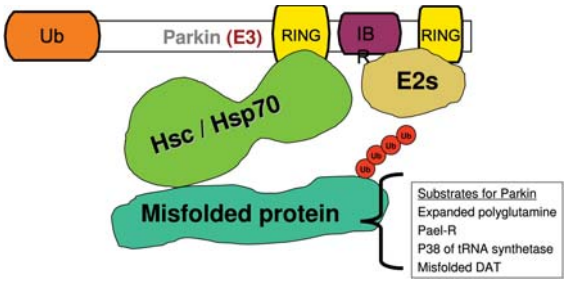

Fig. 9.3. Parkin may recognize a subset of misfolded proteins through Hsc/Hsp70. Parkin interacts with Hsc/Hsp70 through its first RING domain [28]. Parkin may recognize misfolded proteins including Pael-R, and p38 by using Hsc/Hsp70 as a substrate recognition subunit.

9.3.2 The Link between Substrate Accumulation and Cell Death: Pael-R

9.3.2.1 Pael-R and Endoplasmic Reticulum Stress

Pael-R is a putative G-protein-coupled orphan receptor, which is highly expressed in the central nervous system, especially in the substantia nigra [43, 50, 51]. Although the physiological function of Pael-R is implicated in dopamine metabolism, its ligand has yet to be identified [52]. It has been shown that misfolded Pael-R was ubiquitinated by parkin at the level of the endoplasmic reticulum and the disturbance of Pael-R degradation leads to ER stress-induced cell death.

The endoplasmic reticulum (ER) functions as a quality control regulator of membrane and secretory proteins [53]. Newly synthesized secretory proteins are transported to the ER. Inside its lumen, ER chaperones such as BiP/GRP78 bind to these newly synthesized proteins to facilitate their proper folding. After this, proteins enter the conventional secretory pathway. Proteins that are not properly folded are transported back to the cytosol where they are degraded via ubiquitin-proteasomal degradation, a process known as endoplasmic reticulum-associated degradation (ERAD) [54] (Figure 9.4). It has been shown that parkin is an ERAD-related E3 and that Pael-R is a substrate. When insoluble misfolded Pael-R is accumulated in the cells by the inhibition of the proteasome, Pael-R is first accumulated in the ER and then forms a special type of aggregate, known as an aggresome, in the cytoplasm [55]. As these aggresomes form, the cells undergo apoptosis, demonstrating cell death due to the accumulation of Pael-R.

Pael-R-induced cell death was assumed to be mediated by ER stress. Abnormal accumulation of unfolded protein in the ER is a major threat to cell viability, a phenomenon known as ER stress or unfolded protein stress. Cells attempt to adapt to ER stress in several different ways, including transcriptional upregulation of ER chaperones, and suppression of translation. These cellular responses are

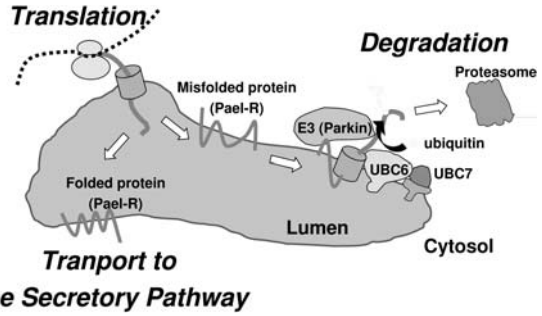

Fig. 9.4. Endoplasmic reticulum-associated degradation (ERAD). ERAD is a protein degradation system for unfolded secretory and membrane proteins. Improperly folded Pael-R is subject to ERAD, and parkin is an E3 involved in ERAD.

collectively known as unfolded protein responses (UPR) [53]. However, when the burden of accumulated protein exceeds these protective mechanisms, cells undergo a death process accompanied by the activation of JNK and caspases as well as upregulation of CHOP [56, 67].

Consistent with the idea that accumulation of misfolded Pael-R contributes to the pathogenesis of AR-JP, the level of detergent-insoluble Pael-R was elevated in the brains of AR-JP patients [43].

9.3.2.2 Pael-R Overexpressing Animals and Dopaminergic Neurodegeneration

The Drosophila model for AR-JP was created by overexpression of Pael-R [58]. When Pael-R was expressed in dopaminergic neurons in Drosophila, the number of dopaminergic neurons observed within the dorsomedial cluster fell to about 50% of that observed in control flies at 40 days of age. Equal numbers of dopaminergic neurons were observed in younger Pael-R and control flies, indicating that the observed cell loss was due to neurodegeneration occurring after birth. Moreover, even when Pael-R expression was driven by a pan-neuronal promoter, only dopaminergic neurons underwent degeneration. This suggests that dopaminergic neurons are selectively vulnerable to Pael-R toxicity.

A recent report showed that Pael-R overexpression in the substantia nigra of mouse brain through adenoviral vectors, resulted in induction of ER stress followed by dopaminergic neuronal death [59]. Pael-R-induced cell death was greatly enhanced in the parkin-deficient mouse and was suppressed by the overexpression of an ER chaperone, ORP150. Moreover, when the animal was pretreated with dopamine synthesis inhibitor, dopaminergic neuronal death was significantly attenuated, indicating that dopamine enhances Pael-R toxicity. It has been reported that dopamine covalently modifies and functionally inactivates Parkin [60]. Although the relationship between Pael-R toxicity and dopamine is still obscure, cellular protective mechanisms against Pael-R toxicity other than Parkin, might also be inactivated by dopamine.

9.3.3
The Link between Substrate Accumulation and Cell Death: CDC-rel1, Synphilin-1, Cyclin E and p38

When CDC-rel1 was introduced into the striatum and the substantia nigra of rat brain by using adeno-associated viral vectors, only dopaminergic cells in the substantia nigra underwent cell death [61]. Since the reduction of dopamine levels by pharmacological treatment alleviated nigral cell death and CDC-rel1 overexpression in PC12 cells decreased the extracellular dopamine level, the accumulation of dopamine by CDC-rel1-mediated exocytosis inhibition is thought to contribute to dopaminergic neuron-selective cell death.

Synphilin-1 is an α-synuclein-interacting protein that promotes the formation of Lewy body-like inclusions in cultured cells [62]. Parkin mediates K63-linked polyubiquitinanation of synphilin-1, apparently contributing to inclusion formation by α-synuclein and synphilin-1 in cultured cells [19]. Moreover, parkin can protect against the toxicity induced by α-synuclein plus synphilin-1 overexpression following proteasome inhibition [39].

Cyclin E has been implicated in glutamate-induced neuronal death, since it is accumulated in primary neuronal cultures in response to glutamanergic excitotoxin kainate. Interestingly, parkin overexpression inhibits the accumulation of cyclin E and cell death induced by kainate treatment, whereas RNAi-mediated parkin downregulation showed the opposite effects. The mechanism underlying cyclin E-induced cell death is not clear.

The p38 subunit plays an essential role in the *in vivo* assembly of the ARS complex [63]. When overexpressed in dopaminergic neuroblastoma cells, it forms aggresomes and induces cell death by unknown mechanisms. Parkin promotes the formation of ubiquitinated p38-positive inclusion bodies and suppresses the p38-induced cell toxicity [42]. It has been noted that only p38 is shown to be upregulated by 15% in the ventral midbrain of the parkin-null mouse among all the substrates identified [48]. The role of p38 in dopaminergic neuronal death should be validated in animal models in the future.

9.4
The Animal Models of AR-JP

To establish animal models of AR-JP, parkin gene deletion mutants for Drosophila and mouse were created [64–69]. However, the phenotypes of parkin-null mutant animals are very different from those of AR-JP patients.

9.4.1
Drosophila Model of AR-JP

The parkin gene deletion mutant Drosophila are small in size, have a short life span and become vulnerable to oxidative stress [64, 65]. The most remarkable

phenotypes of the mutant fly are apoptotic muscle degeneration and disturbances in spermatogenesis, which result in locomotor dysfunction and male sterility respectively [64, 65]. Ultrastructural analysis revealed abnormal mitochondrial morphology in both muscle and sperm [64]. On the other hand, the number of dopaminergic neurons was not reduced, although shrinkage of the cell bodies and decreased tyrosine hydroxylase immunostaining in proximal dendrites of dopaminergic neurons were observed [64].

