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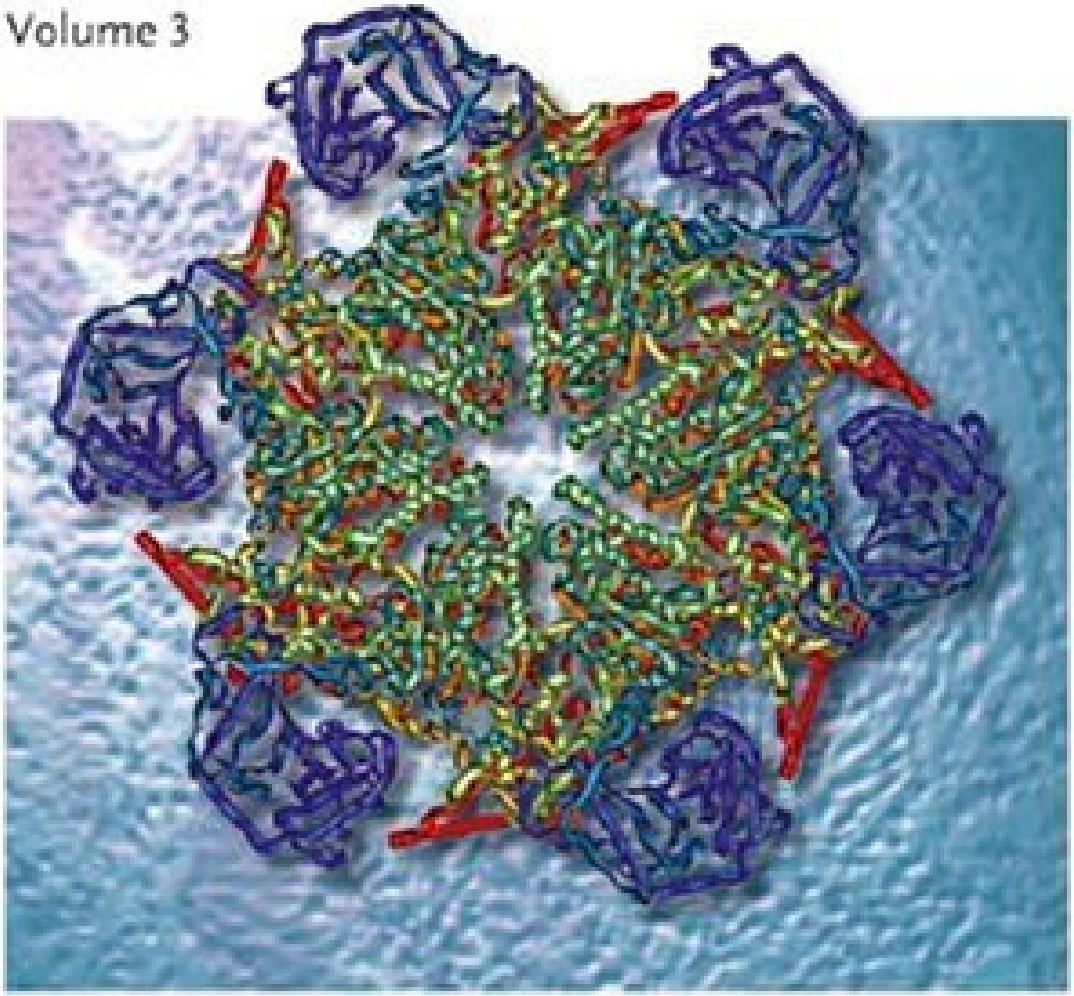
Cell Biology of the Ubiquitin-Proteasome System

Volume 3



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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

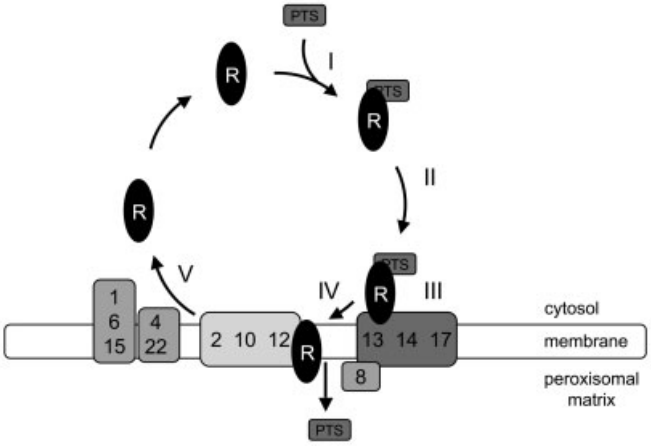

Fig. 1.1. Model for peroxisomal matrix protein import and receptor cycling. The following steps in the receptor cycle have been proposed: (I) binding of the receptor to matrix proteins in the cytosol. (II) Transport of the receptor–cargo complex to the peroxisomal membrane. (III) Docking of the receptor–cargo 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

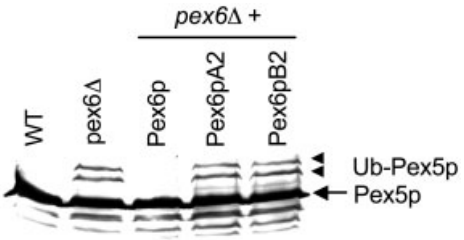

Fig. 1.2. Pex6p ATP-binding and -hydrolysis mutant cells accumulate (poly)ubiquitinated forms of Pex5p. TCA lysates of oleate-induced *pex6*Δ cells expressing wild-type Pex6p, or Pex6p point mutants were analyzed by anti-Pex5p immunoblotting. Lysates of untransformed wild-type and *pex6*Δ cells were 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

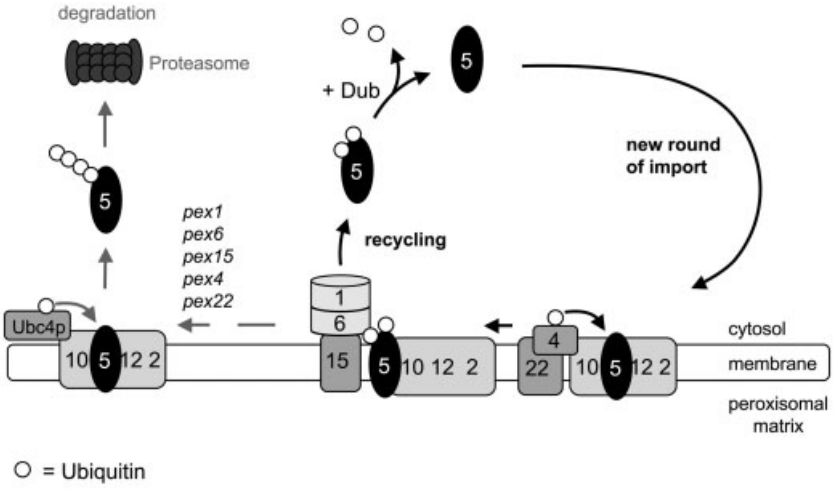

Fig. 1.3. Hypothetical model for Pex5p ubiquitination and ubiquitin-dependent recycling of Pex5p. After release of its cargo, Pex5p is present at the membrane tightly associated with the RING complex, consisting of Pex2p, Pex10p and Pex12p. Subsequently, Pex5p is monoubiquitinated at two different lysine 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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References

1 De Duve, C., and Baudhuin, P. Peroxisomes (microbodies and related particles). *Physiol. Rev.* **1966**, *46*, 323–57.

2 Van den Bosch, H., Schutgens, R. B., Wanders, R. J., and Tager, J. M. Biochemistry of peroxisomes. *Annu. Rev. Biochem.* **1992**, *61*, 157–97.

3 Mannaerts, G. P., and Van Veldhoven, P. P. Metabolic pathways in mammalian peroxisomes. *Biochimie* **1993**, *75*, 147–58.

4 Veenhuis, M., van der Klei, I. J., Titorenko, V., and Harder, W. *Hansenula polymorpha*: an attractive model organism for molecular studies of peroxisome biogenesis and function. *FEMS Microbiol. Lett.* **1992**, *79*, 393–403.

5 Muller, W. H., van der Krift, T. P., Krouwer, A. J., Wosten, H. A., van der Voort, L. H., Smaal, E. B. and Verkleij, A. J. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. *EMBO J.* **1991**, *10*, 489–95.

6 Michels, P. A., Hannaert, V., and Bringaud, F. Metabolic aspects of glycosomes in trypanosomatidae – new data and views. *Parasitol. Today* **2000**, *16*, 482–89.

7 Kindl, H. Fatty acid degradation in plant peroxisomes: function and biosynthesis of the enzymes involved. *Biochimie* **1993**, *75*, 225–30.

8 Weller, S., Gould, S. J., and Valle, D. Peroxisome biogenesis disorders. *Annu. Rev. Genomics Hum. Genet.* **2003**, *4*, 165–211.

9 Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J. A., Kunau, W. H., Lazarow, P. B., Mannaerts, G. P., Moser, H. W., Osumi, T., Rachubinski, R. A., Roscher, A., Subramani, S., Tabak, H. F., Tsukamoto, T., Valle, D., van der Klei, I., van Veldhoven, P. P., and Veenhuis, M. A unified nomenclature for peroxisome biogenesis factors. *J. Cell Biol.* **1996**, *135*, 1–3.

10 Vizeacoumar, F. J., Torres-Guzman, J. C., Bouard, D., Aitchison, J. D., and Rachubinski, R. A. Pex30p, Pex31p, and Pex32p form a family of peroxisomal integral membrane proteins regulating peroxisome size and number in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **2004**, *15*, 665–77.

11 Wiebel, F. F., and Kunau, W. H. The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature* **1992**, *359*, 73–76.

12 Pickart, C. M. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **2001**, *70*, 503–33.

13 Crane, D. I., Kalish, J. E., and Gould, S. J. The *Pichia pastoris PAS4* gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. *J. Biol. Chem.* **1994**, *269*, 21835–44.

14 Purdue, P. E., and Lazarow, P. B. Pex18p is constitutively degraded during peroxisome biogenesis. *J. Biol. Chem.* **2001**, *276*, 47684–89.

15 Kiel, J. A., Emmrich, K., Meyer, H. E., and Kunau, W. H. Ubiquitination of the peroxisomal targeting signal type 1 receptor, Pex5p, suggests the presence of a quality control mechanism during peroxisomal matrix protein import. *J. Biol. Chem.* **2005**, *280*, 1921–30.

16 Kragt, A., Voorn-Brouwer, T., van den Berg, M., and Distel, B. The *Saccharomyces cerevisiae* peroxisomal import receptor Pex5p is monoubiquitinated in wild type cells. *J. Biol. Chem.* **2005**, *280*, 7867–74.

17 Platta, H. W., Girzalsky, W., and Erdmann, R. Ubiquitination of the peroxisomal import receptor Pex5p. *Biochem. J.* **2004**, *384*, 37–45.

18 Purdue, P. E., and Lazarow, P. B. Peroxisome biogenesis. *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 701–52.

19 Swinkels, B. W., Gould, S. J., and Subramani, S. Targeting efficiencies of various permutations of the consensus C-terminal tripeptide

peroxisomal targeting signal. *FEBS Lett.* **1992**, *305*, 133–36.

20 Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* **1989**, *108*, 1657–64.

21 Gietl, C., Faber, K. N., van der Klei, I. J., and Veenhuis, M. Mutational analysis of the N-terminal topogenic signal of watermelon glyoxysomal malate dehydrogenase using the heterologous host *Hansenula polymorpha*. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3151–55.

22 Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J.* **1991**, *10*, 3255–62.

23 Van der Leij, I., Franse, M. M., Elgersma, Y., Distel, B., and Tabak, H. F. PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11782–86.

24 Marzioch, M., Erdmann, R., Veenhuis, M., and Kunau, W. H. PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. *EMBO J.* **1994**, *13*, 4908–18.

25 Gatto, G. J., Jr., Geisbrecht, B. V., Gould, S. J., and Berg, J. M. Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5. *Nat. Struct. Biol.* **2000**, *7*, 1091–95.

26 Klein, A. T., Barnett, P., Bottger, G., Konings, D., Tabak, H. F., and Distel, B. Recognition of peroxisomal targeting signal type 1 by the import receptor Pex5p. *J. Biol. Chem.* **2001**, *276*, 15034–41.

27 Choe, J., Moyersoen, J., Roach, C., Carter, T. L., Fan, E., Michels, P. A., and Hol, W. G. Analysis of the sequence motifs responsible for the interactions of peroxins 14 and 5, which are involved in glycosome biogenesis in *Trypanosoma brucei*. *Biochemistry* **2003**, *42*, 10915–22.

28 Saidowsky, J., Dodt, G., Kirchberg, K., Wegner, A., Nastainczyk, W., Kunau, W. H., and Schliebs, W. The di-aromatic pentapeptide repeats of the human peroxisome import receptor PEX5 are separate high affinity binding sites for the peroxisomal membrane protein PEX14. *J. Biol. Chem.* **2001**, *276*, 34524–29.

29 Otera, H., Setoguchi, K., Hamasaki, M., Kumashiro, T., Shimizu, N., and Fujiki, Y. Peroxisomal targeting signal receptor Pex5p interacts with cargoes and import machinery components in a spatiotemporally differentiated manner: conserved Pex5p WXXXF/Y motifs are critical for matrix protein import. *Mol. Cell Biol.* **2002**, *22*, 1639–55.

30 Barnett, P., Bottger, G., Klein, A. T., Tabak, H. F., and Distel, B. The peroxisomal membrane protein Pex13p shows a novel mode of SH3 interaction. *EMBO J.* **2000**, *19*, 6382–91.

31 Purdue, P. E., Yang, X., and Lazarow, P. B. Pex18p and Pex21p, a novel pair of related peroxins essential for peroxisomal targeting by the PTS2 pathway. *J. Cell Biol.* **1998**, *143*, 1859–69.

32 Einwächter, H., Sowinski, S., Kunau, W. H., and Schliebs, W. *Yarrowia lipolytica* Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway. *EMBO Rep.* **2001**, *2*, 1035–39.

33 Schäfer, A., Kerssen, D., Veenhuis, M., Kunau, W. H., and Schliebs, W. Functional similarity between the peroxisomal PTS2 receptor binding protein Pex18p and the N-terminal half of the PTS1 receptor Pex5p. *Mol. Cell Biol.* **2004**, *24*, 8895–906.

34 Dammai, V., and Subramani, S. The human peroxisomal targeting signal receptor, Pex5p, is translocated into the peroxisomal matrix and recycled to the cytosol. *Cell* **2001**, *105*, 187–96.

35 Kunau, W. H. Peroxisomes: the extended shuttle to the peroxisome matrix. *Curr. Biol.* **2001**, *11*, 659–62.

36 Nair, D. M., Purdue, P. E., and Lazarow, P. B. Pex7p translocates in and out of peroxisomes in *Saccharomyces cerevisiae*. *J. Cell Biol.* **2004**, *167*, 599–604.

37 Hettema, E. H., Girzalsky, W., van den Berg, M., Erdmann, R., and Distel, B. *Saccharomyces cerevisiae* Pex3p and Pex19p are required for proper localization and stability of peroxisomal membrane proteins. *EMBO J.* **2000**, *19*, 223–33.

38 Agne, B., Meindl, N. M., Niederhoff, K., Einwachter, H., Rehling, P., Sickmann, A., Meyer, H. E., Girzalsky, W., and Kunau, W. H. Pex8p: an intraperoxisomal organizer of the peroxisomal import machinery. *Mol. Cell* **2003**, *11*, 635–46.

39 Chang, C. C., Warren, D. S., Sacksteder, K. A., and Gould, S. J. PEX12 interacts with PEX5 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. *J. Cell Biol.* **1999**, *147*, 761–74.

40 Dodt, G., and Gould, S. J. Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J. Cell Biol.* **1996**, *135*, 1763–74.

41 Rehling, P., Skaletz-Rorowski, A., Girzalsky, W., Voorn-Brouwer, T., Franse, M. M., Distel, B., Veenhuis, M., Kunau, W. H., and Erdmann, R. Pex8p, an intraperoxisomal peroxin of *Saccharomyces cerevisiae* required for protein transport into peroxisomes binds the PTS1 receptor pex5p. *J. Biol. Chem.* **2000**, *275*, 3593–602.

42 Koller, A., Snyder, W. B., Faber, K. N., Wenzel, T. J., Rangell, L., Keller, G. A., and Subramani, S. Pex22p of *Pichia pastoris*, essential for peroxisomal matrix protein import, anchors the ubiquitin-conjugating enzyme, Pex4p, on the peroxisomal membrane. *J. Cell Biol.* **1999**, *146*, 99–112.

43 Patel, S., and Latterich, M. The AAA team: related ATPases with diverse functions. *Trends Cell Biol.* **1998**, *8*, 65–71.

44 Vale, R. D. AAA proteins. Lords of the ring. *J. Cell Biol.* **2000**, *150*, 13–19.

45 Hanson, P. I., and Whiteheart, S. W. AAA+ proteins: have engine, will work. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 519–29.

46 Birschmann, I., Stroobants, A. K., van den Berg, M. Schafer, A., Rosenkranz, K., Kunau, W. H., and Tabak, H. F. Pex15p of *Saccharomyces cerevisiae* provides a molecular basis for recruitment of the AAA peroxin Pex6p to peroxisomal membranes. *Mol. Biol. Cell* **2003**, *14*, 2226–36.

47 Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W. B., Distel, B., Subramani, S., and Tabak, H. F. Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S. cerevisiae*, causes proliferation of the endoplasmic reticulum membrane. *EMBO J.* **1997**, *16*, 7326–41.

48 Erdmann, R., Wiebel, F. F., Flessau, A., Rytka, J., Beyer, A., Frohlich, K. U., and Kunau, W. H. *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell* **1991**, *64*, 499–510.

49 Voorn-Brouwer, T., van der Leij, I., Hemrika, W., Distel, B., and Tabak, H. F. Sequence of the PAS8 gene, the product of which is essential for biogenesis of peroxisomes in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1993**, *1216*, 325–28.

50 Matsumoto, N., Tamura, S., and Fujiki, Y. The pathogenic peroxin Pex26p recruits the Pex1p-Pex6p AAA ATPase complexes to peroxisomes. *Nat. Cell Biol.* **2003**, *5*, 454–60.

51 Eckert, J. H., and Johnsson, N. Pex10p links the ubiquitin conjugating enzyme Pex4p to the protein import machinery of the peroxisome. *J. Cell Sci.* **2003**, *116*, 3623–34.

52 Collins, C. S., Kalish, J. E., Morrell, J. C., McCaffery, J. M., and

Gould, S. J. The peroxisome biogenesis factors pex4p, pex22p, pex1p, and pex6p act in the terminal steps of peroxisomal matrix protein import. *Mol. Cell Biol.* **2000**, *20*, 7516–26.

53 Van der Klei, I. J., Hilbrands, R. E., Kiel, J. A., Rasmussen, S. W., Cregg, J. M., and Veenhuis, M. The ubiquitin-conjugating enzyme Pex4p of *Hansenula polymorpha* is required for efficient functioning of the PTS1 import machinery. *EMBO J.* **1998**, *17*, 3608–18.

54 Oliveira, M. E., Gouveia, A. M., Pinto, R. A., Sa-Miranda, C., and Azevedo, J. E. The energetics of Pex5p-mediated peroxisomal protein import. *J. Biol. Chem.* **2003**, *278*, 39483–88.

55 Platta, H. W., Grunau, S., Rosenkranz, K., Girzalsky, W., and Erdmann, R. Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol. *Nat. Cell Biol.* **2005**, *7*, 817–22.

56 Hicke, L. Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 195–201.

57 Fleming, A., and Osley, M. A. Silence of the rings. *Cell* **2004**, *119*, 449–51.

58 Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **2000**, *19*, 94–102.

59 Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **1989**, *243*, 1576–83.

60 Seufert, W., and Jentsch, S. Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *EMBO J.* **1990**, *9*, 543–50.

61 Veenhuis, M., Mateblowski, M., Kunau, W. H., and Harder, W. Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast* **1987**, *3*, 77–84.

62 Ghislain, M., Udvardy, A., and Mann, C. *S. cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. *Nature* **1993**, *366*, 358–62.

63 Lazarow, P. B. Peroxisome biogenesis: advances and conundrums. *Curr. Opin. Cell Biol.* **2003**, *15*, 489–97.

64 Patarca, R., and Fletcher, M. A. Ring finger in the peroxisome assembly factor-1. *FEBS Lett.* **1992**, *312*, 1–2.

65 Kalish, J. E., Theda, C., Morrell, J. C., Berg, J. M., and Gould, S. J. Formation of the peroxisome lumen is abolished by loss of *Pichia pastoris* Pas7p, a zinc-binding integral membrane protein of the peroxisome. *Mol. Cell Biol.* **1995**, *15*, 6406–19.

66 Tan, X., Waterham, H. R., Veenhuis, M., and Cregg, J. M. The *Hansenula polymorpha* PER8 gene encodes a novel peroxisomal integral membrane protein involved in proliferation. *J. Cell Biol.* **1995**, *128*, 307–19.

67 Joazeiro, C. A., and Weissman, A. M. RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **2000**, *102*, 549–52.

68 Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **1999**, *286*, 309–12.

69 Okumoto, K., Abe, I., and Fujiki, Y. Molecular anatomy of the peroxin Pex12p: ring finger domain is essential for Pex12p function and interacts with the peroxisome-targeting signal type 1-receptor Pex5p and a ring peroxin, Pex10p. *J. Biol. Chem.* **2000**, *275*, 25700–10.

70 Albertini, M., Girzalsky, W., Veenhuis, M., and Kunau, W. H. Pex12p of *Saccharomyces cerevisiae* is a component of a multi-protein complex essential for peroxisomal matrix protein import. *Eur. J. Cell Biol.* **2001**, *80*, 257–70.

71 Birschmann, I., Rosenkranz, K., Erdmann, R., and Kunau, W. H. Structural and functional analysis of the interaction of the AAA-peroxins Pex1p and Pex6p. *FEBS J.* **2005**, *272*, 47–58.

72 Moir, D., Stewart, S. E., Osmond,

B. C., and Botstein, D. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudo-reversion studies. *Genetics* **1982**, *100*, 547–63.

73 Fröhlich, K. U. An AAA family tree. *J. Cell Sci.* **2001**, *114*, 1601–02.

74 Woodman, P. G. p97, a protein coping with multiple identities. *J. Cell Sci.* **2003**, *116*, 4283–90.

75 Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **2004**, *429*, 841–47.

76 Bays, N. W., et al. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat. Cell Biol.* **2001**, *3*, 24–29.

77 Tsai, B., Ye, Y., and Rapoport, T. A. Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 246–55.

78 Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* **2005**, *120*, 73–84.

79 Park, S., Isaacson, R., Kim, H. T., Silver, P. A., and Wagner, G. Ufd1 exhibits the AAA-ATPase fold with two distinct ubiquitin interaction sites. *Structure* **2005**, *13*, 995–1005.

80 Dai, R. M., and Li, C. C. Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat. Cell Biol.* **2001**, *3*, 740–44.

81 Meyer, H. H., Wang, Y., and Warren, G. Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. *EMBO J.* **2002**, *21*, 5645–52.

82 Shiozawa, K., Maita, N., Tomii, K., Seto, A., Goda, N., Akiyama, Y., Shimizu, T., Shirakawa, M., and Hiroaki, H. Structure of the N-terminal domain of PEX1 AAA-ATPase. Characterization of a putative adaptor-binding domain. *J. Biol. Chem.* **2004**, *279*, 50060–68.

83 Golbik, R., Lupas, A. N., Koretke, K. K., Baumeister, W., and Peters, J. The Janus face of the archaeal Cdc48/p97 homologue VAT: protein folding versus unfolding. *Biol. Chem.* **1999**, *380*, 1049–62.

84 Gouveia, A. M., Reguenga, C., Oliveira, M. E., Sa-Miranda, C., and Azevedo, J. E. Characterization of peroxisomal Pex5p from rat liver. Pex5p in the Pex5p-Pex14p membrane complex is a transmembrane protein. *J. Biol. Chem.* **2000**, *275*, 32444–51.

85 Kiel, J. A., Otzen, M., Veenhuis, M., and Van der Klei, I. J. Obstruction of polyubiquitination affects PTS1 peroxisomal matrix protein import. *Biochim. Biophys. Acta* **2005**, in press.

86 Zolman, B. K., and Bartel, B. An Arabidopsis indole-3-butyric acid-response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1786–91.

87 Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48 (UFD1/NPL4), a ubiquitin-selective chaperone. *Cell* **2001**, *107*, 667–77.

88 Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H. D., and Jentsch, S. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* **2000**, *102*, 577–86.

89 Kiel, J. A., Hilbrands, R. E., van der Klei, I. J., Rasmussen, S. W., Salomons, F. A., van der Heide, M., Faber, K. N., Cregg, J. M., and Veenhuis, M. *Hansenula polymorpha* Pex1p and Pex6p are peroxisome-associated AAA proteins that functionally and physically interact. *Yeast* **1999**, *15*, 1059–78.

90 Baker, R. T., Tobias, J. W., and Varshavsky, A. Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. *J. Biol. Chem.* **1992**, *267*, 23364–75.

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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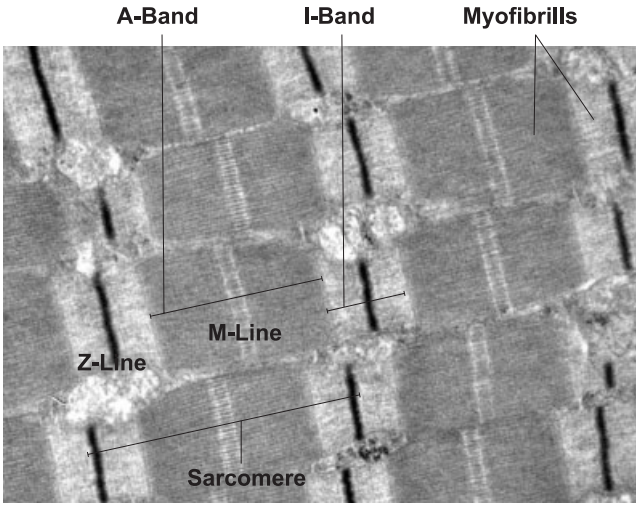

Fig. 2.1. Mouse skeletal muscle viewed by transmission electron microscopy. See text for details Magnification 12 000×. (Courtesy of Dr. Michaela Schweizer)

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

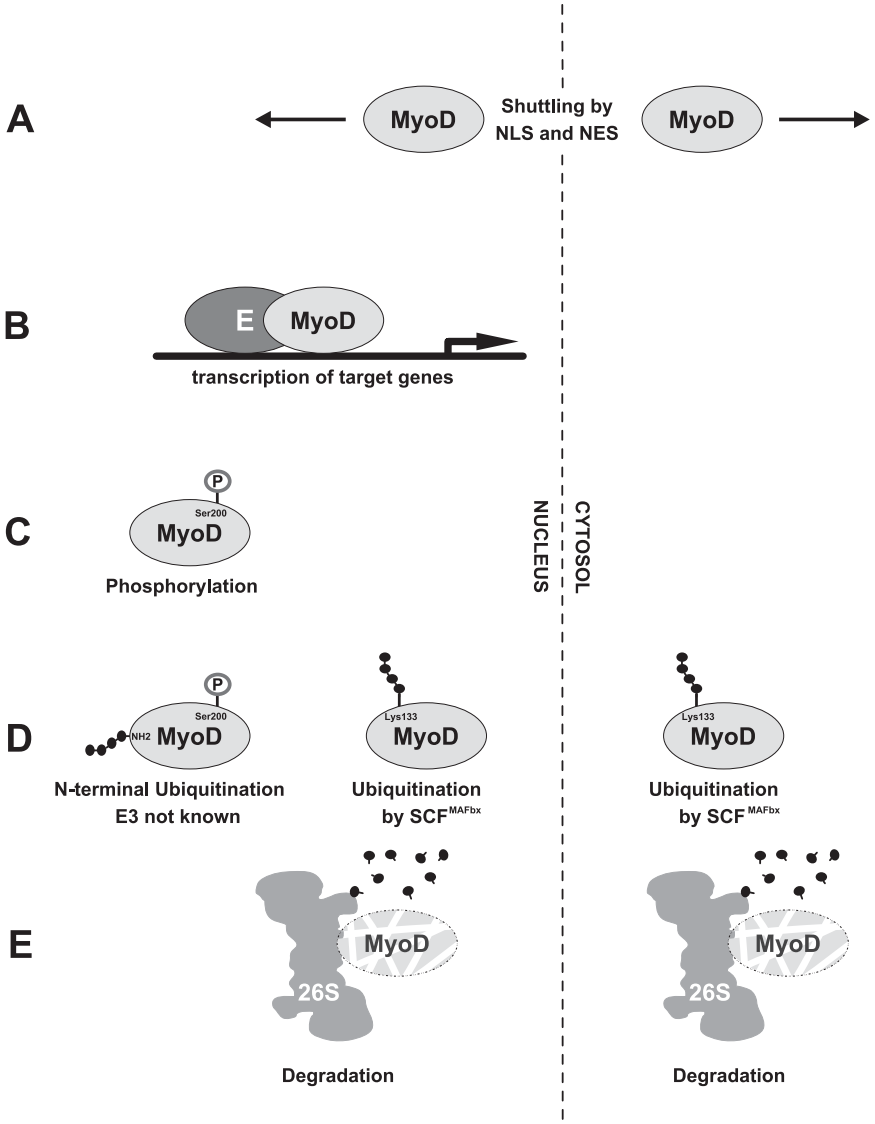

Fig. 2.2. Degradation pathways of MyoD. (A) MyoD is rapidly synthesized in the cytoplasm and can be transported to the nucleus via its intrinsic NLS and/or through the modulating activity of E-proteins and Id proteins. Excess amounts of MyoD protein are shuttled back to the cytosol via its NES. (B) Together 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

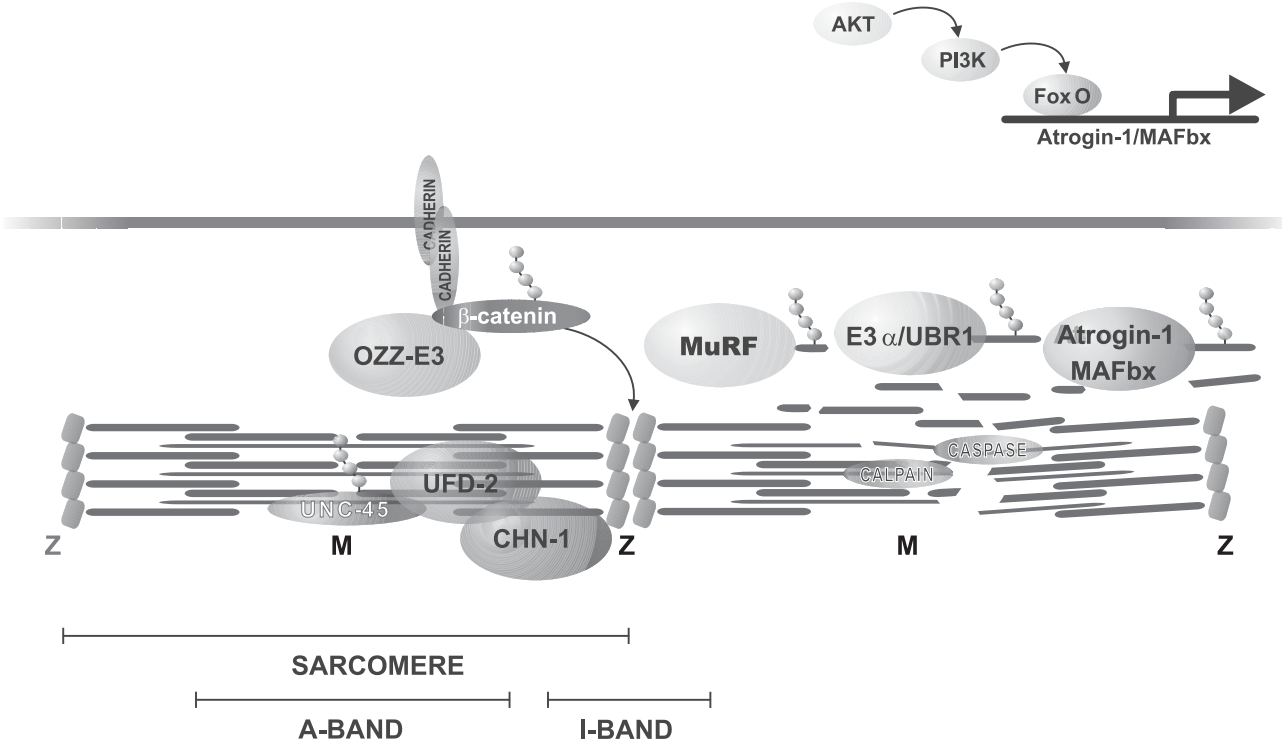

Fig. 2.3. Ubiquitin ligases are involved in the assembly, remodelling and breakdown of the sarcomere. The E3 ligases Ozz-E3 and the E3/E4 complex UFD-2/CHN-1 regulate sarcomere organization and myosin assembly by regulating protein levels of β-catenin or UNC-45, respectively (left). Caspases or calpains 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.

References

1 Voisin, L., Breuille, D., Combaret, L., Pouyet, C., Taillandier, D., Aurousseau, E., Obled, C., and Attaix, D. (**1996**). Muscle wasting in a rat model of long-lasting sepsis results from the activation of lysosomal, Ca^{2+}-activated, and ubiquitin-proteasome proteolytic pathways. *J. Clin. Invest. 1996 Apr* 1;97(7):1610–7.

2 Huang, J., and Forsberg, N.E. (**1998**). Role of calpain in skeletal-muscle protein degradation. *Proc. Natl. Acad. Sci. U.S.A. 1998 Oct* 13;95(21):12100–5.

3 Du, J., Wang, X., Miereles, C., Bailey, J.L., Debigare, R., Zheng, B., Price, S.R., and Mitch, W.E. (**2004**). Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J. Clin. Invest.*, *113*. 115–123.

4 Lee, S.W., Dai, G., Hu, Z., Wang, X., Du, J., and Mitch, W.E. (**2004**). Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. *J. Am. Soc. Nephrol.*, *15*. 1537–1545.

5 Lecker, S.H., Solomon, V., Mitch, W.E., and Goldberg, A.L. (**1999**). Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J. Nutr.*, *129*. 227S–237S.

6 Clark, K.A., McElhinny, A.S., Beckerle, M.C., and Gregorio, C.C. (**2002**). Striated muscle cytoarchitecture: an intricate web of form and function. *Annu. Rev. Cell Dev. Biol.*, *18*. 637–706.

7 Pownall, M.E., Gustafsson, M.K., and Emerson, C.P., Jr. (**2002**). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu. Rev. Cell Dev. Biol.*, Volume 18. 747–783.

8 Parker, M.H., Seale, P., and Rudnicki, M.A. (**2003**). Looking back to the embryo: defining transcriptional networks in adult myogenesis. *Nat. Rev. Genet.*, vol. 4. 497–507.

9 Sabourin, L.A., and Rudnicki, M.A. (**2000**). The molecular regulation of myogenesis. *Clin. Genet.*, *57*. 16–25.

10 Davis, R.L., Weintraub, H., and Lassar, A.B. (**1987**). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, *51*. 987–1000.

11 Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T.K., Turner, D., Rupp, R., Hollenberg, S., and et al. eds. (**1991**). The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* 251(4995):761–6.

12 Choi, J., Costa, M.L., Mermelstein, C.S., Chagas, C., Holtzer, S., and Holtzer, H. (**1990**). MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proc. Natl. Acad. Sci. USA*, *87*. 7988–7992.

13 Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B., and Miller, A.D. (**1989**). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc. Natl. Acad. Sci. USA*, *86*. 5434–5438.

14 Lassar, A.B., Paterson, B.M., and Weintraub, H. (**1986**). Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell*, *47*. 649–656.

15 Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N.J., and Fernandez, A. (**1998**). The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J. Cell Biol.*, *142*. 1447–1459.

16 Gardrat, F., Montel, V., Raymond, J., and Azanza, J.L. (**1997**). Proteasome and myogenesis. *Mol. Biol. Rep.*, *24*. 77–81.

17 Breitschopf, K., Bengal, E., Ziv, T., Admon, A., and Ciechanover, A. (**1998**). A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein. *Embo J*, *17*. 5964–5973.

18 Abu Hatoum, O., Gross-Mesilaty, S., Breitschopf, K., Hoffman, A., Gonen, H., Ciechanover, A., and Bengal, E. (**1998**). Degradation of myogenic transcription factor MyoD by the ubiquitin pathway in vivo and in vitro: regulation by specific DNA binding. *Mol. Cell Biol.*, *18*. 5670–5677.

19 Varshavsky, A. (**1997**). The N-end rule pathway of protein degradation. *Genes Cells*, vol. 2. 13–28.

20 Batonnet, S., Leibovitch, M.P., Tintignac, L., and Leibovitch, S.A. (**2004**). Critical role for lysine 133 in the nuclear ubiquitin-mediated degradation of MyoD. *J. Biol. Chem.*, *279*. 5413–5420.

21 Lingbeck, J.M., Trausch-Azar, J.S., Ciechanover, A., and Schwartz, A.L. (**2003**). Determinants of nuclear and cytoplasmic ubiquitin-mediated degradation of MyoD. *J. Biol. Chem.*, *278*. 1817–1823.

22 Song, A., Wang, Q., Goebl, M.G., and Harrington, M.A. (**1998**). Phosphorylation of nuclear MyoD is required for its rapid degradation. *Mol. Cell Biol.*, *18*. 4994–4999.

23 Ma, P.C., Rould, M.A., Weintraub, H., and Pabo, C.O. (**1994**). Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell*, *77*. 451–459.

24 Langlands, K., Yin, X., Anand, G., and Prochownik, E.V. (**1997**). Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *J. Biol. Chem.*, *272*. 19785–19793.

25 Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (**1991**). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell*, *66*. 305–315.

26 Kadesch, T. (**1993**). Consequences of heteromeric interactions among helix-loop-helix proteins. *Cell Growth Differ.*, *4*. 49–55.

27 Sun, L., Trausch-Azar, J.S., Ciechanover, A., and Schwartz, A.L. (**2005**). Ubiquitin-proteasome-mediated degradation, intracellular localization, and protein synthesis of MyoD and Id1 during muscle differentiation. *J. Biol. Chem.*, *280*. 26448–26456.