9.4.2 Parkin-null Drosophila and Drosophila

Mutations in the PTEN-induced putative kinase 1(PINK1) are responsible for the autosomal recessive form of familial Parkinson's disease termed PARK6 [70]. PINK1 is a putative mitochondrial protein kinase, whose function is totally unknown. Three recent reports have shown that deletion of Drosophila PINK1 leads to an almost identical phenotype to that of Parkin-deficient Drosophila, i.e. it produces mitochondrial dysfunction resulting in male sterility, apoptotic muscle degeneration, and moderate loss of dopaminergic neurons [71–73]. Interestingly, the disease phenotype of PINK1-deleted Drosophila was rescued by Parkin overexpression, but not vice versa, suggesting that Parkin functions downstream of PINK1.

9.4.3 Mouse Model of AR-JP

Parkin knockout mice, in which exon 3 or 7 is deleted, have been described by three different groups [66–68]. Dopaminergic neuronal loss was not observed in any of the reports, even in aged mice. However, regarding the parkin exon-3 deletion mutant mouse, the dopamine level in the limbic system was elevated and the level of dopamine transporters was lowered according to one report, while the extracellular dopamine concentration in the striatum was increased according to a second report [66, 67]. These changes are accompanied by behavioral or electrophysiological alterations. In addition, in the mutant mouse reported by Goldberg et al., mild mitochondrial dysfunction and mild increase of oxidative stress were observed [74].

On the other hand, in exon-7-deleted parkin mutant mice, abnormalities in the nigrostriatal dopaminergic system were not detected [68]. However, noradrenergic neurons in the locus coeruleus were decreased by 20% in 70% of the total number in mice. These mild noradrenergic neuronal losses were detected as early as 2 months after birth and do not appear to progress with further aging. Consistent with this neuronal loss, the level of noradrenalin in the brain and spinal cord was reduced, accompanied by a significant reduction in the noradrenalin-dependent startle response.

In contrast to these reports, a recent extensive analysis of parkin exon-2 deletion mutant mouse revealed that the behavioral profile and cathecholamine levels in

the brain were not different from those of control mice [69]. Moreover these mutant mice were not more sensitive to 6-hydroxydopamine or methamphetamine neurotoxicity, indicating that Parkin-deficient mice are not a robust model of parkinsonism [75].

9.4.4 The Problems with Animal Models of AR-JP

The differences between parkin-deficient fly and mouse models may be explained by the difference in the endogenous substrates or the presence of redundant pathways dealing with parkin substrates in mice. Drosophila and human parkin shows a similar cell protective effect against human Pael-R- and alpha-synuclein-mediated toxicities, suggesting that the substrates are conserved to some extent [58, 76, 77]. It is particularly important to investigate whether the relationship between Parkin and PINK1 is conserved in mice and humans.

The reason why dopaminergic cell loss does not occur in the parkin knockout mouse may be due to the existence of redundant ubiquitination pathways in mice. For example, Pael-R is known to be ubiquitinated by an ER-resident E3, Hrd1 as well as by Parkin [78]. In addition, the absence of dopaminergic cell loss can also probably be attributed to the relatively short lifespan of mice (2–3 years), which would not provide enough time for the toxic substrates to accumulate in concentrations sufficient to cause cell death.

It has been noted that disturbances of the nigrostriatal system, which may represent the early signs of neurodegeneration, are suggested to occur in two exon 3-deletion mutant mice. However, the individual key findings in these papers are not in accord and no dopaminergic phenotype was detected in the exon 7- and exon 2-deletion mutant mice with respect to parkin [68, 69]. Some of the discrepancies in the detection of mild phenotypes might be caused by the different techniques employed or differences in the genetic backgrounds of the mice. Taking these possibilities into consideration, detailed and careful comparison of the phenotypes of these different parkin knockout mice should be carried to identify the real and reproducible phenotype.

9.5 Future Directions

Seven years have passed since parkin was identified as a ubiquitin ligase, and since then 13 different molecules have been isolated as parkin substrates. Some of the substrate molecules appear to explain the pathogenetic mechanisms underlying AR-JP. However, proof of accumulation of known substrates in the parkin knockout mouse brain has not been obtained except for p38, probably because of the relatively short lifespan of the mouse. So, what then is the next step?

One of the potentially promising approaches is to examine whether the nigral dopaminergic neurons in parkin-deficient mice are vulnerable to a specific stress

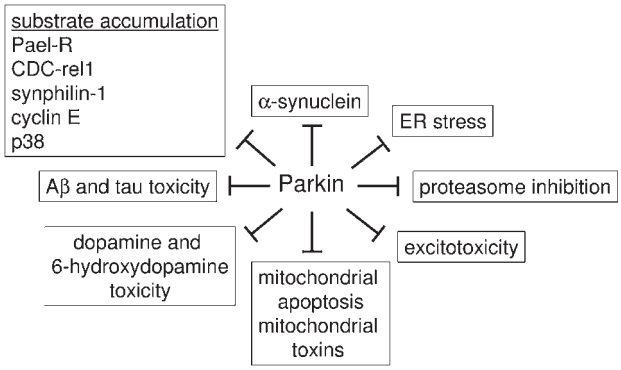

Fig. 9.5. Parkin protects cells from various stresses.

or overexpression of a specific substrate using virus vector or transgenic approaches, as has been applied to Pael-R [59]. On one hand, it is possible that the disease-causing substrate(s) has not been identified and further efforts to identify such a substrate(s) will also be important.

On the other hand, parkin appears to have cell-protective functions against various stresses (Figure 9.5). According to the reports to date, Parkin protects cells against ER stress [13], proteasomal inhibition [77], excitotoxicity [45], ceramide-induced mitochondrial apoptosis [79], mitochondrial toxins [80], intracellular Aβ [80], tau [81, 82], dopamine or 6-hydoxydopamine toxicity [47, 83] and α-synuclein-induced cell death [58, 76, 77, 84]. It is intriguing to ask whether clearance/sequestration of certain parkin substrate(s) contributes to such cell protective effects. Whether the pathways to neurodegeneration caused by parkin mutations are multiple or not should be clarified in the future.

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10
Parkin and Neurodegeneration

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10.1
Introduction

Parkinson's disease (PD) was first described by James Parkinson in his 1817 publication titled "An Essay on the Shaking Palsy" [1], although descriptions of a similar disease are found in ancient Ayurvedic literature in India [2]. PD is estimated to affect about 1–2% of the population over the age of 65 years, with age as the most consistent risk factor [3]. The prevalence of PD amongst different ethnic groups is variable, and the disease has a slightly higher incidence rate in men than women [4]. With a growing aging population and a significantly high mortality rate [5], examining the pathophysiology of this second most common neurodegenerative disorder has received considerable attention.

Clinically, PD is characterized by parkinsonism, which consists of a group of symptoms such as tremor, bradykinesia (slowness of movement), rigidity, and postural instability (difficulty with balance). However, parkinsonism is observed in other brain disorders, making PD diagnosis a challenge in the clinic [3]. In addition, some patients also show signs of elevated anxiety, depression, and dementia. A hallmark feature of PD is the progressive death of selected but heterogeneous populations of neurons throughout the brain, including the substantia nigra pars compacta, coerulus–subcoeruleus complex, brain stem nuclei, nucleus basalis of Meynert, parts of the hypothalamus and cortex, as well as the olfactory bulb [3, 6]. Deficiency of dopamine in the nigrostriatal pathway of the brain is thought to be the major cause of motor dysfunction observed in PD. It is estimated that a loss of 60–70% of dopaminergic neurons in the substantia nigra precedes the onset of symptoms [7]. Functional imaging studies have also been used to follow the nigrostriatal degeneration observed in PD [8]. Another key pathological feature that has classically distinguished PD from other parkinsonism disorders is the presence of dystrophic neurites (Lewy neurites) and eosinophilic cytoplasmic proteinaceous inclusions, called Lewy bodies, in surviving neurons [9].

Primarily a sporadic disease, the etiology of PD is largely unknown. Several environmental factors, including exposure to toxins are associated with an

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Table 10.1. Loci and genes that have been associated with PD.

Locus	Chromosomal location	Gene	Mode of inheritance	Reference
PARK1/PARK4	4q21–q23	*α-synuclein*	AD	20, 26
PARK2	6q25.2–q27	*parkin*	usually AR	21
PARK3	2p13	*unknown*	AD	27
PARK5	4p14	*UCH-L1*	unclear	28
PARK6	1p35–p36	*PINK1*	AR	23
PARK7	1p36	*DJ-1*	AR	22
PARK8	12p11.2–q13.1	*LRRK2*	AD	24, 25
PARK10	1p32	*unknown*	unclear	29
PARK11	2q36–q37	*unknown*	unclear	30

increased risk of PD. Despite some early studies describing monozygotic twins with PD [10, 11], subsequent studies to determine the relative contribution of genetics to the onset of PD concluded that heredity played a negligible role [12–14]. The identification of neurotoxins that selectively damaged dopaminergic neurons and caused parkinsonism symptoms strengthened this theory [15, 16]. However, a later study with a large sample size concluded that while there was little genetic contribution to the development of PD in twins after 50 years of age, genetics contributed significantly to early-onset PD cases [17]. In addition, imaging studies revealed that concordance for nigral pathology may be higher in PD twins than previously described [18]. Over the last decade, genetic susceptibility has been definitively established with the identification of several distinct loci that are strongly associated with familial forms of PD [19–25] (Table 10.1).

The link between rare Mendelian PD and the more widespread sporadic PD is yet to be unequivocally established. However, since clinical and pathological findings overlap noticeably, the assumption that these two forms of the same disease share common causative and pathogenic pathways has encouraged rigorous research in this field [31]. This chapter explores the role of one Mendelian gene, *parkin*, in the pathophysiology of familial and sporadic PD.

10.2 AR JP and Parkin

10.2.1 ARJP: Introduction

Autosomal recessive juvenile parkinsonism (ARJP) is an early-onset, recessively inherited variant of PD with levodopa-responsive classic parkinson's symptoms in addition to some atypical features such as sleep benefit, dystonia (involuntary

muscle contractions) and abnormal gait [32–34]. Pathologically, ARJP is characterized by the lack of Lewy bodies with neuronal loss and gliosis restricted largely to the substantia nigra pars compacta and locus coerulus [35]. Linkage analysis in 13 Japanese families with ARJP resulted in the discovery of a locus on chromosome 6q25 that strongly associated with this familial form of PD [36]. Shortly after, chromosomal deletions in five Japanese patients with ARJP were analyzed to identify the causative gene, whose protein product was denoted "parkin".

Ongoing controversy with regard to whether ARJP is similar to sporadic PD or is its own clinical entity stems from conflicting clinical, imaging and pathological studies in parkin-positive patients. Parkin-linked PD is characterized by significant heterogeneity in clinical symptoms, drug response, ethnicities of patients, age at onset, and progression of disease, with little correlation to the type of mutations identified [37–39]. The wide variation in age at onset, ranging from 7 to 72 years, not only between unrelated patients but also within a single family with the same mutation is remarkable [37, 39–41]. Several positron emission tomography (PET) studies have found little difference in striatal uptake of 18-fluorodopa between patients with sporadic PD and those with mutations in *parkin* [42–44]. However, one PET study suggests that parkin-positive patients with severe clinical manifestations tend to show significant differences in 18-fluorodopa uptake compared to sporadic PD patients [45]. Even more confounding is the observation that two of seven known parkin-linked post-mortem cases have eosinophilic Lewy bodies [46, 47], which is uncharacteristic in ARJP. In addition, one parkin-positive case was reported to have basophilic inclusions that were positive for proteins normally present in Lewy bodies [48]. Due to the slow progression of parkin-linked PD [32], there are limited numbers of cases available for imaging and post-mortem analysis, making conclusive deductions about the pathophysiology of this disease a sizeable challenge.

10.2.2 *PARKIN*: The Gene

Parkin is one of the largest known genes, spanning over 1.4 Mb and comprising 12 exons encoding a short 4.5-kb mRNA transcript that is expressed in several human tissues, including the brain [21]. The gene is extensively conserved among vertebrates and invertebrates, including rodents, fruit flies, birds, frogs and pufferfish [49, 50]. Mutations in *parkin* account for about 50% of early-onset familial PD with recessive inheritance and about 10–20% of early-onset PD cases with no family history [41]. Over 100 different mutations in *parkin* have been described, including point mutations, intra-exonic deletions, single base pair deletions, mutiple exon deletions, exon multiplications, intronic splice site and promoter region variants [51–53] (Figure 10.1). There may be some evidence that recurrent point mutations in *parkin* arise from common founders, but those involving whole exons may be independent events [54]. The majority of the mutations in *parkin* cause premature termination due to a frameshift or nonsense mutations, resulting in non-functional translation products as demonstrated by the exon 4 deletion in

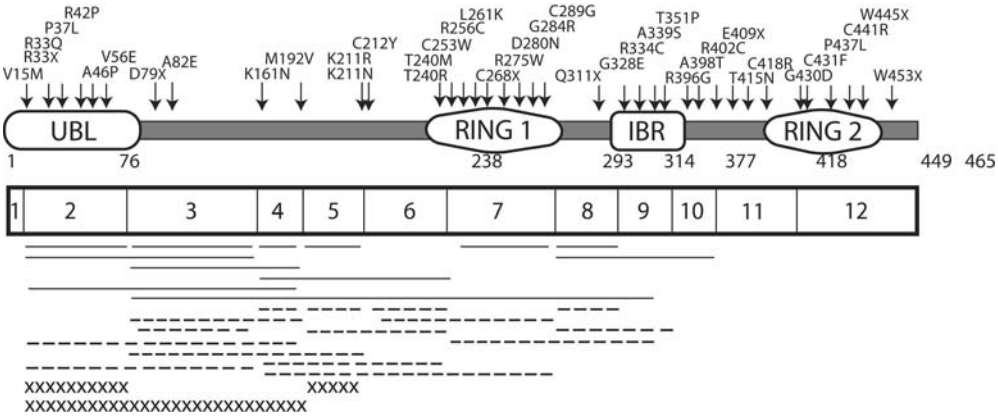

Fig. 10.1. Mutations in Parkin A schematic of pathogenic point mutations (arrows), exon deletions (solid lines), duplications (dashed lines) and triplications (crosses) identified in parkin.

an ARJP patient [55, 56]. On the other hand, missense mutations are hypothesized to destabilize the parkin protein or disable its normal function, resulting in loss-of-function [55]. Polymorphisms in *parkin* were thought to be associated with increased or decreased susceptibility in sporadic PD, but varied results across numerous studies have been inconclusive [57–61].

Mutations in *parkin* mostly show homozygous or compound heterozygous (i.e. different mutations on each allele) inheritance patterns, confirming the autosomal recessive nature of ARJP. However, several published cases were heterozygous for mutations in *parkin* and have forced a paradigm shift in understanding the disease transmission of parkin-linked PD. Some heterozygous mutations have also been associated with increased risk for late-onset PD [62, 63]. More conventional explanations for the presence of heterozygous mutations in *parkin* include haploinsufficiency as a risk factor [47, 53] or that a second mutation was missed either due to the large size and complexity of *parkin* or incomplete screening techniques [53]. Yet another potential explanation is that a single mutation in *parkin* could confer toxic gain-of-function or a dominant-negative function. This may be unlikely, although not improbable, since the described heterozygous mutations have also been found in homozygous or compound heterozygous states, and in some cases, patients with the mutation are asymptomatic [52]. Experimental data from imaging, molecular and biochemical analyses favor the haploinsufficiency model. PET studies in asymptomatic carriers of a single *parkin* mutation show reduced striatal 18-fluorodopa uptake compared to controls [42]. Further, there is evidence for reduced expression due to a single nucleotide polymorphism (–258T/G) in the promoter region of *parkin* in two separate studies involving different ethnicities [64, 65]. Finally, the identification of stress-induced modified parkin with reduced

function in sporadic PD cases further supports the haploinsufficiency model [66–68].