28 Lingbeck, J.M., Trausch-Azar, J.S., Ciechanover, A., and Schwartz, A.L. (**2005**). E12 and E47 modulate cellular localization and proteasome-mediated degradation of MyoD and Id1. *Oncogene*.

29 Trausch-Azar, J.S., Lingbeck, J., Ciechanover, A., and Schwartz, A.L. (**2004**). Ubiquitin-Proteasome-mediated degradation of Id1 is modulated by MyoD. *J. Biol. Chem.*, *279*. 32614–32619.

30 Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B. (**1989**). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell*, *58*. 537–544.

31 Benezra, R., Davis, R.L., Lassar, A., Tapscott, S., Thayer, M., Lockshon, D., and Weintraub, H. (**1990**). Id: a negative regulator of helix-loop-helix DNA binding proteins. Control of terminal myogenic differentiation. *Ann. NY Acad. Sci.*, *599*. 1–11.

32 Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., and Weintraub, H. (**1990**). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell*, *61*. 49–59.

33 Crescenzi, M., Fleming, T.P., Lassar, A.B., Weintraub, H., and Aaronson, S.A. (**1990**). MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl. Acad. Sci. USA*, *87*. 8442–8446.

34 Rao, S.S., Chu, C., and Kohtz, D.S. (**1994**). Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. *Mol. Cell Biol.*, *14*. 5259–5267.

35 Skapek, S.X., Rhee, J., Spicer, D.B., and Lassar, A.B. (**1995**). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science*, *267*. 1022–1024.

36 Kitzmann, M., Vandromme, M., Schaeffer, V., Carnac, G., Labbe, J.C., Lamb, N., and Fernandez, A. (**1999**). cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. *Mol. Cell Biol.*, *19*. 3167–3176.

37 Willems, A.R., Schwab, M., and Tyers, M. (**2004**). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim. BioPhys. Acta*, *1695*. 133–170.

38 Cardozo, T., and Pagano, M. (**2004**). The SCF ubiquitin ligase: insights into a molecular machine. *Nat. Rev. Mol. Cell Biol.*, *5*. 739–751.

39 Tintignac, L.A., Leibovitch, M.P., Kitzmann, M., Fernandez, A., Ducommun, B., Meijer, L., and Leibovitch, S.A. (**2000**). Cyclin E-cdk2 phosphorylation promotes late G1-phase degradation of MyoD in muscle cells. *Exp. Cell Res.*, *259*. 300–307.

40 Tintignac, L.A., Sirri, V., Leibovitch, M.P., Lecluse, Y., Castedo, M., Metivier, D., Kroemer, G., and Leibovitch, S.A. (**2004**). Mutant MyoD lacking Cdc2 phosphorylation sites delays M-phase entry. *Mol. Cell Biol.*, *24*. 1809–1821.

41 Tintignac, L.A., Lagirand, J., Batonnet, S., Sirri, V., Leibovitch, M.P., and Leibovitch, S.A. (**2005**). Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase. *J. Biol. Chem.*, *280*. 2847–2856.

42 Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (**1997**). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*, *91*. 209–219.

43 Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (**2002**). E3 ubiquitin ligase that recognizes sugar chains. *Nature*, *418*. 438–442.

44 Ang, X.L., and Wade Harper, J. (**2005**). SCF-mediated protein degradation and cell cycle control. *Oncogene*, *24*. 2860–2870.

45 Vandromme, M., Cavadore, J.C., Bonnieu, A., Froeschle, A., Lamb, N., and Fernandez, A. (**1995**). Two nuclear localization signals present in the basic-helix 1 domains of MyoD promote its active nuclear translocation and can function independently. *Proc. Natl. Acad. Sci. USA*, *92*. 4646–4650.

46 Wojcik, C., and DeMartino, G.N. (**2003**). Intracellular localization of proteasomes. *Int. J. Biochem. Cell Biol.*, *35*. 579–589.

47 De Conto, F., Missorini, S., Arcangeletti, C., Pinardi, F., Montarras, D., Pinset, C., Vassy, J., Geraud, G., Chezzi, C., and Scherrer, K. (**1997**). Prosome cytodistribution relative to desmin and actin filaments in dividing C2.7 myoblasts and during myotube formation in vitro. *Exp. Cell Res.*, *233*. 99–117.

48 Floyd, Z.E., Trausch-Azar, J.S., Reinstein, E., Ciechanover, A., and Schwartz, A.L. (**2001**). The nuclear ubiquitin-proteasome system degrades MyoD. *J. Biol. Chem.*, *276*. 22468–22475.

49 Lindon, C., Albagli, O., Domeyne, P., Montarras, D., and Pinset, C. (**2000**). Constitutive instability of muscle regulatory factor Myf5 is distinct from its mitosis-specific disappearance, which requires a D-box-like motif overlapping the basic domain. *Mol. Cell Biol.*, *20*. 8923–8932.

50 Vinals, F., and Ventura, F. (**2004**). Myogenin protein stability is decreased by BMP-2 through a mechanism implicating Id1. *J. Biol. Chem.*, *279*. 45766–45772.

51 Heery, D.M., Hoare, S., Hussain, S., Parker, M.G., and Sheppard, H. (**2001**). Core LXXLL motif sequences in CREB-binding protein, SRC1, and RIP140 define affinity and selectivity for steroid and retinoid receptors. *J. Biol. Chem.*, *276*. 6695–6702.

52 Nastasi, T., Bongiovanni, A., Campos, Y., Mann, L., Toy, J.N., Bostrom, J., Rottier, R., Hahn, C., Conaway, J.W., Harris, A.J., and D'Azzo, A. (**2004**). Ozz-E3, a muscle-specific ubiquitin ligase, regulates beta-catenin degradation during myogenesis. *Dev. Cell*, *6*. 269–282.

53 Hoppe, T., Cassata, G., Barral, J.M., Springer, W., Hutagalung, A.H., Epstein, H.F., and Baumeister, R. (**2004**). Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in *C. elegans*. *Cell*, *118*. 337–349.

54 Bienz, M., and Clevers, H. (**2000**). Linking colorectal cancer to Wnt signaling. *Cell*, *103*. 311–320.

55 Gottardi, C.J., and Gumbiner, B.M. (**2001**). Adhesion signaling: how beta-catenin interacts with its partners. *Curr. Biol.*, *11*. R792–794.

56 Wu, G., Xu, G., Schulman, B.A., Jeffrey, P.D., Harper, J.W., and Pavletich, N.P. (**2003**). Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. *Mol. Cell*, *11*. 1445–1456.

57 Polakis, P. (**2002**). Casein kinase 1: a Wnt'er of disconnect. *Curr. Biol.*, *12*. R499–R501.

58 Matsuzawa, S.I., and Reed, J.C. (**2001**). Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol. Cell*, *7*. 915–926.

59 Liu, J., Stevens, J., Rote, C.A., Yost, H.J., Hu, Y., Neufeld, K.L., White, R.L., and Matsunami, N. (**2001**). Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. *Mol. Cell*, *7*. 927–936.

60 Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H.E., Behrens, J., Sommer, T., and Birchmeier, W. (**2002**). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat. Cell Biol.*, *4*. 222–231.

61 Kile, B.T., Schulman, B.A., Alexander, W.S., Nicola, N.A., Martin, H.M., and Hilton, D.J. (**2002**). The SOCS box: a tale of destruction and degradation. *Trends Biochem. Sci.*, *27*. 235–241.

62 Lai, E.C. (**2002**). Protein degradation: four E3s for the notch pathway. *Curr. Biol.*, *12*. R74–78.

63 Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C.J., and Boulianne, G.L. (**2001**). Neuralized functions as an E3 ubiquitin ligase during Drosophila development. *Curr. Biol.*, *11*. 1675–1679.

64 Lai, E.C., Deblandre, G.A., Kintner, C., and Rubin, G.M. (**2001**). Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. *Dev. Cell*, vol. 1. 783–794.

65 Wu, G., Liu, C., and He, X. (**2004**). Ozz; a new name on the long list of beta-catenin's nemeses. *Mol. Cell*, *13*. 451–453.

66 Barral, J.M., Hutagalung, A.H., Brinker, A., Hartl, F.U., and Epstein, H.F. (**2002**). Role of the myosin assembly protein UNC-45 as a molecular chaperone for myosin. *Science*, *295*, 669–671.

67 Hutagalung, A.H., Landsverk, M.L., Price, M.G., and Epstein, H.F. (**2002**). The UCS family of myosin chaperones. *J. Cell Sci.*, *115*, 3983–3990.

68 Epstein, H.F., and Thomson, J.N. (**1974**). Temperature-sensitive mutation affecting myofilament assembly in Caenorhabditis elegans. *Nature*, *250*, 579–580.

69 Barral, J.M., Bauer, C.C., Ortiz, I., and Epstein, H.F. (**1998**). Unc-45 mutations in Caenorhabditis elegans implicate a CRO1/She4p-like domain in myosin assembly. *J. Cell Biol.*, *143*, 1215–1225.

70 Hoppe, T., Cassata, G., Barral, J.M., Springer, W., Hutagalung, A.H., Epstein, H.F., and Baumeister, R. (**2004**). Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in *C. elegans*. *Cell 118*, 337–349.

71 Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (**1999**). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell 96*, 635–644.

72 Hoppe, T. (**2005**). Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. *Trends Biochem. Sci.*, *30*, 183–187.

73 Cyr, D.M., Hohfeld, J., and Patterson, C. (**2002**). Protein quality control: U-box-containing E3 ubiquitin ligases join the fold. *Trends Biochem. Sci.*, *27*, 368–375.

74 Murata, S., Chiba, T., and Tanaka, K. (**2003**). CHIP: a quality-control E3 ligase collaborating with molecular chaperones. *Int. J. Biochem. Cell Biol.*, *35*, 572–578.

75 Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y., and Patterson, C. (**1999**). Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. *Mol. Cell Biol.*, *19*, 4535–4545.

76 Price, M.G., Landsverk, M.L., Barral, J.M., and Epstein, H.F. (**2002**). Two mammalian UNC-45 isoforms are related to distinct cytoskeletal and muscle-specific functions. *J. Cell Sci.*, *115*, 4013–4023.

77 Kaneko, C., Hatakeyama, S., Matsumoto, M., Yada, M., Nakayama, K., and Nakayama, K.I. (**2003**). Characterization of the mouse gene for the U-box-type ubiquitin ligase UFD2a. *Biochem. Biophys. Res. Commun.*, *300*, 297–304.

78 Mahoney, J.A., Odin, J.A., White, S.M., Shaffer, D., Koff, A., Casciola-Rosen, L., and Rosen, A. (**2002**). The human homologue of the yeast polyubiquitination factor Ufd2p is cleaved by caspase 6 and granzyme B during apoptosis. *Biochem. J. 361*, 587–595.

79 Lecker, S.H., Jagoe, R.T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S.R., Mitch, W.E., and Goldberg, A.L. (**2004**). Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *Faseb J. 18*, 39–51.

80 Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K.I. (**2001**). U box proteins as a new family of ubiquitin-protein ligases. *J. Biol. Chem.*, *276*, 33111–33120.

81 Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J., and Patterson, C. (**2001**). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *J. Biol. Chem.*, *276*, 42938–42944.

82 Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (**2001**). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.*, *2*, 1133–1138.

83 Furuno, K., and Goldberg, A.L. (**1986**). The activation of protein degradation in muscle by Ca2+ or muscle injury does not involve a lysosomal mechanism. *Biochem. J*, *237*. 859–864.

84 Medina, R., Wing, S.S., and Goldberg, A.L. (**1995**). Increase in levels of polyubiquitin and proteasome mRNA in skeletal muscle during starvation and denervation atrophy. *Biochem. J*, *307 (Pt 3)*. 631–637.

85 Wing, S.S., and Banville, D. (**1994**). 14-kDa ubiquitin-conjugating enzyme: structure of the rat gene and

regulation upon fasting and by insulin. *Am. J. Physiol, 267.* E39–48.

86 Fischer, D., Sun, X., Gang, G., Pritts, T., and Hasselgren, P.O. (**2000**). The gene expression of ubiquitin ligase E3alpha is upregulated in skeletal muscle during sepsis in rats-potential role of glucocorticoids. *Biochem. Biophys. Res. Commun., 267.* 504–508.

87 Lecker, S.H., Solomon, V., Price, S.R., Kwon, Y.T., Mitch, W.E., and Goldberg, A.L. (**1999**). Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats. *J. Clin. Invest., 104.* 1411–1420.

88 Solomon, V., Baracos, V., Sarraf, P., and Goldberg, A.L. (**1998**). Rates of ubiquitin conjugation increase when muscles atrophy, largely through activation of the N-end rule pathway. *Proc. Natl. Acad. Sci. USA, 95.* 12602–12607.

89 Kwak, K.S., Zhou, X., Solomon, V., Baracos, V.E., Davis, J., Bannon, A.W., Boyle, W.J., Lacey, D.L., and Han, H.Q. (**2004**). Regulation of protein catabolism by muscle-specific and cytokine-inducible ubiquitin ligase E3alpha-II during cancer cachexia. *Cancer Res., 64.* 8193–8198.

90 Koohmaraie, M. (**1992**). The role of Ca(2+)-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. *Biochimie, 74.* 239–245.

91 Solomon, V., and Goldberg, A.L. (**1996**). Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *J. Biol. Chem., 271.* 26690–26697.

92 Williams, A., Sun, X., Fischer, J.E., and Hasselgren, P.O. (**1999**). The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery, 126.* 744–749; discussion 749–750.

93 Taillandier, D., Aurousseau, E., Meynial-Denis, D., Bechet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, X., Guezennec, C.Y., Schmid, H.P. (**1996**). Coordinate activation of lysosomal, Ca 2+-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. *Biochem. J., 316 (Pt 1).* 65–72.

94 Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E., and Meier, P. (**2003**). Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nat. Cell Biol., 5.* 467–473.

95 Ditzel, M., and Meier, P. (**2005**). Ubiquitylation in apoptosis: DIAP1's (N-)en(d)igma. *Cell Death Differ., 12.* 1208–1212.

96 Moretti, A., Weig, H.J., Ott, T., Seyfarth, M., Holthoff, H.P., Grewe, D., Gillitzer, A., Bott-Flugel, L., Schomig, A., Ungerer, M., and Laugwitz, K.L. (**2002**). Essential myosin light chain as a target for caspase-3 in failing myocardium. *Proc. Natl. Acad. Sci. USA, 99.* 11860–11865.

97 Du, J., Wang, X., Miereles, C., Bailey, J.L., Debigare, R., Zheng, B., Price, S.R., and Mitch, W.E. (**2004**). Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J. Clin. Invest., 113.* 115–123.

98 Bodine, S.C., Latres, E., Baumhueter, S., Lai, V.K., Nunez, L., Clarke, B.A., Poueymirou, W.T., Panaro, F.J., Na, E., Dharmarajan, K., Pan, Z.Q., Valenzuela, D.M., DeChiara, T.M., Stitt, T.N., Yancopoulos, G.D., and Glass, D.J. (**2001**). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science, 294.* 1704–1708.

99 Lecker, S.H., Jagoe, R.T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S.R., Mitch, W.E., and Goldberg, A.L. (**2004**). Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *Faseb J., 18.* 39–51.

100 Nikawa, T., Ishidoh, K., Hirasaka, K., Ishihara, I., Ikemoto, M., Kano,

M., Kominami, E., Nonaka, I., Ogawa, T., Adams, G.R., Baldwin, K.M., Yasui, N., Kishi, K., and Takeda, S. (**2004**). Skeletal muscle gene expression in space-flown rats. *Faseb J.*, *18*. 522–524.

101 Centner, T., Yano, J., Kimura, E., McElhinny, A.S., Pelin, K., Witt, C.C., Bang, M.L., Trombitas, K., Granzier, H., Gregorio, C.C., Sorimachi, H., and Labeit, S. (**2001**). Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain. *J. Mol. Biol.*, *306*. 717–726.

102 Pizon, V., Iakovenko, A., Van Der Ven, P.F., Kelly, R., Fatu, C., Furst, D.O., Karsenti, E., and Gautel, M. (**2002**). Transient association of titin and myosin with microtubules in nascent myofibrils directed by the MURF2 RING-finger protein. *J. Cell Sci.*, *115*. 4469–4482.

103 McElhinny, A.S., Perry, C.N., Witt, C.C., Labeit, S., and Gregorio, C.C. (**2004**). Muscle-specific RING finger-2 (MURF-2) is important for microtubule, intermediate filament and sarcomeric M-line maintenance in striated muscle development. *J. Cell Sci.*, *117*. 3175–3188.

104 Spencer, J.A., Eliazer, S., Ilaria, R.L., Jr., Richardson, J.A., and Olson, E.N. (**2000**). Regulation of microtubule dynamics and myogenic differentiation by MURF, a striated muscle RING-finger protein. *J. Cell Biol.*, *150*. 771–784.

105 McElhinny, A.S., Kakinuma, K., Sorimachi, H., Labeit, S., and Gregorio, C.C. (**2002**). Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J. Cell Biol.*, *157*. 125–136.

106 Dai, K.S., and Liew, C.C. (**2001**). A novel human striated muscle RING zinc finger protein, SMRZ, interacts with SMT3b via its RING domain. *J. Biol. Chem.*, *276*. 23992–23999.

107 Kedar, V., McDonough, H., Arya, R., Li, H.H., Rockman, H.A., and Patterson, C. (**2004**). Muscle-specific RING finger 1 is a bona fide ubiquitin ligase that degrades cardiac troponin I. *Proc. Natl. Acad. Sci. USA*, *101*. 18135–18140.

108 Witt, S.H., Granzier, H., Witt, C.C., and Labeit, S. (**2005**). MURF-1 and MURF-2 target a specific subset of myofibrillar proteins redundantly: towards understanding MURF-dependent muscle ubiquitination. *J. Mol. Biol.*, *350*. 713–722.

109 Tskhovrebova, L., and Trinick, J. (**2003**). Titin: properties and family relationships. *Nat. Rev. Mol. Cell Biol.*, vol. 4. 679–689.

110 Kramerova, I., Kudryashova, E., Tidball, J.G., and Spencer, M.J. (**2004**). Null mutation of calpain 3 (p94) in mice causes abnormal sarcomere formation in vivo and in vitro. *Hum. Mol. Genet.*, *13*. 1373–1388.

111 Gomes, M.D., Lecker, S.H., Jagoe, R.T., Navon, A., and Goldberg, A.L. (**2001**). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. USA*, *98*. 14440–14445.

112 Jagoe, R.T., Lecker, S.H., Gomes, M., and Goldberg, A.L. (**2002**). Patterns of gene expression in atrophying skeletal muscles: response to food deprivation. *Faseb J.*, *16*. 1697–1712.

113 Wray, C.J., Mammen, J.M., Hershko, D.D., and Hasselgren, P.O. (**2003**). Sepsis upregulates the gene expression of multiple ubiquitin ligases in skeletal muscle. *Int. J. Biochem. Cell Biol.*, *35*. 698–705.

114 Dehoux, M.J., van Beneden, R.P., Fernandez-Celemin, L., Lause, P.L., and Thissen, J.P. (**2003**). Induction of MafBx and Murf ubiquitin ligase mRNAs in rat skeletal muscle after LPS injection. *FEBS Lett.*, *544*. 214–217.

115 Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S.H., and Goldberg, A.L. (**2004**). Foxo transcription factors induce the atrophy-related ubiquitin ligase

atrogin-1 and cause skeletal muscle atrophy. *Cell*, *117*. 399–412.

116 Doyle, D.A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (**1996**). Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell*, *85*. 1067–1076.

117 Stitt, T.N., Drujan, D., Clarke, B.A., Panaro, F., Timofeyva, Y., Kline, W.O., Gonzalez, M., Yancopoulos, G.D., and Glass, D.J. (**2004**). The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell*, *14*. 395–403.

118 Cao, P.R., Kim, H.J., and Lecker, S.H. (**2005**). Ubiquitin-protein ligases in muscle wasting. *Int. J. Biochem. Cell Biol.*, *37*. 2088–2097.

119 Li, H.H., Kedar, V., Zhang, C., McDonough, H., Arya, R., Wang, D.Z., and Patterson, C. (**2004**). Atrogin-1/muscle atrophy F-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an SCF ubiquitin ligase complex. *J. Clin. Invest.*, *114*. 1058–1071.

120 Serrano, A.L., Murgia, M., Pallafacchina, G., Calabria, E., Coniglio, P., Lomo, T., and Schiaffino, S. (**2001**). Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc. Natl. Acad. Sci. USA*, *98*. 13108–13113.

121 Marinovic, A.C., Zheng, B., Mitch, W.E., and Price, S.R. (**2002**). Ubiquitin (UbC) expression in muscle cells is increased by glucocorticoids through a mechanism involving Sp1 and MEK1. *J. Biol. Chem.*, *277*. 16673–16681.

122 Dardevet, D., Sornet, C., Vary, T., and Grizard, J. (**1996**). Phosphatidylinositol 3-kinase and p70 s6 kinase participate in the regulation of protein turnover in skeletal muscle by insulin and insulin-like growth factor I. *Endocrinology*, *137*. 4087–4094.

123 Price, S.R., Bailey, J.L., Wang, X., Jurkovitz, C., England, B.K., Ding, X., Phillips, L.S., and Mitch, W.E. (**1996**). Muscle wasting in insulinopenic rats results from activation of the ATP-dependent, ubiquitin-proteasome proteolytic pathway by a mechanism including gene transcription. *J. Clin. Invest.*, *98*. 1703–1708.

124 Mitch, W.E., and Price, S.R. (**2001**). Mechanisms activated by kidney disease and the loss of muscle mass. *Am. J. Kidney Dis.*, *38*. 1337–1342.

125 Kwon, Y.T., Xia, Z., Davydov, I.V., Lecker, S.H., and Varshavsky, A. (**2001**). Construction and analysis of mouse strains lacking the ubiquitin ligase UBR1 (E3alpha) of the N-end rule pathway. *Mol. Cell Biol.*, *21*. 8007–8021.

126 Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A.M., Andersen, J.S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (**1998**). Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature*, *396*. 590–594.

127 Chen, Z.J. (**2005**). Ubiquitin signalling in the NF-kappaB pathway. *Nat. Cell Biol.*, *7*. 758–765.

128 Cai, D., Frantz, J.D., Tawa, N.E., Jr., Melendez, P.A., Oh, B.C., Lidov, H.G., Hasselgren, P.O., Frontera, W.R., Lee, J., Glass, D.J., and Shoelson, S.E. (**2004**). IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell*, *119*. 285–298.

129 Whitehouse, A.S., Khal, J., and Tisdale, M.J. (**2003**). Induction of protein catabolism in myotubes by 15(S)-hydroxyeicosatetraenoic acid through increased expression of the ubiquitin-proteasome pathway. *Br. J. Cancer*, *89*. 737–745.

130 Li, Y.P., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D.L., and Reid, M.B. (**2005**). TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *Faseb J.*, *19*. 362–370.

131 Tracey, K.J. (**2002**). Lethal weight loss: the focus shifts to signal transduction. *Sci. STKE*, *2002*. PE21.

132 Koistinen, H.A., Chibalin, A.V., and Zierath, J.R. (**2003**). Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from Type 2 diabetic patients. *Diabetologia*, *46*. 1324–1328.

133 Williamson, D., Gallagher, P., Harber, M., Hollon, C., and Trappe, S. (**2003**). Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. *J. Physiol.*, *547*. 977–987.

134 Di Giovanni, S., Molon, A., Broccolini, A., Melcon, G., Mirabella, M., Hoffman, E.P., and Servidei, S. (**2004**). Constitutive activation of MAPK cascade in acute quadriplegic myopathy. *Ann. Neurol*, *55*. 195–206.

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).

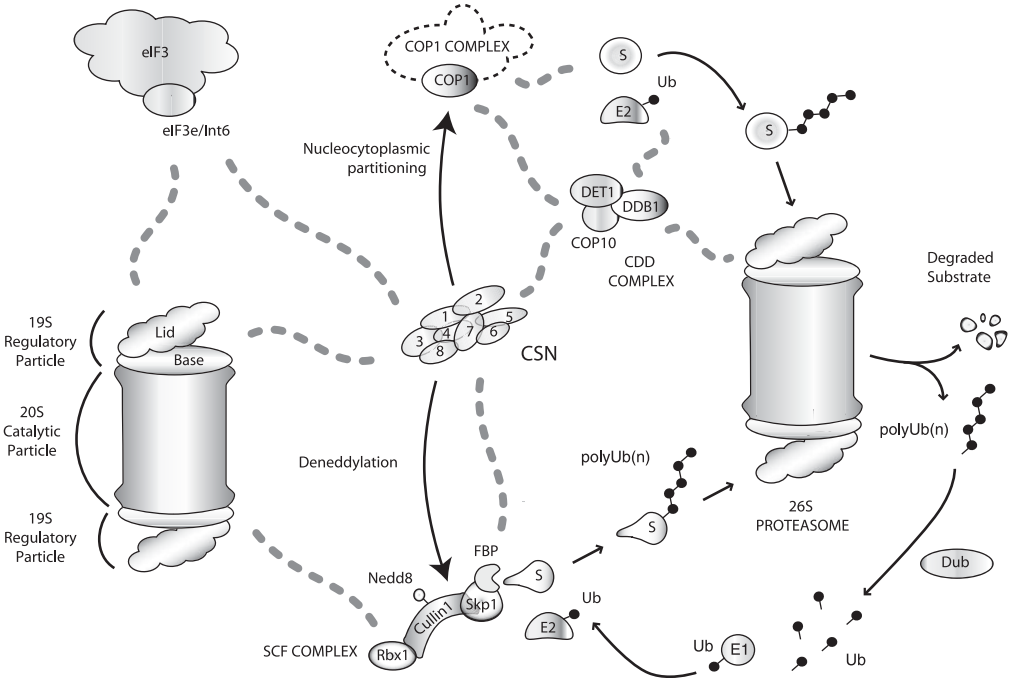

Fig. 3.1. *Arabidopsis* CSN biochemical activities and map of interactions with components of the ubiquitin/26S proteasome pathway. *Arabidopsis* CSN shows two different biochemical activities, represented with large headed arrows, directed towards the E3 ubiquitin–protein ligases: Nedd8–Cullin deconjugation (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-

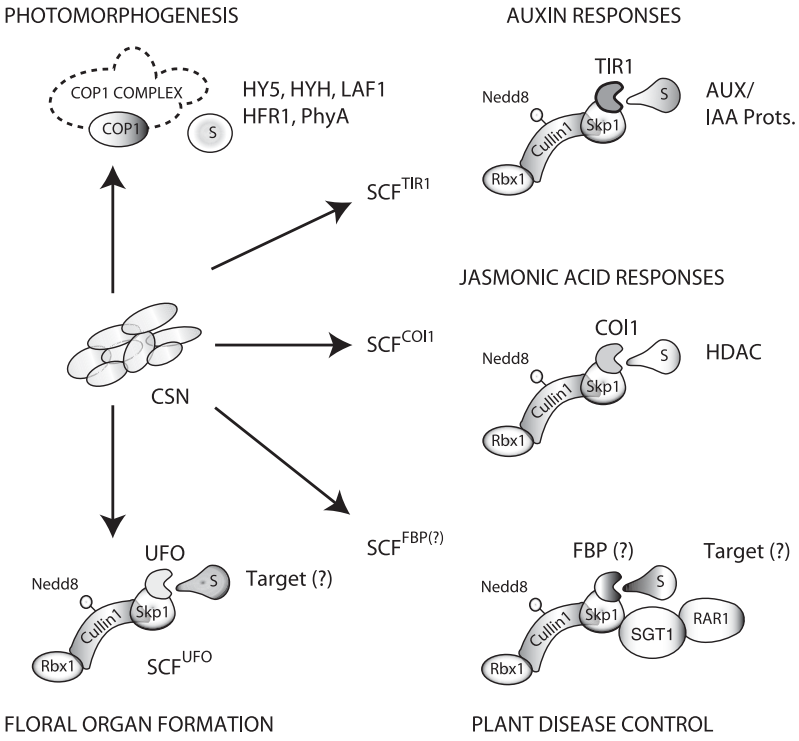

Fig. 3.2. *Arabidopsis* CSN functions in plant developmental processes. The CSN regulates E3 ubiquitin–protein ligases mediating different aspects of plant biology, including floral development, photomorphogenesis, and plant response to pathogens and hormones. The mechanism of action of the CSN involves 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.

References

Alonso, J.M. and Stepanova, A.N. (**2004**). The ethylene signaling pathway. *Science 306*: 1513–1515.

Aravind, L. and Ponting, C.P. (**1998**). Homologues of 26S proteasome subunits are regulators of transcription and translation. *Protein Sci. 7*: 1250–1254.

Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (**2002**). The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. *Science 295*: 2073–2076.

Benvenuto, G., Formiggini, F., Laflamme, P., Malakhov, M., and Bowler, C. (**2002**). The photomorphogenesis regulator DET1 binds the amino-terminal tail of histone H2B in a nucleosome context. *Curr. Biol. 12*: 1529–1534.

Buche, C., Poppe, C., Schafer, E., and Kretsch, T. (**2000**). *eid1*: a new *Arabidopsis* mutant hypersensitive in phytochrome A-dependent high-irradiance responses. *Plant Cell 12*: 547–558.

Busch, S., Eckert, S.E., Krappmann, S., and Braus, G.H. (**2003**). The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans*. *Mol. Microbiol. 49*: 717–730.

Caballero, O.L., Resto, V., Patturajan, M., Meerzaman, D., Guo, M.Z., Engles, J., Yochem, R., Ratovitski, E., Sidransky, D., and Jen, J. (**2002**). Interaction and colocalization of PGP9.5 with JAB1 and p27(Kip1). *Oncogene 21*: 3003–3010.

Chamovitz, D.A., Wei, N., Osterlund, M.T., von Arnim, A.G., Staub, J.M., Matsui, M., and Deng, X.W. (**1996**). The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. *Cell 86*: 115–121.

Cheng, Y., Dai, X., and Zhao, Y. (**2004**). AtCAND1, a HEAT-repeat protein that participates in auxin signaling in *Arabidopsis*. *Plant Physiol. 135*: 1020–1026.

Chuang, H.W., Zhang, W., and Gray, W.M. (**2004**). *Arabidopsis* ETA2, an apparent ortholog of the human cullin-interacting protein CAND1, is required for auxin responses mediated by the SCF(TIR1) ubiquitin ligase. *Plant Cell 16*: 1883–1897.

Coen, E.S. and Meyerowitz, E.M. (**1991**). The war of the whorls: genetic interactions controlling flower development. *Nature 353*: 31–37.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V., and Deshaies, R.J. (**2002**). Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science 298*: 608–611.

Deshaies, R.J. (**1999**). SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol. 15*: 435–467.

Deng, X.W., Matsui, M., Wei, N., Wagner, D., Chu, A.M., Feldmann, K.A., and Quail, P.H. (**1992**). COP1, an *Arabidopsis* regulatory gene, encodes a protein with both a zinc-binding motif and a G beta homologous domain. *Cell 71*: 791–801.

Deng, X.W., Dubiel, W., Wei, N., Mundt, K., Colicelli, J., Kato, J.Y., Naumann, M., Segal, D., Seeger, M., Carr, A., Glickman, M., and Chamovitz, D.A. (**2000**). Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. *Trend Genet. 16*: 202–203.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G. (**2002**). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. *Plant J. 32*: 457–466.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (**2005**). The F-box protein TIR1 is an auxin receptor. *Nature 435*: 441–445.

Dias, D.C., Dolios, G., Wang, R., and Pan, Z.Q. (**2002**). CUL7: A DOC domain-containing cullin selectively binds Skp1.Fbx29 to form an SCF-like complex. *Proc. Natl. Acad. Sci. USA 99*: 16601–16606.

Dieterle, M., Thomann, A., Renou, J.P.,

Parmentier, Y., Cognat, V., Lemonnier, G., Muller, R., Shen, W.H., Kretsch, T., and Genschik, P. (**2005**). Molecular and functional characterization of *Arabidopsis* Cullin 3A. *Plant J. 41*: 386–399.

Dieterle, M., Zhou, Y.C., Schafer, E., Funk, M., and Kretsch, T. (**2001**). EID1, an F-box protein involved in phytochrome A-specific light signaling. *Genes Dev. 15*: 939–944.

Dohmann, E.M., Kuhnle, C., and Schwechheimer, C. (**2005**). Loss of the CONSTITUTIVE PHOTOMORPHOGENIC9 Signalosome Subunit 5 Is Sufficient to Cause the cop/det/fus Mutant Phenotype in *Arabidopsis*. *Plant Cell 17*: 1967–1978.

Doronkin, S., Djagaeva, I., and Beckendorf, S.K. (**2003**). The COP9 signalosome promotes degradation of Cyclin E during early Drosophila oogenesis. *Dev. Cell 4*: 699–710.

Dunand-Sauthier, I., Walker, C., Wilkinson, C., Gordon, C., Crane, R., Norbury, C., and Humphrey, T. (**2002**). Sum1, a component of the fission yeast eIF3 translation initiation complex, is rapidly relocalized during environmental stress and interacts with components of the 26S proteasome. *Mol. Biol. Cell 13*: 1626–1640.

Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (**2003**). The COP9 Signalosome interacts physically with SCFCOI1 and modulates jasmonate responses. *Plant Cell 15*: 1083–1094.

Feng, S., Shen, Y., Sullivan, J.A., Rubio, V., Xiong, Y., Sun, T.P., and Deng, X.W. (**2004**). *Arabidopsis* CAND1, an unmodified CUL1-interacting protein, is involved in multiple developmental pathways controlled by ubiquitin/proteasome-mediated protein Degradation. *Plant Cell 16*: 1870–1882.

Figueroa, P., Gusmaroli, G., Serino, G., Habashi, J., Ma, L., Shen, Y., Feng, S., Bostick, M., Callis, J., Hellmann, H., and Deng, X.W. (**2005**). *Arabidopsis* has two redundant Cullin 3 proteins that are essential for embryo development and that interact with RBX1 and BTB proteins to form multisubunit E3 ubiquitin ligase complexes in vivo. *Plant Cell 17*: 1180–1195.

Fleet, C.M. and Sun, T.P. (**2005**). A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Curr. Opin. Plant Biol. 8*: 77–85.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D., and Chamovitz, D.A. (**1999**). The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr. Biol. 9*: 1187–1190.

Fu, H., Reis, N., Lee, Y., Glickman, M.H., and Vierstra, R.D. (**2001**). Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *EMBO J. 20*: 7096–7107.

Furukawa, M., Zhang, Y., McCarville, J., Ohta, T., and Xiong, Y. (**2000**). The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. *Mol. Cell Biol. 20*: 8185–8197.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M., and Vierstra, R.D. (**2002**). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, *99*: 11519–11524.

Gan-Erdene, T., Nagamalleswari, K., Yin, L., Wu, K., Pan, Z.Q., and Wilkinson, K.D. (**2003**). Identification and characterization of DEN1, a deneddylase of the ULP family. *J. Biol. Chem. 278*: 28892–28900.

Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D.A. (**2003**). BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. *Mol. Cell 12*: 783–790.

Gingerich, D.J., Gagne, J.M., Salter, D.W., Hellmann, H., Estelle, M., Ma, L., and Vierstra, R.D. (**2005**). Cullins 3a and 3b assemble with members of the broad complex/tramtrack/bric-a-brac (BTB) protein family to form essential ubiquitin-protein ligases (E3s) in *Arabidopsis*. *J. Biol. Chem. 280*: 18810–18821.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (**1998**). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell 94*: 615–623.

Goldenberg, S.J., Cascio, T.C., Shumway, S.D., Garbutt, K.C., Liu, J., Xiong, Y., and

Zheng, N. (**2004**). Structure of the Cand1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell 119*: 517–528.

Gong, L. and Yeh, E.T. (**1999**). Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. *J. Biol. Chem. 274*: 12036–12042.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (**2001**). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature 414*: 271–276.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K., and Nakatani, Y. (**2003**). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell 113*: 357–367.

Guo, H. and Ecker, J.R. (**2003**). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell 115*: 667–677.

Gusmaroli, G., Feng, S., and Deng, X.W. (**2004**). The *Arabidopsis* CSN5A and CSN5B subunits are present in distinct COP9 signalosome complexes, and mutations in their JAMM domains exhibit differential dominant negative effects on development. *Plant Cell 16*: 2984–3001.

Halliday, K.J. (**2004**). Plant hormones: the interplay of brassinosteroids and auxin. *Curr. Biol. 14*: R1008–R1010.

Hammond-Kosack, K.E. and Jones, J.D.G. (**1996**). Resistance gene–dependent plant defense responses. *Plant Cell 8*: 1773–1791.

Hardtke, C.S., Gohda, K., Osterlund, M.T., Oyama, T., Okada, K., Deng, X.W. (**2000**). HY5 stability and activity in *Arabidopsis* is regulated by phosphorylation in its COP1 binding domain. *EMBO J. 19*: 4997–5006.

He, J.X., Gendron, J.M., Sun, Y., Gampala, S.S., Gendron, N., Sun, C.Q., and Wang, Z.Y. (**2005**). BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. *Science 307*: 1634–1638.

He, J.X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.Y. (**2002**). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA 99*: 10185–10190.

Hershko, A. and Ciechanover, A. (**1998**). The ubiquitin system. *Annu. Rev. Biochem. 67*: 425–479.

Hoareau Alves, K., Bochard, V., Rety, S., and Jalinot, P. (**2002**). Association of the mammalian proto-oncoprotein Int-6 with the three protein complexes eIF3, COP9 signalosome and 26S proteasome. *FEBS Lett. 527*: 15–21.

Hofmann, K. and Bucher, P. (**1998**). The PCI domain: a common theme in three multiprotein complexes. *Trends Biochem. Sci. 23*: 204–205.

Holm, M., Ma, L.G., Qu, L.J., and Deng, X.W. (**2002**). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in *Arabidopsis*. *Genes Dev. 16*: 1247–1259.

Huang, X., Hetfeld, B.K., Seifert, U., Kahne, T., Kloetzel, P.M., Naumann, M., Bech-Otschir, D., and Dubiel, W. (**2005**). Consequences of COP9 signalosome and 26S proteasome interaction. *FEBS J. 272*: 3909–3917.

Jang, I.C., Yang, J.Y., Seo, H.S., and Chua, N.H. (**2005**). HFR1 is targeted by COP1 E3 ligase for post-translational proteolysis during phytochrome A signaling. *Genes Dev. 19*: 593–602.

Jones, D.A. and Takemoto, D. (**2004**). Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules. *Curr. Opin. Immunol. 16*: 48–62.

Kamura, T., Conrad, M.N., Yan, Q., Conaway, R.C., and Conaway, J.W. (**1999**). The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev. 13*: 2928–2933.

Kapelari, B., Bech-Otschir, D., Hegerl, R., Schade, R., Dumdey, R., and Dubiel, W. (**2000**). Electron microscopy and subunit-subunit interaction studies reveal a first architecture of COP9 signalosome. *J. Mol. Biol. 300*: 1169–1178.

Karniol, B., Malec, P., and Chamovitz, D.A. (**1999**). *Arabidopsis* FUSCA5 encodes a novel phosphoprotein that is a component of the COP9 complex. *Plant Cell 11*: 839–848.

Karniol, B., Yahalom, A., Kwok, S.F., Tsuge, T., Matsui, M., Deng, X.W., and Chamovitz, D.A. (**1998**). The *Arabidopsis*

homologue of an eIF3 complex subunit associates with the COP9 complex. *FEBS Lett. 439*: 173–179.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., Omata, M., and Tanaka, K. (**2001**). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J. 20*: 4003–4012.

Kepinski, S. and Leyser, O. (**2005**). The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature 435*: 446–451.

Kim, S., Choi, H.I., Ryu, H.J., Park, J.H., Kim, M.D., and Kim, S.Y. (**2004**). ARIA, an *Arabidopsis* arm repeat protein interacting with a transcriptional regulator of abscisic acid-responsive gene expression, is a novel abscisic acid signaling component. *Plant Physiol. 136*: 3639–3648.

Kim, T., Hofmann, K., von Arnim, A.G., and Chamovitz, D.A. (**2001**). PCI complexes: pretty complex interactions in diverse signaling pathways. *Trends Plant Sci. 6*: 379–386.

Kitagawa, K., Skowyra, D., Elledge, S.J., Harper, J.W., and Hieter, P. (**1999**). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. *Mol. Cell 4*: 21–33.

Kwok, S.F., Solano, R., Tsuge, T., Chamovitz, D.A., Ecker, J.R., Matsui, M., and Deng, X.W. (**1998**). *Arabidopsis* homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. *Plant Cell 10*: 1779–1790.

Kwok, S.F., Staub, J.M., and Deng, X.W. (**1999**). Characterization of two subunits of *Arabidopsis* 19S proteasome regulatory complex and its possible interaction with the COP9 complex. *J. Molec. Biol. 285*: 85–95.

Lammer, D., Mathias, N., Laplaza, J.M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (**1998**). Modification of yeast Cdc53p by the ubiquitin-related protein Rub1p affects function of the SCFCdc4 complex. *Genes Dev. 12*: 914–926.

Li, L., Peng, Z., and Deng, X.W. (**2003**). The COP9 signalosome: an alternative lid for the 26S proteasome? *Trends Cell Biol. 13*: 507–509.

Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (**1998**). A novel protein modification pathway related to the ubiquitin system. *EMBO J. 17*: 2208–2214.

Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (**2002**). NEDD8 modification of CUL1 dissociates p120 (CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol. Cell 10*: 1511–1518.

Liu, Y., Schiff, M., Serino, G., Deng, X.W., and Dinesh-Kumar, S.P. (**2002**). Role of SCF ubiquitin-ligase and the COP9 signalosome in the *N* gene-mediated resistance response to *tobacco mosaic virus*. *Plant Cell 14*: 1483–1496.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A., and Deshaies, R.J. (**2001**). Promotion of NEDD8-CUL1 conjugate cleavage by COP9 signalosome. *Science 292*: 1382–1385.

Lykke-Andersen, K., Schaefer, L., Menon, S., Deng, X.W., Miller, J.B., and Wei, N. (**2003**). Disruption of the COP9 signalosome Csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin E, and early embryonic death. *Mol. Cell Biol. 23*: 6790–6797.

Ma, L., Li, J., Qu, L., Hager, J., Chen, Z., Zhao, H., and Deng, X.W. (**2001**). Light control of *Arabidopsis* development entails coordinated regulation of genome expression and cellular pathways. *Plant Cell 13*: 2589–2607.

Mauch-Mani, B. and Mauch, F. (**2005**). The role of abscisic acid in plant-pathogen interactions. *Curr. Opin. Plant Biol. 8*: 409–414.

Maytal-Kivity, V., Piran, R., Pick, E., Hofmann, K., and Glickman, M.H. (**2002a**). COP9 signalosome components play a role in the mating pheromone response of *S. cerevisiae*. *EMBO Rep. 3*: 1215–1221.

Maytal-Kivity, V., Reis, N., Hofmann, K., and Glickman, M.H. (**2002b**). MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. *BMC Biochem. 3*: 28.

McDowell, J.M. and Dangl, J.L. (**2000**). Signal transduction in the plant immune response. *Trends Biochem. Sci. 25*: 79–82.

Mendoza, H.M., Shen, L.N., Botting, C., Lewis, A., Chen, J., Ink, B., and Hay, R.T. (**2003**). NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. *J. Biol. Chem. 278*: 25637–25643.

Min, K.W., Kwon, M.J., Park, H.S., Park, Y., Yoon, S.K., and Yoon, J.B. (**2005**). CAND1 enhances deneddylation of CUL1 by COP9 signalosome. *Biochem. Biophys. Res. Commun. 334*: 867–874.

Morimoto, M., Nishida, T., Honda, R., and Yasuda, H. (**2000**). Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). *Biochem. Biophys. Res. Commun. 270*: 1093–1096.

Mundt, K.E., Liu, C., and Carr, A.M. (**2002**). Deletion mutants in COP9/signalosome subunits in fission yeast *Schizosaccharomyces pombe* display distinct phenotypes. *Mol. Biol. Cell 13*: 493–502.

Mundt, K.E., Porte, J., Murray, J.M., Brikos, C., Christensen, P.U., Caspari, T., Hagan, I.M., Millar, J.B., Simanis, V., Hofmann, K., and Carr, A.M. (**1999**). The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. *Curr. Biol. 9*: 1427–1430.

Ng, M. and Yanofsky, M.F. (**2000**). Three ways to learn the ABCs. *Curr. Opin. Plant Biol. 3*: 47–52.

Oron, E., Mannervik, M., Rencus, S., Harari-Steinberg, O., Neuman-Silberberg, S., Segal, D., and Chamovitz, D.A. (**2002**). COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*. *Development. 29*: 4399–4409.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E.A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (**2000**). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *EMBO J. 19*: 3475–3484.

Oshikawa, K., Matsumoto, M., Yada, M., Kamura, T., Hatakeyama, S., and Nakayama, K.I. (**2003**). Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. *Biochem. Biophys. Res. Commun. 303*: 1209–1216.

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (**2000**). Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. *Nature 405*: 462–6.

Peng, Z., Serino, G., and Deng, X.W. (**2001a**). A role of *Arabidopsis* COP9 signalosome in multifaceted developmental processes revealed by the characterization of its subunit 3. *Development. 128*: 4277–4288.

Peng, Z., Serino, G., and Deng, X.W. (**2001b**). Molecular characterization of subunit 6 of the COP9 signalosome and its role in multifaceted developmental processes in *Arabidopsis*. *Plant Cell 13*: 2393–2407.

Peng, Z., Shen, Y., Feng, S., Wang, X., Chitteti, B.N., Vierstra, R.D., and Deng, X.W. (**2003**). Evidence for a physical association of the COP9 signalosome, the proteasome, and specific E3 ligases *in vivo*. *Curr. Biol. 13*, R504–R505.

Pintard, L., Willis, J.H., Willems, A., Johnson, J.L., Srayko, M., Kurz, T., Glaser, S., Mains, P.E., Tyers, M., Bowerman, B., and Peter, M. (**2003**). The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature 425*: 311–316.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (**2003**). EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F box proteins: EBF1 and EBF2. *Cell 115*: 679–689.

Read, M.A., Brownell, J.E., Gladysheva, T.B., Hottelet, M., Parent, L.A., Coggins, M.B., Pierce, J.W., Podust, V.N., Luo, R.S., Chau, V., and Palombella, V.J. (**2000**). Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. *Mol. Cell Biol. 20*: 2326–2333.

Saijo, Y., Sullivan, J.A., Wang, H., Yang, J., Shen, Y., Rubio, V., Ma, L., Hoecker, U., and Deng, X.W. (**2003**). The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. *Genes Dev. 17*: 2642–2647.

Samach, A., Klenz, J.E., Kohalmi, S.E., Risseeuw, E., Haughn, G.W., and Crosby, W.L. (**1999**). The UNUSUAL FLORAL ORGANS gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. *Plant J. 20*: 433–445.

Schwechheimer, C. (**2004**). The COP9 signalosome (CSN): an evolutionary conserved proteolysis regulator in eukaryotic development. *Biochim Biophys Acta. 1695*: 45–54.

Schwechheimer, C. and Villalobos, L.I. (**2004**). Cullin-containing E3 ubiquitin ligases in plant development. *Curr. Opin. Plant Biol. 7*: 677–686.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.W. (**2001**). Interactions of the COP9 signalosome with the E3 ubiquitin-ligase SCF^{TIR1} in mediating auxin response. *Science 292*: 1379–1382.

Schroeder, D.F., Gahrtz, M., Maxwell, B.B., Cook, R.K., Kan, J.M., Alonso, J.M., Ecker, J.R., and Chory, J. (**2002**). De-etiolated 1 and damaged DNA binding protein 1 interact to regulate *Arabidopsis* photomorphogenesis. *Curr. Biol. 12*: 1462–1472.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M., and Dubiel, W. (**1998**). A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *FASEB J. 12*: 469–478.

Seo, H.S., Watanabe, E., Tokutomi, S., Nagatani, A., and Chua, N.H. (**2004**). Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. *Genes Dev. 18*: 617–622.

Seo, H.S., Yang, J.Y., Ishikawa, M., Bolle, C., Ballesteros, M.L., and Chua, N.H. (**2003**). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. *Nature 423*: 995–999.

Serino, G. and Deng, X.W. (**2003**). The COP9 signalosome: regulating plant development through the control of proteolysis. *Ann Rev. Plant Biol. 54*: 65–82.

Serino, G., Tsuge, T., Kwok, S., Matsui, M., Wei, N., and Deng, X.W. (**1999**). *Arabidopsis* cop8 and fus4 mutations define the same gene that encodes subunit 4 of the COP9 signalosome. *Plant Cell 11*: 1967–1980.

Sullivan, J.A. and Deng, X.W. (**2003**). From seed to seed: the role of photoreceptors in *Arabidopsis* development. *Dev. Biol. 260*: 289–297.

Sullivan, J.A., Shirasu, K., Deng, X.W. (**2003**). The diverse roles of ubiquitin and the 26S proteasome in the life of plants. *Nat. Rev. Genet. 4*: 948–958.

Suzuki, G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. (**2002**). *Arabidopsis* COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. *Genes Dev. 16*: 554–559.

Tepperman, J.M., Zhu, T., Chang, H.S., Wang, X., and Quail, P.H. (**2001**). Multiple transcription-factor genes are early targets of phytochrome A signaling. *Proc. Natl. Acad. Sci. USA 98*: 9437–9442.

Thomann, A., Brukhin, V., Dieterle, M., Gheyeselinck, J., Vantard, M., Grossniklaus, U., and Genschik, P. (**2005a**). *Arabidopsis* CUL3A and CUL3B genes are essential for normal embryogenesis. *Plant J. 43*: 437–448.

Thomann, A., Dieterle, M., and Genschik, P. (**2005b**). Plant CULLIN-based E3s: phytohormones come first. *FEBS Lett. 579*: 3239–3245.

Tomoda, K., Kubota, Y., and Kato, J. (**1999**). Degradation of the cyclin-dependent-kinase inhibitor $p27^{Kip1}$ is instigated by Jab1. *Nature 398*: 160–165.

Tomoda, K., Kubota, Y., Arata, Y., Mori, S., Maeda, M., Tanaka, T., Yoshida, M., Yoneda-Kato, N., and Kato, J.Y. (**2002**). The cytoplasmic shuttling and subsequent degradation of $p27^{kip1}$ mediated by Jab1/CSN5 and the COP9 signalosome complex. *J. Biol. Chem. 277*: 2302–2310.

Tsuge, T., Matsui, M., and Wei, N. (**2001**). The subunit 1 of the COP9 signalosome suppresses gene expression through its N-terminal domain and incorporates into the complex through the PCI domain. *J. Mol. Biol. 305*: 1–9.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.Y., Hsing, Y.I., Kitano, H., Yamaguchi, I., and Matsuoka, M. (**2005**). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature 437*: 693–698.

Uhle, S., Medalia, O., Waldron, R., Dumdey, R., Henklein, P., Bech-Otschir, D., Huang, X., Berse, M., Sperling, J., Schade, R., and Dubiel, W. (**2003**). Protein kinase CK2 and protein kinase D are

associated with the COP9 signalosome. *EMBO J. 22*: 1302–1312.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (**1999**). Activation and repression of transcription by auxin-response factors. *Proc. Natl. Acad. Sci. USA. 96*: 5844–5849.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R. 3rd, Koonin, E.V., and Deshaies, R.J. (**2002**). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science 298*: 611–615.

von Arnim, A.G. and Chamovitz, D.A. (**2003**). Protein homeostasis: a degrading role for Int6/eIF3e. *Curr. Biol. 13*: R323–R325.

von Arnim, A.G., and Deng, X.W. (**1994**). Light inactivation of *Arabidopsis* photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. *Cell 79*: 1035–1045.

von Arnim, A.G., Osterlund, M.T., Kwok, S.F., and Deng, X.W. (**1997**). Genetic and developmental control of nuclear accumulation of COP1, a repressor of photomorphogenesis in *Arabidopsis*. *Plant Physiol. 114*: 779–788.

Wang, H., Ma, L., Li, J.M., Zhao, H.Y., and Deng, X.W. (**2001**). Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. *Science 294*: 154–158.

Wang, K.L., Yoshida, H., Lurin, C., and Ecker, J.R. (**2004**). Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. *Nature 428*: 945–950.

Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W., and Wei, N. (**2003**), The COP9 signalosome interacts with SCF UFO and participates in Arabidopsis flower development. *Plant Cell 15*: 1071–1082.

Wang, X., Kang, D., Feng, S., Serino, G., Schwechheimer, C., and Wei, N. (**2002**). CSN1 N-terminal-dependent activity is required for *Arabidopsis* development but not for Rub1/Nedd8 deconjugation of cullins: a structure-function study of CSN1 subunit of COP9 signalosome. *Mol. Biol. Cell 13*: 646–655.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., and Chory, J. (**2002**). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Dev. Cell 2*: 505–513.

Weber, H., Bernhardt, A., Dieterle, M., Hano, P., Mutlu, A., Estelle, M., Genschik, P., and Hellmann, H. (**2005**). *Arabidopsis* AtCUL3a and AtCUL3b form complexes with members of the BTB/POZ-MATH protein family. *Plant Physiol. 137*: 83–93.

Wee, S., Hetfeld, B., Dubiel, W., and Wolf, D.A. (**2002**). Conservation of the COP9/signalosome in budding yeast. *BMC Genet. 3*: 15.

Wei, N. and Deng, X.W. (**1996**). The role of the COP/DET/FUS genes in light control of *Arabidopsis* seedling development. *Plant Physiol. 112*: 871–878.

Wei, N. and Deng, X.W. (**2003**). The COP9 signalosome. *Annu. Rev. Cell Dev. Biol. 19*: 261–286.

Wei, N., Chamovitz, D.A., and Deng, X.W. (**1994**). *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell 78*: 117–124.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M., and Deng, X.W. (**1998**). The COP9 complex is conserved between plants and animals and is related to the 26S proteasome regulatory complex. *Curr. Biol. 8*: 919–922.

Weigel, D. and Meyerowitz, E.M. (**1994**). The ABCs of Floral Homeotic Genes. *Cell 78*: 203–209.

Wertz, I.E., O'Rourke, K.M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R.J., and Dixit, V.M. (**2004**). Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science 303*: 1371–1374.

Wu, J.T., Lin, H.C., Hu, Y.C., and Chien, C.T. (**2005**). Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation. *Nat. Cell Biol.* (Published online ahead of print on August, 28th).

Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C.G., Wei, N., Wilkinson, K.D., Wang, R., and Pan, Z.Q. (**2003**). DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *J. Biol. Chem. 278*: 28882–28891.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro,

M., and Turner, J.G. (**1998**). COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science 280*: 1091–1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (**2002**). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. *Plant Cell 14*: 1919–1935.

Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T.H., Vidal, M., Elledge, S.J., and Harper, J.W. (**2003**). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. *Nature 425*: 316–321.

Yahalom, A., Kim, T.H., Winter, E., Karniol, B., von Arnim, A.G., and Chamovitz, D.A. (**2001**). *Arabidopsis* eIF3e (INT-6) associates with both eIF3c and the COP9 signalosome subunit CSN7. *J. Biol. Chem. 276*: 334–340.

Yan, J., Walz, K., Nakamura, H., Carattini-Rivera, S., Zhao, Q., Vogel, H., Wei, N., Justice, M.J., Bradley, A., and Lupski, J.R. (**2003**). COP9 signalosome subunit 3 is essential for maintenance of cell proliferation in the mouse embryonic epiblast. *Mol. Cell Biol. 23*: 6798–6808.

Yanagawa, Y., Sullivan, J.A., Komatsu, S., Gusmaroli, G., Suzuki, G., Yin, J., Ishibashi, T., Saijo, Y., Rubio, V., Kimura, S., Wang, J., and Deng, X.W. (**2004**). *Arabidopsis* COP10 forms a complex with DDB1 and DET1 in vivo and enhances the activity of ubiquitin conjugating enzymes. *Genes Dev. 18*: 2172–2181.

Yang, H.Y., Zhou, B.P., Hung, M.C., and Lee, M.H. (**2000**). Oncogenic signals of HER-2/neu in regulating the stability of the cyclin-dependent kinase inhibitor p27. *J. Biol. Chem. 275*: 24735–24739.

Yang, J., Lin, R., Sullivan, J., Hoecker, U., Liu, B., Xu, L., Deng, X.W., and Wang, H. (**2005**). Light regulates COP1-mediated degradation of HFR1, a transcription factor essential for light signaling in *Arabidopsis*. *Plant Cell 17*: 804–821.

Yang, X., Lee, S., So, J.H., Dharmasiri, S., Dharmasiri, N., Ge, L., Jensen, C., Hangarter, R., Hobbie, L., and Estelle, M. (**2004**). The IAA1 protein is encoded by AXR5 and is a substrate of SCF(TIR1). *Plant J. 40*: 772–782.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Xiao, D., Wang, X., Rodriguez-Suarez, R.J., Zhang, H., and Wei, N. (**2002**). The COP9 signalosome inhibits $p27^{kip1}$ degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. *Curr. Biol. 12*: 667–672.

Yen, H.C., Gordon, C., and Chang, E.C. (**2003**). *Schizosaccharomyces pombe* Int6 and Ras homologs regulate cell division and mitotic fidelity via the proteasome. *Cell 112*: 207–217.

Zheng, J., Yang, X., Harrell, J.M., Ryzhikov, S., Shim, E.H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (**2002**). CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol. Cell 10*: 1519–1526.

Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, G., Deshaies, R.J., and Wolf, D.A. (**2001**). The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. *BioMed. Central Biochem. 2*: 7.

Zhou, C., Wee, S., Rhee, E., Naumann, M., Dubiel, W., and Wolf, D.A. (**2003**). Fission yeast COP9/signalosome suppresses cullin activity through recruitment of the deubiquitylating enzyme Ubp12p. *Mol. Cell 11*: 927–938.

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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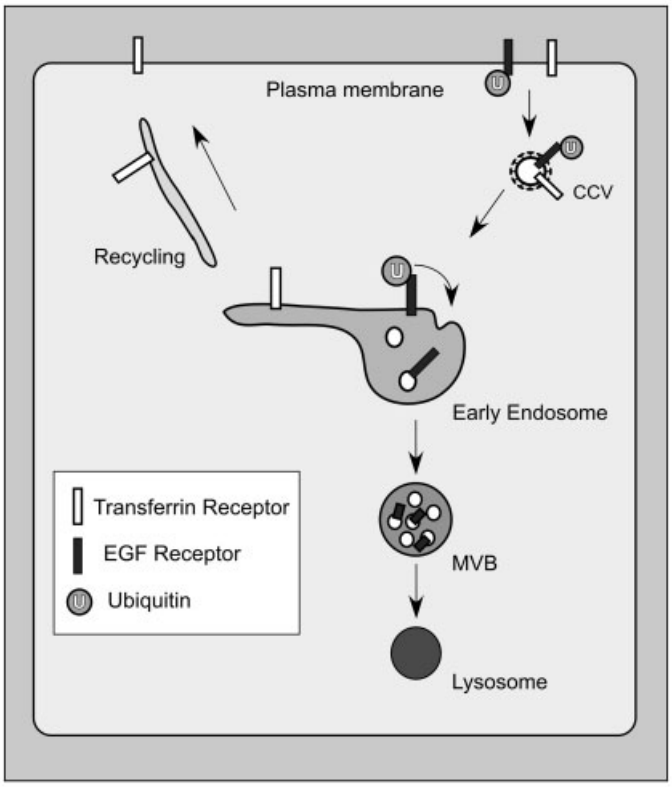

Fig. 4.1. Protein sorting in the endocytic pathway. Upon ligand activation (not shown), growth factor receptors (e.g., EGF receptor) are internalized via Clathrin-coated vesicles (CCV) and delivered to the tubulo-vesicular early endosome. Here, ubiquitinated receptors are sequestered into the lumenal 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

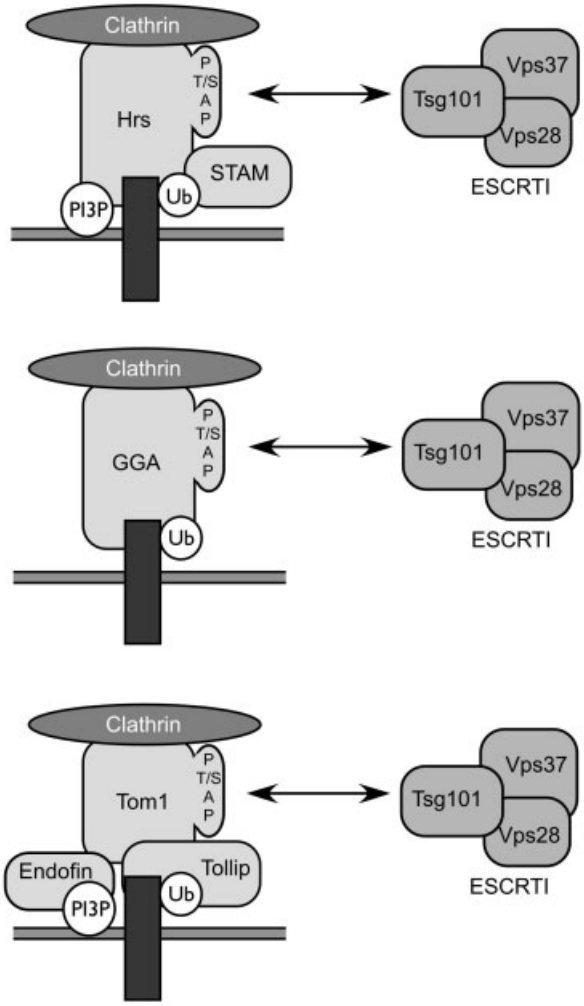

Fig. 4.2. Ubiquitin-binding adapter complexes implicated in MVB sorting. Three endosomal adapter complexes have been implicated in the sorting of proteins from the endosome to the lysosome. Each of these complexes interacts with clathrin, which decorates the endosomal membrane in discrete patches. Ubiquitin 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).

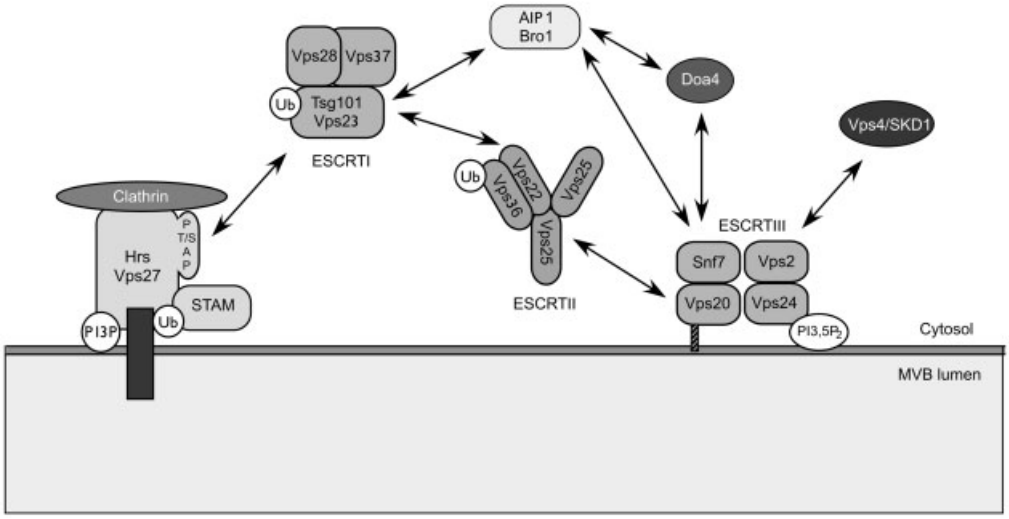

Fig. 4.3. Interactions of the MVB sorting machinery. The first point of engagement of ubiquitinated proteins with the MVB sorting machinery is the Hrs–STAM complex. Hrs (Vps27 in yeast) associates with the endosomal membrane through interaction of its FYVE-domain with PtdIns3*P*. Both Hrs and STAM (HseI 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).

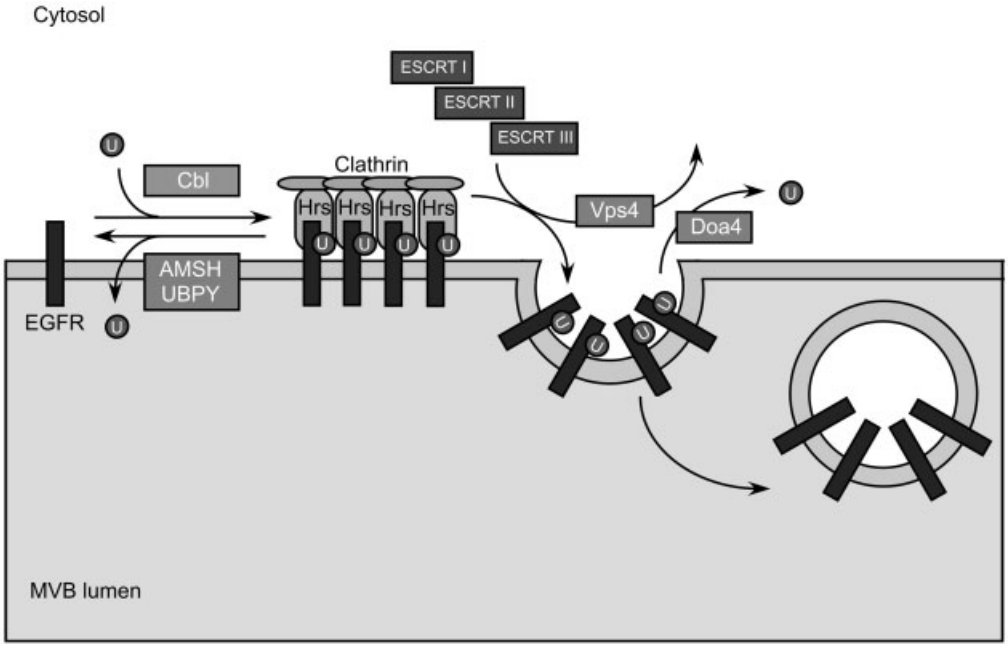

Fig. 4.4. Sorting of ubiquitinated receptors at the endosome. Ubiquitination of growth factor receptors (e.g., EGF receptor (EGFR)), by an E3 ligase (e.g. Cbl) promotes their interaction with Hrs in a Clathrin-coated microdomain. DUBs (e.g., AMSH and UBPY) may counteract Cbl activity and oppose MVB sorting 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-

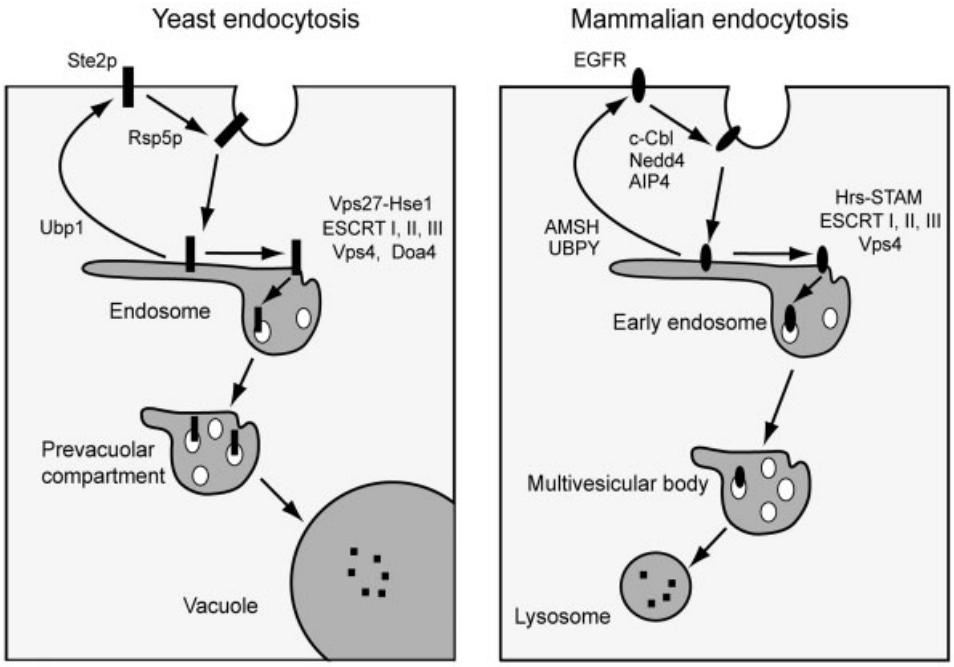

Fig. 4.5. The endocytic pathway in yeast and mammalian cells. Endocytosed proteins (e.g., Ste2 and EGFR) are sorted at the early endosome by multiple components of the multivesicular body (MVB) sorting machinery (Hrs–STAM, Vps27–HseI, ESCRTI–III). From here, cargo can be either recycled back to the plasma 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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References

Alam, S. L., Sun, J., Payne, M., Welch, B. D., Blake, B. K., Davis, D. R., Meyer, H. H., Emr, S. D., and Sundquist, W. I. (**2004**). Ubiquitin interactions of NZF zinc fingers. *Embo J. 23*, 1411–21.

Amerik, A. Y., Nowak, J., Swaminathan, S.,

and Hochstrasser, M. (**2000**). The DoA4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. *Mol. Biol. Cell 11*, 3365–3380.

Angers, A., Ramjaun, A. R., and McPherson, P. S. (**2004**). The HECT domain ligase itch ubiquitinates endophilin and localizes to the trans-Golgi network and endosomal system. *J. Biol. Chem. 279*, 11471–9.

Asada, H., Ishii, N., Sasaki, Y., Endo, K., Kasai, H., Tanaka, N., Takeshita, T., Tsuchiya, S., Konno, T., and Sugamura, K. (**1999**). Grf40, A novel Grb2 family member, is involved in T cell signaling through interaction with SLP-76 and LAT. *J. Exp. Med. 189*, 1383–90.

Babst, M., Katzmann, D. J., Estepa-Sabal, E. J., Meerloo, T., and Emr, S. D. (**2002a**). Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev. Cell 3*, 271–82.

Babst, M., Katzmann, D. J., Snyder, W. B., Wendland, B., and Emr, S. D. (**2002b**). Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev. Cell 3*, 283–9.

Babst, M., Odorizzi, G., Estepa, E. J., and Emr, S. D. (**2000**). Mammalian tumour susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. *Traffic 1*, 248–258.

Babst, M., Wendland, B., Estepa, E. J., and Emr, S. D. (**1998**). The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. *Embo J. 17*, 2982–93.

Bache, K. G., Brech, A., Mehlum, A., and Stenmark, H. (**2003a**). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J. Cell Biol. 162*, 435–42.

Bache, K. G., Raiborg, C., Mehlum, A., and Stenmark, H. (**2003b**). STAM and Hrs are subunits of a multivalent ubiquitin-binding complex on early endosomes. *J. Biol. Chem. 278*, 12513–21.

Bache, K. G., Slagsvold, T., Cabezas, A., Rosendal, K. R., Raiborg, C., and Stenmark, H. (**2004**). The growth-regulatory protein HCRP1/hVps37A is a subunit of mammalian ESCRT-I and mediates receptor down-regulation. *Mol. Biol. Cell 15*, 4337–46.

Bankaitis, V. A., Johnson, L. M., and Emr, S. D. (**1986**). Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc. Natl. Acad. Sci. USA 83*, 9075–9079.

Bilodeau, P. S., Urbanowski, J. L., Winistorfer, S. C., and Piper, R. C. (**2002**). The Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein sorting. *Nat. Cell Biol. 4*, 534–9.

Bilodeau, P. S., Winistorfer, S. C., Allaman, M. M., Surendhran, K., Kearney, W. R., Robertson, A. D., and Piper, R. C. (**2004**). The GAT domains of clathrin-associated GGA proteins have two ubiquitin-binding motifs. *J. Biol. Chem. 279*, 54808–16.

Bishop, N., Horman, A., and Woodman, P. (**2002**). Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates. *J. Cell Biol. 157*, 91–101.

Blondel, M. O., Morvan, J., Dupre, S., Urban-Grimal, D., Haguenauer-Tsapis, R., and Volland, C. (**2004**). Direct sorting of the yeast uracil permease to the endosomal system is controlled by uracil binding and Rsp5p-dependent ubiquitylation. *Mol. Biol. Cell 15*, 883–95.

Bonifacino, J. S. (**2004**). The GGA proteins: adaptors on the move. *Nat. Rev. Mol. Cell Biol. 5*, 23–32.

Bonifacino, J. S., and Weissman, A. (**1998**). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu. Rev. Cell. Dev. Biol. 14*, 19–57.

Bowers, K., Lottridge, J., Helliwell, S. B., Goldthwaite, L. M., Luzio, J. P., and Stevens, T. H. (**2004**). Protein-protein interactions of ESCRT complexes in the yeast *Saccharomyces cerevisiae*. *Traffic 5*, 194–210.

Bowers, K., Piper, S. C., Edeling, M. A., Gray, S. R., Owen, D. J., Lehner, P. J., and Luzio, J. P. (**2005**). Degradation of endocytosed epidermal growth factor and virally-ubiquitinated MHC class I is independent of mammalian ESCRTII. *J. Biol. Chem. 281*, 5094–5105.

Carter, S., Urbe, S., and Clague, M. J. (**2004**). The met receptor degradation pathway: Requirement for K48-linked

polyubiquitin independent of proteasome activity. *J. Biol. Chem. 281*, 5094–5105.

Cenciarelli, C., Hou, D., Hsu, K. C., Rellahan, B. L., Wiest, D. L., Smith, H. T., Fried, V. A., and Weissman, A. M. (**1992**). Activation-induced ubiquitination of the T cell antigen receptor. *Science 257*, 795–7.

Ciechanover, A. (**2005**). Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol. 6*, 79–86.

Clague, M. J. (**1998**). Molecular aspects of the endocytic pathway. *Biochem. J. 336*, 271–282.

Clague, M. J. (**2002**). Membrane transport: a coat for ubiquitin. *Curr. Biol. 12*, R529–R531.

Clague, M. J. and Urbe, S. (**2001**). The interface of receptor trafficking and signalling. *J. Cell Sci. 114*, 3075–81.

Clague, M. J. and Urbe, S. (**2003**). Hrs function: viruses provide the clue. *Trends Cell Biol. 13*, 603–6.

Colledge, M., Snyder, E. M., Crozier, R. A., Soderling, J. A., Jin, Y., Langeberg, L. K., Lu, H., Bear, M. F., and Scott, J. D. (**2003**). Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron 40*, 595–607.

Confalonieri, S., Salcini, A. E., Puri, C., Tacchetti, C., and Di Fiore, P. P. (**2000**). Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. *J. Cell Biol. 150*, 905–12.

de Melker, A. A., van der Horst, G., and Borst, J. (**2004**). Ubiquitin ligase activity of c-Cbl guides the epidermal growth factor receptor into clathrin-coated pits by two distinct modes of Eps15 recruitment. *J. Biol. Chem. 279*, 55465–73.

Dell'Angelica, E. C., Puertollano, R., Mullins, C., Aguilar, R. C., Vargas, J. D., Hartnell, L. M., and Bonifacino, J. S. (**2000**). GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J. Cell Biol. 149*, 81–94.

Di Fiore, P. P., Polo, S., and Hofmann, K. (**2003**). When ubiquitin meets ubiquitin receptors: a signalling connection. *Nat. Rev. Mol. Cell Biol. 4*, 491–7.

Duan, L., Miura, Y., Dimri, M., Majumder, B., Dodge, I. L., Reddi, A. L., Ghosh, A., Fernandes, N., Zhou, P., Mullane-Robinson, K., Rao, N., Donoghue, S., Rogers, R. A., Bowtell, D., Naramura, M., Gu, H., Band, V., and Band, H. (**2003**). Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. *J. Biol. Chem. 278*, 28950–60.

Dunn, R., Klos, D. A., Adler, A. S., and Hicke, L. (**2004**). The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J. Cell Biol. 165*, 135–44.

Dupre, S., Urban-Grimal, D., and Haguenauer-Tsapis, R. (**2004**). Ubiquitin and endocytic internalization in yeast and animal cells. *Biochim. Biophys. Acta 1695*, 89–111.

Fazioli, F., Minichiello, L., Matoskova, W. T., Wong, W. T., and Di Fiore, P. P. (**1993**). Eps15, a novel tyrosine kinase substrate exhibits transforming activity. *Mol. Cell Biol. 13*, 5814–5828.

Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J., and Hopkins, C. R. (**1990**). Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell 61*, 623–634.

Fisher, R. D., Wang, B., Alam, S. L., Higginson, D. S., Robinson, H., Sundquist, W. I., and Hill, C. P. (**2003**). Structure and ubiquitin binding of the ubiquitin-interacting motif. *J. Biol. Chem. 278*, 28976–84.