10.2.3 *PARKIN*: Localization and Regulation

In situ hybridization studies show widespread expression of parkin in the rat brain [69]. In dopaminergic neurons of human substantia nigra, parkin mRNA is robustly expressed in a similar pattern as α-synuclein, another PD-linked gene [70]. Although the amount of parkin mRNA is comparable in various regions of the human brain, there seems to be a relative abundance of parkin in the substantia nigra [56]. The parkin protein is also widely expressed in neurons and glia of rodents [71, 72]. Predominantly localized to the cytosol, parkin may be associated with cellular organelles and structures, including the Golgi complex, endoplasmic reticulum, neurites, cytoplasmic and synaptic vesicles [56, 71, 73]. Parkin has also been shown to localize to lipid rafts and postsynaptic densities in rat brain, suggesting a role in synaptic transmission and plasticity [74]. Another study describes parkin immunoreactivity around synaptic vesicles in presynaptic elements of some axons, further supporting a role for parkin in normal synaptic function [75]. Contradictory reports about the presence of parkin in Lewy bodies of sporadic PD patients using different antibodies to parkin, have made it difficult to ascertain a role for parkin in the formation of these inclusion bodies [56, 76–81]. However, the specificity of parkin antibodies has recently been questioned as the majority of parkin antibodies recognize a nonspecific protein of the same molecular weight in *parkin*-null mice. Thus definitive localization of parkin will require re-assessment with specific antibodies.

Regulation of parkin levels occurs primarily at the transcriptional level, although there is some evidence of control at the protein level by degradation. Characterization of the *parkin* promoter region led to the identification of a CpG island that is involved in bi-directional transcriptional regulation of parkin and a second gene, PACRG [82]. This shared promoter contains a conserved binding motif for myc-like proteins, through which N-myc has been shown to repress transcription at the *parkin* promoter, plausibly regulating parkin expression during development [83]. The *parkin* promoter variant (–258T/G) that affects gene transcription also affects the physiologic response of the promoter to various cell stressors [64]. *In vitro* luciferase assays to assess the transcriptional activity of the wild-type *parkin* promoter shows significant upregulation under oxidative and proteasomal stress, unlike the –258G variant [64]. Parkin expression is largely absent during embryogenesis, but becomes apparent towards the later stages of development and is predominant in adult cells [49, 83–86]. Levels of exogenous parkin protein may be tightly regulated via rapid degradation by the ubiquitin–proteasome system (UPS) [81]. In addition, there is *in vitro* evidence that the ubiquitin-protein isopeptidase ligase, Nrdp1, may promote parkin turnover [87]. Exogenously expressed truncations were used to demonstrate that its first six residues are involved in regulating cellular levels of parkin [88]. Tunicamycin-induced unfolded protein stress (UPS),

but not other types of stress such as H_2O_2, heat shock or ultraviolet light, cause elevated parkin mRNA and protein levels in SH-SY5Y neuroblastoma cells [89]. In addition, tunicamycin treatment in rat primary astrocyte, but not cortical neuronal, cultures results in a modest increase in parkin protein levels [90]. However, another study with SH-SY5Y cells did not show any change in parkin mRNA and protein levels after tunicamycin treatment [91], resulting in some confusion about the association between parkin and the UPS. Parkin mRNA expression increased several fold in response to rotenone, iron and paraquat treatments and parkin becomes insoluble in response to these stressors [92]. The propensity of parkin to become more insoluble and therefore functionally unavailable in aged human tissue, under conditions of stress or mutations supports the loss-of-function and haploinsufficiency hypothesis in parkin-linked PD [76, 92, 93].

10.3
Parkin in the Ubiquitin–Proteasome Pathway

The parkin protein contains an N-terminal ubiquitin-like domain (UBL) and a C-terminal RBR domain, comprising of two RING fingers separated by an in-between-RING (IBR) domain [21]. The RING motif is common to several ubiquitin E3 ligase enzymes that catalyze the conjugation of activated ubiquitin to target substrates [94]. Soon after its discovery, several studies identified parkin as an E2-dependent RING-type E3 ubiquitin ligase in the ubiquitin–proteasome pathway (UPP) [89, 95, 96]. Early studies proposed that there were mutational hotspots, particularly in the functionally significant exon 7 that translate into RING domains [51, 55]. However, compiling all known mutations to date suggests that while there may be some clustering of missense mutations in the functional domains of parkin, deletion mutations are not limited to any one region.

10.3.1
The Ubiquitin–Proteasome Pathway

The UPP is a temporally-regulated highly specific intracellular process, which rapidly catalyzes the turnover of proteins through ubiquitination and proteasome-mediated degradation [97]. Ubiquitin, a highly conserved 76-amino acid protein, acts as functionally distinct signals for proteasomal and lysosomal proteolysis as well as a non-proteolytic signal in protein trafficking and DNA repair [98]. Conjugation of ubiquitin to protein substrates involves three sequential steps: an ATP-dependent activation step catalyzed by the ubiquitin-activating enzyme (E1) that forms a thio-ester bond between ubiquitin and E1; an intermediate step, in which the activated ubiquitin is covalently linked to a ubiquitin-conjugating enzyme (E2) via a similar thio-ester linkage; and finally transferring the ubiquitin molecule to a lysine residue of the substrate in a reaction mediated by a ubiquitin ligase (E3). In addition, an ubiquitin elongation factor (E4) may be recruited to catalyze multiple cycles so four or more ubiquitin molecules can be linked together in a

polyubiquitin chain on the substrate. The ubiquitin molecule contains seven lysine (K) residues at amino acid positions 6, 11, 27, 29, 33, 48 and 63. Polyubiquitin chains are synthesized by covalently linking one ubiquitin monomer to the next via iso-peptide bonds between the C-terminus glycine residue of each ubiquitin unit and a specific lysine residue of the previous ubiquitin. Thus, substrates can be modified by mono-ubiquitination, multiple mono-ubiquitinations, or polyubiquitination and the different chains, depending on size and linkage type, provide distinct intracellular signals [99]. In humans, a single E1 enzyme activates ubiquitin for the entire cascade of downstream E2s; about 50 E2s serve multiple E3s to execute distinct biological functions; and hundreds to thousands of E3 enzymes confer specificity to the UPP process by recognizing a limited set of substrates. Regulating E3–substrate or E3–E2 interactions through motif recognition, conformational or covalent modifications provides ample opportunity to tightly control the rate and timing of proteolysis in the cell [100]. Elucidating the mechanism by which an E3 ligase selectively recognizes a particular substrate is a subject of intense research in the field.

Based on the type of conserved domains they contain, the known E3s can be categorized into one of three ubiquitin ligase families: Homologous to E6AP Carboxy Terminus (HECT), Really Interesting New Gene (RING), and UFD2-homology (U-box) proteins [101]. The HECT-type E3s typically contain a domain that is capable of binding activated ubiquitin via a thio-ester bond and serves as a direct intermediate in the transfer of ubiquitin from the E2 to the substrate [101]. The RING-type and U-box-type E3s serve as "bridging" molecules that act as scaffolds to facilitate the transfer of ubiquitin by bringing a ubiquitin-conjugated E2 into close proximity with the target substrate. The RING domain consists of a short stretch of amino acids that is rich in cysteine and histidine residues, and the RING-type E3s are further classified into three sub-families, based on the number and spacing of these conserved residues [101]. Also RING-type E3s may function as single subunit enzymes or co-exist with combinations of other proteins to form a multi-subunit enzyme with more opportunity to dictate substrate specificity [98].

10.3.2
PARKIN: An E3 Ubiquitin Ligase

Parkin contains the characteristic RING-IBR-RING (RBR) domain and has been shown to exist as both a single subunit ligase, and in a multi-subunit Skp1-Cullin-F-box (SCF) complex as well [102]. A number of E2s have been shown to associate with parkin, with UbcH7 and UbcH8 being the most common under physiological conditions, although sufficient debate persists on which E2 is preferred conjugating enzyme for parkin [95, 96]. Under conditions of unfolded protein stress, parkin is served by the endoplasmic reticulum membrane (ER)-associated E2s, Ubc6 and Ubc7 [103]. Under these conditions, parkin interacts with Hsp70 and the U-box E3 ligase, CHIP, which modulate the E3 ligase activity of parkin [104]. A similar complex of parkin with Hsp70 and expanded poly-glutamine proteins has also

been reported [105]. Further, parkin interacts in a complex with Hsp70 and BAG5, a protein that is upregulated during dopaminergic neuron injury [106]. Parkin localization at post-synaptic densities (PSD) prompted additional investigation, which suggests that parkin interacts with a large multimeric protein complex, implicated in NMDA trafficking, scaffolding, and signaling at the PSD [74]. It remains unclear whether parkin interacts with these complexes preferentially under varying physiological or stress conditions and how these different proteins may modulate its substrate specificity.