Fujita, H., Yamanaka, M., Imamura, K., Tanaka, Y., Nara, A., Yoshimori, T., Yokota, S., and Himeno, M. (**2003**). A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: class E vps phenotype in mammalian cells. *J. Cell Sci. 116*, 401–14.

Galan, J. M. and Haguenauer-Tsapis, R. (**1997**). Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *Embo J. 16*, 5847–54.

Galan, J. M., Moreau, V., Andre, B., Volland, C., and Haguenauer-Tsapis, R. (**1996**). Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J. Biol. Chem. 271*, 10946–52.

Gillooly, D. J., Morrow, I. C., Lindsay, M.,

Gould, R., Bryant, N. J., Gaullier, J. M., Parton, R. G., and Stenmark, H. (**2000**). Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J. 19*, 4577–4588.

Gnesutta, N., Ceriani, M., Innocenti, M., Mauri, I., Zippel, R., Sturani, E., Borgonovo, B., Berruti, G., and Martegani, E. (**2001**). Cloning and characterization of mouse UBPy, a deubiquitinating enzyme that interacts with the ras guanine nucleotide exchange factor CDC25(Mm)/Ras-GRF1. *J. Biol. Chem. 276*, 39448–54.

Gorden, P., Carpentier, J. L., Cohen, S., and Orci, L. (**1978**). Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc. Natl. Acad. Sci. USA 75*, 5025–9.

Grenson, M. (**1983**). Inactivation-reactivation process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem. 133*, 135–9.

Haas, A. L. and Bright, P. M. (**1987**). The dynamics of ubiquitin pools within cultured human lung fibroblasts. *J. Biol. Chem. 262*, 345–51.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (**2003**). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat. Cell Biol. 5*, 461–6.

Haigler, H. T., McKanna, J. A., and Cohen, S. (**1979**). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J. Cell Biol. 81*, 382–95.

Hammond, D. E., Carter, S., McCullough, J., Urbe, S., Vande Woude, G., and Clague, M. J. (**2003**). Endosomal dynamics of met determine signaling output. *Mol. Biol. Cell 14*, 1346–54.

Hammond, D. E., Urbe, S., Vande Woude, G. F., and Clague, M. J. (**2001**). Down-regulation of MET, the receptor for hepatocyte growth factor. *Oncogene 20*, 2761–70.

Hein, C., Springael, J. Y., Volland, C., Haguenauer-Tsapis, R., and Andre, B. (**1995**). NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. *Mol. Microbiol. 18*, 77–87.

Hicke, L. and Dunn, R. (**2003**). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol. 19*, 141–72.

Hicke, L. and Riezman, H. (**1996**). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell 84*, 277–287.

Hierro, A., Sun, J., Rusnak, A. S., Kim, J., Prag, G., Emr, S. D., and Hurley, J. H. (**2004**). Structure of the ESCRT-II endosomal trafficking complex. *Nature 431*, 221–5.

Hofmann, K. and Bucher, P. (**1996**). The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. *Trends Biochem. Sci. 21*, 172–3.

Hopkins, C. R. (**1983**). Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell 35*, 321–330.

Hofmann, K., and Falquet, L. (**2001**). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. *TIBS. 26*, 347–350.

Howard, T. L., Stauffer, D. R., Degnin, C. R., and Hollenberg, S. M. (**2001**). CHMP1 functions as a member of a newly defined family of vesicle trafficking proteins. *J. Cell Sci. 114*, 2395–404.

Huang, F. and Sorkin, A. (**2005**). Grb2-mediated Recruitment of the RING Domain of Cbl to the EGF Receptor Is Essential and Sufficient to Support Receptor Endocytosis. *Mol. Biol. Cell. 16*, 1268–1281.

Ingham, R. J., Gish, G., and Pawson, T. (**2004**). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene 23*, 1972–84.

Ishii, N., Owada, Y., Yamada, M., Miura, S., Murata, K., Asao, H., Kondo, H., and Sugamura, K. (**2001**). Loss of neurons in the hippocampus and cerebral cortex of AMSH-deficient mice. *Mol. Cell Biol. 21*, 8626–37.

Itoh, F., Asao, H., Sugamura, K., Heldin, C. H., ten Dijke, P., and Itoh, S. (**2001**).

Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. *Embo J. 20*, 4132–42.

Kaneko, T., Kumasaka, T., Ganbe, T., Sato, T., Miyazawa, K., Kitamura, N., and Tanaka, N. (**2003**). Structural insight into modest binding of a non-PXXP ligand to the signal transducing adaptor molecule-2 Src homology 3 domain. *J. Biol. Chem. 278*, 48162–8.

Kang, R. S., Daniels, C. M., Francis, S. A., Shih, S. C., Salerno, W. J., Hicke, L., and Radhakrishnan, I. (**2003**). Solution structure of a CUE-ubiquitin complex reveals a conserved mode of ubiquitin binding. *Cell 113*, 621–30.

Kato, M., Miyazawa, K., and Kitamura, N. (**2000**). A de-ubiquitinating enzyme UBPY interacts with the SH3 domain of Hrs binding protein via a novel binding motif Px(V/I)(D/N)RxxKP. *J. Biol. Chem. 275*, 37481–37487.

Katoh, K., Shibata, H., Suzuki, H., Nara, A., Ishidoh, K., Kominami, E., Yoshimori, T., and Maki, M. (**2003**). The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. *J. Biol. Chem. 278*, 39104–13.

Katoh, Y., Shiba, Y., Mitsuhashi, H., Yanagida, Y., Takatsu, H., and Nakayama, K. (**2004**). Tollip and Tom1 form a complex and recruit ubiquitin-conjugated proteins onto early endosomes. *J. Biol. Chem. 279*, 24435–43.

Katz, M., Shtiegman, K., Tal-Or, P., Yakir, L., Mosesson, Y., Harari, D., Machluf, Y., Asao, H., Jovin, T., Sugamura, K., and Yarden, Y. (**2002**). Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4. *Traffic 3*, 740–51.

Katzmann, D. J., Odorizzi, G., and Emr, S. D. (**2002**). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell Biol. 3*, 893–905.

Kee, Y., Lyon, N., and Huibregtse, J. M. (**2005**). The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. *Embo J. 24*, 2414–24.

Kolling, R. and Hollenberg, C. P. (**1994**). The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants. *Embo J. 13*, 3261–71.

Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., and Wood, W. I. (**1987**). Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature 330*, 537–43.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (**1998**). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev. 12*, 3663–3674.

Li, H. and Seth, A. (**2004**). An RNF11: Smurf2 complex mediates ubiquitination of the AMSH protein. *Oncogene 23*, 1801–8.

Li, L. and Cohen, S. N. (**1996**). Tsg101: a novel tumor susceptibility gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. *Cell 85*, 319–29.

Lin, Y., Kimpler, L. A., Naismith, T. V., Lauer, J. M., and Hanson, P. I. (**2005**). Interaction of the mammalian ESCRT-III protein hSnf7–1 with itself, membranes, and the AAA+ ATPase SKD1. *J. Biol. Chem. 280*, 12799–12809.

Lloyd, T. E., Atkinson, R., Wu, M. N., Zhou, Y., Pennetta, G., and Bellen, H. J. (**2002**). Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in Drosophila. *Cell 108*, 261–269.

Lu, Q., Hope, L. W., Brasch, M., Reinhard, C., and Cohen, S. N. (**2003**). TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation. *Proc. Natl. Acad. Sci. USA 100*, 7626–31.

Luhtala, N. and Odorizzi, G. (**2004**). Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes. *J. Cell Biol. 166*, 717–29.

Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., and Benovic, J. L. (**2003**). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev. Cell 5*, 709–22.

Maytal-Kivity, V., Reis, N., Hofmann, K., and Glickman, M. H. (**2002**). MPN+, a

putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. *BMC Biochem. 3*, 28.

McCullough, J., Clague, M. J., and Urbe, S. (**2004**). AMSH is an endosome-associated ubiquitin isopeptidase. *J. Cell Biol. 166*, 487–92.

Miranda, M., Wu, C. C., Sorkina, T., Korstjens, D., and Sorkin, A. (**2005**). Enhanced ubiquitylation and accelerated degradation of the dopamine transporter mediated by protein kinase C. *J. Biol. Chem. 280*, 35617–35624.

Mizuno, E., Iura, T., Mukai, A., Yoshimori, T., Kitamura, N., and Komada, M. (**2005**). Regulation of EGF receptor down-regulation by UBPY-mediated deubiquitination at endosomes. *Mol. Biol. Cell 16*, 5163–5174.

Morvan, J., Froissard, M., Haguenauer-Tsapis, R., and Urban-Grimal, D. (**2004**). The ubiquitin ligase Rsp5p is required for modification and sorting of membrane proteins into multivesicular bodies. *Traffic 5*, 383–92.

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J., and Yarden, Y. (**2003**). Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. *J. Biol. Chem. 278*, 21323–6.

Mueller, T. D. and Feigon, J. (**2002**). Solution structures of UBA domains reveal a conserved hydrophobic surface for protein-protein interactions. *J. Mol. Biol. 319*, 1243–55.

Naviglio, S., Mattecucci, C., Matoskova, B., Nagase, T., Nomura, N., Di Fiore, P. P., and Draetta, G. F. (**1998**). UBPY: a growth-regulated human ubiquitin isopeptidase. *Embo J. 17*, 3241–50.

Odorizzi, G., Katzmann, D. J., Babst, M., Audhya, A., and Emr, S. D. (**2003**). Bro1 is an endosome-associated protein that functions in the MVB pathway in *Saccharomyces cerevisiae*. *J. Cell Sci. 116*, 1893–903.

Papa, F. R. and Hochstrasser, M. (**1993**). The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. *Nature 366*, 313–9.

Papa, F. R., Amerik, A. Y., and Hochstrasser, M. (**1999**). Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome. *Mol. Biol. Cell 10*, 741–56.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (**2003**). A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol. 21*, 921–6.

Perier, F., Coulter, K. L., Liang, H., Radeke, C. M., Gaber, R. F., and Vandenberg, C. A. (**1994**). Identification of a novel mammalian member of the NSF/CDC48p/Pas1p/TBP-1 family through heterologous expression in yeast. *FEBS Lett. 351*, 286–90.

Perry, W. L., Hustad, C. M., Swing, D. A., O'Sullivan, T. N., Jenkins, N. A., and Copeland, N. G. (**1998**). The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nat. Genet. 18*, 143–6.

Peschard, P. and Park, M. (**2003**). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell 3*, 519–23.

Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. (**2001**). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol. Cell 8*, 995–1004.

Petrelli, A., Gilestro, G. F., Lanzardo, S., Comoglio, P. M., Migone, N., and Giordano, S. (**2002**). The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature 416*, 187–190.

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P. P. (**2002**). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature 416*, 451–455.

Pornillos, O., Alam, S. L., Rich, R. L., Myszka, D. G., Davis, D. R., and Sundquist, W. I. (**2002**). Structure and functional interactions of the Tsg101 UEV domain. *Embo J. 21*, 2397–406.

Pornillos, O., Higginson, D. S., Stray, K. M., Fisher, R. D., Garrus, J. E., Payne, M., He, G. P., Wang, H. E., Morham, S. G., and Sundquist, W. I. (**2003**). HIV Gag mimics the Tsg101-recruiting activity of

the human Hrs protein. *J. Cell Biol. 162*, 425–34.

Prag, G., Misra, S., Jones, E. A., Ghirlando, R., Davies, B. A., Horazdovsky, B. F., and Hurley, J. H. (**2003**). Mechanism of ubiquitin recognition by the CUE domain of Vps9p. *Cell 113*, 609–20.

Puertollano, R. (**2005**). Interactions of TOM1L1 with the multivesicular body sorting machinery. *J. Biol. Chem. 280*, 9258–64.

Puertollano, R. and Bonifacino, J. S. (**2004**). Interactions of GGA3 with the ubiquitin sorting machinery. *Nat. Cell Biol. 6*, 244–51.

Puertollano, R., Randazzo, P. A., Presley, J. F., Hartnell, L. M., and Bonifacino, J. S. (**2001**). The GGAs promote ARF-dependent recruitment of clathrin to the TGN. *Cell 105*, 93–102.

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T., and Liu, Y. C. (**2000**). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J. Biol. Chem. 275*, 35734–7.

Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E., and Stenmark, H. (**2002**). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nature Cell Biol. 4*, 394–398.

Raiborg, C., Gronvold Bache, K., Mehlum, A., Stang, E., and Stenmark, H. (**2001**). Hrs recruits clathrin to early endosomes. *EMBO J. 20*, 5008–5021.

Reggiori, F. and Pelham, H. R. (**2001**). Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. *EMBO J. 20*, 5176–5186.

Rocca, A., Lamaze, C., Subtil, A., and Dautry-Varsat, A. (**2001**). Involvement of the ubiquitin/proteasome system in sorting of the Interleukin 2 receptor β chain to late endocytic compartments. *Mol. Biol. Cell 12*, 1293–1301.

Rotin, D., Kanelis, V., and Schild, L. (**2001**). Trafficking and cell surface stability of ENaC. *Am. J. Physiol. Renal Physiol. 281*, F391–9.

Row, P. E., Clague, M. J., and Urbe, S. (**2005**). Growth factors induce differential phosphorylation profiles of the Hrs-Stam complex: a common node in signaling networks with signal specific properties. *Biochem. J. 389*, 629–636.

Sachse, M., Strous, G. J., and Klumperman, J. (**2004**). ATPase-deficient hVPS4 impairs formation of internal endosomal vesicles and stabilizes bilayered clathrin coats on endosomal vacuoles. *J. Cell Sci. 117*, 1699–708.

Sachse, M., Urbé, S., Oorschot, V., Strous, G. J., and Klumperman, J. (**2002**). Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes. *Mol. Biol. Cell 13*, 1313–1328.

Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K., and Hayashi, S. (**2004**). Drosophila Nedd4 regulates endocytosis of notch and suppresses its ligand-independent activation. *Curr. Biol. 14*, 2228–36.

Schmitz, C., Kinner, A., and Kolling, R. (**2005**). The deubiquitinating enzyme Ubp1 affects sorting of the ABC-transporter Ste6 in the endocytic pathway. *Mol. Biol. Cell. 16*, 1319–1329.

Scott, P. M., Bilodeau, P. S., Zhdankina, O., Winistorfer, S. C., Hauglund, M. J., Allaman, M. M., Kearney, W. R., Robertson, A. D., Boman, A. L., and Piper, R. C. (**2004**). GGA proteins bind ubiquitin to facilitate sorting at the trans-Golgi network. *Nat. Cell Biol. 6*, 252–9.

Seet, L. F., Liu, N., Hanson, B. J., and Hong, W. (**2004**). Endofin recruits TOM1 to endosomes. *J. Biol. Chem. 279*, 4670–9.

Shekhtman, A. and Cowburn, D. (**2002**). A ubiquitin-interacting motif from Hrs binds to and occludes the ubiquitin surface necessary for polyubiquitination in monoubiquitinated proteins. *Biochem. Biophys. Res. Commun. 296*, 1222–7.

Shiba, Y., Katoh, Y., Shiba, T., Yoshino, K., Takatsu, H., Kobayashi, H., Shin, H. W., Wakatsuki, S., and Nakayama, K. (**2004**). GAT (GGA and Tom1) domain responsible for ubiquitin binding and ubiquitination. *J. Biol. Chem. 279*, 7105–11.

Shih, S. C., Katzmann, D. J., Schnell, J. D., Sutanto, M., Emr, S. D., and Hicke, L. (**2002**). Epsins and Vs27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nature Cell Biol. 4*, 389–393.

Shih, S. C., Sloper-Mould, K. E., and Hicke, L. (**2000**). Monoubiquitin carries a

novel internalization signal that is appended to activated receptors. *Embo J. 19*, 187–98.

Slagsvold, T., Aasland, R., Hirano, S., Bache, K. G., Raiborg, C., Trambaiano, D., Wakatsuki, S., and Stenmark, H. (**2005**). Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. *J. Biol. Chem. 280*, 19600–19606.

Soares, L., Seroogy, C., Skrenta, H., Anandasabapathy, N., Lovelace, P., Chung, C. D., Engleman, E., and Fathman, C. G. (**2004**). Two isoforms of otubain 1 regulate T cell energy via GRAIL. *Nat. Immunol. 5*, 45–54.

Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W. Y., and Dikic, I. (**2002**). Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature 416*, 183–7.

Springael, J. Y. and Andre, B. (**1998**). Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*. *Mol. Biol. Cell 9*, 1253–63.

Springael, J. Y., Galan, J. M., Haguenauer-Tsapis, R., and Andre, B. (**1999**). NH4+-induced down-regulation of the *Saccharomyces cerevisiae* Gap1p permease involves its ubiquitination with lysine-63-linked chains. *J. Cell Sci.* **112** (Pt **9**), 1375–83.

Stuchell, M. D., Garrus, J. E., Muller, B., Stray, K. M., Ghaffarian, S., McKinnon, R., Krausslich, H. G., Morham, S. G., and Sundquist, W. I. (**2004**). The human endosomal sorting complex required for transport (ESCRT-I) and its role in HIV-1 budding. *J. Biol. Chem. 279*, 36059–71.

Sundquist, W. I., Schubert, H. L., Kelly, B. N., Hill, G. C., Holton, J. M., and Hill, C. P. (**2004**). Ubiquitin recognition by the human TSG101 protein. *Mol. Cell 13*, 783–9.

Swaminathan, S., Amerik, A. Y., and Hochstrasser, M. (**1999**). The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. *Mol. Biol. Cell 10*, 2583–94.

Swanson, K. A., Kang, R. S., Stamenova, S. D., Hicke, L., and Radhakrishnan, I. (**2003**). Solution structure of Vps27 UIM-ubiquitin complex important for endosomal sorting and receptor downregulation. *Embo J. 22*, 4597–606.

Tanaka, N., Kaneko, K., Asao, H., Kasai, H., Endo, Y., Fujita, T., Takeshita, T., and Sugamura, K. (**1999**). Possible involvement of a novel STAM-associated molecule "AMSH" in intracellular signal transduction mediated by cytokines. *J. Biol. Chem. 274*, 19129–35.

Teo, H., Perisic, O., Gonzalez, B., and Williams, R. L. (**2004**). ESCRT-II, an endosome-associated complex required for protein sorting: crystal structure and interactions with ESCRT-III and membranes. *Dev. Cell 7*, 559–69.

Terrel, J., Shih, S., Dunn, R., and Hicke, L. (**1998**). *Mol. Cell 1*, 193–202.

Thien, C. B. F. and Langdon, W. Y. (**2001**). Cbl: many adaptions to regulate protein tyrosine kinases. *Nature Rev. 2*, 294–305.

Thien, C. B. F., Walker, F., and Langdon, W. Y. (**2001**). Ring finger mutations that abolish c-Cbl directed polyubiquitination and down-regulation of the EGF receptor are insufficient for cell transformation. *Mol. Cell 7*, 355–365.

Torrisi, M. R., Lotti, L. V., Belleudi, F., Gradini, R., Salcini, A. E., Confalonieri, S., Pelicci, P. G., and Di Fiore, P. P. (**1999**). Eps15 is recruited to the plasma membrane upon epidermal growth factor receptor activation and localizes to components of the endocytic pathway during receptor internalization. *Mol. Biol. Cell 10*, 417–34.

Traub, L. M. (**2003**). Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. *J. Cell Biol. 163*, 203–8.

Urbanowski, J. L. and Piper, R. C. (**2001**). Ubiquitin sorts proteins into the intralumenal degradative compartment of the late endosome/vacuole. *Traffic 2*, 622–630.

Urbé, S., Mills, I. G., Stenmark, H., Kitamura, N., and Clague, M. J. (**2000**). Endosomal localization and receptor dynamics determine tyrosine phosphorylation of hepatocyte growth factor-regulated tyrosine kinase substrate. *Mol. Cell Biol. 20*, 7685–92.

Urbé, S., Sachse, M., Row, P. E., Preisinger, C., Barr, F. A., Strous, G., Klumperman, J., and Clague, M. J. (**2003**). The UIM domain of Hrs couples receptor sorting to vesicle formation. *J. Cell Sci. 116*, 4169–4179.

Vadlamudi, R. K., Joung, I., Strominger, J. L., and Shin, J. (**1996**). p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. *J. Biol. Chem. 271*, 20235–7.

van Kerkhof, P., Govers, R., Alves dos Santos, C. M., and Strous, G. J. (**2000**). Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. *J. Biol. Chem. 275*, 1575–1580.

Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (**2002**). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science 298*, 611–15.

von Schwedler, U. K., Stuchell, M., Muller, B., Ward, D. M., Chung, H. Y., Morita, E., Wang, H. E., Davis, T., He, G. P., Cimbora, D. M., Scott, A., Kransslich, H. G., Kaplan, J., Morham, S. G., and Sundquist, W. J. (**2003**). The protein network of HIV budding. *Cell 114*, 701–13.

Wang, G., McCaffery, J. M., Wendland, B., Dupre, S., Haguenauer-Tsapis, R., and Huibregtse, J. M. (**2001**). Localization of the Rsp5p ubiquitin-protein ligase at multiple sites within the endocytic pathway. *Mol. Cell Biol. 21*, 3564–75.

Whitley, P., Reaves, B. J., Hashimoto, M., Riley, A. M., Potter, B. V., and Holman, G. D. (**2003**). Identification of mammalian Vps24p as an effector of phosphatidylinositol 3,5-bisphosphate-dependent endosome compartmentalization. *J. Biol. Chem. 278*, 38786–95.

Wu, X., Yen, L., Irwin, L., Sweeney, C., and Carraway, K. L., 3rd. (**2004**). Stabilization of the E3 ubiquitin ligase Nrdp1 by the deubiquitinating enzyme USP8. *Mol. Cell Biol. 24*, 7748–57.

Yamakami, M., Yoshimori, T., and Yokosawa, H. (**2003**). Tom1, a VHS domain-containing protein, interacts with tollip, ubiquitin, and clathrin. *J. Biol. Chem. 278*, 52865–72.

Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U. et al. (**1986**). Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature 323*, 226–32.

Yoshimori, T., Yamagata, F., Yamamoto, A., Mizushima, N., Kabeya, Y., Nara, A., Miwako, I., Ohashi, M., Ohsumi, M., and Ohsumi, Y. (**2000**). The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol. Biol. Cell 11*, 747–63.

Zhu, Y., Doray, B., Poussu, A., Lehto, V. P., and Kornfeld, S. (**2001**). Binding of GGA2 to the lysosomal enzyme sorting motif of the mannose 6-phosphate receptor. *Science 292*, 1716–8.

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).

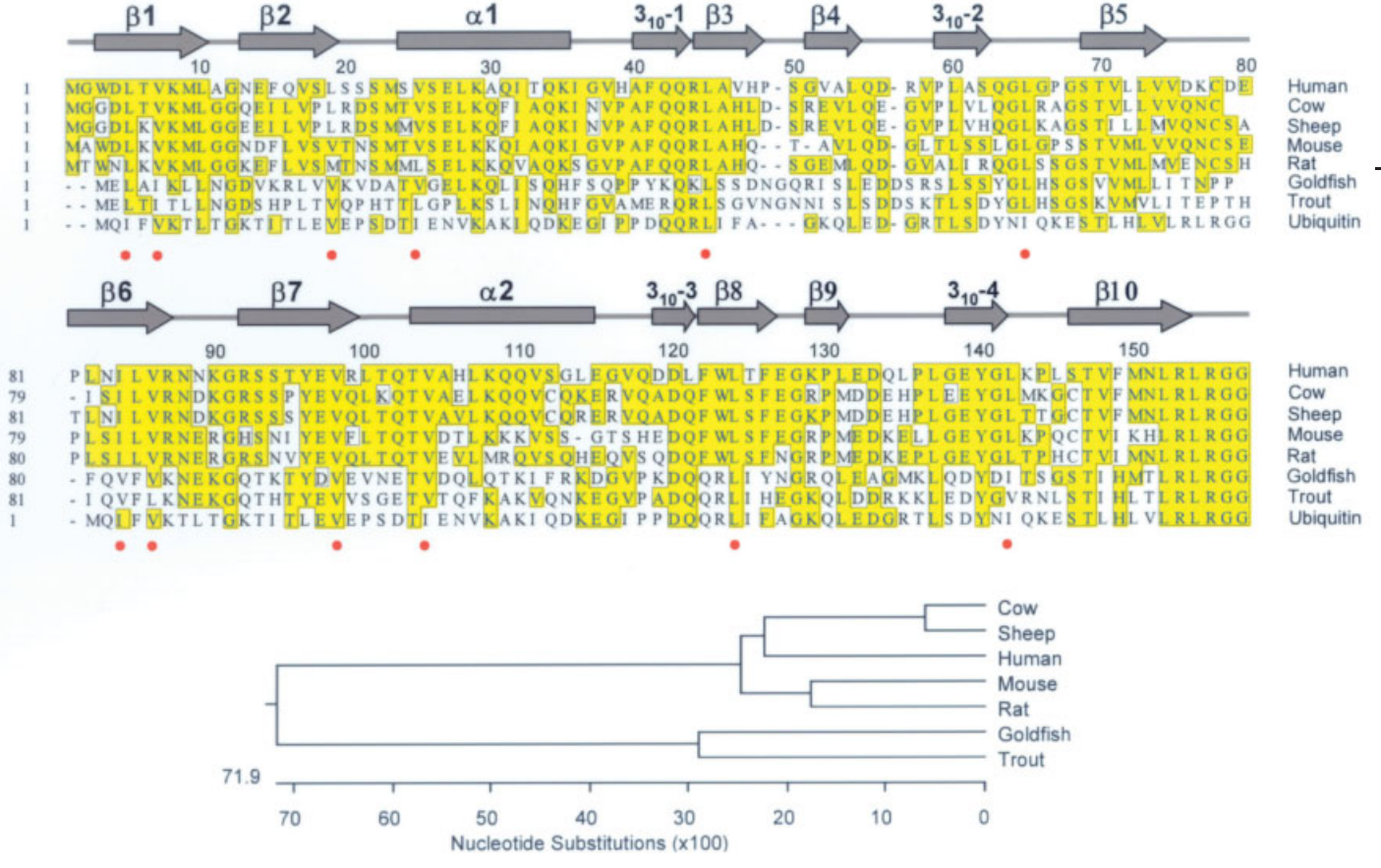

Fig. 5.1. Sequence comparison of ISG15 orthologs to ubiquitin. Upper panel: Sequences for the extant ISG15 orthologs are compared to human ubiquitin using Clustal W and a PAM250 residue weight table. Sequences are segregated into the two ubiquitin-like domains based on sequence homology. Secondary structural 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

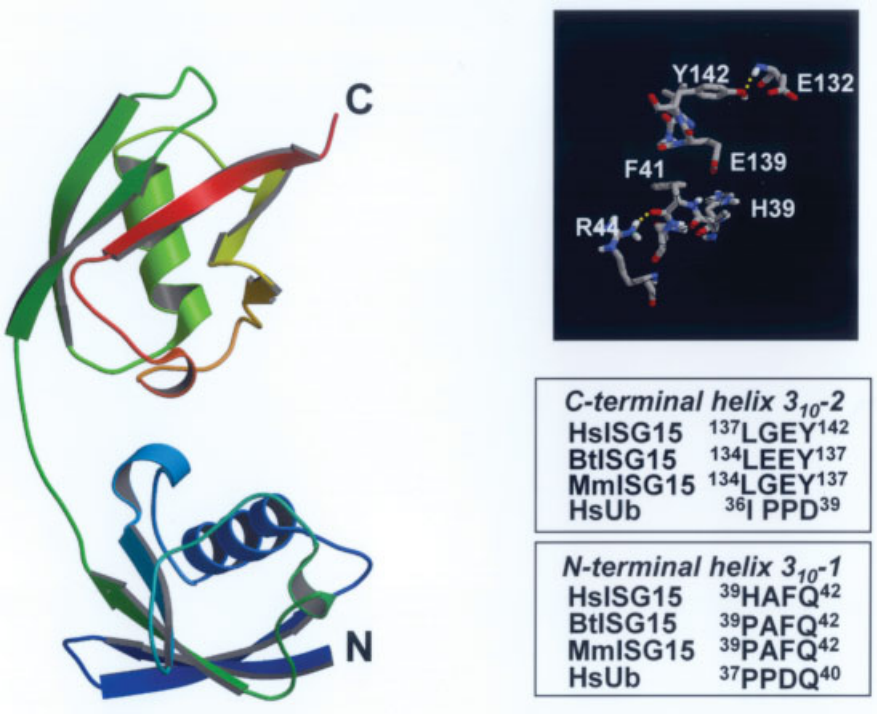

Fig. 5.2. Crystal structure of human ISG15. Left panel: The 2.4 Å crystal structure for Human ISG15 harbouring a C78S point mutation to enhance solution stability against spontaneous denaturation [28]. Colours for the ribbon diagram range from dark blue for the amino terminus to red for the carboxyl 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-

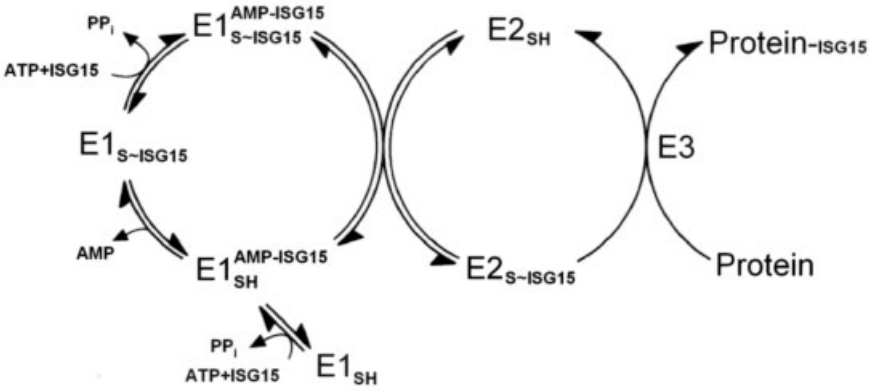

Fig. 5.3. Mechanism of ISG15 conjugation. The mechanism of ISG15 conjugation is composed of three enzymes as described in the text. E1: ISG15 activating enzyme; E2: ISG15 carrier protein (UbcH8); E3: ISG15 isopeptide ligase.

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

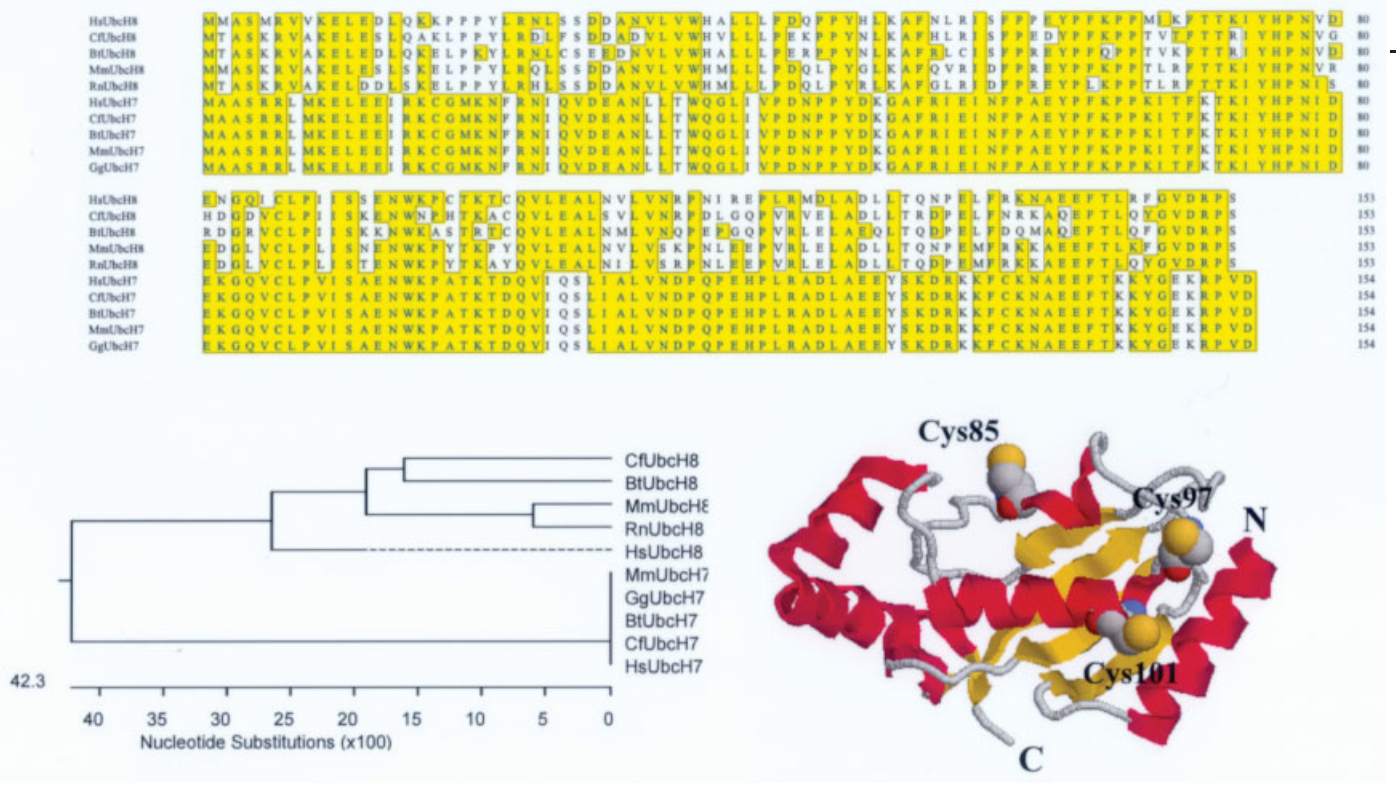

Fig. 5.4. Sequence comparison of UbcH7 and UbcH8 orthologs. Top panel: Sequence comparison of extant UbcH7 and UbcH8 orthologs by the Clustal W method using a PAM250 residue weight table. Lower left panel: Phylogenetic comparison of UbcH8 and UbcH7 orthologs. Lower right panel: Crystal structure of human 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.

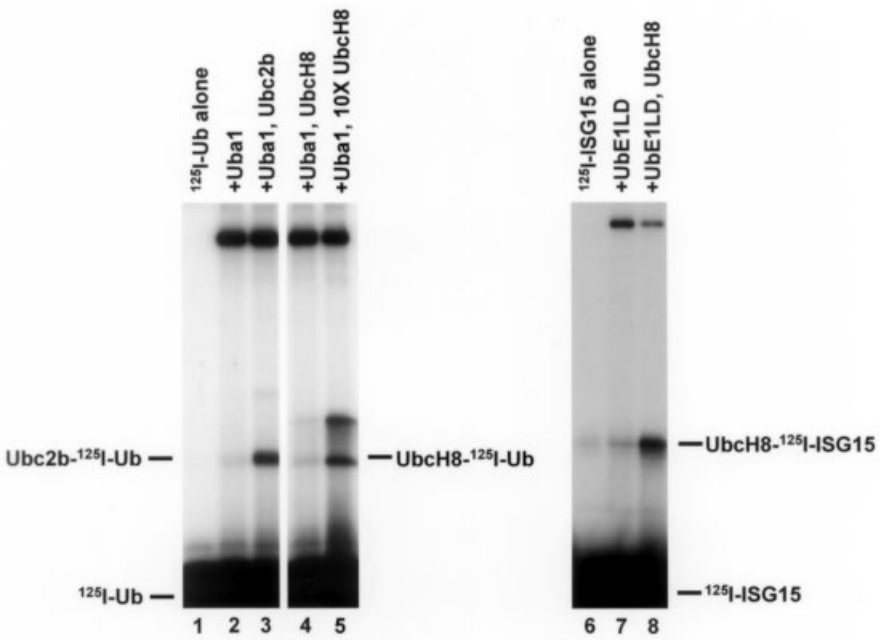

Fig. 5.5. Functional analysis of UbcH8. Autoradiogram of end-point functional thiolester assays of Uba1- (left panel, lanes 1–5) or UbE1L- (right panel, lanes 6–8) catalyzed ^{125}I-ubiquitin (left panel) or ^{125}I-ISG15 transthiolation to recombinant UbcH8. Assays were conducted as described previously 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

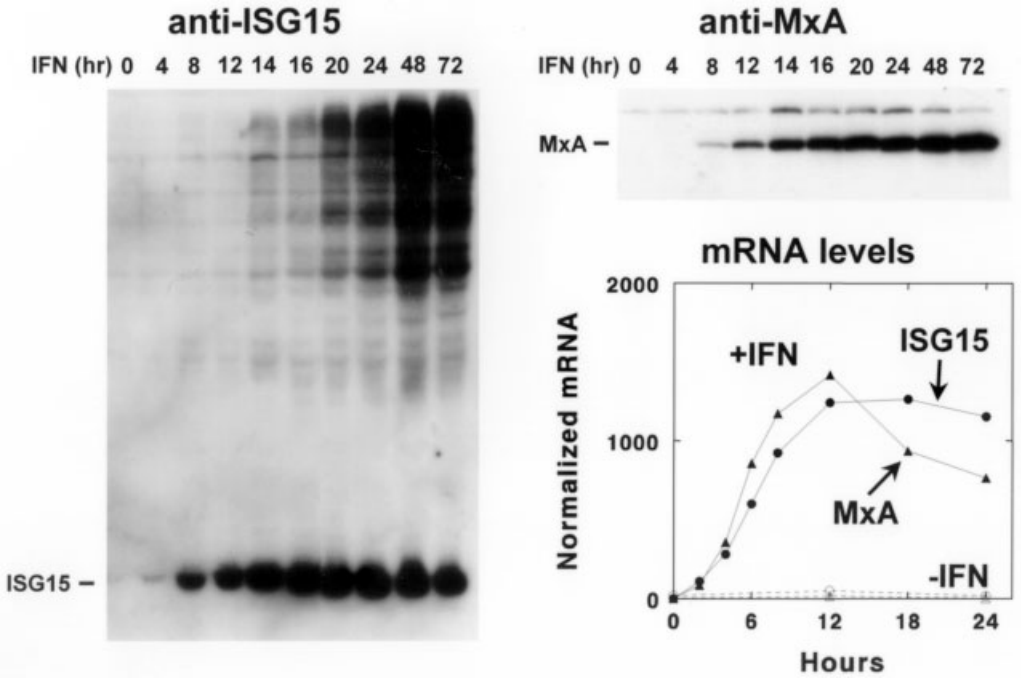

Fig. 5.6. Biphasic induction of ISG15 pools. Left panel: Western blot showing the biphasic induction of free and conjugated ISG15 pools following addition of IFNβ (10^3 IU ml^{-1}) to confluent A549 cultures [21]. Upper right panel: Western blot showing the induction of antiviral MxA protein in the same 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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References

1 A. Ciechanover, H. Heller, S. Elias, A. L. Haas, A. Hershko *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 1365–1368.
2 A. Hershko, A. Ciechanover, H. Heller, A. L. Haas, I. A. Rose *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 1783–1786.
3 K. D. Wilkinson, M. K. Urban, A. L. Haas *J. Biol. Chem.* **1980**, *255*, 7529–7532.