10.3.3 Parkin and Lewy Bodies

An increasing number of human diseases are being discovered that are caused by a dysfunctional ubiquitination system. The UPP, along with chaperones, are thought to maintain cell survival and homeostasis by preventing the accumulation of abnormal or toxic proteins that are misfolded or damaged. The well-characterized K48-linked ubiquitin chain on substrates is known to target them for clearance via the 26S proteasome [107, 108]. Therefore a mutation or post-translational modification inhibiting an E3 enzyme or its substrate results in an excess accumulation of the substrate, which may have deleterious consequences. Inclusion bodies are a pathological hallmark of neurodegenerative diseases, such as Lewy bodies in PD, which contain abnormally folded or aggregated disease-associated proteins as well as components of protein quality control machinery, including ubiquitin, proteasome subunits and chaperones [109]. While the mechanism for formation of Lewy bodies still remains unknown 93 years after they were first discovered, there is intense debate about whether these protein-sequestering bodies are neurotoxic or neuroprotective [110]. The discovery that parkin is an E3 ubiquitin ligase, combined with the lack of Lewy bodies in all but two cases of ARJP, implicates a strong role for anomalies in protein homeostasis and UPP in the pathogenesis of parkin-associated PD.

10.3.4 Parkin Substrates

Identification of parkin substrates that may be neurotoxic at elevated steady-state levels is critical to elucidating the underlying neurodegenerative mechanisms in parkin-linked PD. The first parkin substrate to be identified is parkin itself; when exogenously expressed, the E3 ligase can auto-ubiquitinate and promotes its own degradation [96]. In the same study, a yeast two-hybrid screen with full-length parkin yielded another potential substrate: a synaptic vesicle-enriched GTPase called Cell Division Control-Related protein 1 (CDCrel-1) [96]. Parkin binds, ubiquitinates and promotes the proteasome-dependent degradation of CDCrel-1, while pathogenic parkin mutants were unable to turnover the substrate [96]. A later study identified CDCrel-2a as another putative substrate for parkin and showed increased steady-state levels of CDCrel-1 and CDCrel-2a in brains of ARJP patients [111].

Another synaptic vesicle-associated protein, synaptotagmin XI, whose function is unknown, was also described as a parkin substrate [112]. The implication that the parkin substrates, synaptotagmin XI and septin family proteins may be involved in synaptic vesicle transport, docking, and fusion or recycling in the brain, generated immense interest in the role of parkin at the synapse and in pre-synaptic neurotransmission. In a separate study, another yeast two-hybrid screen using full-length parkin as bait revealed a putative G protein-coupled integral membrane polypeptide, named *P*arkin-*a*ssociated *e*ndothelin-*l*ike *R*eceptor (Pael-R), which is degraded by parkin-mediated ubiquitination [103]. Further, this study provides the earliest evidence that parkin has a cytoprotective function under adverse conditions, specifically unfolded protein stress, since abnormally folded Pael-R causes ER stress [103]. Pael-R is accumulated in the detergent-insoluble fraction of ARJP patient brains, suggesting that parkin is crucial for the turnover of this ER-associated substrate [103]. A follow-up study by the same group showed that during unfolded protein response, CHIP promotes the ubiquitination and degradation of Pael-R by parkin [104]. Next, one group demonstrated that parkin interacts with UbcH7 and ubiquitinates a rare O-glycosylated-α-synuclein variant in human brain [113], but the more prevalent non-glycosylated α-synuclein is not a parkin substrate [114]. However, these data have not been replicated in other systems, seriously questioning the physiological relevance of this finding. The lack of altered α-synuclein steady-state levels, where parkin is overexpressed or deficient further supports the idea that parkin has no effect on α-synuclein metabolism [115–119].

While assessing a role for parkin in the ubiquitination of proteins in the Lewy body, since there is high ubiquitin immunoreactivity in these inclusions, the α-synuclein-interacting protein, synphilin-1, was identified as a parkin substrate [114]. When parkin is co-expressed with synphilin-1 and α-synuclein, ubiquitin-positive cytoplasmic inclusions are formed, but familial-linked parkin mutants disrupt ubiquitination of synphilin-1 and the formation of inclusions [114]. This finding is of immense interest since synphilin-1 is found in Lewy bodies [120]. Subsequent studies revealed that synphilin-1 is ubiquitinated by parkin in a non-classical proteasome-independent manner that involves the formation of K63-linked polyubiquitin chains, without appreciable degradation of synphilin-1 by parkin [121]. The ability of parkin to function as a dual ubiquitin ligase, catalyzing the formation of both K48- and K63-type polyubiquitin chains was further confirmed by a study that showed that parkin, in the presence of α-synuclein, promotes the formation of K63-linked chains [122]. Parkin interacts with the E2 complex, comprising UbcH13 and Uev1a, to mediate K63-linkages, supporting the hypothesis that the fate of substrate targets rests on the specific E3–E2 interactions [122]. The relevance of parkin-mediated K63 ubiquitination remains to be clarified. Since K63-linked chains can interact with the proteasome [123], it is plausible that excessive K63 polyubiquitination may interfere with substrate proteolysis and result in accumulated proteins. On the other hand, K63 polyubiquitination may represent an alternate pathway in cells that are stressed with proteasomal overload, diverting the substrates into aggregates. Consistent with the latter hypothesis is

the observation that parkin-mediated K63-linked ubiquitination of synphilin-1 enhances the formation of cytoplasmic inclusions, when parkin is co-expressed with synphilin-1 and α-synuclein [121]. These data strongly suggest that the proteasome-independent K63-linked ubiquitination may play a role in inclusion formation in PD as well as other neurodegenerative disease and warrants further study [124].

The race to discover neurotoxic substrates of parkin led to the identification of cell cycle-regulating cyclin E, cytoskeleton-associated α/β tubulin, aminoacyl-tRNA synthetase complex subunit AIMP2 (p38/JTV-1), neuron-specific dopamine transporter, E3 SUMO ligase RanBP2 and the expanded poly-glutamine ataxin-3 fragment [102, 105, 125–128]. An SCF-like ubiquitin ligase complex, comprising parkin, hSel10 and Cul1, is involved in the ubiquitination of parkin and cyclin E [102]. In addition, this report provided evidence for parkin regulation of cyclin E levels in ARJP and PD brains as well as in kainate-induced neuronal apoptosis by overexpression and knockdown of parkin [102]. Strong binding and co-localization between parkin and microtubules was demonstrated in rat cortical neurons and cell lines, with no localization of parkin to the post-synaptic densities, in contrast to prior reports [74, 125]. Furthermore parkin ubiquitinates and accelerates the degradation of α/β tubulin *in vitro* and *in vivo* [125]. Since PD-linked neurotoxins, such as MPP^+ and rotenone, can induce depolymerization of microtubules, and misfolded tubulin may be toxic in the cell, the ability of parkin to degrade these key structural components may be crucial to neuronal survival [125]. A separate study found that parkin strongly associates with and stabilizes microtubules, a process which was not affected by PD-linked mutations, suggesting that the E3 ligase and microtubule-binding activities of parkin are independent [129]. Parkin may recognize misfolded proteins through its association with Hsp70 and mediate ubiquitination and proteasome-dependent degradation of these proteins, as is seen with expanded poly-glutamine ataxin-3 [105]. Another study reports the ability of parkin to preferentially ubiquitinate and degrade misfolded dopamine transporter (DAT), thus preventing misfolded DAT from oligomerizing with properly folded DAT and ensuring sufficient cell surface expression of native DAT [127]. In yet another yeast two-hybrid screen with full-length parkin, the most recent substrate discovered is nuclear membrane-associated RanBP2, which sumolyates the histone deacetylase, HDAC4 [128]. The significance of this novel substrate and the consequential modulation of HDAC4 levels by parkin to PD pathogenesis are far from established.