4 M. W. Lake, M. M. Wuebbens, K. V. Rajagopalan, H. Schindelin *Nature* **2001**, *414*, 325–329.

5 M. J. Rudolph, M. M. Wuebbens, K. V. Rajagopalan, H. Schindelin *Nat. Struct. Biol.* **2001**, *8*, 42–46.

6 M. J. Rudolph, M. M. Wuebbens, O. Turque, K. V. Rajagopalan, H. Schindelin *J. Biol. Chem.* **2003**, *278*, 14514–14522.

7 C. N. Larsen, H. Wang *J. Proteome Res.* **2002**, *1*, 411–419.

8 A. L. Haas, T. J. Siepmann *Faseb J.* **1997**, *11*, 1257–1268.

9 E. S. Johnson, R. J. Schwienhorst, R. J. Dohmen, G. Blobel *Embo J.* **1997**, *16*, 5509–5519.

10 T. Okuma, R. Honda, G. Ichikawa, N. Tsumagari, H. Yasuda *Biochem. Biophys. Res. Commun.* **1999**, *254*, 693–698.

11 D. Liakopoulos, G. Doenges, K. Matuschewski, S. Jentsch *Embo J.* **1998**, *17*, 2208–2214.

12 F. Osaka, H. Kawasaki, N. Aida, M. Saeki, T. Chiba, S. Kawashima, K. Tanaka, S. Kato *Genes Devel.* **1998**, *12*, 2263–2268.

13 G. A. Dittmar, C. R. Wilkinson, P. T. Jedrzejewski, D. Finley *Science* **2002**, *295*, 2442–2446.

14 I. Tanida, N. Mizushima, M. Kiyooka, M. Ohsumi, T. Ueno, Y. Ohsumi, E. Kominami *Mol. Biol. Cell* **1999**, *10*, 1367–1379.

15 I. Tanida, E. Tanida-Miyake, T. Ueno, E. Kominami *J. Biol. Chem.* **2001**, *276*, 1701–1706.

16 I. Tanida, M. Komatsu, T. Ueno, E. Kominami *Biochem. Biophys. Res. Commun.* **2003**, *300*, 637–644.

17 K. Furukawa, N. Mizushima, T. Noda, Y. Ohsumi *J. Biol. Chem.* **2000**, *275*, 7462–7465.

18 Y. C. Liu, J. Pan, C. Zhang, W. Fan, M. Collinge, J. R. Bender, S. M. Weissman *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4313–4318.

19 S. Raasi, G. Schmidtke, M. Groettrup *J. Biol. Chem.* **2001**, *276*, 35334–35343.

20 A. L. Haas, P. Ahrens, P. M. Bright, H. Ankel *J. Biol. Chem.* **1987**, *262*, 11315–11323.

21 K. R. Loeb, A. L. Haas *J. Biol. Chem.* **1992**, *267*, 7806–7813.

22 P. J. Farrell, R. J. Broeze, P. Lengyel *Nature* **1979**, *279*, 523–525.

23 B. D. Korant, D. C. Blomstrom, G. J. Jonak, E. Knight, Jr. *J. Biol. Chem.* **1984**, *259*, 14835–14839.

24 D. C. Blomstrom, D. Fahey, R. Kutny, B. D. Korant, E. Knight, Jr. *J. Biol. Chem.* **1986**, *261*, 8811–8816.

25 E. Knight, Jr., B. Cordova *J. Immunol.* **1991**, *146*, 2280–2284.

26 N. Reich, B. Evans, D. Levy, D. Fahey, E. J. Knight, J. E. J. Darnell *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6394–6398.

27 C. N. Pace, B. A. Shirley, J. A. Thomson "Measuring the conformational stability of a protein." In: *Protein Structure: A Practical Approach*; T. E. Creighton, ed. IRL Press-Oxford University Press, Oxford, **1989**, pp 311–330.

28 J. Narasimhan, M. Wang, Z. Fu, J. M. Klein, A. L. Haas, J. J. Kim *J. Biol. Chem.* **2005**, *280*, 27356–27365.

29 J. Narasimhan, J. L. Potter, A. L. Haas *J. Biol. Chem.* **1996**, *271*, 324–330.

30 R. Day, D. A. Beck, R. S. Armen, V. Daggett *Protein Sci.* **2003**, *12*, 2150–2160.

31 K. J. Ritchie, D. E. Zhang *Semin. Cell Dev. Biol.* **2004**, *15*, 237–246.

32 J. L. Potter, J. Narasimhan, L. Mende-Mueller, A. L. Haas *J. Biol. Chem.* **1999**, *274*, 25061–25068.

33 C. Schmitz, A. Kinner, R. Kölling *Mol. Biol. Cell* **2005**, *16*, 1319–1329.

34 M. P. Malakhov, O. A. Malakhova, K. I. Kim, K. J. Ritchie, D. E. Zhang *J. Biol. Chem.* **2002**, *277*, 9976–9981.

35 K. D. Wilkinson *Semin. Cell Dev. Biol.* **2000**, *11*, 141–148.

36 C. Zhao, C. Denison, J. M. Huibregtse, S. Gygi, R. M. Krug *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10200–10205.

37 N. V. Giannakopoulos, J. K. Luo, V. Papov, W. Zou, D. J. Lenschow, B. S. Jacobs, E. C. Borden, J. Li, H. W. Virgin, D. E. Zhang *Biochem. Biophys. Res. Commun.* **2005**, *336*, 496–506.

38 C. M. Pickart *Annu. Rev. Biochem.* **2001**, *70*, 503–533.

39 R. L. Welchman, C. Gordon, R. J. Mayer *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 599–609.

40 A. L. Haas, J. V. Warms, A. Hershko, I. A. Rose *J. Biol. Chem.* **1982**, *257*, 2543–2548.

41 A. L. Haas, I. A. Rose *J. Biol. Chem.* **1982**, *257*, 10329–10337.

42 R. N. Bohnsack, A. L. Haas *J. Biol. Chem.* **2003**, *278*, 26823–26830.

43 W. Yuan, R. M. Krug *Embo J.* **2001**, *20*, 362–371.

44 B. Carritt, K. Kok, A. van den Berg, J. Osinga, A. Pilz, R. M. Hofstra, M. B. Davis, A. Y. van der Veen, P. H. Rabbitts, K. Gulati *Cancer Res.* **1992**, *52*, 1536–1541.

45 K. Kok, R. Hofstra, A. Pilz, A. van den Berg, P. Terpstra, C. H. Buys, B. Carritt *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 6071–6075.

46 P. M. McLaughlin, W. Helfrich, K. Kok, M. Mulder, S. W. Hu, M. G. Brinker, M. H. Ruiters, L. F. de Leij, C. H. Buys *Int. J. Cancer* **2000**, *85*, 871–876.

47 P. M. Handley-Gearhart, A. G. Stephen, J. S. Trausch-Azar, A. Ciechanover, A. L. Schwartz *J. Biol. Chem.* **1994**, *269*, 33171–33178.

48 I. Pitha-Rowe, W. J. Petty, Q. Feng, P. H. Koza-Taylor, D. A. Dimattia, L. Pinder, K. H. Dragnev, N. Memoli, V. Memoli, T. Turi, J. Beebe, S. Kitareewan, E. Dmitrovsky *Cancer Res.* **2004**, *64*, 8109–8115.

49 F. G. Whitby, G. Xia, C. M. Pickart, C. P. Hill *J. Biol. Chem.* **1998**, *273*, 34983–34991.

50 H. Walden, M. S. Podgorski, D. T. Huang, D. W. Miller, R. J. Howard, D. L. Minor, J. M. Holton, B. A. Schulman *Mol. Cell* **2003**, *12*, 1427–1437.

51 L. M. Lois, C. D. Lima *Embo J.* **2005**, *24*, 439–451.

52 H. Walden, M. S. Podgorski, B. A. Schulman *Nature* **2003**, *422*, 330–334.

53 T. J. Siepmann, R. N. Bohnsack, Z. Tokgöz, O. V. Baboshina, A. L. Haas *J. Biol. Chem.* **2003**, *278*, 9448–9457.

54 K. I. Kim, N. V. Giannakopoulos, H. W. Virgin, D. E. Zhang *Mol. Cell Biol.* **2004**, *24*, 9592–9600.

55 T. A. Nyman, S. Matikainen, T. Sareneva, I. Julkunen, N. Kalkkinen *Eur. J. Biochem.* **2000**, *267*, 4011–4019.

56 C. Zhao, S. L. Beaudenon, M. L. Kelley, M. B. Waddell, W. Yuan, B. A. Schulman, J. M. Huibregtse, R. M. Krug *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7578–7582.

57 I. Pitha-Rowe, B. A. Hassel, E. Dmitrovsky *J. Biol. Chem.* **2004**, *279*, 18178–18187.

58 S. Kitareewan, I. Pitha-Rowe, D. Sekula, C. H. Lowrey, M. J. Nemeth, T. R. Golub, S. J. Freemantle, E. Dmitrovsky *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 3806–3811.

59 K. Kok, A. van den Berg, P. M. Veldhuis, A. Y. van der Veen, M. Franke, E. F. Schoenmakers, M. M. Hulsbeek, A. H. van der Hout, L. de Leij, W. van de Ven *Cancer Res.* **1994**, *54*, 4183–4187.

60 B. Sarcevic, A. Mawson, R. T. Baker, R. L. Sutherland *Embo J.* **2002**, *21*, 2009–2018.

61 S. Kumar, W. H. Kao, P. M. Howley *J. Biol. Chem.* **1997**, *272*, 13548–13554.

62 P. Kaiser, W. Seufert, L. Hofferer, B. Kofler, C. Sachsenmaier, H. Herzog, S. Jentsch, M. Schweiger, R. Schneider *J. Biol. Chem.* **1994**, *269*, 8797–8802.

63 S. Qin, B. Nakajima, M. Nomura, S. M. Arfin *J. Biol. Chem.* **1991**, *266*, 15549–15554.

64 T. P. Moynihan, H. C. Ardley, J. P. Leek, J. Thompson, N. S. Brindle, A. F. Markham, P. A. Robinson *Mammalian Genome* **1996**, *7*, 520–525.

65 U. Nuber, S. Schwarz, P. Kaiser, R. Schneider, M. Scheffner *J. Biol. Chem.* **1996**, *271*, 2795–2800.

66 A. I. Su, M. P. Cooke, K. A. Ching, Y. Hakak, J. R. Walker, T. Wiltshire, A. P. Orth, R. G. Vega, L. M. Sapinoso, A. Moqrich, A. Patapoutian, G. M. Hampton, P. G.

Schultz, J. B. Hogenesch *Proc. Natl. Acad. Sci. USA* **2002**.
67 M. Kimura, T. Hattori, Y. Matsuda, T. Yoshioka, N. Sumi, Y. Umeda, S. Nakashima, Y. Okano *Cytogenet. Cell Genet.* **1997**, *78*, 107–111.
68 J. M. Huibregtse, M. Scheffner, P. M. Howley *Embo J.* **1991**, *10*, 4129–4135.
69 J. M. Huibregtse, M. Scheffner, P. M. Howley *Mol. Cell. Biol.* **1993**, *13*, 775–784.
70 Q. Gao, L. Singh, A. Kumar, S. Srinivasan, D. E. Wazer, V. Band *J. Virol.* **2001**, *75*, 4459–4466.
71 Y. Zhang, J. Gao, K. K. Chung, H. Huang, V. L. Dawson, T. M. Dawson *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13354–13359.
72 K. K. Chung, Y. Zhang, K. L. Lim, Y. Tanaka, H. Huang, J. Gao, C. A. Ross, V. L. Dawson, T. M. Dawson *Nat. Med.* **2001**, *7*, 1144–1150.
73 J. Niwa, S. Ishigaki, N. Hishikawa, M. Yamamoto, M. Doyu, S. Murata, K. Tanaka, N. Taniguchi, G. Sobue *J. Biol. Chem.* **2002**, *277*, 36793–36798.
74 L. S. Chin, J. P. Vavalle, L. Li *J. Biol. Chem.* **2002**, *277*, 35071–35079.
75 M. K. Tripathi, G. Chaudhuri *Biochem. Biophys. Res. Commun.* **2005**, *328*, 43–48.
76 A. L. Haas, P. M. Bright *J. Biol. Chem.* **1988**, *263*, 13258–13267.
77 S. Jentsch, J. P. McGrath, A. Varshavsky *Nature* **1987**, *329*, 131–134.
78 A. L. Haas, P. B. Reback, V. Chau *J. Biol. Chem.* **1991**, *266*, 5104–5112.
79 K. L. Lorick, J. P. Jensen, S. Fang, A. M. Ong, S. Hatakeyama, A. M. Weissman *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11364–11369.
80 C. A. Joazeiro, A. M. Weissman *Cell* **2000**, *102*, 549–552.
81 Y. Zhang, J. Gao, K. K. Chung, H. Huang, V. L. Dawson, T. M. Dawson *Proc. Natl. Acad. Sci. USA* **2005**, *97*, 13354–13359.
82 T. C. Wheeler, L. S. Chin, Y. Li, F. L. Roudabush, L. Li *J. Biol. Chem.* **2002**, *277*, 10273–10282.
83 J. Niwa, S. Ishigaki, M. Doyu, T. Suzuki, K. Tanaka, G. Sobue *Biochem. Biophys. Res. Commun.* **2001**, *281*, 706–713.
84 A. Dastur, S. Beaudenon, M. Kelley, R. M. Krug, J. M. Huibregtse *J. Biol. Chem.* **2006**, *281*, 4334–4338.
85 T. Urano, T. Saito, T. Tsukui, M. Fujita, T. Hosoi, M. Muramatsu, Y. Ouchi, S. Inoue *Nature* **2002**, *417*, 871–875.
86 W. Zou, D. E. Zhang *J. Biol. Chem.* **2006**, *281*, 3989–3994.
87 M. Scheffner, J. M. Huibregtse, R. D. Vierstra, P. M. Howley *Cell* **1993**, *75*, 495–505.
88 T. Suzuki, T. Urano, T. Tsukui, K. Horie-Inoue, T. Moriya, T. Ishida, M. Muramatsu, Y. Ouchi, H. Sasano, S. Inoue *Clin. Cancer Res.* **2005**, *11*, 6148–6154.
89 K. Hochrainer, H. Mayer, U. Baranyi, B. Binder, J. Lipp, R. Kroismayr *Genomics* **2005**, *85*, 153–164.
90 H. D. Ulrich *Trends. Cell Biol.* **2005**, *15*, 525–532.
91 L. Haracska, C. A. Torres-Ramos, R. E. Johnson, S. Prakash, L. Prakash *Mol. Cell Biol.* **2004**, *24*, 4267–4274.
92 S. D. Desai, A. L. Haas, L. M. Wood, Y. C. Tsai, S. Pestka, E. H. Rubin, A. Saleem, E. K. Nur, L. F. Liu *Cancer Res.* **2006**, *66*, 921–928.
93 M. Liu, X. L. Li, B. A. Hassel *J. Biol. Chem.* **2003**, *278*, 1594–1602.
94 T. Ito, J. Niwa, N. Hishikawa, S. Ishigaki, M. Doyu, G. Sobue *J. Biol. Chem* **2003**, *278*, 29106–29114.
95 H. Takeuchi, J. Niwa, N. Hishikawa, S. Ishigaki, F. Tanaka, M. Doyu, G. Sobue *J. Neurochem.* **2004**, *89*, 64–72.
96 E. Poutiainen, L. Hokkanen, M. L. Niemi, M. Farkkila *Pharmacol. Biochem. Behav.* **1994**, *47*, 901–905.
97 J. Huang, L. G. Xu, T. Liu, Z. Zhai, H. B. Shu *FEBS Lett.* **2006**, *580*, 940–947.
98 A. Y. Nikolaev, M. Li, N. Puskas, J. Qin, W. Gu *Cell* **2003**, *112*, 29–40.
99 I. Márin, J. I. Lucas, A. C. Gradilla, A. Ferrús *Physiol. Genomics.* **2004**, *17*, 253–263.
100 C. C. Ng, H. Arakawa, S. Fukuda, H. Kondoh, Y. Nakamura *Oncogene* **2003**, *22*, 4449–4458.

101 T. P. Moynihan, H. C. Ardley, U. Nuber, S. A. Rose, P. F. Jones, A. F. Markham, M. Scheffner, P. A. Robinson *J. Biol. Chem.* **1999**, *274*, 30963–30968.

102 J. M. Huibregtse, M. Scheffner, S. Beaudenon, P. M. Howley *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2563–2567.

103 M. Scheffner, B. A. Werness, J. M. Huibregtse, A. J. Levine, P. M. Howley *Cell* **1990**, *63*, 1129–1136.

104 A. L. Haas, P. M. Bright *J. Biol. Chem.* **1985**, *260*, 12464–12473.

105 A. Haas "Immunochemical probes of ubiquitin pool dynamics." In: *Ubiquitin*; M. Rechsteiner, ed. Plenum Press, New York, **1988**, 173–206.

106 A. L. Haas, P. M. Bright *J. Biol. Chem.* **1987**, *262*, 345–351.

107 R. Medina, S. S. Wing, A. Haas, A. L. Goldberg *Biomed. Biochim. Acta* **1991**, *50*, 347–356.

108 J. Lowe, H. McDermott, K. Loeb, M. Landon, A. L. Haas, R. J. Mayer *J. Pathology* **1995**, *177*, 163–169.

109 K. R. Loeb, A. L. Haas *Mol. Cell. Biol.* **1994**, *14*, 8408–8419.

110 A. L. Haas, I. A. Rose *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6845–6848.

111 L. Q. Liu, R. J. Ilaria, P. D. Kingsley, A. Iwama, R. A. van Etten, J. Palis, D. E. Zhang *Mol. Cell Biol.* **1999**, *19*, 3029–3038.

112 O. Malakhova, M. Malakhov, C. Hetherington, D. E. Zhang *J. Biol. Chem.* **2002**, *277*, 14703–14711.

113 H. Schwer, L. Q. Liu, L. Zhou, M. T. Little, Z. Pan, C. J. Hetherington, D. E. Zhang *Genomics* **2000**, *65*, 44–52.

114 O. A. Malakhova, M. Yan, M. P. Malakhov, Y. Yuan, K. J. Ritchie, K. I. Kim, L. F. Peterson, K. Shuai, D. E. Zhang *Genes Dev.* **2003**, *17*, 455–460.

115 K. J. Ritchie, C. S. Hahn, K. I. Kim, M. Yan, D. Rosario, L. Li, J. C. de la Torre, D. E. Zhang *Nat. Med.* **2004**, *10*, 1374–1378.

116 K. I. Kim, O. A. Malakhova, K. Hoebe, M. Yan, B. Beutler, D. E. Zhang *J. Immunol.* **2005**, *175*, 847–854.

117 S. Tokarz, C. Berset, J. La Rue, K. Friedman, K. Nakayama, D. E. Zhang, S. Lanker *J. Biol. Chem.* **2004**, *279*, 46424–46430.

118 A. Osiak, O. Utermöhlen, S. Niendorf, I. Horak, K. P. Knobeloch *Mol. Cell Biol.* **2005**, *25*, 6338–6345.

119 K. P. Knobeloch, O. Utermohlen, A. Kisser, M. Prinz, I. Horak *Mol. Cell Biol.* **2005**, *25*, 11030–11034.

120 K. I. Kim, M. Yan, O. Malakhova, J. K. Luo, M. F. Shen, W. Zou, J. C. de la Torre, D. E. Zhang *Mol. Cell Biol.* **2006**, *26*, 472–479.

121 J. D'Cunha, S. Ramanujam, R. J. Wagner, P. L. Witt, E. J. Knight, E. C. Borden *J. Immunol.* **1996**, *157*, 4100–4108.

122 J. L. Taylor, J. D'Cunha, P. Tom, W. J. O'Brien, E. C. Borden *J. Interfer. Cytokine Res.* **1996**, *16*, 937–940.

123 K. Ishii, M. Kurita-Taniguchi, M. Aoki, T. Kimura, Y. Kashiwazaki, M. Matsumoto, T. Seya *Immunol. Lett.* **2005**, *98*, 280–290.

124 S. G. Park, H. J. Kim, Y. H. Min, E. C. Choi, Y. K. Shin, B. J. Park, S. W. Lee, S. Kim *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 6356–6361.

125 M. Recht, E. C. Borden, E. Knight, Jr. *J. Immunol.* **1991**, *147*, 2617–2623.

126 J. D'Cunha, E. J. Knight, A. L. Haas, R. L. Truitt, E. C. Borden *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 211–215.

127 E. Padovan, L. Terracciano, U. Certa, B. Jacobs, A. Reschner, M. Bolli, G. C. Spagnoli, E. C. Borden, M. Heberer *Cancer Res.* **2002**, *62*, 3453–3458.

128 S. Jamal, R. J. Schneider *J. Clin. Invest.* **2002**, *110*, 443–452.

129 M. Owhashi, Y. Taoka, K. Ishii, S. Nakazawa, H. Uemura, H. Kambara *Biochem. Biophys. Res. Commun.* **2003**, *309*, 533–539.

130 G. Goldstein, M. Scheid, U. Hammerling, E. A. Boyse, D. H. Schlesinger, H. D. Niall *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 11–15.

131 W. A. Kagan, G. J. O'Neill, G. S. Incefy, G. Goldstein, R. A. Good *Blood* **1977**, *50*, 275–288.

132 D. H. Schlesinger, G. Goldstein,

H. D. Niall *Biochemistry* **1975**, *14*, 2214–2218.
133 D. H. Schlesinger, G. Goldstein, M. P. Scheid, M. Bitensky *Experientia* **1978**, *34*, 703–704.
134 C. Daly, N. C. Reich *J. Biol. Chem.* **1995**, *270*, 23739–23746.
135 B. T. Hummer, X. L. Li, B. A. Hassel *J. Virol.* **2001**, *75*, 7774–7777.
136 M. J. Kim, A. G. Latham, R. M. Krug *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 10096–10101.
137 G. C. MacQuillan, C. Mamotte, W. D. Reed, G. P. Jeffrey, J. E. Allan *J. Med. Virol.* **2003**, *70*, 219–227.
138 J. Zhang, S. C. Das, C. Kotalik, A. K. Pattnaik, L. Zhang *J. Biol. Chem.* **2004**, *279*, 46335–46342.
139 D. J. Lenschow, N. V. Giannakopoulos, L. J. Gunn, C. Johnston, A. K. O'guin, R. E. Schmidt, B. Levine, H. W. Virgin *J. Virol.* **2005**, *79*, 13974–13983.
140 J. Li, G. W. Peet, D. Balzarano, X. Li, P. Massa, R. W. Barton, K. B. Marcu *J. Biol. Chem.* **2001**, *276*, 18579–18590.
141 M. Masilamani, D. Kassahn, S. Mikkat, M. O. Glocker, H. Illges *Eur. J. Immunol.* **2003**, *33*, 2391–2397.
142 P. M. Martensen, J. Justesen *J. Interfer. Cytokine Res.* **2004**, *24*, 1–19.
143 X. L. Li, M. Boyanapalli, X. Weihua, D. V. Kalvakolanu, B. A. Hassel *J. Interfer. Cytokine Res.* **1998**, *18*, 947–952.
144 R. Kroismayr, U. Baranyi, C. Stehlik, A. Dorfleutner, B. R. Binder, J. Lipp *J. Cell Sci.* **2004**, *117*, 4749–4756.
145 W. Yuan, J. M. Aramini, G. T. Montelione, R. M. Krug *Virology* **2002**, *304*, 291–301.
146 M. S. Kunzi, P. M. Pitha *J. Interfer Cytokine Res.* **1996**, *16*, 919–927.
147 A. Okumura, G. Lu, I. Pitha-Rowe, P. M. Pitha *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 1440–1445.
148 A. Ploubidou, M. Way *Curr. Opin. Cell Biol.* **2001**, *13*, 97–105.
149 C. Bebington, S. C. Bell, F. J. Doherty, A. T. Fazleabas, S. D. Fleming *Biol. Reprod.* **1999**, *60*, 920–928.
150 C. DeRenzo, G. Seydoux *Trends Cell Biol.* **2004**, *14*, 420–426.
151 K. J. Austin, S. K. Ward, M. G. Teixeira, V. C. Dean, D. W. Moore, T. R. Hansen *Biol. Reprod.* **1996**, *54*, 600–606.
152 D. P. Barlow, B. J. Randle, D. C. Burke *Differentiation* **1984**, *27*, 229–235.
153 R. M. Roberts, J. C. Cross, D. W. Leaman *Endocr. Rev.* **1992**, *13*, 432–452.
154 K. J. Austin, B. M. Bany, E. L. Belden, L. A. Rempel, J. C. Cross, T. R. Hansen *Endocrinology* **2003**, *144*, 3107–3113.
155 A. Cencic, M. Guillomot, S. Koren, C. La Bonnardière *Placenta*. **2003**, *24*, 862–869.
156 L. A. Rempel, B. R. Francis, K. J. Austin, T. R. Hansen *Biol. Reprod.* **2005**, *72*, 365–372.
157 G. A. Johnson, K. J. Austin, E. A. Van Kirk, T. R. Hansen *Biol. Reprod.* **1998**, *58*, 898–904.
158 K. L. Staggs, K. J. Austin, G. A. Johnson, M. G. Teixeira, C. T. Talbott, V. A. Dooley, T. R. Hansen *Biol. Reprod.* **1998**, *59*, 293–297.
159 G. A. Johnson, T. E. Spencer, T. R. Hansen, K. J. Austin, R. C. Burghardt, F. W. Bazer *Biol. Reprod.* **1999**, *61*, 312–318.
160 M. Slater, C. R. Murphy, J. A. Barden *Histochem. J.* **2002**, *34*, 13–19.
161 M. M. Joyce, F. J. White, R. C. Burghardt, J. J. Muniz, T. E. Spencer, F. W. Bazer, G. A. Johnson *Endocrinology* **2005**, *146*, 675–684.

6
The Role of the Ubiquitin–Proteasome Pathway in the Regulation of the Cellular Hypoxia Response

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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].

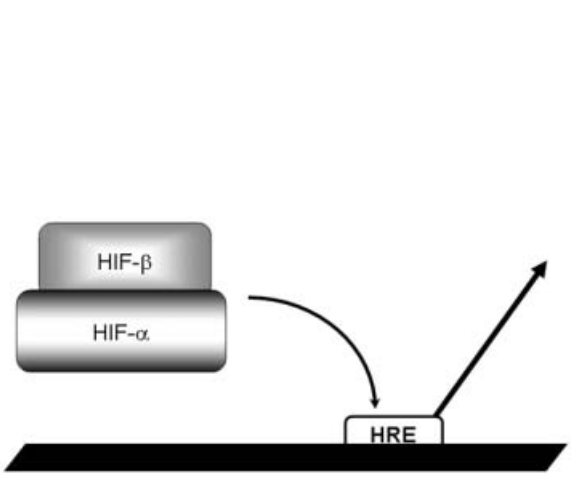
Hypoxia results in reduced oxidative phosphorylation and a shift towards glycolysis as the primary means of ATP production. To facilitate this change, cells upregulate expression of genes that encode glycolytic enzymes and glucose transporters [12, 13]. Tissues and cells that experience reduced oxygen 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–

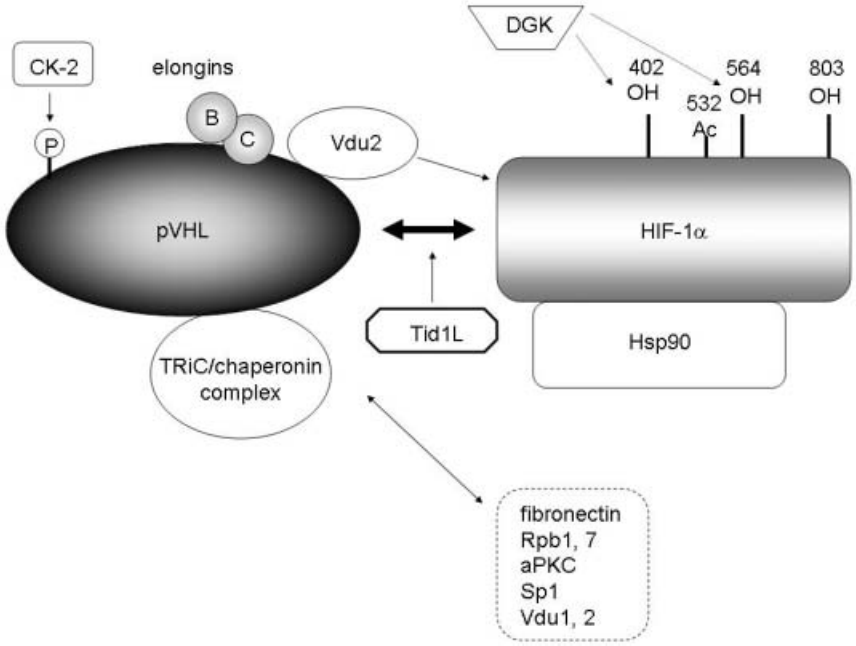


Fig. 6.2. Regulation of the pVHL–HIF pathway by multiple molecules. HIF-1α is post-translationally modified by hydroxylation on proline residues at positions 402 and 564. This hydroxylation serves as a docking site for the pVHL which will lead to its degradation. It also appears that acetylation on lysine 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

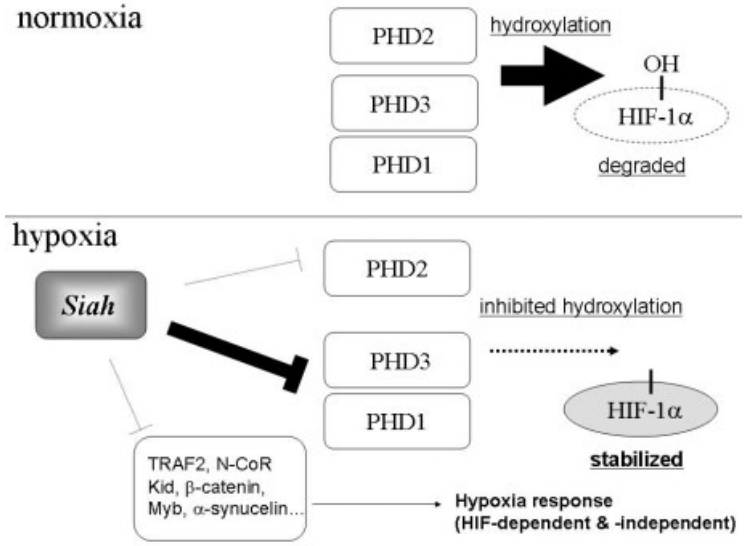


Fig. 6.3. Siah2-dependent regulation of PHDs leads to HIF-1α stabilization in hypoxia. In normoxia PHDs hydroxylate HIF-1α to enable pVHL association which results in its degradation. In hypoxia Siah2 is induced and actively targets PHD3 and 1 for degradation, therefore limiting the availability of PHD 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.

References

1 C. Koumenis, C. Naczki, M. Koritzinsky, S. Rastani, A. Diehl, N. Sonenberg, A. Koromilas, B. G. Wouters. *Mol. Cell. Biol.* **2002**, *22*, 7405–7416.

2 K. J. Lang, A. Kappel, G. J. Goodall. *Mol. Biol. Cell.* **2002**, *13*, 1792–1801.

3 N. S. Chandel, E. Maltepe, E. Goldwasser, C. E. Mathieu, M. C. Simon, P. T. Schumacker. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11715–11720.

4 B. A. Freeman, J. D. Crapo. *J. Biol. Chem.* **1981**, *256*, 10986–10992.

5 F. H. Agani, P. Pichiule, J. C. Chavez, J. C. LaManna. *J. Biol. Chem.* **2000**, *275*, 35863–35867.

6 F. H. Agani, M. Puchowicz, J. C. Chavez, P. Pichiule, J. LaManna. *Am. J. Physiol. Cell. Physiol.* **2002**, *283*, C178–186.

7 N. S. Chandel, D. S. McClintock, C. E. Feliciano, T. M. Wood, J. A. Melendez, A. M. Rodriguez, P. T. Schumacker. *J. Biol. Chem.* **2000**, *275*, 25130–25138.

8 A. Kulisz, N. Chen, N. S. Chandel, Z. Shao, P. T. Schumacker. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2002**, *282*, L1324–L1329.

9 D. P. Pearlstein, M. H. Ali, P. T. Mungai, K. L. Hynes, B. L. Gewertz, P. T. Schumacker. *Arterioscler. Thromb. Vasc. Biol.* **2002**, *22*, 566–573.

10 R. Dirmeier, K. M. O'Brien, M. Engle, A. Dodd, E. Spears, R. O. Poyton. *J. Biol. Chem.* **2002**, *277*, 34773–34784.

11 A. Carriere, M. C. Carmona, Y. Fernandez, M. Rigoulet, R. H. Wenger, L. Penicaud, L. Casteilla. *J. Biol. Chem.* **2004**, *279*, 40462–40469.

12 M. L. Macheda, S. Rogers, J. D. Best. *J. Cell. Physiol.* **2005**, *202*, 654–662.

13 R. A. Gatenby, R. J. Gillies. *Nat. Rev. Cancer.* **2004**, *4*, 891–899.

14 C. K. Sen, S. Khanna, B. M. Babior, T. K. Hunt, E. C. Ellison, S. Roy. *J. Biol. Chem.* **2002**, *277*, 33284–33290.

15 E. A. O'Toole, M. P. Marinkovich, C. L. Peavey, M. R. Amieva, H. Furthmayr, T. A. Mustoe, D. T. Woodley. *J. Clin. Invest.* **1997**, *100*, 2881–2891.

16 G. L. Wang, G. L. Semenza. *J. Biol. Chem.* **1995**, *270*, 1230–1237.

17 G. L. Wang, B. H. Jiang, E. A. Rue, G. L. Semenza. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 5510–5514.

18 R. H. Wenger, D. P. Stiehl, G. Camenisch. *Sci. STKE.* **2005**, *2005*, re12.

19 Y. Z. Gu, S. M. Moran, J. B. Hogenesch, L. Wartman, C. A. Bradfield. *Gene Expr.* **1998**, *7*, 205–213.

20 C. J. Hu, L. Y. Wang, L. A. Chodosh, B. Keith, M. C. Simon. *Mol. Cell. Biol.* **2003**, *23*, 9361–9374.

21 R. R. Raval, K. W. Lau, M. G. Tran, H. M. Sowter, S. J. Mandriota, J. L. Li, C. W. Pugh, P. H. Maxwell, A. L. Harris, P. J. Ratcliffe. *Mol. Cell. Biol.* **2005**, *25*, 5675–5686.

22 Y. Makino, R. Cao, K. Svensson, G. Bertilsson, M. Asman, H. Tanaka, Y. Cao, A. Berkenstam, L. Poellinger. *Nature* **2001**, *414*, 550–554.

23 Y. Makino, A. Kanopka, W. J. Wilson, H. Tanaka, L. Poellinger. *J Biol. Chem.* **2002**, *277*, 32405–32408.

24 P. J. Kallio, I. Pongratz, K. Gradin, J. McGuire, L. Poellinger. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5667–5672.

25 B. H. Jiang, G. Jiang, J. Z. Zheng, Z. Lu, T. Hunter, P. K. Vogt. *Cell Growth Differ.* **2001**, *12*, 363–369.

26 H. Zhong, K. Chiles, D. Feldser, E. Laughner, C. Hanrahan, M. M. Georgescu, J. W. Simons, G. L. Semenza. *Cancer Res.* **2000**, *60*, 1541–1545.

27 W. Zundel, C. Schindler, D. Haas-Kogan, A. Koong, F. Kaper, E. Chen, A. R. Gottschalk, H. E. Ryan, R. S. Johnson, A. B. Jefferson, D. Stokoe, A. J. Giaccia. *Genes Dev.* **2000**, *14*, 391–396.

28 D. Feldser, F. Agani, N. V. Iyer, B. Pak, G. Ferreira, G. L. Semenza. *Cancer Res.* **1999**, *59*, 3915–3918.

29 Y. M. Li, B. P. Zhou, J. Deng, Y. Pan, N. Hay, M. C. Hung. *Cancer Res.* **2005**, *65*, 3257–3263.

30 N. Masson, C. Willam, P. H. Maxwell, C. W. Pugh, P. J. Ratcliffe. *EMBO J.* **2001**, *20*, 5197–5206.

31 A. C. Epstein, J. M. Gleadle, L. A. McNeill, K. S. Hewitson, J. O'Rourke, D. R. Mole, M. Mukherji, E. Metzen, M. I. Wilson, A. Dhanda, Y. M. Tian, N. Masson, D. L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P. H. Maxwell, C. W. Pugh, C. J. Schofield, P. J. Ratcliffe. *Cell* **2001**, *107*, 43–54.

32 R. K. Bruick, S. L. McKnight. *Science* **2001**, *294*, 1337–1340.

33 P. Jaakkola, D. R. Mole, Y. M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. Kriegsheim, H. F. Hebestreit, M. Mukherji, C. J. Schofield, P. H. Maxwell, C. W. Pugh, P. J. Ratcliffe. *Science* **2001**, *292*, 468–472.

34 M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane, W. G. Kaelin, Jr. *Science* **2001**, *292*, 464–468.

35 F. Yu, S. B. White, Q. Zhao, F. S. Lee. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9630–9635.

36 F. Oehme, P. Ellinghaus, P. Kolkhof, T. J. Smith, S. Ramakrishnan, J. Hutter, M. Schramm, I. Flamme. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 343–349.

37 C. L. Cioffi, X. Q. Liu, P. A. Kosinski, M. Garay, B. R. Bowen. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 947–953.

38 E. Berra, E. Benizri, A. Ginouves, V. Volmat, D. Roux, J. Pouyssegur. *EMBO J.* **2003**, *22*, 4082–4090.

39 R. J. Appelhoff, Y. M. Tian, R. R. Raval, H. Turley, A. L. Harris, C. W. Pugh, P. J. Ratcliffe, J. M. Gleadle. *J. Biol. Chem.* **2004**, *279*, 38458–38465.

40 E. Metzen, U. Berchner-Pfannschmidt, P. Stengel, J. H. Marxsen, I. Stolze, M. Klinger, W. Q. Huang, C. Wotzlaw, T. Hellwig-Burgel, W. Jelkmann, H. Acker, J. Fandrey. *J. Cell Sci.* **2003**, *116*, 1319–1326.

41 J. Huang, Q. Zhao, S. M. Mooney, F. S. Lee. *J. Biol. Chem.* **2002**, *277*, 39792–39800.

42 J. Myllyharju, K. I. Kivirikko. *Trends Genet.* **2004**, *20*, 33–43.

43 N. Arquier, P. Vigne, E. Duplan, T. Hsu, P. P. Therond, C. Frelin, G. D'Angelo. *Biochem. J.* **2005**, doi:10.1042/BJ20050675.

44 K. K. To, L. E. Huang. *J. Biol. Chem.* **2005**, *280*, 38102–38107.

45 M. Callapina, J. Zhou, S. Schnitzer, E. Metzen, C. Lohr, J. W. Deitmer, B. Brune. *Exp. Cell Res.* **2005**, *306*, 274–284.

46 D. Lando, D. J. Peet, J. J. Gorman, D. A. Whelan, M. L. Whitelaw, R. K. Bruick. *Genes Dev.* **2002**, *16*, 1466–1471.

47 T. Kamura, D. M. Koepp, M. N. Conrad, D. Skowyra, R. J. Moreland, O. Iliopoulos, W. S. Lane, W. G. Kaelin, Jr., S. J. Elledge, R. C. Conaway, J. W. Harper, J. W. Conaway. *Science* **1999**, *284*, 657–661.

48 J. Lisztwan, G. Imbert, C. Wirbelauer, M. Gstaiger, W. Krek. *Genes Dev.* **1999**, *13*, 1822–1833.

49 J. T. Winston, P. Strack, P. Beer-Romero, C. Y. Chu, S. J. Elledge, J. W. Harper. *Genes Dev.* **1999**, *13*, 270–283.

50 A. Yaron, A. Hatzubai, M. Davis, I. Lavon, S. Amit, A. M. Manning, J. S. Andersen, M. Mann, F. Mercurio, Y. Ben-Neriah. *Nature* **1998**, *396*, 590–594.

51 W. G. Kaelin, Jr. *Clin. Cancer Res.* **2004**, *10*, 6290S–6295S.

52 P. H. Maxwell, M. S. Wiesener, G. W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher, P. J. Ratcliffe. *Nature* **1999**, *399*, 271–275.

53 J. H. Min, H. Yang, M. Ivan, F. Gertler, W. G. Kaelin, Jr., N. P. Pavletich. *Science* **2002**, *296*, 1886–1889.

54 W. C. Hon, M. I. Wilson, K. Harlos, T. D. Claridge, C. J. Schofield, C. W. Pugh, P. H. Maxwell, P. J. Ratcliffe, D. I. Stuart, E. Y. Jones. *Nature* **2002**, *417*, 975–978.

55 B. H. Jiang, G. L. Semenza, C. Bauer, H. H. Marti. *Am. J. Physiol.* **1996**, *271*, C1172–1180.

56 O. Iliopoulos, A. Kibel, S. Gray, W. G. Kaelin, Jr. *Nat. Med.* **1995**, *1*, 822–826.

57 T. Kamura, S. Sato, K. Iwai, M. Czyzyk-Krzeska, R. C. Conaway, J. W. Conaway. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10430–10435.

58 A. Kibel, O. Iliopoulos, J. A. DeCaprio, W. G. Kaelin, Jr. *Science* **1995**, *269*, 1444–1446.

59 T. Kishida, T. M. Stackhouse, F. Chen, M. I. Lerman, B. Zbar. *Cancer Res.* **1995**, *55*, 4544–4548.

60 M. Ohh, Y. Takagi, T. Aso, C. E. Stebbins, N. P. Pavletich, B. Zbar, R. C. Conaway, J. W. Conaway, W. G. Kaelin, Jr. *J. Clin. Invest.* **1999**, *104*, 1583–1591.

61 A. R. Schoenfeld, E. J. Davidowitz, R. D. Burk. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8507–8512.

62 M. K. Bae, J. W. Jeong, S. H. Kim, S. Y. Kim, H. J. Kang, D. M. Kim, S. K. Bae, I. Yun, G. A. Trentin, M. Rozakis-Adcock, K. W. Kim. *Cancer Res.* **2005**, *65*, 2520–2525.

63 Z. Li, D. Wang, E. M. Messing, G. Wu. *EMBO Rep.* **2005**, *6*, 373–378.

64 K. Mekhail, M. Khacho, A. Carrigan, R. R. Hache, L. Gunaratnam, S. Lee. *J. Cell Biol.* **2005**, *170*, 733–744.

65 M. P. Lolkema, M. L. Gervais, C. M. Snijckers, R. P. Hill, R. H. Giles, E. E. Voest, M. Ohh. *J. Biol. Chem.* **2005**, *280*, 22205–22211.

66 E. Temes, S. Martin-Puig, B. Acosta-Iborra, M. C. Castellanos, M. Feijoo-Cuaresma, G. Olmos, J. Aragones, M. O. Landazuri. *J. Biol. Chem.* **2005**, *280*, 24238–24244.

67 Y. Tanaka, H. Kanno, M. Dezawa, T. Mimura, A. Kubo, I. Yamamoto. *Neurosci. Lett.* **2005**, *383*, 28–32.

68 J. A. LaIuppa, E. T. Papoutsakis, W. M. Miller. *Exp. Hematol.* **1998**, *26*, 835–843.

69 D. L. Hevehan, E. T. Papoutsakis, W. M. Miller. *Exp. Hematol.* **2000**, *28*, 267–275.

70 O. Genbacev, Y. Zhou, J. W. Ludlow, S. J. Fisher. *Science* **1997**, *277*, 1669–1672.

71 D. M. Adelman, M. Gertsenstein, A. Nagy, M. C. Simon, E. Maltepe. *Genes Dev.* **2000**, *14*, 3191–3203.

72 S. A. Gebb, P. L. Jones. *Adv. Exp. Med. Biol.* **2003**, *543*, 117–125.

73 H. Okuda, K. Saitoh, S. Hirai, K. Iwai, Y. Takaki, M. Baba, N. Minato, S. Ohno, T. Shuin. *J. Biol. Chem.* **2001**, *276*, 43611–43617.

74 J. Zhou, T. Schmid, R. Frank, B. Brune. *J. Biol. Chem.* **2004**, *279*, 13506–13513.

75 D. M. Katschinski, L. Le, D. Heinrich, K. F. Wagner, T. Hofer, S. G. Schindler, R. H. Wenger. *J. Biol. Chem.* **2002**, *277*, 9262–9267.

76 J. S. Isaacs, Y. J. Jung, E. G. Mimnaugh, A. Martinez, F. Cuttitta, L. M. Neckers. *J. Biol. Chem.* **2002**, *277*, 29936–29944.

77 H. van der Wel, A. Ercan, C. M. West. *J. Biol. Chem.* **2005**, *280*, 14645–14655.

78 N. G. Della, P. V. Senior, D. D. Bowtell. *Development* **1993**, *117*, 1333–1343.

79 S. Li, Y. Li, R. W. Carthew, Z. C. Lai. *Cell* **1997**, *90*, 469–478.

80 A. H. Tang, T. P. Neufeld, E. Kwan, G. M. Rubin. *Cell* **1997**, *90*, 459–467.

81 H. Okabe, S. Satoh, Y. Furukawa, T. Kato, S. Hasegawa, Y. Nakajima, Y. Yamaoka, Y. Nakamura. *Cancer Res.* **2003**, *63*, 3043–3048.

82 F. Relaix, X. Wei, W. Li, J. Pan, Y. Lin, D. D. Bowtell, D. A. Sassoon, X. Wu. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2105–2110.

83 J. Zhang, M. G. Guenther, R. W. Carthew, M. A. Lazar. *Genes Dev.* **1998**, *12*, 1775–1780.

84 J. Tanikawa, E. Ichikawa-Iwata, C. Kanei-Ishii, A. Nakai, S. Matsuzawa, J. C. Reed, S. Ishii. *J. Biol. Chem.* **2000**, *275*, 15578–15585.

85 R. Tiedt, B. A. Bartholdy, G. Matthias, J. W. Newell, P. Matthias. *EMBO J.* **2001**, *20*, 4143–4152.

86 J. Boehm, Y. He, A. Greiner, L. Staudt, T. Wirth. *EMBO J.* **2001**, *20*, 4153–4162.

87 M. Fanelli, A. Fantozzi, P. De Luca, S. Caprodossi, S. Matsuzawa, M. A. Lazar, P. G. Pelicci, S. Minucci. *J. Biol. Chem.* **2004**, *279*, 5374–5379.

88 L. Susini, B. J. Passer, N. Amzallag-Elbaz, T. Juven-Gershon, S. Prieur, N. Privat, M. Tuynder, M. C. Gendron, A. Israel, R. Amson, M. Oren, A. Telerman. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 15067–15072.

89 A. Germani, A. Prabel, S. Mourah, M. P. Podgorniak, A. Di Carlo, R. Ehrlich, S. Gisselbrecht, N. Varin-Blank, F. Calvo, H. Bruzzoni-Giovanelli. *Oncogene* **2003**, *22*, 8845–8851.

90 J. P. Venables, C. Dalgliesh, M. P. Paronetto, L. Skitt, J. K. Thornton, P. T. Saunders, C. Sette, K. T. Jones, D. J. Elliott. *Hum. Mol. Genet.* **2004**, *13*, 1525–1534.

91 A. Germani, H. Bruzzoni-Giovanelli, A. Fellous, S. Gisselbrecht, N. Varin-Blank, F. Calvo. *Oncogene* **2000**, *19*, 5997–6006.

92 T. C. Wheeler, L. S. Chin, Y. Li, F. L. Roudabush, L. Li. *J. Biol. Chem.* **2002**, *277*, 10273–10282.

93 K. Moriyoshi, K. Iijima, H. Fujii, H. Ito, Y. Cho, S. Nakanishi. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8614–8619.

94 E. Liani, A. Eyal, E. Avraham, R. Shemer, R. Szargel, D. Berg, A. Bornemann, O. Riess, C. A. Ross, R. Rott, S. Engelender. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5500–5505.

95 Y. Nagano, H. Yamashita, T. Takahashi, S. Kishida, T. Nakamura, E. Iseki, N. Hattori, Y. Mizuno, A. Kikuchi, M. Matsumoto. *J. Biol. Chem.* **2003**, *278*, 51504–51514.

96 A. Bursen, S. Moritz, A. Gaussmann, T. Dingermann, R. Marschalek. *Oncogene* **2004**, *23*, 6237–6249.

97 P. L. Oliver, E. Bitoun, J. Clark, E. L. Jones, K. E. Davies. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14901–14906.

98 H. Habelhah, A. Laine, H. Erdjument-Bromage, P. Tempst, M. E. Gershwin, D. D. Bowtell, Z. Ronai. *J. Biol. Chem.* **2004**, *279*, 53782–53788.

99 E. Santelli, M. Leone, C. Li, T. Fukushima, N. E. Preece, A. J. Olson, K. R. Ely, J. C. Reed, M. Pellecchia, R. C. Liddington, S. Matsuzawa. *J. Biol. Chem.* **2005**, *280*, 34278–34287.

100 G. Polekhina, C. M. House, N. Traficante, J. P. Mackay, F. Relaix,

D. A. Sassoon, M. W. Parker, D. D. Bowtell. *Nat. Struct. Biol.* **2002**, *9*, 68–75.

101 S. I. Matsuzawa, J. C. Reed. *Mol. Cell.* **2001**, *7*, 915–926.

102 K. Nakayama, I. J. Frew, M. Hagensen, M. Skals, H. Habelhah, A. Bhoumik, T. Kadoya, H. Erdjument-Bromage, P. Tempst, P. B. Frappell, D. D. Bowtell, Z. Ronai. *Cell* **2004**, *117*, 941–952.

103 I. J. Frew, V. E. Hammond, R. A. Dickins, J. M. Quinn, C. R. Walkley, N. A. Sims, R. Schnall, N. G. Della, A. J. Holloway, M. R. Digby, P. W. Janes, D. M. Tarlinton, L. E. Purton, M. T. Gillespie, D. D. Bowtell. *Mol. Cell Biol.* **2003**, *23*, 9150–9161.

104 C. Koumenis, R. Alarcon, E. Hammond, P. Sutphin, W. Hoffman, M. Murphy, J. Derr, Y. Taya, S. W. Lowe, M. Kastan, A. Giaccia. *Mol. Cell Biol.* **2001**, *21*, 1297–1310.

105 W. G. An, M. Kanekal, M. C. Simon, E. Maltepe, M. V. Blagosklonny, L. M. Neckers. *Nature* **1998**, *392*, 405–408.

106 R. Alarcon, C. Koumenis, R. K. Geyer, C. G. Maki, A. J. Giaccia. *Cancer Res.* **1999**, *59*, 6046–6051.

107 L. Zhang, R. P. Hill. *Cancer Res.* **2004**, *64*, 4180–4189.

108 J. I. Bardos, N. M. Chau, M. Ashcroft. *Mol. Cell Biol.* **2004**, *24*, 2905–2914.

109 A. L. Nieminen, S. Qanungo, E. A. Schneider, B. H. Jiang, F. H. Agani. *J. Cell Physiol.* **2005**, *204*, 364–369.

110 D. Chen, M. Li, J. Luo, W. Gu. *J. Biol. Chem.* **2003**, *278*, 13595–13598.

111 A. Di Carlo, R. De Mori, F. Martelli, G. Pompilio, M. C. Capogrossi, A. Germani. *J. Biol. Chem.* **2004**, *279*, 16332–16338.

112 C. T. Taylor, G. T. Furuta, K. Synnestvedt, S. P. Colgan. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12091–12096.

113 R. Shao, F. P. Zhang, F. Tian, P. Anders Friberg, X. Wang, H. Sjoland, H. Billig. *FEBS Lett.* **2004**, *569*, 293–300.

114 K. M. Comerford, M. O. Leonard, J. Karhausen, R. Carey, S. P. Colgan, C. T. Taylor. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 986–991.

115 M. Tojo, K. Matsuzaki, T. Minami, Y. Honda, H. Yasuda, T. Chiba, H. Saya, Y. Fujii-Kuriyama, M. Nakao. *J. Biol. Chem.* **2002**, *277*, 46576–46585.

116 P. Gubellini, G. M. Bisso, A. Ciofi-Luzzatto, S. Fortuna, P. Lorenzini, H. Michalek, G. Scarsella. *Neurochem. Res.* **1997**, *22*, 93–100.

117 S. J. Vannucci, R. Mummery, R. B. Hawkes, C. C. Rider, P. W. Beesley. *J. Cereb. Blood Flow Metab.* **1998**, *18*, 376–385.

118 A. R. Schoenfeld, E. J. Davidowitz, R. D. Burk. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8507–8512.

119 D. E. Feldman, C. Spiess, D. E. Howard, J. Frydman. *Mol. Cell* **2003**, *12*, 1213–1224.

120 K. Mekhail, L. Gunaratnam, M. E. Bonicalzi, S. Lee. *Nat. Cell Biol.* **2004**, *6*, 642–647.

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-

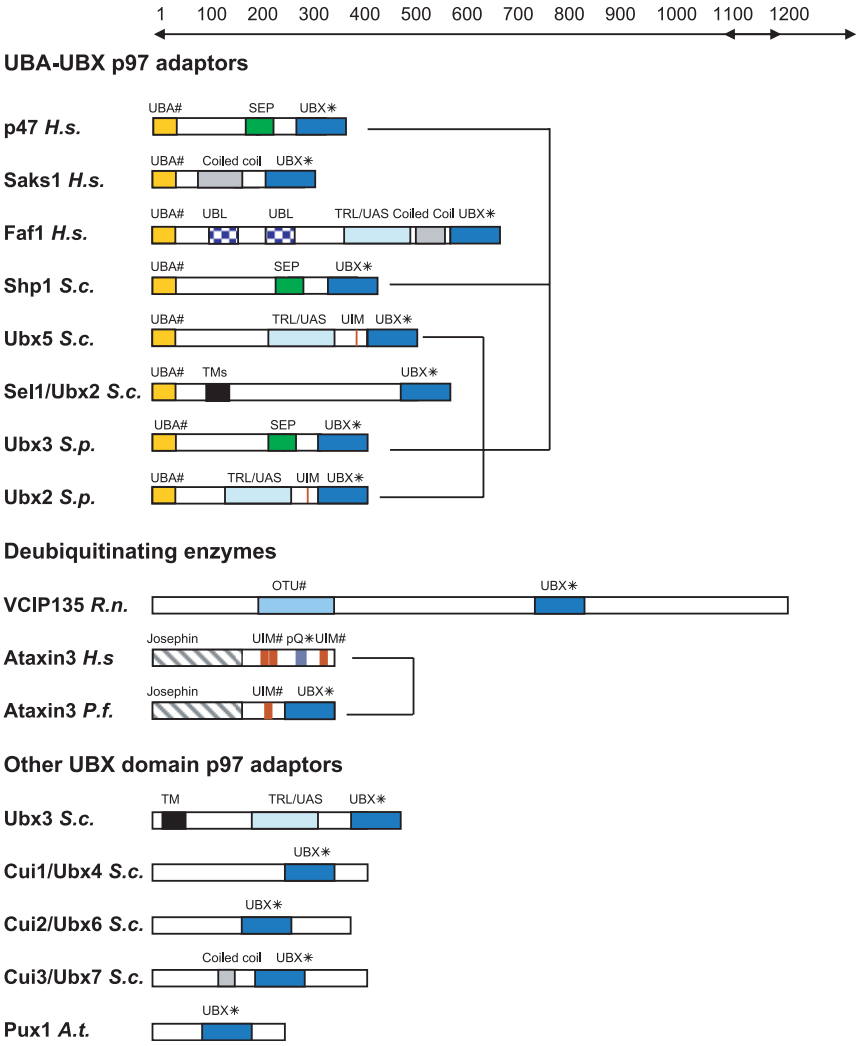


Fig. 7.1. Representations of different p97/CDC48/VCP interactors and assorted proteins important for the ubiquitin–proteasome system, showing their predicted and/or known domain structures. *: the part of the protein that was mapped to interact with p97/CDC48/VCP. #: ubiquitin-binding domains. Domain 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.

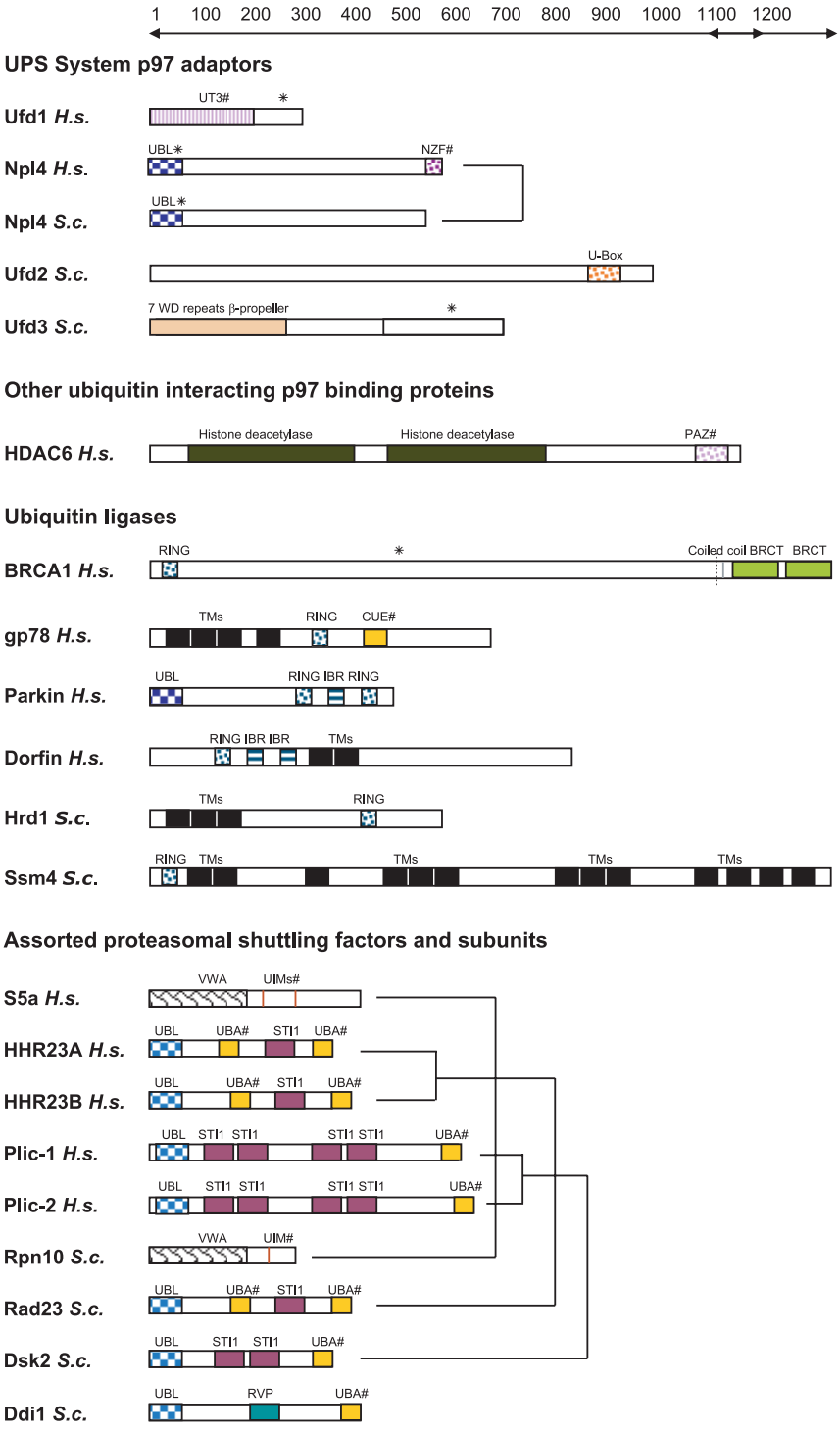


Fig. 7.1 (continued)

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

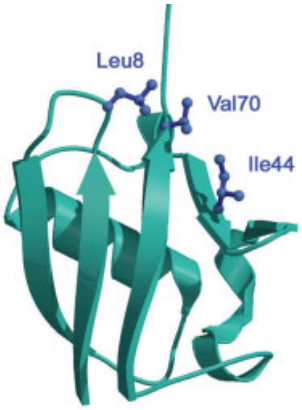


Fig. 7.2. The structure of ubiquitin. Key interacting residues are shown in ball and stick form.

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

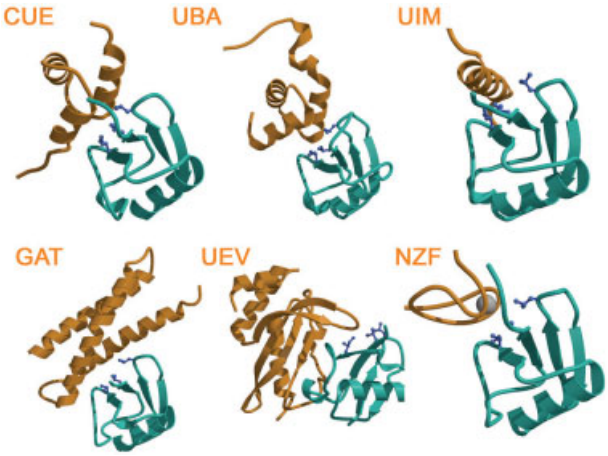


Fig. 7.3. Structure gallery of ubiquitin with residues of its hydrophobic patch in ball and stick representation (turquoise) and structures of its complexes with ubiquitin-binding domains (orange): CUE–ubiquitin (pdb code 1OTR), UBA–ubiquitin (pdb code 1WR1), UIM–ubiquitin (pdb code 1Q0W), GAT–ubiquitin (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

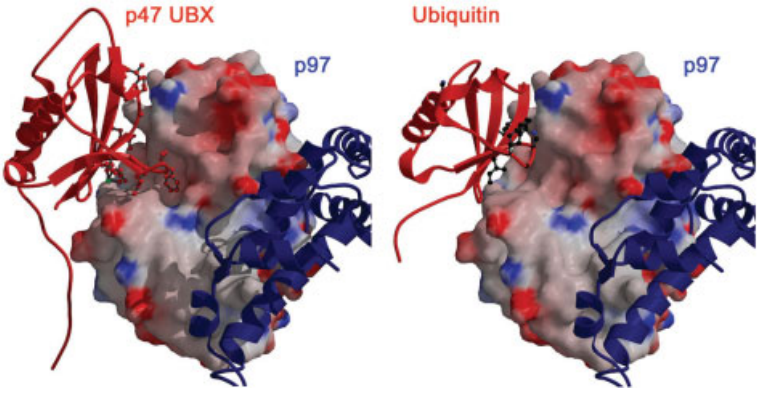


Fig. 7.4. Electrostatic surface representation of the p97 N domain in complex with p47 UBX (red) in comparison with ubiquitin binding modelled with a similar interface. Note the significantly shorter S3/S4 loop in ubiquitin.

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-

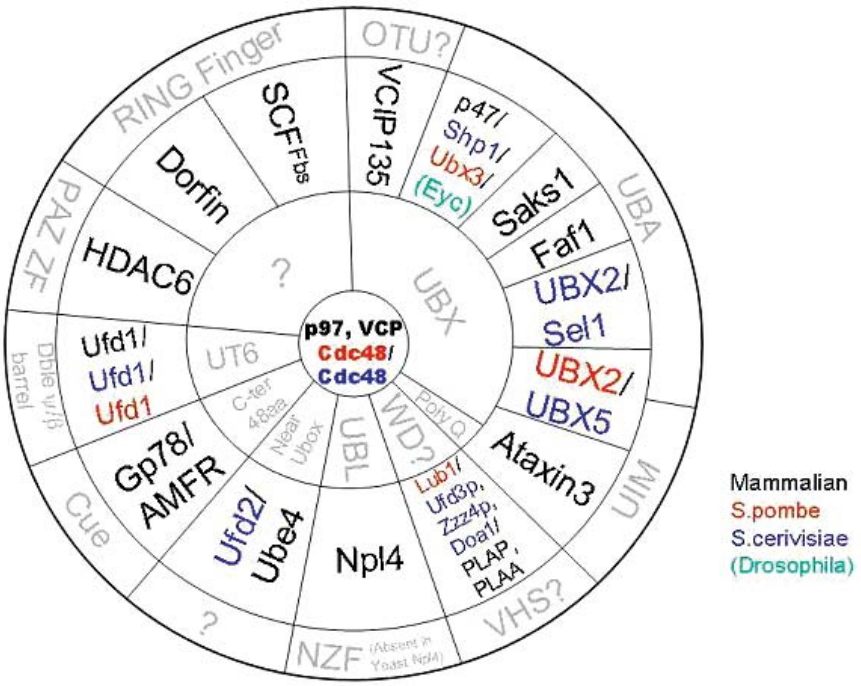


Fig. 7.5. Classification of proteins that interact with both p97 and ubiquitin according to their structural domain of interaction. From the centre (p97) are, first, the domains that interact with p97, then the proteins (colour-coded homologues according to origin), and finally the outer circle, which 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

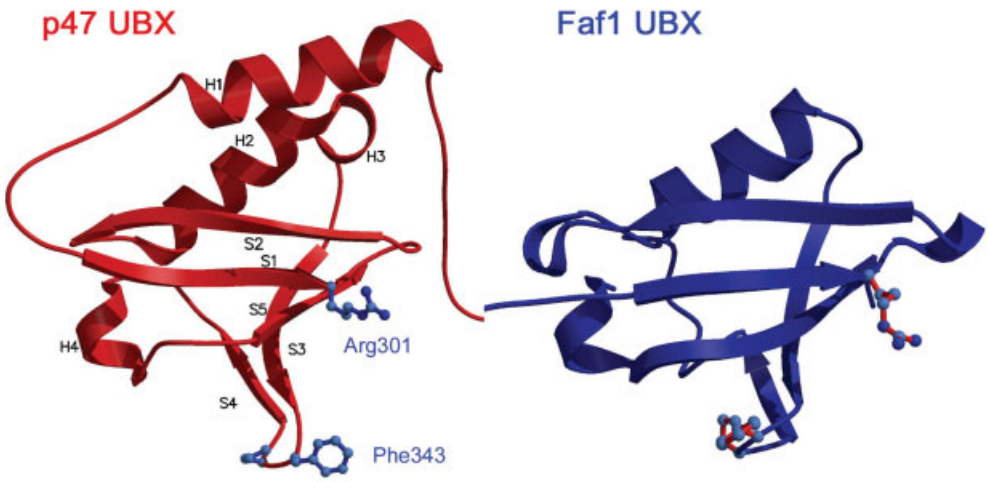


Fig. 7.6. Structures of the UBX domains of p47 (red, pdb code 1S3S, [39]) and FAF1 (blue, pdb code 1H8C, [73]). Conserved residues in the S3/S4 loop and the conserved arginine are represented in ball and stick form.

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.

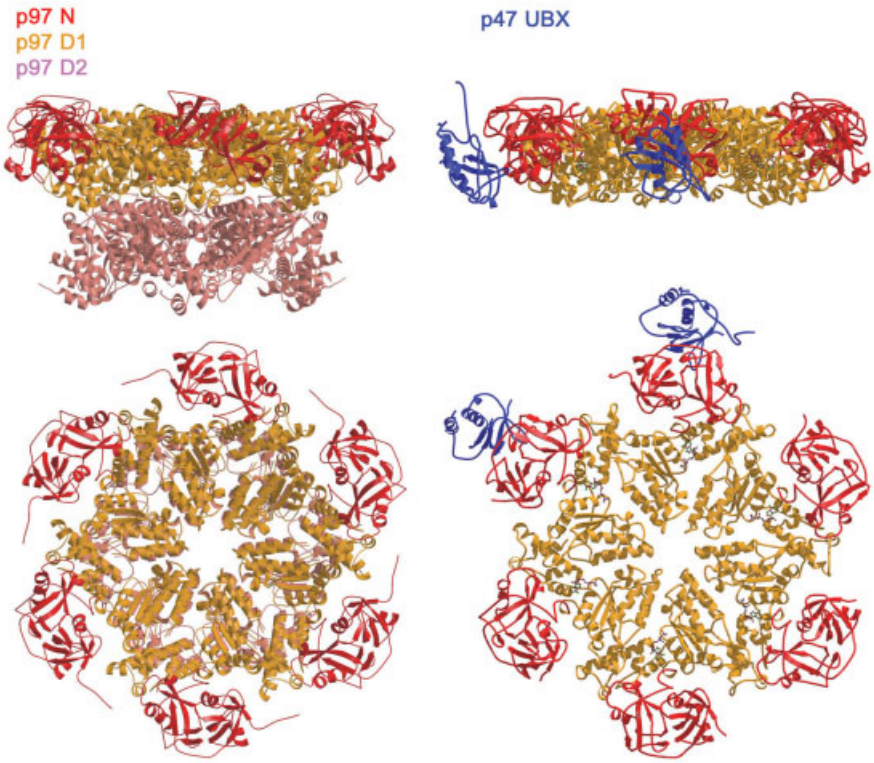


Fig. 7.7. Left: Ribbon representation (top and side views) of full-length p97 (pdb code 1R7R, [141]) (in red, orange and salmon). Right: Corresponding views of p97-ND1 complexed with p47-UBX (pdb code 1JRU, [39]).

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].

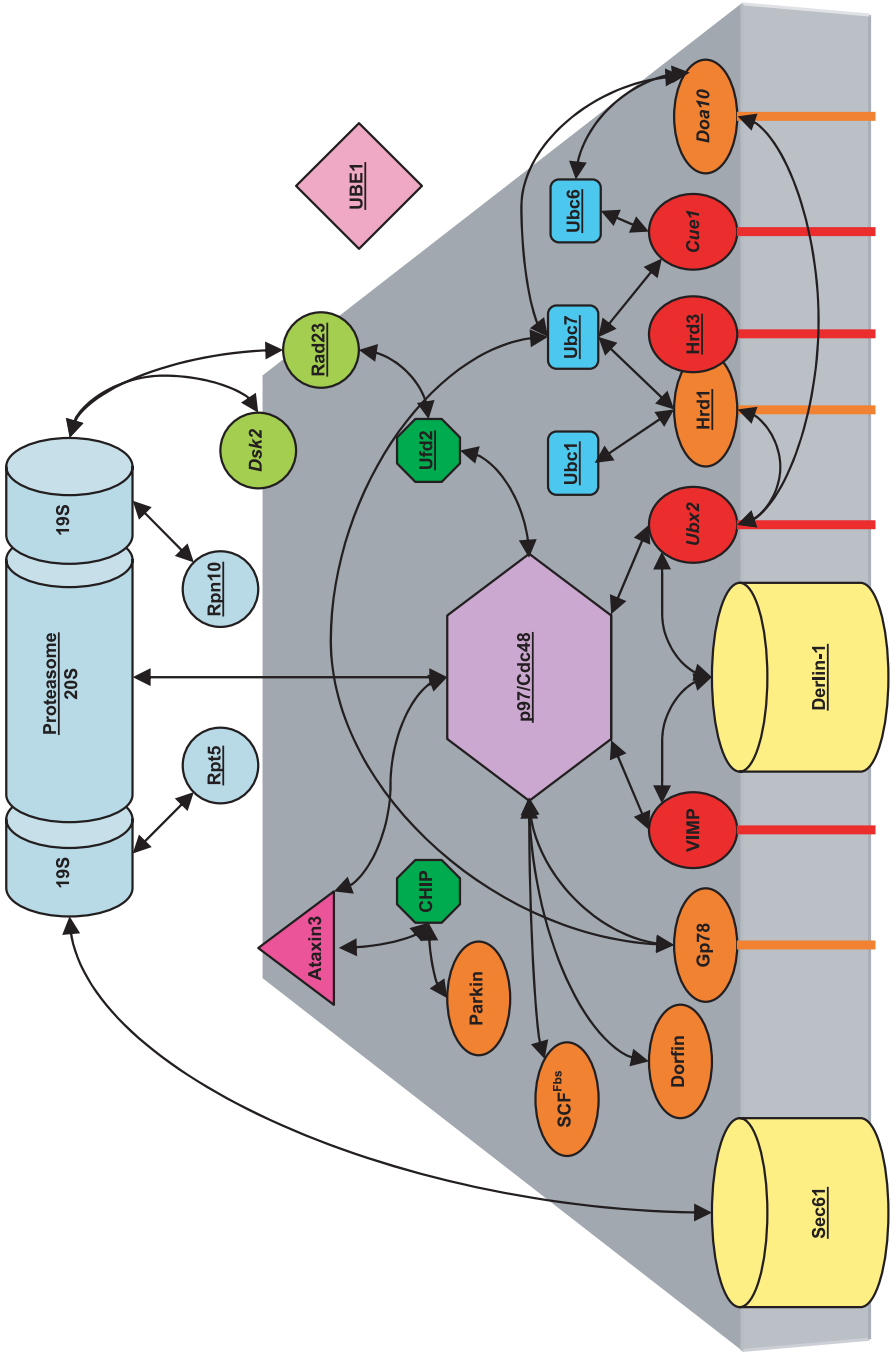
Accumulation of misfolded, aggregated polypeptides leads to toxic protein aggregation events, inactivation of functional proteins and ultimately cell death, all of which lead to many diseases (discussed in Section 7.5). Conversely, the premature degradation of key proteins can also be a cause of disease, 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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References

1 Moir, D., Stewart, S. E., Osmond, B. C., and Botstein, D. (**1982**) Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics 100*, 547–563.

2 Schmidt, W. E., Mutt, V., Carlquist, M., Kratzin, H., Conlon, J. M., and Creutzfeldt, W. (**1985**) Valosin: isolation and characterization of a novel peptide from porcine intestine. *FEBS Lett. 191*, 264–268.

3 Koller, K. J. and Brownstein, M. J. (**1987**) Use of a cDNA clone to identify a supposed precursor protein containing valosin. *Nature 325*, 542–545.

4 Gill, J. S., Ghatei, M. A., Domin, J., and Bloom, S. R. (**1989**) The generation of valosin-like peptides from a precursor protein in vitro as an extraction artifact. *Life Sci. 44*, 483–491.

5 Fröhlich, K. U., Fries, H. W., Rudiger, M., Erdmann, R., Botstein, D., and Mecke, D. (**1991**) Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J. Cell Biol. 114*, 443–453.

6 Erdmann, R., Wiebel, F. F., Flessau, A., Rytka, J., Beyer, A., Frohlich, K. U., and Kunau, W. H. (**1991**) PAS1, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell 64*, 499–510.

7 Ghislain, M., Dohmen, R. J., Levy, F., and Varshavsky, A. (**1996**) Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *Embo J. 15*, 4884–4899.

8 Dai, R. M., Chen, E., Longo, D. L., Gorbea, C. M., and Li, C. C. (**1998**) Involvement of valosin-containing protein, an ATPase Co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. *J. Biol. Chem. 273*, 3562–3573.

9 Acharya, U., Jacobs, R., Peters, J. M., Watson, N., Farquhar, M. G., and Malhotra, V. (**1995**) The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. *Cell 82*, 895–904.

10 Rabouille, C., Levine, T. P., Peters, J. M., and Warren, G. (**1995**) An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell 82*, 905–914.

11 Ogura, T. and Wilkinson, A. J.

(**2001**) AAA+ superfamily ATPases: common structure – diverse function. *Genes Cells 6*, 575–597.

12 Ogura, T., Whiteheart, S. W., and Wilkinson, A. J. (**2004**) Conserved arginine residues implicated in ATP hydrolysis, nucleotide-sensing, and inter-subunit interactions in AAA and AAA+ ATPases. *J. Struct. Biol. 146*, 106–112.

13 Pickart, C. M. and Eddins, M. J. (**2004**) Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta 1695*, 55–72.

14 Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (**1999**) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell 96*, 635–644.

15 Rechsteiner, M. and Hill, C. P. (**2005**) Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. *Trends Cell Biol. 15*, 27–33.