The p38 subunit of aminoacyl-tRNA synthetase complex, AIMP2, is a parkin substrate, as demonstrated by two separate groups [118, 126]. This co-factor is a key scaffolding component for assembly of the multi-tRNA synthetase complex. As part of a multi-protein complex with Hsp70 and CHIP, parkin interacts with and ubiquitinates AIMP2, promoting its proteasomal degradation [118]. Excessive AIMP2 is toxic, but the AIMP2-induced toxicity is mitigated by exogenously expressed parkin [118, 126]. Overexpression of AIMP2 results in the formation of cytoplasmic AIMP2- and ubiquitin-positive inclusions that is enhanced in the presence of parkin, suggesting that the non-ubiquitinated non-aggregated form of

AIMP2 is deleterious [126]. Interestingly, AIMP2-positive immunoreactivity was observed in Lewy bodies[118, 126]; only three other parkin substrates – synphilin-1, Pael-R, and synaptotagmin XI – have been shown to co-localize in Lewy bodies [78, 112, 120, 130]. In *parkin*-null mice, since parkin dysfunction impairs its ability to effectively mediate the degradation of substrates, authentic substrates of parkin should accumulate [131]. A comprehensive study to assess the steady-state levels of numerous published substrates of parkin revealed that AIMP2 is upregulated in the midbrain and hindbrain of *parkin*-null mice, while none of the other substrates, including CDCrel-1, synphilin-1, Pael-R, cyclin E, and synaptotagmin XI, were upregulated [118]. Parkin mediates proteasome-independent K63-ubiquitination of synphilin-1 (see above), providing a likely explanation for the unchanged steady-state levels of synphilin-1 in *parkin*-null mice. Accumulation of AIMP2 is observed in ARJP patient brains as well as in sporadic PD brains with functional inactivation of parkin due to oxidative, nitrosative and dopaminergic stress [118]. Although other putative substrates have been reported to be upregulated in AR-JP brains [102, 103, 111, 118], AIMP2 is the only substrate that is consistently upregulated in *parkin*-null mice, AR-JP brains and sporadic PD brains; thus, it appears to be the only pathogenic parkin substrate identified to date.

10.4 Parkin in Neuroprotection

10.4.1 Toxic Parkin Substrates

The fundamental question that is yet to be credibly addressed in neurodegenerative diseases is the mechanism by which a selective subset of neurons becomes susceptible to the pathological effects of a mutant gene product. In parkin-linked PD, it is hypothesized that absence of parkin or mutations that abolish its enzymatic activity result in an accumulation of parkin substrates that lead to the specific degeneration of dopaminergic neurons, the most affected neuronal subtype in PD. The most apparent solution to this conundrum would be the identification of a substrate that is expressed only in these neurons. However, only one of the identified putative substrates is exclusively expressed in dopaminergic neurons. The dopamine transporter (DAT) is only found on the cell surface of dopaminergic neurons and is responsible for rapid re-uptake of released dopamine into the neuron [127]. There is *in vitro* evidence that parkin ubiquitinates primarily misfolded DAT in the presence of tunicamycin, which induces unfolded protein stress [127]. The authors propose that dysfunctional parkin causes increased misfolded DAT that interferes with the cell surface expression of normal DAT and dopamine re-uptake, thus sensitizing neurons to extracellular dopamine stress [127]. However, it is imperative to have *in vivo* data to support this hypothesis before any conclusive inference can be made. Alternatively, it is possible that the toxicity threshold tolerated by dopaminergic neurons under certain stress conditions is

significantly lower than that of other cell types. Only a few of the identified substrates have been implicated in dopaminergic neuron death. Overexpression of Pael-R in *Drosophila* causes dopaminergic neuron-specific toxicity, which is exacerbated when the expression of endogenous parkin is inhibited by RNAi interference and alleviated by overexpression of parkin in flies [132]. Interestingly, reduced expression of endogenous *Drosophila* parkin alone is not sufficient to cause pathology [132]; it seems that stress, such as that induced by Pael-R overexpression, coupled with parkin insufficiency is necessary for dopaminergic cell death *in vitro*. Another putative parkin substrate, cyclin E, is elevated in primary midbrain dopaminergic cultures when parkin levels are "knocked down" using parkin-specific siRNA and the cultures are treated with the excitotoxin, kainate [102]. The study found that parkin deficiency preferentially sensitizes midbrain dopaminergic neurons, versus midbrain GABAergic cultures, to kainate excitotoxicity, but not MPP^+ toxicity [102]. However, since cyclin E upregulation in toxicity models is not limited to the brain [133], the molecular mechanism underlying dopamine neuron-specific susceptibility needs to be elucidated.

A third parkin substrate, AIMP2, sensitizes human neuroblastoma cells to TNF-α toxicity, which is alleviated by parkin overexpression [118]. Adenovirus-mediated overexpression of AIMP2 in the substantia nigra of mice induces significant dopaminergic neuron loss *in vivo* [118]. Adeno-associated viral delivery of the parkin substrate, CDCrel-1, in the substantia nigra of rats causes significant degeneration of dopamine neurons, but has no effect on neurons in another brain region, the globus pallidus [134]. This site-specific neurodegeneration is prevented by inhibiting dopamine synthesis, suggesting a role for CDCrel-1 in dopamine biochemistry [134]. While both the afore-mentioned studies on viral-mediated overexpression of parkin substrates in rodents show compelling data on selective degeneration, behavioral analyses as well as the therapeutic potential of increasing parkin expression in these animals have yet to be reported upon. Thus, dopaminergic neurons in the substantia nigra may have an increased susceptibility to substrate accumulation. However, PD pathology in the brain extends beyond the substantia nigra and none of the substrate toxicity studies have addressed this issue.

10.4.2 Stress-mediated Toxicity

The protective function of parkin extends beyond preventing the accumulation of its putative substrates. Modulation of parkin levels during unfolded protein stress [89, 135], and the evidence from biochemical studies that parkin interacts with molecular chaperones to preferentially ubiquitinate and degrade misfolded proteins [103, 105, 127], suggest a strong role for parkin in protection against unfolded protein stress. Proteasomal defects apparent in the substantia nigra of sporadic PD patients [136], combined with the presence of UPP components in Lewy bodies [109], have prompted a number of cell culture studies on the effect of parkin in proteolytic stress. In mouse midbrain cultures, proteasome inhibition with

MG-132 and lactacystin resulted in decreased numbers of TH-positive neurons, which was restored by overexpression of parkin [117]. Further, *in vitro* studies show that proteasome inhibition causes endogenous parkin to be recruited into perinuclear microtubule-dependent aggresome-like structures, primarily localized to the centrosome [78, 137, 138]. Overexpression of parkin reduces the MG-132-induced aggresome-like bodies, while protecting the cells from MG-132-induced toxicity [137]. Aggresomes are large non-toxic inclusions formed at the centrosome that sequester misfolded and/or deleterious proteins and are proposed to be involved in the biogenesis of Lewy bodies [139]. Treating cell cultures with a variety of PD-associated toxins, such as manganese and rotenone, induces the formation of similar parkin-positive perinuclear inclusions, which segregates parkin from its normal cellular localization [92, 140]. Parkin overexpression protects dopaminergic cell lines from manganese-induced toxicity, independent of the proteasome system [140]. On the other hand, rotenone treatment causes dose-dependent impairment in proteasome activity, which is relieved by parkin overexpression [92]. Inhibiting the proteasome also abrogates parkin protection against ceramide-mediated cell death *in vitro* [141], supporting the premise that the protective function of parkin against certain stressors may be modulated through maintaining proteasome function. Another study showed that parkin protection against caspase-dependent cell death induced by dopamine treatment is mediated by its ubiquitination/degradation function, although the molecular mechanism is not known [142]. Reduction of endogenous parkin in a glial-like cell line resulted in increased susceptibility to dopamine-induced caspase-dependent as well as H_2O_2-induced caspase-independent cell death, however parkin overexpression did not protect against stress-induced toxicity. The astonishingly large number and variety of parkin substrates, along with the range of cellular stressors that parkin protects against, suggest a vital versatile neuroprotective role for parkin in the survival of dopaminergic neurons.

10.5
Parkin and Other PD-linked Genes

10.5.1
α-Synuclein

In attempting to connect the first two identified PD-linked genes in a common pathogenic pathway, a number of *in vitro* and *in vivo* studies on the ability of parkin to suppress α-synuclein toxicity have been undertaken. Abnormal accumulation of α-synuclein in Lewy bodies is considered to be a pathological hallmark of PD. Unlike parkin, α-synuclein is associated with an autosomal dominant form of PD with Lewy body pathology [19]. Overexpression of wild-type and mutant α-synuclein induces toxicity in cell lines as well as primary midbrain cultures, which is rescued by parkin overexpression in these cultures [117, 143, 144]. While parkin

protection in one study is associated with the appearance of high molecular weight non-ubiquitinated α-synuclein species [143], another study argues that parkin mediates protection in a non-proteasomal manner by enhancing the protease activity of calpain to cleave α-synuclein [144]. Parkin overexpression in *Drosophila* mitigates α-synuclein-induced pathology and toxicity [132]. Lentiviral-mediated co-expression of parkin with a α-synuclein pathogenic mutant in rats reduces the number of α-synuclein-induced dopaminergic neuron losses in the substantia nigra, while increasing the amount of phosphorylated α-synuclein inclusions, which is typically found in Lewy bodies [145]. Interestingly, overexpression of glial cell line-derived neurotrophic factor (GDNF), which represents a promising neuroprotective target for PD, was unable to mitigate α-synuclein toxicity in these rats [145]. This suggests a unique role for parkin in neuroprotection against α-synuclein, potentially linking the two familial-linked proteins in a common pathway. However, we have recently shown that there is no observable synergistic effect of parkin deficiency and overexpression of mutant α-synuclein in mice, suggesting that these two genes may have independent mechanisms of pathogenesis [119].

10.5.2 DJ-1

Mutations in *DJ-1* are associated with autosomal recessive early-onset PD, similar to parkin, although it appears to be a rare cause of familial PD [146]. The versatile DJ-1 protein may possess chaperone and oxidative stress-sensing activities, which is confirmed by *in vitro* studies [146]. Parkin interacts selectively but differentially with pathogenic DJ-1 mutants, as well as with wild-type DJ-1 following oxidative stress in cell culture overexpression studies [146]. However, parkin does not ubiquitinate and augment the degradation of mutant DJ-1 as well as wild-type DJ-1 after oxidative stress [146]. In dissecting the biological relevance of the interaction between parkin and DJ-1, we found that in enhancing DJ-1 stability, parkin is part of a larger complex, comprising of CHIP and Hsp70, which can independently associate with the highly unstable pathogenic DJ-1 mutant. This ubiquitination-independent stabilizing effect of parkin is further supported by the significantly increased levels of DJ-1 in the detergent-insoluble fraction of post-mortem PD cortex brains and the marked reduction in DJ-1 levels in the detergent-insoluble fraction of the parkin-deficient post-mortem ARJP cortex brains [146]. Thus the oxidative stress- and mutation-induced association between parkin and DJ-1 may represent a common molecular pathway in the pathogenesis of PD that warrants further study.

10.5.3 LRRK2

Leucine-rich repeat kinase 2 (*LRRK2*) is the most recent gene to be associated with autosomal-dominant PD. A gigantic protein of 2527 amino acids, this mixed-lineage kinase has several predicted functional domains, with point mutations

found in almost all the identified domains. Parkin interacts with LRRK2 via its C-terminal RING2 domain in cell culture overexpression studies, an association that is not altered by pathogenic mutants of LRRK2 [147]. This interaction is specific to parkin as LRRK2 does not bind to other known PD-linked genes, including DJ-1, α-synuclein, and tau [147]. Further parkin co-expression increases the number of ubiquitin-positive cytoplasmic aggregates of LRRK2, although there is no evidence that parkin directly ubiquitinates LRRK2 [147]. As more detailed mechanisms of the degenerative pathways involving PD-linked genes are revealed, the relevance of the interactions between these gene products will become more apparent.

10.6 Mechanisms of Parkin Dysfunction

10.6.1 Pathogenic Mutations

To understand how the variety of mutations in *parkin* contribute to its dysfunction to result in a shared pathogenic outcome, cell culture-based overexpression studies in various cell lines have been conducted. Since parkin is associated with recessive forms of PD, it was hypothesized that mutations in *parkin* result in the loss of E3 ligase activity that would translate into accumulated cytotoxic substrates. However, early studies showed that some parkin mutants retain or may have partially disrupted ubiquitination activity [89, 114, 126, 148]. An extensive investigation of several parkin mutants shows that each mutant may be defective in one or more aspects of the ubiquitination/degradation process or is abnormally localized, which manifests as an apparent loss-of-function [131, 149]. Some mutants have completely abolished enzymatic activity, but other mutants are relatively unaffected or have increased activity, as demonstrated by parkin auto-ubiquitination as well as ubiquitination of two established parkin substrates: synphilin-1 and AIMP2 [131]. Regardless of the alternate mechanisms of disruption in the process, all studied parkin mutants, unlike wild-type parkin, were unable to effectively reduce the steady-state levels of substrates [131]. The function of the N-terminal UBL domain in parkin has not as yet been established; although one NMR study suggested that parkin may bind the proteasome via its UBL domain, an interaction that may be abolished by a known pathogenic mutation in the domain [150]. Interestingly, another study reported that the C-terminal IBR-RING domain is essential in mediating the interaction with the 20S proteasomal subunit α4 [151]. Pathogenic mutations in the UBL domain of parkin result in highly unstable protein products that are rapidly degraded, compared to wild type, resulting in less functional parkin in the cell [152].

A consistent finding across several groups is the altered solubility and localization with overexpressed pathogenic parkin mutants [93, 131, 148, 152, 153]. While some parkin mutants, especially those that are localized to the RING finger, reliably aggregate into cytoplasmic inclusions, slight discrepancies exist amongst

other mutants that may be attributed to the various cell lines used. It is thought that sequestering enzymatically-active mutants into these aggresome-like bodies would preclude them from ubiquitinating substrates and therefore manifest as loss-of-function mutants. Exogenous PD-associated stress, including oxidative and dopamine stress, result in similar misfolding and aggregation of wild-type parkin protein, suggesting that this mechanism may account for some parkin dysfunction in sporadic PD, where parkin is not mutated. Although they provide substantial insight into the mechanisms of abnormal parkin function and localization, these studies are limited in their interpretation until they are extended into *in vivo* models. We recently showed a significant increase in detergent-insoluble parkin in mouse brain after treatment with the PD toxin, MPTP [92]. These observations, coupled with the presence of increased age-dependent insoluble parkin in human brains, which is marginally enhanced in PD patients [76, 92], strengthens this proposed mechanism of abrogated neuroprotection by parkin through progressive depletion of functionally available parkin.

10.6.2
Cellular Regulators of Parkin

Recent studies emphasize numerous environmentally-inflicted stress conditions that inactivate the enzymatic activity of parkin, plausibly predisposing heterozygous carriers and non-carriers of parkin mutations to PD [66–68, 106, 154]. In cell culture, treatment with inducers of apoptosis, such as the kinase inhibitor, staurosporine, resulted in proteolytic caspase-dependent truncations of parkin that are predicted to be non-functional [154]. This is in line with reported toxicity experiments using staurosporine, where parkin was unable to exert any protection [141]. The *bcl-2-associated athanogene 5* gene product, BAG5, is a molecular mediator of dopaminergic neurodegeneration localized to Lewy bodies, whose expression is induced after dopaminergic neuron injury [106]. BAG5 interacts directly with Hsp70 and parkin, negatively regulating their respective cellular activities as well as the protective function of parkin [106]. The study further suggests that BAG5, through its inhibition of Hsp70, can promote aggregation of overexpressed parkin and presents evidence for BAG5-mediated dopaminergic neurodegeneration in an *in vivo* model of PD [106]. Another reported molecular regulator of parkin function is 14-3-3$^{\eta}$, a member of the 14-3-3 family of proteins that is found in Lewy bodies [155]. 14-3-3$^{\eta}$ binds and suppresses the ubiquitin ligase activity of parkin, but α-synuclein overexpression abrogates the inhibition of parkin function by 14-3-3$^{\eta}$, yet again functionally linking these two PD-linked gene products [155].