16 Pickart, C. M. and Fushman, D. (**2004**) Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol. 8*, 610–616.

17 Wilkinson, K. D., Ventii, K. H., Friedrich, K. L., and Mullally, J. E. (**2005**) The ubiquitin signal: assembly, recognition and termination. *EMBO Rep. 6*, 815–820.

18 Shang, F., Deng, G., Liu, Q., Guo, W., Haas, A. L., Crosas, B., Finley, D., and Taylor, A. (**2005**) Lys6-modified ubiquitin inhibits ubiquitin-dependent protein degradation. *J. Biol. Chem. 280*, 20365–20374.

19 Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (**2001**) HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol. Biol. Cell 12*, 4114–4128.

20 Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (**2001**) Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. *Cell 107*, 667–677.

21 Hitchcock, A. L., Krebber, H., Frietze, S., Lin, A., Latterich, M., and Silver, P. A. (**2001**) The conserved npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. *Mol. Biol. Cell 12*, 3226–3241.

22 Dreveny, I., Pye, V. E., Beuron, F., Briggs, L. C., Isaacson, R. L., Matthews, S. J., McKeown, C., Yuan, X., Zhang, X., and Freemont, P. S. (**2004**) p97 and close encounters of every kind: a brief review. *Biochem. Soc. Trans. 32*, 715–720.

23 Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (**2000**) A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *Embo J. 19*, 2181–2192.

24 Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (**1997**) p47 is a cofactor for p97-mediated membrane fusion. *Nature 388*, 75–78.

25 Cao, K., Nakajima, R., Meyer, H. H., and Zheng, Y. (**2003**) The AAA-ATPase Cdc48/p97 regulates spindle disassembly at the end of mitosis. *Cell 115*, 355–367.

26 Hetzer, M., Meyer, H. H., Walther, T. C., Bilbao-Cortes, D., Warren, G., and Mattaj, I. W. (**2001**) Distinct AAA-ATPase p97 complexes function in discrete steps of nuclear assembly. *Nat. Cell Biol. 3*, 1086–1091.

27 Ye, Y., Meyer, H. H., and Rapoport, T. A. (**2001**) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature 414*, 652–656.

28 Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (**2005**) A series of ubiquitin-binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell 120*, 73–84.

29 Elkabetz, Y., Shapira, I., Rabinovich, E., and Bar-Nun, S. (**2004**) Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplamic reticulum-bound

p97/Cdc48p and proteasome. *J. Biol. Chem. 279*, 3980–3989.

30 Madura, K. (**2004**) Rad23 and Rpn10: perennial wallflowers join the melee. *Trends Biochem. Sci. 29*, 637–640.

31 Meyer, H. H. (**2005**) Golgi reassembly after mitosis: the AAA family meets the ubiquitin family. *Biochim. Biophys. Acta 1744*, 481–492.

32 Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P., and Kondo, H. (**2002**) VCIP*135*, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly in vivo. *J. Cell Biol. 159*, 855–866.

33 Thoms, S. (**2002**) Cdc48 can distinguish between native and non-native proteins in the absence of cofactors. *FEBS Lett. 520*, 107–110.

34 Mizuno, Y., Hori, S., Kakizuka, A., and Okamoto, K. (**2003**) Vacuole-creating protein in neurodegenerative diseases in humans. *Neurosci. Lett. 343*, 77–80.

35 Dalal, S., Rosser, M. F., Cyr, D. M., and Hanson, P. I. (**2004**) Distinct roles for the AAA ATPases NSF and p97 in the secretory pathway. *Mol. Biol. Cell 15*, 637–648.

36 Fu, X., Ng, C., Feng, D., and Liang, C. (**2003**) Cdc48p is required for the cell cycle commitment point at Start via degradation of the G1-CDK inhibitor Far1p. *J. Cell Biol. 163*, 21–26.

37 Watts, G. D., Wymer, J., Kovach, M. J., Mehta, S. G., Mumm, S., Darvish, D., Pestronk, A., Whyte, M. P., and Kimonis, V. E. (**2004**) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat. Genet. 36*, 377–381.

38 Jentsch, S. and Pyrowolakis, G. (**2000**) Ubiquitin and its kin: how close are the family ties? *Trends Cell Biol. 10*, 335–342.

39 Dreveny, I., Kondo, H., Uchiyama, K., Shaw, A., Zhang, X., and Freemont, P. S. (**2004**) Structural basis of the interaction between the AAA ATPase p97/VCP and its adaptor protein p47. *Embo J. 23*, 1030–1039.

40 Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (**1994**) A 26 S protease subunit that binds ubiquitin conjugates. *J. Biol. Chem. 269*, 7059–7061.

41 Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M., and Pickart, C. (**1996**) Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting. *Proc. Natl. Acad. Sci. USA 93*, 861–866.

42 Meyer, H. H., Wang, Y., and Warren, G. (**2002**) Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. *Embo J. 21*, 5645–5652.

43 Slagsvold, T., Aasland, R., Hirano, S., Bache, K. G., Raiborg, C., Trambaiolo, D., Wakatsuki, S., and Stenmark, H. (**2005**) Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. *J. Biol. Chem. 280*, 19600–19606.

44 Hofmann, R. M. and Pickart, C. M. (**1999**) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell 96*, 645–653.

45 Sancho, E., Vila, M. R., Sanchez-Pulido, L., Lozano, J. J., Paciucci, R., Nadal, M., Fox, M., Harvey, C., Bercovich, B., Loukili, N., Ciechanover, A., Lin, S. L., Sanz, F., Estivill, X., Valencia, A., and Thomson, T. M. (**1998**) Role of UEV-1, an inactive variant of the E2 ubiquitin-conjugating enzymes, in in vitro differentiation and cell cycle behavior of HT-29-M6 intestinal mucosecretory cells. *Mol. Cell Biol. 18*, 576–589.

46 Donaldson, K. M., Yin, H., Gekakis, N., Supek, F., and Joazeiro, C. A. (**2003**) Ubiquitin signals protein trafficking via interaction with a novel ubiquitin binding domain in the membrane fusion regulator, Vps9p. *Curr. Biol. 13*, 258–262.

47 Ponting, C. P. (**2000**) Proteins of the endoplasmic-reticulum-associated

degradation pathway: domain detection and function prediction. *Biochem. J. 351 Pt 2*, 527–535.

48 Shih, S. C., Prag, G., Francis, S. A., Sutanto, M. A., Hurley, J. H., and Hicke, L. (**2003**) A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. *Embo J. 22*, 1273–1281.

49 Bertolaet, B. L., Clarke, D. J., Wolff, M., Watson, M. H., Henze, M., Divita, G., and Reed, S. I. (**2001**) UBA domains mediate protein-protein interactions between two DNA damage-inducible proteins. *J. Mol. Biol. 313*, 955–963.

50 Hofmann, K. and Bucher, P. (**1996**) The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. *Trends Biochem. Sci. 21*, 172–173.

51 Vadlamudi, R. K., Joung, I., Strominger, J. L., and Shin, J. (**1996**) p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. *J. Biol. Chem. 271*, 20235–20237.

52 Dell'Angelica, E. C., Puertollano, R., Mullins, C., Aguilar, R. C., Vargas, J. D., Hartnell, L. M., and Bonifacino, J. S. (**2000**) GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J. Cell Biol. 149*, 81–94.

53 Shiba, Y., Katoh, Y., Shiba, T., Yoshino, K., Takatsu, H., Kobayashi, H., Shin, H. W., Wakatsuki, S., and Nakayama, K. (**2004**) GAT (GGA and Tom1) domain responsible for ubiquitin binding and ubiquitination. *J. Biol. Chem. 279*, 7105–7111.

54 Mizuno, E., Kawahata, K., Kato, M., Kitamura, N., and Komada, M. (**2003**) STAM proteins bind ubiquitinated proteins on the early endosome via the VHS domain and ubiquitin-interacting motif. *Mol. Biol. Cell 14*, 3675–3689.

55 Hook, S. S., Orian, A., Cowley, S. M., and Eisenman, R. N. (**2002**) Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes. *Proc. Natl. Acad. Sci. USA 99*, 13425–13430.

56 Hicke, L., Schubert, H. L., and Hill, C. P. (**2005**) Ubiquitin-binding domains. *Nat. Rev. Mol. Cell Biol. 6*, 610–621.

57 Park, S., Isaacson, R., Kim, H. T., Silver, P. A., and Wagner, G. (**2005**) Ufd1 Exhibits the AAA-ATPase Fold with Two Distinct Ubiquitin Interaction Sites. *Structure (Cambridge) 13*, 995–1005.

58 Sloper-Mould, K. E., Jemc, J. C., Pickart, C. M., and Hicke, L. (**2001**) Distinct functional surface regions on ubiquitin. *J. Biol. Chem. 276*, 30483–30489.

59 Young, P., Deveraux, Q., Beal, R. E., Pickart, C. M., and Rechsteiner, M. (**1998**) Characterization of two polyubiquitin binding sites in the 26S protease subunit 5a. *J. Biol. Chem. 273*, 5461–5467.

60 Mueller, T. D. and Feigon, J. (**2003**) Structural determinants for the binding of ubiquitin-like domains to the proteasome. *Embo J. 22*, 4634–4645.

61 Elsasser, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Muller, B., Feng, M. T., Tubing, F., Dittmar, G. A., and Finley, D. (**2002**) Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nat. Cell Biol. 4*, 725–730.

62 Sundquist, W. I., Schubert, H. L., Kelly, B. N., Hill, G. C., Holton, J. M., and Hill, C. P. (**2004**) Ubiquitin recognition by the human TSG101 protein. *Mol. Cell 13*, 783–789.

63 Mattera, R., Puertollano, R., Smith, W. J., and Bonifacino, J. S. (**2004**) The trihelical bundle subdomain of the GGA proteins interacts with multiple partners through overlapping but distinct sites. *J. Biol. Chem. 279*, 31409–31418.

64 Raasi, S., Varadan, R., Fushman, D., and Pickart, C. M. (**2005**) Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat. Struct. Mol. Biol. 12*, 708–714.

65 Elsasser, S. and Finley, D. (**2005**) Delivery of ubiquitinated substrates to

protein-unfolding machines. *Nat. Cell Biol. 7*, 742–749.

66 Dai, R. M. and Li, C. C. (**2001**) Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat. Cell Biol. 3*, 740–744.

67 Hartmann-Petersen, R., Wallace, M., Hofmann, K., Koch, G., Johnsen, A. H., Hendil, K. B., and Gordon, C. (**2004**) The ubx2 and ubx3 cofactors direct cdc48 activity to proteolytic and nonproteolytic ubiquitin-dependent processes. *Curr. Biol. 14*, 824–828.

68 Bruderer, R. M., Brasseur, C., and Meyer, H. H. (**2004**) The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism. *J. Biol. Chem. 279*, 49609–49616.

69 Russell, N. S. and Wilkinson, K. D. (**2004**) Identification of a novel 29-linked polyubiquitin binding protein, Ufd3, using polyubiquitin chain analogues. *Biochemistry 43*, 4844–4854.

70 Decottignies, A., Evain, A., and Ghislain, M. (**2004**) Binding of Cdc48p to a ubiquitin-related UBX domain from novel yeast proteins involved in intracellular proteolysis and sporulation. *Yeast 21*, 127–139.

71 Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (**2004**) Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. *EMBO Rep. 5*, 818–824.

72 Soukenik, M., Diehl, A., Leidert, M., Sievert, V., Bussow, K., Leitner, D., Labudde, D., Ball, L. J., Lechner, A., Nagler, D. K., and Oschkinat, H. (**2004**) The SEP domain of p47 acts as a reversible competitive inhibitor of cathepsin L. *FEBS Lett. 576*, 358–362.

73 Buchberger, A., Howard, M. J., Proctor, M., and Bycroft, M. (**2001**) The UBX domain: a widespread ubiquitin-like module. *J. Mol. Biol. 307*, 17–24.

74 Hatakeyama, S., Matsumoto, M., Yada, M., and Nakayama, K. I. (**2004**) Interaction of U-box-type ubiquitin-protein ligases (E3s) with molecular chaperones. *Genes Cells 9*, 533–548.

75 Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., and Hampton, R. Y. (**2001**) Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat. Cell Biol. 3*, 24–29.

76 Swanson, R., Locher, M., and Hochstrasser, M. (**2001**) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev. 15*, 2660–2674.

77 Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S., and Weissman, A. M. (**2001**) The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA 98*, 14422–14427.

78 Imai, Y., Soda, M., and Takahashi, R. (**2000**) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem. 275*, 35661–35664.

79 Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (**2002**) E3 ubiquitin ligase that recognizes sugar chains. *Nature 418*, 438–442.

80 Yoshida, Y., Tokunaga, F., Chiba, T., Iwai, K., Tanaka, K., and Tai, T. (**2003**) Fbs2 is a new member of the E3 ubiquitin ligase family that recognizes sugar chains. *J. Biol. Chem. 278*, 43877–43884.

81 Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (**2005**) Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat. Cell Biol. 10*, 993–998.

82 Schuberth, C. and Buchberger, A. (**2005**) Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nat. Cell Biol. 7*, 999–1006.

83 Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr,

D. M. (**2001**) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol. 3*, 100–105.

84 Jana, N. R., Dikshit, P., Goswami, A., Kotliarova, S., Murata, S., Tanaka, K., and Nukina, N. (**2005**) Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. *J. Biol. Chem. 280*, 11635–11640.

85 Zhang, H., Wang, Q., Kajino, K., and Greene, M. I. (**2000**) VCP, a weak ATPase involved in multiple cellular events, interacts physically with BRCA1 in the nucleus of living cells. *DNA Cell Biol. 19*, 253–263.

86 Ishigaki, S., Hishikawa, N., Niwa, J., Iemura, S., Natsume, T., Hori, S., Kakizuka, A., Tanaka, K., and Sobue, G. (**2004**) Physical and functional interaction between Dorfin and Valosin-containing protein that are colocalized in ubiquitylated inclusions in neurodegenerative disorders. *J. Biol. Chem. 279*, 51376–51385.

87 Yoshida, Y., Adachi, E., Fukiya, K., Iwai, K., and Tanaka, K. (**2005**) Glycoprotein-specific ubiquitin ligases recognize N-glycans in unfolded substrates. *EMBO Rep. 6*, 239–244.

88 Zhong, X., Shen, Y., Ballar, P., Apostolou, A., Agami, R., and Fang, S. (**2004**) AAA ATPase p97/VCP interacts with gp78: a ubiquitin ligase for ER-associated degradation. *J. Biol. Chem.*

89 Kaneko, C., Hatakeyama, S., Matsumoto, M., Yada, M., Nakayama, K., and Nakayama, K. I. (**2003**) Characterization of the mouse gene for the U-box-type ubiquitin ligase UFD2a. *Biochem. Biophys. Res. Commun. 300*, 297–304.

90 Amerik, A. Y., and Hochstrasser, M. (**2004**) Mechanism and function of deubiquitinating enzymes. *Biochim. Biophys. Acta 1695*, 189–207.

91 Doss-Pepe, E. W., Stenroos, E. S., Johnson, W. G., and Madura, K. (**2003**) Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. *Mol. Cell Biol. 23*, 6469–6483.

92 Albrecht, M., Golatta, M., Wullner, U., and Lengauer, T. (**2004**) Structural and functional analysis of ataxin-2 and ataxin-3. *Eur. J. Biochem. 271*, 3155–3170.

93 Romisch, K. (**2005**) Endoplasmic reticulum-associated degradation. *Annu. Rev. Cell Dev. Biol. 21*, 435–456.

94 Alder, N. N., Shen, Y., Brodsky, J. L., Hendershot, L. M., and Johnson, A. E. (**2005**) The molecular mechanisms underlying BiP-mediated gating of the Sec61 translocon of the endoplasmic reticulum. *J. Cell Biol. 168*, 389–399.

95 Lilley, B. N. and Ploegh, H. L. (**2004**) A membrane protein required for dislocation of misfolded proteins from the ER. *Nature 429*, 834–840.

96 Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (**2004**) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature 429*, 841–847.

97 Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (**2005**) Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat. Cell Biol. 7*, 993–998.

98 Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (**2000**) A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat. Cell Biol. 2*, 379–384.

99 Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R. Y. (**2000**) Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J. Cell Biol. 151*, 69–82.

100 Lenk, U., Yu, H., Walter, J., Gelman, M. S., Hartmann, E., Kopito, R. R., and Sommer, T. (**2002**) A role for mammalian Ubc6 homologues in ER-associated protein degradation. *J. Cell Sci. 115*, 3007–3014.

101 Walter, J., Urban, J., Volkwein, C., and Sommer, T. (**2001**) Sec61p-

independent degradation of the tail-anchored ER membrane protein Ubc6p. *Embo J. 20*, 3124–3131.

102 Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (**2002**) Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. *Embo J. 21*, 615–621.

103 Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (**2000**) Recognition of the polyubiquitin proteolytic signal. *Embo J. 19*, 94–102.

104 Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K. I. (**2001**) U box proteins as a new family of ubiquitin-protein ligases. *J. Biol. Chem. 276*, 33111–33120.

105 Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L. Y., and Patterson, C. (**1999**) Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. *Mol. Cell Biol. 19*, 4535–4545.

106 Imai, J., Hasegawa, H., Maruya, M., Koyasu, S., and Yahara, I. (**2005**) Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. *Int. Immunol. 17*, 45–53.

107 Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K. I., and Takahashi, R. (**2002**) CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell 10*, 55–67.

108 Ye, Y., Meyer, H. H., and Rapoport, T. A. (**2001**) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature 414*, 652–656.

109 Ye, Y., Meyer, H. H., and Rapoport, T. A. (**2003**) Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J. Cell Biol. 162*, 71–84.

110 Elsasser, S., Chandler-Militello, D., Muller, B., Hanna, J., and Finley, D. (**2004**) Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. *J. Biol. Chem. 279*, 26817–26822.

111 Fu, H., Reis, N., Lee, Y., Glickman, M. H., and Vierstra, R. D. (**2001**) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *Embo J. 20*, 7096–7107.

112 Medicherla, B., Kostova, Z., Schaefer, A., and Wolf, D. H. (**2004**) A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep. 5*, 692–697.

113 Seeger, M., Hartmann-Petersen, R., Wilkinson, C. R., Wallace, M., Samejima, I., Taylor, M. S., and Gordon, C. (**2003**) Interaction of the anaphase-promoting complex/cyclosome and proteasome protein complexes with multiubiquitin chain-binding proteins. *J. Biol. Chem. 278*, 16791–16796.

114 Saeki, Y., Sone, T., Toh-e, A., and Yokosawa, H. (**2002**) Identification of ubiquitin-like protein-binding subunits of the 26S proteasome. *Biochem. Biophys. Res. Commun. 296*, 813–819.

115 Seeger, M., Ferrell, K., and Dubiel, W. (**1997**) The 26S proteasome: a dynamic structure. *Mol. Biol. Rep. 24*, 83–88.

116 Verma, R., Oania, R., Graumann, J., and Deshaies, R. J. (**2004**) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell 118*, 99–110.

117 Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A., and Finley, D. (**1998**) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell 94*, 615–623.

118 Saeki, Y., Toh-e, A., and Yokosawa, H. (**2000**) Rapid isolation and characterization of the yeast proteasome

regulatory complex. *Biochem. Biophys. Res. Commun. 273*, 509–515.

119 Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (**2002**) A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature 416*, 763–767.

120 Kalies, K. U., Allan, S., Sergeyenko, T., Kroger, H., and Romisch, K. (**2005**) The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. *Embo J. 24*, 2284–2293.

121 Rape, M. and Jentsch, S. (**2004**) Productive RUPture: activation of transcription factors by proteasomal processing. *Biochim. Biophys. Acta 1695*, 209–213.

122 Mao, Y., Senic-Matuglia, F., Di Fiore, P. P., Polo, S., Hodsdon, M. E., and De Camilli, P. (**2005**) Deubiquitinating function of ataxin-3: insights from the solution structure of the Josephin domain. *Proc. Natl. Acad. Sci. USA 102*, 12700–12705.

123 Burnett, B. G. and Pittman, R. N. (**2005**) The polyglutamine neurodegenerative protein ataxin 3 regulates aggresome formation. *Proc. Natl. Acad. Sci. USA 102*, 4330–4335.

124 Seigneurin-Berny, D., Verdel, A., Curtet, S., Lemercier, C., Garin, J., Rousseaux, S., and Khochbin, S. (**2001**) Identification of Components of the Murine Histone Deacetylase 6 Complex: Link between Acetylation and Ubiquitination Signaling Pathways. *Mol. Cell Biol. 21*, 8035–8044.

125 McNeill, H., Knebel, A., Arthur, J. S., Cuenda, A., and Cohen, P. (**2004**) A novel UBA and UBX domain protein that binds polyubiquitin and VCP and is a substrate for SAPKs. *Biochem. J. 384*, 391–400.

126 Song, E. J., Yim, S. H., Kim, E., Kim, N. S., and Lee, K. J. (**2005**) Human Fas-associated factor 1, interacting with ubiquitinated proteins and valosin-containing protein, is involved in the ubiquitin-proteasome pathway. *Mol. Cell Biol. 25*, 2511–2524.

127 Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (**2002**) AAA-ATPase p97/Cdc48p, a Cytosolic Chaperone Required for Endoplasmic Reticulum-Associated Protein Degradation. *Mol. Cell Biol. 22*, 626–634.

128 Woodman, P. G. (**2003**) p97, a protein coping with multiple identities. *J. Cell Sci. 116*, 4283–4290.

129 Latterich, M., Frohlich, K. U., and Schekman, R. (**1995**) Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. *Cell 82*, 885–893.

130 Wang, Q., Song, C., and Li, C. C. (**2004**) Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *J. Struct. Biol. 146*, 44–57.

131 Kano, F., Kondo, H., Yamamoto, A., Kaneko, Y., Uchiyama, K., Hosokawa, N., Nagata, K., and Murata, M. (**2005**) NSF/SNAPs and p97/p47/VCIP135 are sequentially required for cell cycle-dependent reformation of the ER network. *Genes Cells 10*, 989–999.

132 Enenkel, C., Lehmann, A., and Kloetzel, P. M. (**1999**) GFP-labelling of 26S proteasomes in living yeast: insight into proteasomal functions at the nuclear envelope/rough ER. *Mol. Biol. Rep. 26*, 131–135.

133 Babst, M. (**2005**) A protein's final ESCRT. *Traffic 6*, 2–9.

134 Song, C., Wang, Q., and Li, C. C. (**2003**) ATPase activity of p97-valosin-containing protein (VCP). D2 mediates the major enzyme activity, and D1 contributes to the heat-induced activity. *J. Biol. Chem. 278*, 3648–3655.

135 Rouiller, I., DeLaBarre, B., May, A. P., Weis, W. I., Brunger, A. T., Milligan, R. A., and Wilson-Kubalek, E. M. (**2002**) Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle. *Nat. Struct. Biol. 9*, 950–957.

136 Rouiller, I., Butel, V. M., Latterich, M., Milligan, R. A., and Wilson-Kubalek, E. M. (**2000**) A major conformational change in p97 AAA ATPase upon ATP binding. *Mol. Cell 6*, 1485–1490.

137 Beuron, F., Flynn, T. C., Ma, J., Kondo, H., Zhang, X., and Freemont, P. S. (**2003**) Motions and negative cooperativity between p97 domains revealed by cryo-electron microscopy and quantised elastic deformational model. *J. Mol. Biol. 327*, 619–629.

138 DeLaBarre, B., and Brunger, A. T. (**2005**) Nucleotide dependent motion and mechanism of action of p97/VCP. *J. Mol. Biol. 347*, 437–452.

139 Davies, J. M., Tsuruta, H., May, A. P., and Weis, W. I. (**2005**) Conformational changes of p97 during nucleotide hydrolysis determined by small-angle X-Ray scattering. *Structure (Cambridge) 13*, 183–195.

140 Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., and Freemont, P. S. (**2000**) Structure of the AAA ATPase p97. *Mol. Cell 6*, 1473–1484.

141 Huyton, T., Pye, V. E., Briggs, L. C., Flynn, T. C., Beuron, F., Kondo, H., Ma, J., Zhang, X., and Freemont, P. S. (**2003**) The crystal structure of murine p97/VCP at 3.6A. *J. Struct. Biol. 144*, 337–348.

142 DeLaBarre, B. and Brunger, A. T. (**2003**) Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nat. Struct. Biol. 10*, 856–863.

143 Golbik, R., Lupas, A. N., Koretke, K. K., Baumeister, W., and Peters, J. (**1999**) The Janus face of the archaeal Cdc48/p97 homologue VAT: protein folding versus unfolding. *Biol. Chem. 380*, 1049–1062.

144 Brunger, A. T. and DeLaBarre, B. (**2003**) NSF and p97/VCP: similar at first, different at last. *FEBS Lett. 555*, 126–133.

145 Whiteheart, S. W. and Matveeva, E. A. (**2004**) Multiple binding proteins suggest diverse functions for the *N*-ethylmaleimide sensitive factor. *J. Struct. Biol. 146*, 32–43.

146 Beuron, F., Dreveny, I., Yuan, X., Pye, V. E., Mckeown, C., Briggs, L. C., Cliff, M. J., Kaneko, Y., Wallis, R., Isaacson, R. L., Ladbury, J. E., Matthews, S. J., Kondo, H., Zhang, X., Freemont, P. S. (**2006**) Conformational changes in the AAA ATPase p97-p47 adaptor complex. *EMBO J. 25*, 1967–1976.

147 Osborne, A. R., Rapoport, T. A., and van den Berg, B. (**2005**) Protein Translocation by the Sec61/Secy Channel. *Annu. Rev. Cell Dev. Biol. 21*, 529–550.

148 Hirabayashi, M., Inoue, K., Tanaka, K., Nakadate, K., Ohsawa, Y., Kamei, Y., Popiel, A. H., Sinohara, A., Iwamatsu, A., Kimura, Y., Uchiyama, Y., Hori, S., and Kakizuka, A. (**2001**) VCP/p97 in abnormal protein aggregates, cytoplasmic vacuoles, and cell death, phenotypes relevant to neurodegeneration. *Cell Death Differ. 8*, 977–984.

149 Perutz, M. F., Finch, J. T., Berriman, J., and Lesk, A. (**2002**) Amyloid fibers are water-filled nanotubes. *Proc. Natl. Acad. Sci. USA 99*, 5591–5595.

150 Cummings, C. J., Mancini, M. A., Antalffy, B., DeFranco, D. B., Orr, H. T., and Zoghbi, H. Y. (**1998**) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet. 19*, 148–154.

151 Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (**1997**) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell 90*, 537–548.

152 Chai, Y., Koppenhafer, S. L., Shoesmith, S. J., Perez, M. K., and Paulson, H. L. (**1999**) Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. *Hum. Mol. Genet. 8*, 673–682.

153 Nan, L., Wu, Y., Bardag-Gorce, F., Li, J., French, B. A., Wilson, L. T.,

Khanh Nguyen, S., and French, S. W. (**2005**) RNA interference of VCP/p97 increases Mallory body formation. *Exp. Mol. Pathol. 78*, 1–9.

154 Yamanaka, K., Okubo, Y., Suzaki, T., and Ogura, T. (**2004**) Analysis of the two p97/VCP/Cdc48p proteins of *Caenorhabditis elegans* and their suppression of polyglutamine-induced protein aggregation. *J. Struct. Biol. 146*, 242–250.

155 Yamamoto, S., Tomita, Y., Nakamori, S., Hoshida, Y., Iizuka, N., Okami, J., Nagano, H., Dono, K., Umeshita, K., Sakon, M., Ishikawa, O., Ohigashi, H., Aozasa, K., and Monden, M. (**2004**) Valosin-containing protein (p97) and Ki-67 expression is a useful marker in detecting malignant behavior of pancreatic endocrine neoplasms. *Oncology 66*, 468–475.

156 Yamamoto, S., Tomita, Y., Hoshida, Y., Iizuka, N., Kidogami, S., Miyata, H., Takiguchi, S., Fujiwara, Y., Yasuda, T., Yano, M., Nakamori, S., Sakon, M., Monden, M., and Aozasa, K. (**2004**) Expression level of valosin-containing protein (p97) is associated with prognosis of esophageal carcinoma. *Clin. Cancer Res. 10*, 5558–5565.

157 Yamamoto, S., Tomita, Y., Hoshida, Y., Iizuka, N., Monden, M., Yamamoto, S., Iuchi, K., and Aozasa, K. (**2004**) Expression level of valosin-containing protein (p97) is correlated with progression and prognosis of non-small-cell lung carcinoma. *Ann. Surg. Oncol. 11*, 697–704.

158 Yamamoto, S., Tomita, Y., Hoshida, Y., Sakon, M., Kameyama, M., Imaoka, S., Sekimoto, M., Nakamori, S., Monden, M., and Aozasa, K. (**2004**) Expression of valosin-containing protein in colorectal carcinomas as a predictor for disease recurrence and prognosis. *Clin. Cancer Res. 10*, 651–657.

159 Yamamoto, S., Tomita, Y., Hoshida, Y., Takiguchi, S., Fujiwara, Y., Yasuda, T., Yano, M., Nakamori, S., Sakon, M., Monden, M., and Aozasa, K. (**2003**) Expression level of valosin-containing protein is strongly associated with progression and prognosis of gastric carcinoma. *J. Clin. Oncol. 21*, 2537–2544.

160 Yamamoto, S., Tomita, Y., Nakamori, S., Hoshida, Y., Nagano, H., Dono, K., Umeshita, K., Sakon, M., Monden, M., and Aozasa, K. (**2003**) Elevated expression of valosin-containing protein (p97) in hepatocellular carcinoma is correlated with increased incidence of tumor recurrence. *J. Clin. Oncol. 21*, 447–452.

161 Kang, R. S., Daniels, C. M., Francis, S. A., Shih, S. C., Salerno, W. J., Hicke, L., and Radhakrishnan, I. (**2003**) Solution structure of a CUE-ubiquitin complex reveals a conserved mode of ubiquitin binding. *Cell 113*, 621–630.

162 Ohno, A., Jee, J., Fujiwara, K., Tenno, T., Goda, N., Tochio, H., Kobayashi, H., Hiroaki, H., and Shirakawa, M. (**2005**) Structure of the UBA domain of Dsk2p in complex with ubiquitin molecular determinants for ubiquitin recognition. *Structure (Cambridge) 13*, 521–532.

163 Swanson, K. A., Kang, R. S., Stamenova, S. D., Hicke, L., and Radhakrishnan, I. (**2003**) Solution structure of Vps27 UIM-ubiquitin complex important for endosomal sorting and receptor downregulation. *Embo J. 22*, 4597–4606.

164 Prag, G., Lee, S., Mattera, R., Arighi, C. N., Beach, B. M., Bonifacino, J. S., and Hurley, J. H. (**2005**) Structural mechanism for ubiquitinated-cargo recognition by the Golgi-localized, gamma-ear-containing, ADP-ribosylation-factor-binding proteins. *Proc. Natl. Acad. Sci. USA 102*, 2334–2339.

165 Alam, S. L., Sun, J., Payne, M., Welch, B. D., Blake, B. K., Davis, D. R., Meyer, H. H., Emr, S. D., and Sundquist, W. I. (**2004**) Ubiquitin interactions of NZF zinc fingers. *Embo J. 23*, 1411–1421.

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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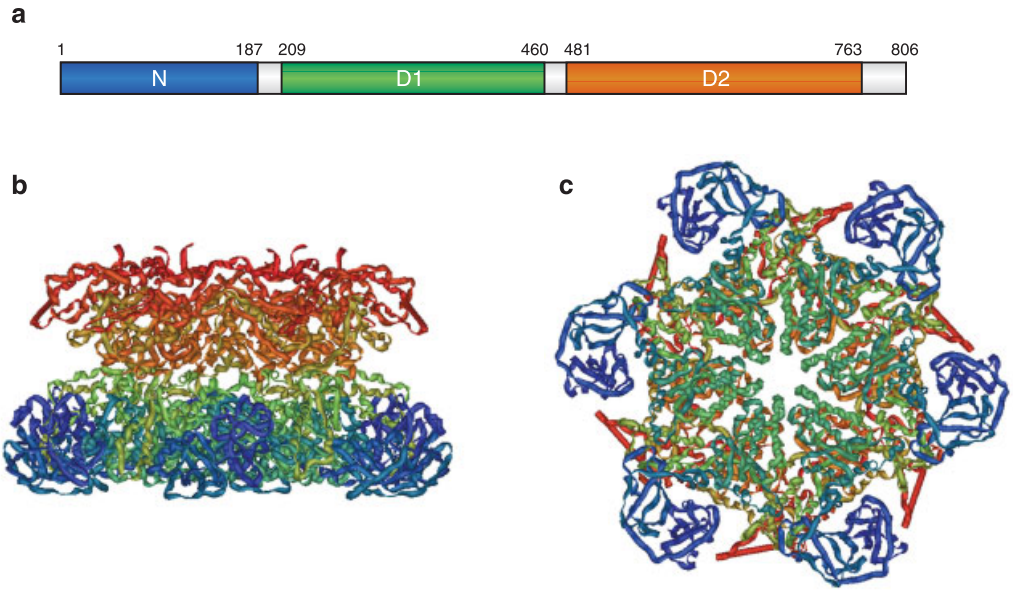

Fig. 8.1. Structure and domain composition of p97. (a) Schematic view of p97 domain composition. The domain borders of the N, D1, and D2 domains are indicated, and the domain colours were chosen to closely match the colour scheme in (b) and (c). (b) Side view of the three-dimensional structure of p97 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).

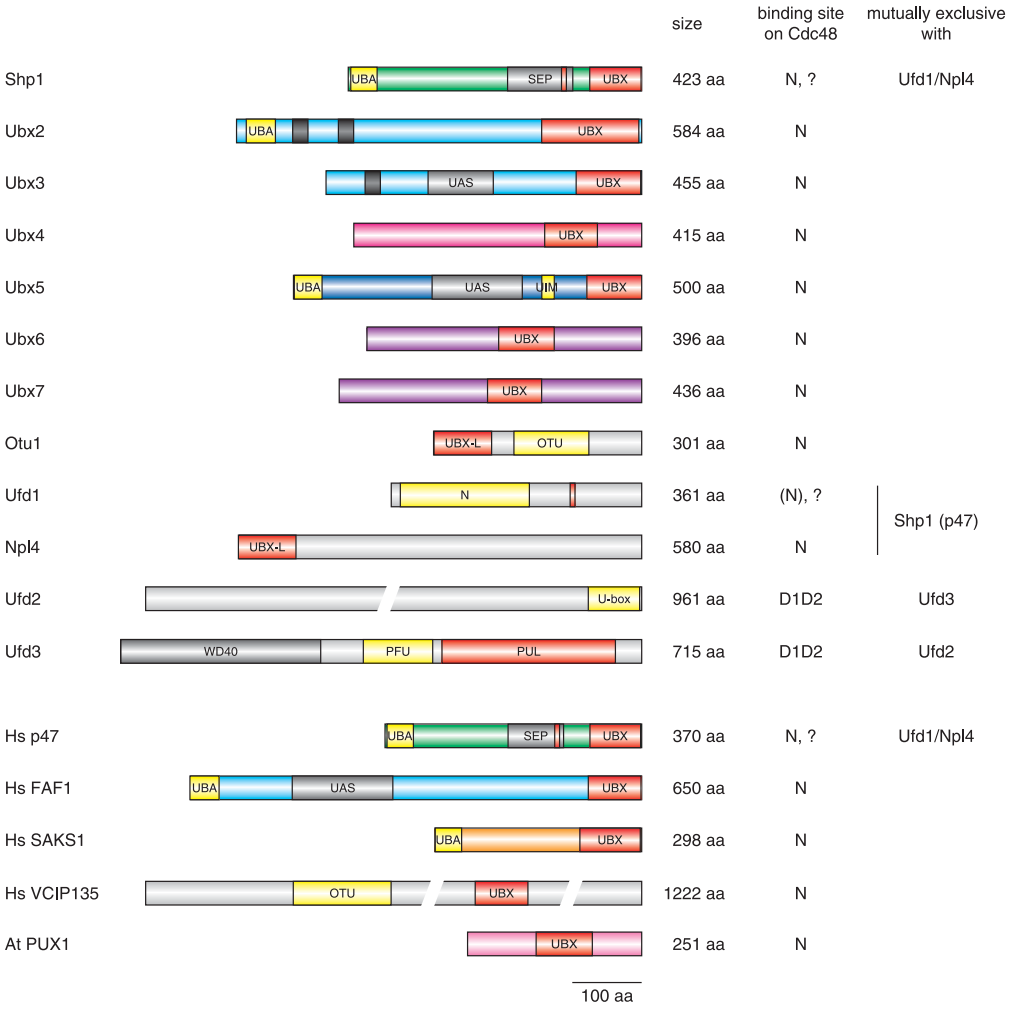

Fig. 8.2. Cdc48 cofactors. The figure shows all known cofactors from *Saccharomyces cerevisiae* (top) and selected cofactors from other species that are discussed in the text (bottom). Cdc48 binding modules including the UBX and UBX-like (UBX-L) domains, the PUL domain, and a short binding motif found 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-

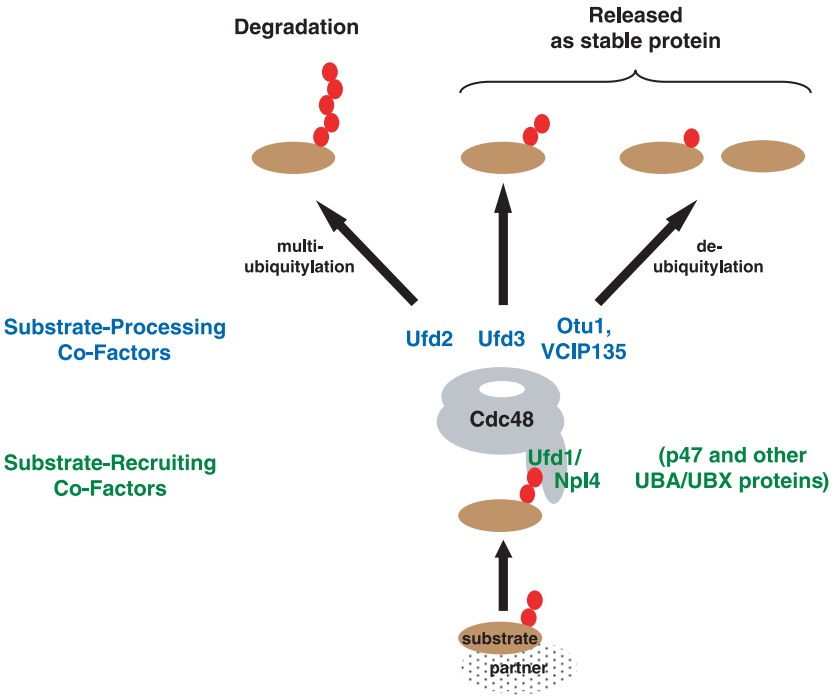

Fig. 8.3. Cdc48 cofactors determine the fate of substrates. Substrate marked by short ubiquitin chains (red circles) is recognized by substrate-recruiting cofactors including Ufd1/Npl4, p47, or other UBA/UBX domain containing proteins (green) and segregated from their partner protein(s). Substrate-processing 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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References

1 Woodman, P. G. (**2003**). p97, a protein coping with multiple identities. *J. Cell Sci. 116*, 4283–4290.

2 Rabouille, C., Kondo, H., Newman, R., Hui, N., Freemont, P., and Warren, G. (**1998**). Syntaxin 5 is a common component of the NSF- and p97-mediated reassembly pathways of Golgi cisternae from mitotic Golgi fragments in vitro. *Cell 92*, 603–610.