10.6.3
Post-translational Regulation of Parkin

Environmental stressors are known to cause intracellular changes that can induce post-translational modifications of key proteins. Neurodegenerative pathologic

conditions, such as PD, are associated with high levels nitrosative stress [156], therefore it was logical to assess whether parkin is modified under these conditions. *S*-nitrosylation of parkin on select cysteine residues in the RING domain is evident *in vitro* and *in vivo* [66, 68]. This unique post-translational modification of parkin results in a bi-phasic alteration, with an initial spike followed by a gradual decrease, in its enzymatic activity [66, 68]. The pathological relevance of this finding was demonstrated by the detection of *S*-nitrosylated parkin in MPTP- and rotenone-treated mice brain as well as in post-mortem brain tissue of sporadic PD patients [66, 68]. *S*-nitrosylation of parkin ultimately leads to loss of E3 ligase activity and loss of its protective function [66]. Interestingly, AIMP2 accumulates in PD patients with increased nitrosative stress, providing further support for AIMP2 being an authentic parkin substrate [118]. A recent study reports the discovery of dopamine-mediated covalently modified parkin that has reduced solubility and ubiquitin ligase activity *in vitro* and in post-mortem sporadic PD brains [67]. Although this may represent only a small fraction of dysfunctional parkin, the close ties to dopamine oxidation that is restricted to dopaminergic neurons renders it a possible relevant pathological modification. Finally, a small reduction in parkin enzymatic activity as a consequence of serine-phosphorylation *in vitro* has been reported, though the physiological relevance of this modification is not known [157].

10.7
Animal Models of Parkin Deficiency

10.7.1
Drosophila Models

Since loss of parkin function is strongly associated with PD in humans, studying the effects of parkin deficiency *in vivo* would be expected to provide clues to understanding the pathogenesis of parkin-linked PD. Parkin is evolutionarily conserved across several species, allowing the generation of several animal models. A *Drosophila* model of ARJP with targeted disruption of the *Drosophila parkin* (*dParkin*) ortholog resulted in viable, but short-lived flies, with male sterility, and locomotor defects due to mitochondrial pathology and/or muscle degeneration [86]. However, there was no apparent neurodegeneration, other than a marginal shrinkage of dopaminergic cell bodies [86]. A follow-up study on the same *Drosophila* line, using different quantification techniques, suggests that a subset of dopaminergic neurons degenerates in *dParkin* mutants, which is rescued by overexpression of glutathione-*S*-transferase, a protein implicated in cellular response to oxidative stress [158]. An independent study confirmed the observed phenotype in loss-of-function mutations in *dParkin*, and further suggests that these mutant flies have increased sensitivity to stress [159].

10.7.2 Mouse Models

A number of mouse models have been generated, where different exons of *parkin* are deleted by homologous recombination. A *parkin* knockout mouse model, with deletion of exon 3, shows no pathology or neurodegeneration, but displays behavioral, biochemical and electrophysiological dopamine-related alternations [160]. A notable increase in monoamine oxidase activity, which catalyzes dopamine oxidation, in these *parkin*-null mice may be explained by a recent report on the ubiquitination-independent ability of parkin to reduce monoamine oxidase mRNA levels [161]. In another independent *parkin* knockout mouse line, with an exon 3 deletion, some mild nigrostriatal deficits, but no profound loss of dopaminergic neurons, were observed [115]. Interestingly, the authors describe increased extracellular dopamine in the striatum of these mice, and ascribe it to increased dopamine release from nigral neurons [115]. The subsequent discovery that parkin regulates levels of misfolded DAT and cell surface expression of native DAT to ensure functional dopamine re-uptake may help clarify the presence of the excess extracellular dopamine observed [127]. Further proteomic analyses of these mice showed that *parkin*-null mice exhibited changes in abundance of several proteins, a large majority of which are associated with normal mitochondrial and antioxidant function [162]. A third mouse model, with an exon 7 deletion to ensure removal of the first RING finger domain, shows loss of catecholaminergic neurons in the locus coeruleus, a brain region affected in PD, along with loss of norepinephrine in certain brain regions [163]. Accompanying the loss of locus coeruleus neurons and norepinephrine is the marked reduction of acoustic startle, a non-epinephrine-dependent process in rodents. Consistent reports of minor deficits that are classically associated with AR-JP [164] supports the hypothesis that loss of parkin alone is insufficient to cause disease in rodents; perhaps the interaction of parkin with other PD-linked genes or environmental factors, such as stress, play a more significant role than was previously envisaged. The generation of animal models that closely resemble parkin-related PD is vital to comprehending this elusive protein's role in neurodegeneration.

10.8 Concluding Remarks

In the 8 years since the discovery of the *parkin* gene, we have come a long way in understanding parkin genetics and biochemistry (Figure 10.2); however, these interesting insights have only resulted in more questions that need to be addressed. Is the E3 ligase activity of parkin its only function in the cell? Does it truly mediate neuroprotection *in vivo*? Can we develop animal models to efficiently test parkin-related hypotheses? And most enigmatic of them all: what is the pathogenic mechanism by which parkin deficiency causes selective degeneration in the brain? The drive to find a solution and utilize emergent biotechnology to design effective

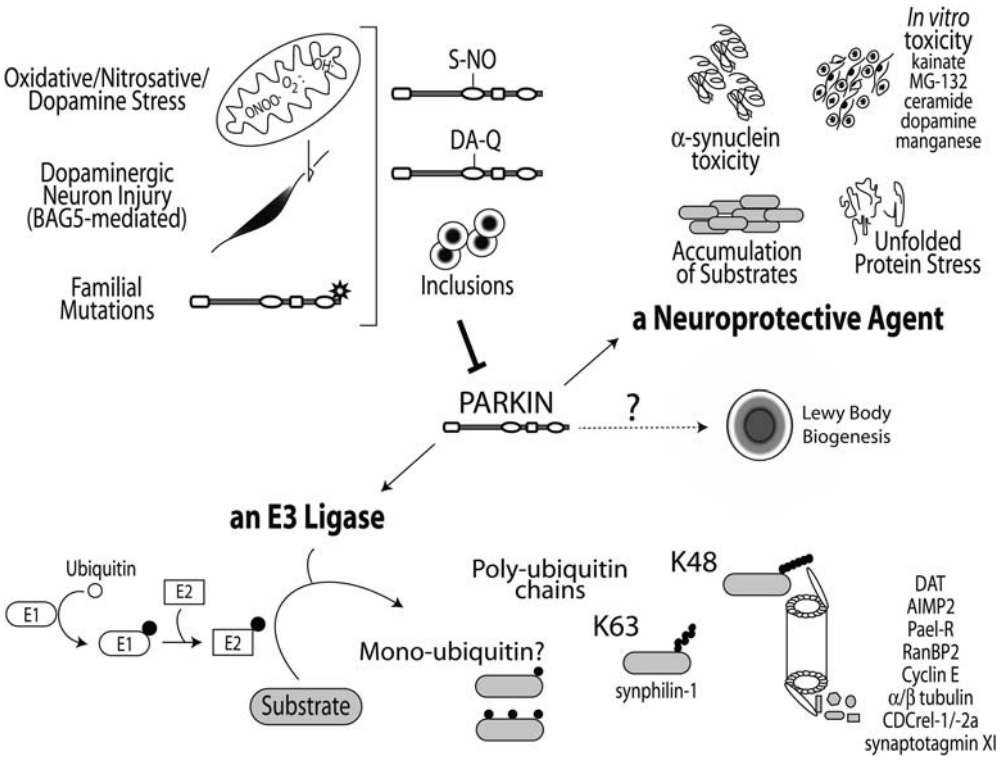

Fig.10.2. Parkin plays a central role as an E3 ligase and a versatile neuroprotective agent. As an E3 ligase, parkin can catalyze K48-linked, K63-linked and mono-ubiquitination of several putative substrates. In addition, parkin protects from toxicity induced by accumulation of some substrates, unfolded protein stress, and overexpression of α-synuclein. A number of *in vitro* studies show that parkin overexpression also protects against other cellular stressors. Mechanisms of parkin dysfunction include familial mutations that disrupt its function by inhibiting its E3 ligase activity or inducing aggregate formation; stress-mediated modifications such as *S*-nitrosylation and dopamine–quinone adduct formation; and negative regulators of parkin activity, such as BAG5, which is induced upon dopaminergic neuron injury.

therapeutics for AR JP, and subsequently for PD, motivates research in this field.

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