3 Thoms, S. (**2002**). Cdc48 can distinguish between native and non-native proteins in the absence of cofactors. *FEBS Lett. 520*, 107–110.

4 Ye, Y., Meyer, H. H., and Rapoport, T. A. (**2003**). Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J. Cell Biol. 162*, 71–84.

5 Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (**2001**). HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol. Biol. Cell 12*, 4114–4128.

6 Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (**2002**). Role of the ubiquitin-selective CDC48(UFD1/NPL4) chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J. 21*, 615–621.

7 Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (**2002**). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol. Cell Biol. 22*, 626–634.

8 Ye, Y., Meyer, H. H., and Rapoport, T. A. (**2001**). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature 414*, 652–656.

9 Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (**2002**). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat. Cell Biol. 4*, 134–139.

10 Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H. D., and Jentsch, S. (**2000**). Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell 102*, 577–586.

11 Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (**2001**). Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. *Cell 107*, 667–677.

12 Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (**1995**). A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem. 270*, 17442–17456.

13 Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (**1999**). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell 96*, 635–644.

14 Ghislain, M., Dohmen, R. J., Levy, F., and Varshavsky, A. (**1996**). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *EMBO J. 15*, 4884–4899.

15 Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D.,

Freemont, P., and Warren, G. (**1997**). p47 is a cofactor for p97-mediated membrane fusion. *Nature 388*, 75–78.

16 Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (**2004**). Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. *EMBO Rep. 5*, 818–824.

17 Decottignies, A., Evain, A., and Ghislain, M. (**2004**). Binding of Cdc48p to a ubiquitin-related UBX domain from novel yeast proteins involved in intracellular proteolysis and sporulation. *Yeast 21*, 127–139.

18 Hartmann-Petersen, R., Wallace, M., Hofmann, K., Koch, G., Johnsen, A. H., Hendil, K. B., and Gordon, C. (**2004**). The Ubx2 and Ubx3 cofactors direct Cdc48 activity to proteolytic and nonproteolytic ubiquitin-dependent processes. *Curr. Biol. 14*, 824–828.

19 McNeill, H., Knebel, A., Arthur, J. S., Cuenda, A., and Cohen, P. (**2004**). A novel UBA and UBX domain protein that binds polyubiquitin and VCP and is a substrate for SAPKs. *Biochem. J. 384*, 391–400.

20 Song, E. J., Yim, S. H., Kim, E., Kim, N. S., and Lee, K. J. (**2005**). Human Fas-associated factor 1, interacting with ubiquitinated proteins and valosin-containing protein, is involved in the ubiquitin-proteasome pathway. *Mol. Cell Biol. 25*, 2511–2524.

21 Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P., and Kondo, H. (**2002**). VCIP135, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly in vivo. *J. Cell Biol. 159*, 855–866.

22 Bruderer, R. M., Brasseur, C., and Meyer, H. H. (**2004**). The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism. *J. Biol. Chem. 279*, 49609–49616.

23 Buchberger, A., Howard, M. J., Proctor, M., and Bycroft, M. (**2001**). The UBX domain: a widespread ubiquitin-like module. *J. Mol. Biol. 307*, 17–24.

24 Yuan, X., Shaw, A., Zhang, X., Kondo, H., Lally, J., Freemont, P. S., and Matthews, S. (**2001**). Solution structure and interaction surface of the C-terminal domain from p47: a major p97-cofactor involved in SNARE disassembly. *J. Mol. Biol. 311*, 255–263.

25 Dreveny, I., Kondo, H., Uchiyama, K., Shaw, A., Zhang, X., and Freemont, P. S. (**2004**). Structural basis of the interaction between the AAA ATPase p97/VCP and its adaptor protein p47. *EMBO J. 23*, 1030–1039.

26 Hofmann, K. and Falquet, L. (**2001**). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. *Trends Biochem. Sci. 26*, 347–350.

27 Hofmann, K. and Bucher, P. (**1996**). The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. *Trends Biochem. Sci. 21*, 172–173.

28 Suyama, M., Doerks, T., Braun, I. C., Sattler, M., Izaurralde, E., and Bork, P. (**2000**). Prediction of structural domains of TAP reveals details of its interaction with p15 and nucleoporins. *EMBO Rep. 1*, 53–58.

29 Buchberger, A. (**2002**). From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol. 12*, 216–221.

30 Meyer, H. H., Wang, Y., and Warren, G. (**2002**). Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. *EMBO J. 21*, 5645–5652.

31 Schuberth, C. and Buchberger, A. (**2005**). Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nat. Cell Biol. 7*, 999–1006.

32 Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (**2000**). A complex of

mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J. 19*, 2181–2192.

33 Rumpf, S. and Jentsch, S. (**2006**). Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. *Mol. Cell 21*, 261–269.

34 Hitt, R. and Wolf, D. H. (**2004**). Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. *FEMS Yeast Res. 4*, 721–729.

35 Park, S., Isaacson, R., Kim, H. T., Silver, P. A., and Wagner, G. (**2005**). Ufd1 exhibits the AAA-ATPase fold with two distinct ubiquitin interaction sites. *Structure 13*, 995–1005.

36 Coles, M., Diercks, T., Liermann, J., Groger, A., Rockel, B., Baumeister, W., Koretke, K. K., Lupas, A., Peters, J., and Kessler, H. (**1999**). The solution structure of VAT-N reveals a 'missing link' in the evolution of complex enzymes from a simple betaalphabetabeta element. *Curr. Biol. 9*, 1158–1168.

37 Mullally, J. E., Chernova, T., and Wilkinson, K. D. (**2006**). Doa1 is a Cdc48 adapter that possesses a novel ubiquitin binding domain. *Mol. Cell Biol. 26*, 822–830.

38 Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (**2005**). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell 120*, 73–84.

39 Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (**2004**). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature 429*, 841–847.

40 Nagahama, M., Suzuki, M., Hamada, Y., Hatsuzawa, K., Tani, K., Yamamoto, A., and Tagaya, M. (**2003**). SVIP is a novel VCP/p97-interacting protein whose expression causes cell vacuolation. *Mol. Biol. Cell 14*, 262–273.

41 Katiyar, S., Li, G., and Lennarz, W. J. (**2004**). A complex between peptide: N-glycanase and two proteasome-linked proteins suggests a mechanism for the degradation of misfolded glycoproteins. *Proc. Natl. Acad. Sci. USA 101*, 13774–13779.

42 Blom, D., Hirsch, C., Stern, P., Tortorella, D., and Ploegh, H. L. (**2004**). A glycosylated type I membrane protein becomes cytosolic when peptide: N-glycanase is compromised. *EMBO J. 23*, 650–658.

43 Li, G., Zhou, X., Zhao, G., Schindelin, H., and Lennarz, W. J. (**2005**). Multiple modes of interaction of the deglycosylation enzyme, mouse peptide N-glycanase, with the proteasome. *Proc. Natl. Acad. Sci. USA 102*, 15809–15814.

44 Suzuki, T., Park, H., Till, E. A., and Lennarz, W. J. (**2001**). The PUB domain: a putative protein-protein interaction domain implicated in the ubiquitin-proteasome pathway. *Biochem. Biophys. Res. Commun. 287*, 1083–1087.

45 Wang, Y., Satoh, A., Warren, G., and Meyer, H. H. (**2004**). VCIP135 acts as a deubiquitinating enzyme during p97-p47-mediated reassembly of mitotic Golgi fragments. *J. Cell Biol. 164*, 973–978.

46 Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (**2005**). Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat. Cell Biol. 7*, 993–998.

47 Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K. D., Cohen, R. E., and Pickart, C. M. (**1997**). Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. *J. Biol. Chem. 272*, 23712–23721.

48 Suzuki, T., Park, H., and Lennarz, W. J. (**2002**). Cytoplasmic peptide: N-glycanase (PNGase) in eukaryotic cells: occurrence, primary structure, and potential functions. *FASEB J. 16*, 635–641.

49 Katiyar, S., Joshi, S., and Lennarz, W. J. (**2005**). The retrotranslocation protein Derlin-1 binds peptide: N-glycanase to the endoplasmic reticulum. *Mol. Biol. Cell 16*, 4584–4594.

50 Hirsch, C., Misaghi, S., Blom, D., Pacold, M. E., and Ploegh, H. L. (**2004**). Yeast N-glycanase distinguishes between native and non-native glycoproteins. *EMBO Rep. 5*, 201–206.

51 Joshi, S., Katiyar, S., and Lennarz, W. J. (**2005**). Misfolding of glycoproteins is a prerequisite for peptide: N-glycanase mediated deglycosylation. *FEBS Lett. 579*, 823–826.

52 Kim, I., Ahn, J., Liu, C., Tanabe, K., Apodaca, J., Suzuki, T., and Rao, H. (**2006**). The Png1-Rad23 complex regulates glycoprotein turnover. *J. Cell Biol. 172*, 211–219.

53 Meyer, H. H., Kondo, H., and Warren, G. (**1998**). The p47 co-factor regulates the ATPase activity of the membrane fusion protein, p97. *FEBS Lett. 437*, 255–257.

54 Rancour, D. M., Park, S., Knight, S. D., and Bednarek, S. Y. (**2004**). Plant UBX domain-containing protein 1, PUX1, regulates the oligomeric structure and activity of arabidopsis CDC48. *J. Biol. Chem. 279*, 54264–54274.

55 Lambertson, D., Chen, L., and Madura, K. (**1999**). Pleiotropic defects caused by loss of the proteasome-interacting factors Rad23 and Rpn10 of *Saccharomyces cerevisiae*. *Genetics 153*, 69–79.

56 Rao, H. and Sastry, A. (**2002**). Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *J. Biol. Chem. 277*, 11691–11695.

57 Ravid, T., Kreft, S. G., and Hochstrasser, M. (**2006**). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *EMBO J.*

58 Saeki, Y., Tayama, Y., Toh-e, A., and Yokosawa, H. (**2004**). Definitive evidence for Ufd2-catalyzed elongation of the ubiquitin chain through Lys48 linkage. *Biochem. Biophys. Res. Commun. 320*, 840–845.

59 Kim, I., Mi, K., and Rao, H. (**2004**). Multiple interactions of Rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. *Mol. Biol. Cell 15*, 3357–3365.

60 Medicherla, B., Kostova, Z., Schaefer, A., and Wolf, D. H. (**2004**). A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep. 5*, 692–697.

61 Dai, R. M., Chen, E., Longo, D. L., Gorbea, C. M., and Li, C. C. (**1998**). Involvement of valosin-containing protein, an ATPase Co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. *J. Biol. Chem. 273*, 3562–3573.

62 Dai, R. M. and Li, C. C. (**2001**). Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat. Cell Biol. 3*, 740–744.

63 Moir, D., Stewart, S. E., Osmond, B. C., and Botstein, D. (**1982**). Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics 100*, 547–563.

64 Fu, X., Ng, C., Feng, D., and Liang, C. (**2003**). Cdc48p is required for the cell cycle commitment point at Start via degradation of the G1-CDK inhibitor Far1p. *J. Cell Biol. 163*, 21–26.

65 Archambault, V., Chang, E. J., Drapkin, B. J., Cross, F. R., Chait, B. T., and Rout, M. P. (**2004**). Targeted proteomic study of the cyclin-Cdk module. *Mol. Cell 14*, 699–711.

66 Cao, K., Nakajima, R., Meyer, H. H., and Zheng, Y. (**2003**). The AAA-ATPase Cdc48/p97 regulates spindle disassembly at the end of mitosis. *Cell 115*, 355–367.

67 Vong, Q. P., Cao, K., Li, H. Y., Iglesias, P. A., and Zheng, Y. (**2005**). Chromosome alignment and segregation regulated by ubiquitination of survivin. *Science 310*, 1499–1504.

68 Yuasa, T., Hayashi, T., Ikai, N., Katayama, T., Aoki, K., Obara, T.,

Toyoda, Y., Maruyama, T., Kitagawa, D., Takahashi, K., Nagao, K., Nakaseko, Y., and Yanagida, M. (**2004**). An interactive gene network for securin-separase, condensin, cohesin, Dis1/Mtc1 and histones constructed by mass transformation. *Genes Cells 9*, 1069–1082.

69 Rabouille, C., Levine, T. P., Peters, J. M., and Warren, G. (**1995**). An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell 82*, 905–914.

70 Acharya, U., Jacobs, R., Peters, J. M., Watson, N., Farquhar, M. G., and Malhotra, V. (**1995**). The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. *Cell 82*, 895–904.

71 Latterich, M., Frohlich, K. U., and Schekman, R. (**1995**). Membrane fusion and the *cell* cycle: Cdc48p participates in the fusion of ER membranes. *Cell 82*, 885–893.

72 Roy, L., Bergeron, J. J., Lavoie, C., Hendriks, R., Gushue, J., Fazel, A., Pelletier, A., Morre, D. J., Subramaniam, V. N., Hong, W., and Paiement, J. (**2000**). Role of p97 and syntaxin 5 in the assembly of transitional endoplasmic reticulum. *Mol. Biol. Cell 11*, 2529–2542.

73 Kano, F., Kondo, H., Yamamoto, A., Tanaka, A. R., Hosokawa, N., Nagata, K., and Murata, M. (**2005**). The maintenance of the endoplasmic reticulum network is regulated by p47, a cofactor of p97, through phosphorylation by cdc2 kinase. *Genes Cells 10*, 333–344.

74 Hetzer, M., Meyer, H. H., Walther, T. C., Bilbao-Cortes, D., Warren, G., and Mattaj, I. W. (**2001**). Distinct AAA-ATPase p97 complexes function in discrete steps of nuclear assembly. *Nat. Cell Biol. 3*, 1086–1091.

75 Seeley, E. S., Kato, M., Margolis, N., Wickner, W., and Eitzen, G. (**2002**). Genomic analysis of homotypic vacuole fusion. *Mol. Biol. Cell 13*, 782–794.

76 Meyer, H. H. (**2005**). Golgi reassembly after mitosis: The AAA family meets the ubiquitin family. *Biochim. Biophys. Acta 1744*, 108–119.

77 Uchiyama, K. and Kondo, H. (**2005**). p97/p47-Mediated biogenesis of Golgi and ER. *J. Biochem (Tokyo) 137*, 115–119.

78 Patel, S. K., Indig, F. E., Olivieri, N., Levine, N. D., and Latterich, M. (**1998**). Organelle membrane fusion: a novel function for the syntaxin homolog Ufe1p in ER membrane fusion. *Cell 92*, 611–620.

79 Dalal, S., Rosser, M. F., Cyr, D. M., and Hanson, P. I. (**2004**). Distinct roles for the AAA ATPases NSF and p97 in the secretory pathway. *Mol. Biol. Cell 15*, 637–648.

80 Raasi, S., Varadan, R., Fushman, D., and Pickart, C. M. (**2005**). Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat. Struct. Mol. Biol. 12*, 708–714.

81 Wojcik, C., Yano, M., and DeMartino, G. N. (**2004**). RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis. *J. Cell Sci. 117*, 281–292.

82 Madeo, F., Frohlich, E., and Frohlich, K. U. (**1997**). A yeast mutant showing diagnostic markers of early and late apoptosis. *J. Cell Biol. 139*, 729–734.

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.

References

1 Pickart CM, Eddins MJ. (**2004**) Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta 1695*:55–72.

2 Devoy A, Soane T, Welchman R, Mayer RJ. (**2005**). The ubiquitin–proteasome system and cancer. *Essays Biochem. 41*:187–203.

3 Layfield R, Lowe J, Bedford L. (**2005**) The ubiquitin-proteasome system and neurodegenerative disorders. *Essays Biochem. 41*:157–171.

4 Varshavsky A. (**2005**) Regulated protein degradation. *Trends Biochem. Sci. 30*:283–286.

5 Finley D, Bartel B, Varshavsky A. (**1989**) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature 338*:394–401.

6 Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP. (**2003**) A proteomics approach to understanding protein ubiquitination. *Nat.Biotechnol. 21*:921–926.

7 Hicke L, Dunn R. (**2003**) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol. 19*:141–172.

8 Dupre S, Urban-Grimal D, Haguenauer-Tsapis R. (**2004**) Ubiquitin and endocytic internalization in yeast and animal cells. *Biochim. Biophys. Acta 1695*:89–111.

9 Urbe S. (**2005**) Ubiquitin and endocytic protein sorting. *Essays Biochem. 41*:81–98.

10 Pickart CM, Fushman D. (**2004**) Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol. 8*:610–616.

11 Baker RT, Tobias JT, Varshavsky A. (**1992**) Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. *J. Biol. Chem. 267*:23364–23375.

12 Kim JH, Park KC, Chung SS, Bang O, Chung CH. (**2003**) Deubiquitinating enzymes as cellular regulators. *J. Biochem. (Tokyo) 134*:9–18.

13 Fischer JA. (**2003**) Deubiquitinating enzymes: their roles in development,

differentiation, and disease. *Int. Rev. Cytol. 229*:43–72.

14 Wing SS. (**2003**) Deubiquitinating enzymes: the importance of driving in reverse along the ubiquitin-proteasome pathway. *Int. J. Biochem. Cell Biol. 35*:590–605.

15 Amerik AY, Hochstrasser M. (**2004**) Mechanism and function of deubiquitinating enzymes. *Biochim. Biophys. Acta 1695*:189–207.

16 Soboleva TA, Baker RT. (**2004**) Deubiquitinating enzymes: their functions and substrate specificity. *Curr. Protein Pept. Sci. 5*:191–200.

17 Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R. (**2005**) A genomic and functional inventory of deubiquitinating enzymes. *Cell 123*:773–786.

18 Wilkinson KD. (**1997**) Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J. 11*:1245–1256.

19 Papa FR, Hochstrasser M. (**1993**) The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 Oncogene *Nature 366*:313–319.

20 Huebner K, Cannizzaro LA, Nakamura T, Hillova J, Mariage-Samson R, Hecht F, Hill M, Croce CM. (**1988**) A rearranged transforming gene, tre, is made up of human sequences derived from chromosome regions 5q, 17q and 18q. *Oncogene 3*:449–455.

21 Nakamura T, Hillova J, Mariage-Samson R, Hill M. (**1988**) Molecular cloning of a novel oncogene generated by DNA recombination during transfection. *Oncogene Res. 2*:357–370.

22 Nakamura T, Hillova J, Mariage-Samson R, Onno M, Huebner K, Cannizzaro LA, Boghosian-Sell L, Croce CM, Hill M. (**1992**) A novel transcriptional unit of the tre oncogene widely expressed in human cancer cells. *Oncogene 7*:733–741.

23 Onno M, Nakamura T, Mariage-Samson R, Hillova J, Hill M. (**1993**) Human TRE17 oncogene is generated from a family of homologous polymorphic sequences by single-base changes. *DNA Cell Biol. 12*:107–118.

24 Paulding CA, Ruvolo M, Haber DA. (**2003**) The Tre2 (USP6) oncogene is a hominoid-specific gene. *Proc. Natl. Acad. Sci. USA 100*:2507–2511.

25 Martinu L, Masuda-Robens JM, Robertson SE, Santy LC, Casanova JE, Chou MM. (**2004**) The TBC (Tre-2/Bub2/Cdc16) domain protein TRE17 regulates plasma membrane-endosomal trafficking through activation of Arf6. *Mol. Cell Biol. 24*:9752–9762.

26 Cohen M, Stutz F, Belgareh N, Haguenauer-Tsapis R, Dargemont C. (**2003**) Ubp3 requires a cofactor, Bre5, to specifically de-ubiquitinate the COPII protein, Sec23. *Nat. Cell Biol. 5*:661–667.

27 Murray RZ, Jolly LA, Wood SA. (**2004**) The FAM deubiquitylating enzyme localizes to multiple points of protein trafficking in epithelia, where it associates with E-cadherin and beta-catenin. *Mol. Biol. Cell 15*:1591–1599.

28 Shen C, Ye Y, Robertson SE, Lau AW, Mak DO, Chou MM. (**2005**) Calcium/calmodulin regulates ubiquitination of the ubiquitin-specific protease TRE17/USP6. *J. Biol. Chem. 280*:35967–35973.

29 Oliveira AM, Hsi BL, Weremowicz S, Rosenberg AE, Dal Cin P, Joseph N, Bridge JA, Perez-Atayde AR, Fletcher JA. (**2004**) USP6 (Tre2) fusion oncogenes in aneurysmal bone cyst. *Cancer Res. 64*:1920–1923.

30 Oliveira AM, Perez-Atayde AR, Inwards CY, Medeiros F, Derr V, Hsi BL, Gebhardt MC, Rosenberg AE, Fletcher JA. (**2004**) USP6 and CDH11 oncogenes identify the neoplastic cell in primary aneurysmal bone cysts and are absent in so-called secondary aneurysmal bone cysts. *Am. J. Pathol. 165*:1773–1780.

31 Oliveira AM, Perez-Atayde AR, Dal Cin P, Gebhardt MC, Chen CJ, Neff JR, Demetri GD, Rosenberg AE, Bridge JA, Fletcher JA. (**2005**) Aneurysmal bone cyst variant translocations upregulate USP6 transcription by promoter swapping

with the ZNF9, COL1A1, TRAP150, and OMD genes. *Oncogene 24*:3419–3426.

32 Gupta K, Copeland NG, Gilbert DJ, Jenkins NA, Gray DA. (**1993**) Unp, a mouse gene related to the tre Oncogene. *Oncogene 8*:2307–2310.

33 Gilchrist CA, Gray DA, Baker RT. (**1997**) A ubiquitin-specific protease that efficiently cleaves the ubiquitin-proline bond. *J. Biol. Chem. 272*: 32280–32285.

34 Gilchrist CA, Baker RT. (**2000**). Characterization of the ubiquitin-specific protease activity of the mouse/human Unp/Unph oncoprotein. *Biochem. Biophys. Acta 1481*:297–309.

35 Gupta K, Chevrette M, Gray DA. (**1994**) The Unp proto-oncogene encodes a nuclear protein. *Oncogene 9*:1729–1731.

36 Blanchette P, Gilchrist CA, Baker RT, Gray DA. (**2001**) Association of UNP, a ubiquitin-specific protease, with the pocket proteins pRb, p107 and p130. *Oncogene 20*:5533–5537.

37 DeSalle LM, Latres E, Lin D, Graner E, Montagnoli A, Baker RT, Pagano M, Loda M. (**2001**) The de-ubiquitinating enzyme Unp interacts with the retinoblastoma protein. *Oncogene 20*:5538–5542.

38 Gray DA, Inazawa J, Gupta K, Wong A, Ueda R, Takahashi T. (**1995**) Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. *Oncogene 10*:2179–2183.

39 Frederick A, Rolfe M, Chiu MI. (**1998**) The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines. *Oncogene 16*:153–165.

40 Soboleva TA, Jans DA, Johnson-Saliba M, Baker RT. (**2005**) Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme. *J. Biol. Chem. 280*:745–752.

41 Velazquez-Fernandez D, Laurell C, Geli J, Hoog A, Odeberg J, Kjellman M, Lundeberg J, Hamberger B, Nilsson P, Backdahl M. (**2005**) Expression profiling of adrenocortical neoplasms suggests a molecular signature of malignancy. *Surgery 138*:1087–1094.

42 Laroia G, Sarkar B, Schneider RJ. (**2002**) Ubiquitin-dependent mechanism regulates rapid turnover of AU-rich cytokine mRNAs. *Proc. Natl. Acad. Sci. USA 99*:1842–1846.

43 Di Donato F, Chan EK, Askanase AD, Miranda-Carus M, Buyon JP. (**2001**) Interaction between 52 kDa SSA/Ro and deubiquitinating enzyme UnpEL: a clue to function. *Int. J. Biochem. Cell Biol. 33*:924–934.

44 Fukuda-Kamitani T, Kamitani T. (**2002**) Ubiquitination of Ro52 autoantigen. *Biochem. Biophys. Res. Commun. 295*:774–778.

45 Wada K, Tanji K, Kamitani T. (**2006**) Oncogenic protein UnpEL/Usp4 deubiquitinates Ro52 by its isopeptidase activity. *Biochem. Biophys. Res. Commun. 339*:731–736.

46 Milojevic T, Reiterer V, Stefan E, Korkhov VM, Dorostkar MM, Ducza E, Ogris E, Boehm S, Freissmuth M, Nanoff C. (**2006**) The ubiquitin-specific protease Usp4 regulates the cell surface level of the A2A-receptor. *Mol. Pharmacol. 69*:1083–1094.

47 Hershfield MS. (**2005**) New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. *Eur. J. Immunol. 35*:25–30.

48 Bignell GR, Warren W, Seal S, Takahashi M, Rapley E, Barfoot R, Green H, Brown C, Biggs PJ, Lakhani SR, Jones C, Hansen J, Blair E, Hofmann B, Siebert R, Turner G, Evans DG, Schrander-Stumpel C, Beemer FA, van Den Ouweland A, Halley D, Delpech B, Cleveland MG, Leigh I, Leisti J, Rasmussen S. (**2000**) Identification of the familial cylindromatosis tumour-suppressor gene. *Nat. Genet. 25*:160–165.

49 Borodovsky A, Ovaa H, Kolli N, Gan-Erdene T, Wilkinson KD, Ploegh HL, Kessler BM. (**2002**)

Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem. Biol. 9*:1149–1159.

50 Brummelkamp TR, Nijman SM, Dirac AM, Bernards R. (**2003**) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. *Nature 424*:797–801.

51 Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G. (**2003**) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature 424*:801–805.

52 Trompouki E, Hatzivassiliou E, Tsichritzis T, Farmer H, Ashworth A, Mosialos G. (**2003**) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature 424*:793–796.

53 Reiley W, Zhang M, Sun SC. (**2004**) Negative regulation of JNK signaling by the tumor suppressor CYLD. *J. Biol. Chem. 279*:55161–55167.

54 Balakirev MY, Tcherniuk SO, Jaquinod M, Chroboczek J. (**2003**) Otubains: a new family of cysteine proteases in the ubiquitin pathway. *EMBO Rep. 4*:517–522.

55 Evans PC, Smith TS, Lai MJ, Williams MG, Burke DF, Heyninck K, Kreike MM, Beyaert R, Blundell TL, Kilshaw PJ. (**2003**) A novel type of deubiquitinating enzyme. *J. Biol. Chem. 278*:23180–23186.

56 Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM. (**2004**) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature 430*:694–699.

57 Chen ZJ. (**2005**) Ubiquitin signalling in the NF-kappaB pathway. *Nat. Cell Biol. 7*:758–765.

58 Krappmann D, Scheidereit C. (**2005**) A pervasive role of ubiquitin conjugation in activation and termination of IkappaB kinase pathways. *EMBO Rep. 6*:321–326.

59 Sun L, Chen ZJ. (**2004**) The novel functions of ubiquitination in signaling. *Curr. Opin. Cell Biol. 16*:119–126.

60 Orth K, Xu Z, Mudgett MB, Bao ZQ, Palmer LE, Bliska JB, Mangel WF, Staskawicz B, Dixon JE. (**2000**) Disruption of signaling by Yersinia effector YopJ, a ubiquitin-like protein protease. *Science 290*:1594–1597.

61 Zhou H, Monack DM, Kayagaki N, Wertz I, Yin J, Wolf B, Dixit VM. (**2005**) Yersinia virulence factor YopJ acts as a deubiquitinase to inhibit NF-{kappa}B activation. *J. Exp. Med. 202*:1327–1332.

62 Zapata JM, Pawlowski K, Haas E, Ware CF, Godzik A, Reed JC. (**2001**) A diverse family of proteins containing tumor necrosis factor receptor-associated factor domains. *J. Biol. Chem. 276*:24242–24252.

63 Everett RD, Meredith M, Orr A, Cross A, Kathoria M, Parkinson J. (**1997**) A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J. 16*:566–577. Corrected and republished in: *EMBO J.* (**1997**) *16*:1519–1530.

64 Baker RT, Wang X-W, Woollatt E, White J, Sutherland GR. (**1999**) Identification, functional characterization and chromosomal localisation of USP15, a novel human ubiquitin-specific protease related to the Unp/USP4 oncoprotein, and a systematic nomenclature proposal for human ubiquitin-specific proteases. *Genomics 59*:264–274.

65 Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. (**2002**) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature 416*:648–653.

66 Yoo KJ, Lee HJ, Lee H, Lee KY, Lee SH, Chung HM, Baek KH. (**2005**) Expression and functional analyses of mHAUSP regulating apoptosis of cervical adenocarcinoma cells. *Int. J. Oncol. 27*:97–104.

67 Li M, Brooks CL, Kon N, Gu W. (**2004**) A dynamic role of HAUSP in

the p53-Mdm2 pathway. *Mol. Cell 13*:879–886.

68 Cummins JM, Rago C, Kohli M, Kinzler KW, Lengauer C, Vogelstein B. (**2004**) Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature 428*:1 p following 486.

69 Meulmeester E, Pereg Y, Shiloh Y, Jochemsen AG. (**2005**) ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation. *Cell Cycle 4*:1166–1170.

70 Masuya D, Huang C, Liu D, Nakashima T, Yokomise H, Ueno M, Nakashima N, Sumitomo S. (**2006**) The HAUSP gene plays an important role in non-small cell lung carcinogenesis through p53-dependent pathways. *J. Pathol. 208*:724–732.

71 Brooks CL, Gu W. (**2006**) p53 Ubiquitination: Mdm2 and Beyond. *Mol. Cell 21*:307–315.

72 Boutell C, Everett RD. (**2003**) The herpes simplex virus type 1 (HSV-1) regulatory protein ICP0 interacts with and Ubiquitinates p53. *J. Biol. Chem. 278*:36596–36602.

73 Boutell C, Canning M, Orr A, Everett RD. (**2005**) Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7. *J. Virol. 79*:12342–12354.

74 Holowaty MN, Zeghouf M, Wu H, Tellam J, Athanasopoulos V, Greenblatt J, Frappier L. (**2003**) Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. *J. Biol. Chem. 278*:29987–29994.

75 Holowaty MN, Sheng Y, Nguyen T, Arrowsmith C, Frappier L. (**2003**) Protein interaction domains of the ubiquitin-specific protease, USP7/HAUSP. *J. Biol. Chem. 278*:47753–47761.

76 Saridakis V, Sheng Y, Sarkari F, Holowaty MN, Shire K, Nguyen T, Zhang RG, Liao J, Lee W, Edwards AM, Arrowsmith CH, Frappier L. (**2005**) Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. *Mol. Cell 18*:25–36.

77 Li Z, Na X, Wang D, Schoen SR, Messing EM, Wu G. (**2002**) Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel-Lindau tumor suppressor protein. *J. Biol. Chem. 277*:4656–4662.

78 Li Z, Wang D, Na X, Schoen SR, Messing EM, Wu G. (**2002**) Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumor suppressor. *Biochem. Biophys. Res. Commun. 294*:700–709.

79 Semenza GL. (**2003**) Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer 3*:721–732.

80 Kaelin WG Jr. (**2002**) Molecular basis of the VHL hereditary cancer syndrome. *Nat. Rev. Cancer 2*:673–682.

81 Li Z, Wang D, Messing EM, Wu G. (**2005**) VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1alpha. *EMBO Rep. 6*:373–378.

82 Curcio-Morelli C, Zavacki AM, Christofollete M, Gereben B, de Freitas BC, Harney JW, Li Z, Wu G, Bianco AC. (**2003**) Deubiquitination of type 2 iodothyronine deiodinase by von Hippel-Lindau protein-interacting deubiquitinating enzymes regulates thyroid hormone activation. *J. Clin. Invest. 112*:189–196.

83 D'Andrea AD. (**2003**). The Fanconi road to cancer. *Genes Dev. 17*:1933–1936.

84 Kennedy RD, D'Andrea AD. (**2005**) The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev. 19*:2925–2940.

85 Nijman SM, Huang TT, Dirac AM, Brummelkamp TR, Kerkhoven RM, D'Andrea AD, Bernards R. (**2005**) The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol. Cell 17*:331–339.

86 Jensen DE, Proctor M, Marquis ST, Gardner HP, Ha SI, Chodosh LA, Ishov AM, Tommerup N, Vissing H, Sekido Y, Minna J, Borodovsky A, Schultz DC, Wilkinson KD, Maul GG, Barlev N, Berger SL, Prendergast GC, Rauscher FJ 3rd. (**1998**) BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene 16*:1097–1112.

87 Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. (**1999**) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. USA 96*:11364–11369.

88 Hashizume R, Fukuda M, Maeda I, Nishikawa H, Oyake D, Yabuki Y, Ogata H, Ohta T. (**2001**) The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J. Biol. Chem. 276*:14537–14540.

89 Mallery DL, Vandenberg CJ, Hiom K. (**2002**) Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J. 21*:6755–6762.

90 Wu-Baer F, Lagrazon K, Yuan W, Baer R. (**2003**) The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin. *J. Biol. Chem. 278*:34743–34746.

91 Nishikawa H, Ooka S, Sato K, Arima K, Okamoto J, Klevit RE, Fukuda M, Ohta T. (**2004**) Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. *J. Biol. Chem. 279*:3916–3924.

92 Coupier I, Cousin PY, Hughes D, Legoix-Ne P, Trehin A, Sinilnikova OM, Stoppa-Lyonnet D. (**2005**) BAP1 and Breast Cancer Risk. *Fam. Cancer 4*:273–277.

93 Schoenfeld AR, Apgar S, Dolios G, Wang R, Aaronson SA. (**2004**) BRCA2 is ubiquitinated in vivo and interacts with USP11, a deubiquitinating enzyme that exhibits prosurvival function in the cellular response to DNA damage. *Mol. Cell Biol. 24*:7444–7455.

94 Ideguchi H, Ueda A, Tanaka M, Yang J, Tsuji T, Ohno S, Hagiwara E, Aoki A, Ishigatsubo Y. (**2002**) Structural and functional characterization of the USP11 deubiquitinating enzyme, which interacts with the RanGTP-associated protein RanBPM. *Biochem. J. 367*:87–95.

95 Chen J, Silver DP, Walpita D, Cantor SB, Gazdar AF, Tomlinson G, Couch FJ, Weber BL, Ashley T, Livingston DM, Scully R. (**1998**) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol. Cell 2*:317–328.

96 Dong Y, Hakimi MA, Chen X, Kumaraswamy E, Cooch NS, Godwin AK, Shiekhattar R. (**2003**) Regulation of BRCC, a holoenzyme complex containing BRCA1 and BRCA2, by a signalosome-like subunit and its role in DNA repair. *Mol. Cell 12*:1087–1099.

97 Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD. (**2001**) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell 7*:249–262.

98 Zhu Y, Carroll M, Papa FR, Hochstrasser M, D'Andrea AD. (**1996**) DUB-1, a deubiquitinating enzyme with growth-suppressing activity. *Proc. Natl. Acad. Sci. USA 93*:3275–3279.

99 Zhu Y, Lambert K, Corless C, Copeland NG, Gilbert DJ, Jenkins NA, D'Andrea AD. (**1997**) DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes. *J. Biol. Chem. 272*:51–57.

100 Migone TS, Humbert M, Rascle A, Sanden D, D'Andrea A, Johnston JA. (**2001**) The deubiquitinating enzyme DUB-2 prolongs cytokine-induced signal transducers and activators of transcription activation

and suppresses apoptosis following cytokine withdrawal. *Blood 98*:1935–1941.

101 Baek KH, Mondoux MA, Jaster R, Fire-Levin E, D'Andrea AD. (**2001**) DUB-2A, a new member of the DUB subfamily of hematopoietic deubiquitinating enzymes. *Blood 98*:636–642.

102 Baek KH, Kim MS, Kim YS, Shin JM, Choi HK. (**2004**) DUB-1A, a novel deubiquitinating enzyme subfamily member, is polyubiquitinated and cytokine-inducible in B-lymphocytes. *J. Biol. Chem. 279*:2368–2376.

103 Saitoh Y, Miyamoto N, Okada T, Gondo Y, Showguchi-Miyata J, Hadano S, Ikeda J-E. (**2000**) The RS447 human megasatellite tandem repetitive sequence encodes a novel deubiquitinating enzyme with a functional promoter. *Genomics 67*:291–300.

104 Burrows JF, McGrattan MJ, Johnston JA. (**2005**) The DUB/USP17 deubiquitinating enzymes, a multigene family within a tandemly repeated sequence. *Genomics 85*:524–529.

105 Burrows JF, McGrattan MJ, Rascle A, Humbert M, Baek KH, Johnston JA. (**2004**) DUB-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. *J. Biol. Chem. 279*:13993–14000.

106 Gesbert F, Malarde V, Dautry-Varsat A. (**2005**) Ubiquitination of the common cytokine receptor gammaC and regulation of expression by an ubiquitination/deubiquitination machinery. *Biochem. Biophys. Res. Commun. 334*:474–480.

107 Zhu Y, Pless M, Inhorn R, Mathey-Prevot B, D'Andrea AD. (**1996**) The murine DUB-1 gene is specifically induced by the beta(c) subunit of interleukin-3 receptor. *Mol. Cell Biol. 16*:4808–4817.

108 Hershko A. (**2005**). The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. *Cell Death Differ. 12*:1191–1197.

109 Mani A, Gelmann EP. (**2005**) The ubiquitin-proteasome pathway and its role in cancer. *J. Clin. Oncol. 23*:4776–4789.

110 Semple CA; RIKEN GER Group; GSL Members. (**2003**) The comparative proteomics of ubiquitination in mouse. *Genome Res. 13*:1389–1394.

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