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Review

Mechanism and function of deubiquitinating enzymes

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Abstract

Attachment of ubiquitin to proteins is a crucial step in many cellular regulatory mechanisms and contributes to numerous biological processes, including embryonic development, the cell cycle, growth control, and prevention of neurodegeneration. In these diverse regulatory settings, the most widespread mechanism of ubiquitin action is probably in the context of protein degradation. Polyubiquitin attachment targets many intracellular proteins for degradation by the proteasome, and (mono)ubiquitination is often required for down-regulating plasma membrane proteins by targeting them to the vacuole (lysosome). Ubiquitin–protein conjugates are highly dynamic structures. While an array of enzymes directs the conjugation of ubiquitin to substrates, there are also dozens of deubiquitinating enzymes (DUBs) that can reverse the process. Several lines of evidence indicate that DUBs are important regulators of the ubiquitin system. These enzymes are responsible for processing inactive ubiquitin precursors, proofreading ubiquitin–protein conjugates, removing ubiquitin from cellular adducts, and keeping the 26S proteasome free of inhibitory ubiquitin chains. The present review focuses on recent discoveries that have led to a better understanding the mechanisms and physiological roles of this diverse and still poorly understood group of enzymes. We also discuss briefly some of the proteases that act on ubiquitin-like protein (UBL) conjugates and compare them to DUBs.

Keywords: Ubiquitin; Proteasome; Deubiquitinating enzyme

1. Introduction

During the past 10 years, conjugation of ubiquitin and ubiquitin-like proteins (UBLs) to intracellular proteins has emerged as an important mechanism for regulating numerous cellular processes. These include cell cycle progression and signal transduction, transport across the plasma membrane, protein quality control in the endoplasmic reticulum, transcriptional regulation, and growth control. The role of ubiquitination in most of these processes is to promote the degradation of specific proteins. A complex enzymatic system is responsible for attaching ubiquitin to and removing it from protein substrates [1–5].

Conjugation of ubiquitin to a substrate requires at least three different enzymes. The first enzyme, E1 or ubiquitin-activating enzyme, carries out the ATP-dependent activation of the C-terminus of ubiquitin, forming a covalently bound intermediate with ubiquitin in which the terminal glycine of ubiquitin is linked to the thiol group of a cysteine residue in the E1 active site. Ubiquitin is then transferred to the active site cysteine residue of a ubiquitin-conjugating enzyme or E2. Finally, a third factor, E3 or ubiquitin–protein ligase, catalyzes the transfer of ubiquitin to a lysine residue in the protein substrate (or in cases the N-terminal α-amino group), forming an amide bond. Proteins can be modified on a single or multiple lysine residues by a single ubiquitin or by ubiquitin oligomers. The fate of a ubiquitin–protein conjugate depends in part on the length of the ubiquitin oligomer(s) and on the configuration of ubiquitin–ubiquitin linkages in the ubiquitin chain. Chains of four or more ubiquitins, in which the C-terminus of one ubiquitin is attached to Lys48 of the next ubiquitin, efficiently promote binding of the modified protein to the 26S proteasome, with

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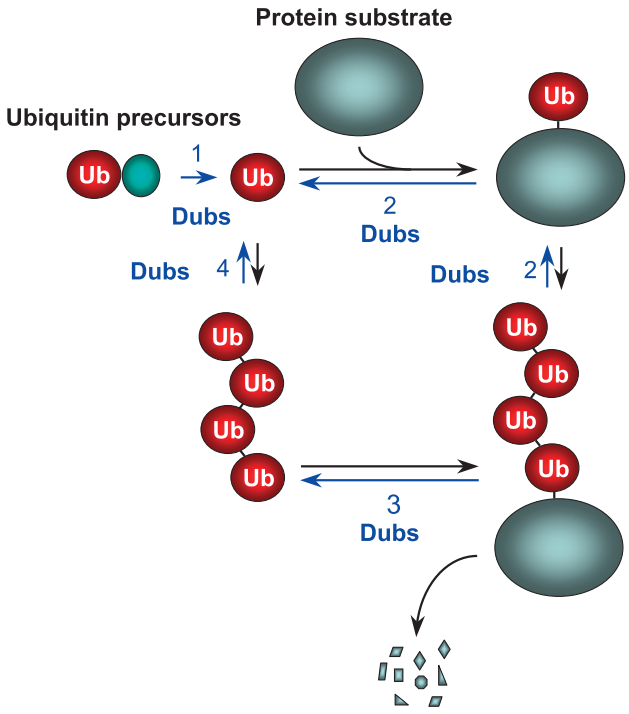


Fig. 1. Functions of DUBs in the ubiquitin pathway. Processing of ubiquitin precursors (1). Editing or rescue of ubiquitin conjugates, which are generally adducts to other proteins in the cell but can also be ligated to abundant small nucleophiles such as glutathione (2). Recycling of ubiquitin or ubiquitin oligomers from ubiquitin–protein conjugates targeted for degradation (3). Disassembly of unanchored ubiquitin oligomers (4).

subsequent degradation of the substrate to small peptides but recycling of the ubiquitins [4]. In contrast, monoubiquitination or attachment of short Lys63-linked ubiquitin chains to a protein can have a variety of consequences that do not include proteasomal degradation. For example, many plasma membrane proteins, particularly in yeast, can be monoubiquitinated, resulting in their endocytosis and trafficking to the vacuole (lysosome) for degradation [6,7].

Despite its covalent linkage to many rapidly degraded cellular proteins, ubiquitin itself is a surprisingly long-lived protein in vivo [8,9]. This is the result of efficient removal of ubiquitin from its conjugates by deubiquitinating enzymes (DUBs) prior to proteolysis of the conjugated protein. Protein deubiquitination is important for several reasons. When it occurs before the commitment of a substrate to either proteasomal or vacuolar proteolysis, it negatively regulates protein degradation. A proofreading mechanism wherein ubiquitin is removed from proteins inappropriately targeted to the proteasome has also been suggested [10]. Conversely, deubiquitination of proteolytic substrates of the ubiquitin system is necessary for sustaining normal rates of proteolysis by helping to maintain a sufficient pool of free ubiquitin within the cell. Moreover, DUBs are responsible for processing inactive ubiquitin precursors, and for keeping the 26S proteasome free of unanchored ("free") ubiquitin chains that can compete with ubiquitinated substrates for ubiquitin-binding sites (Fig. 1).

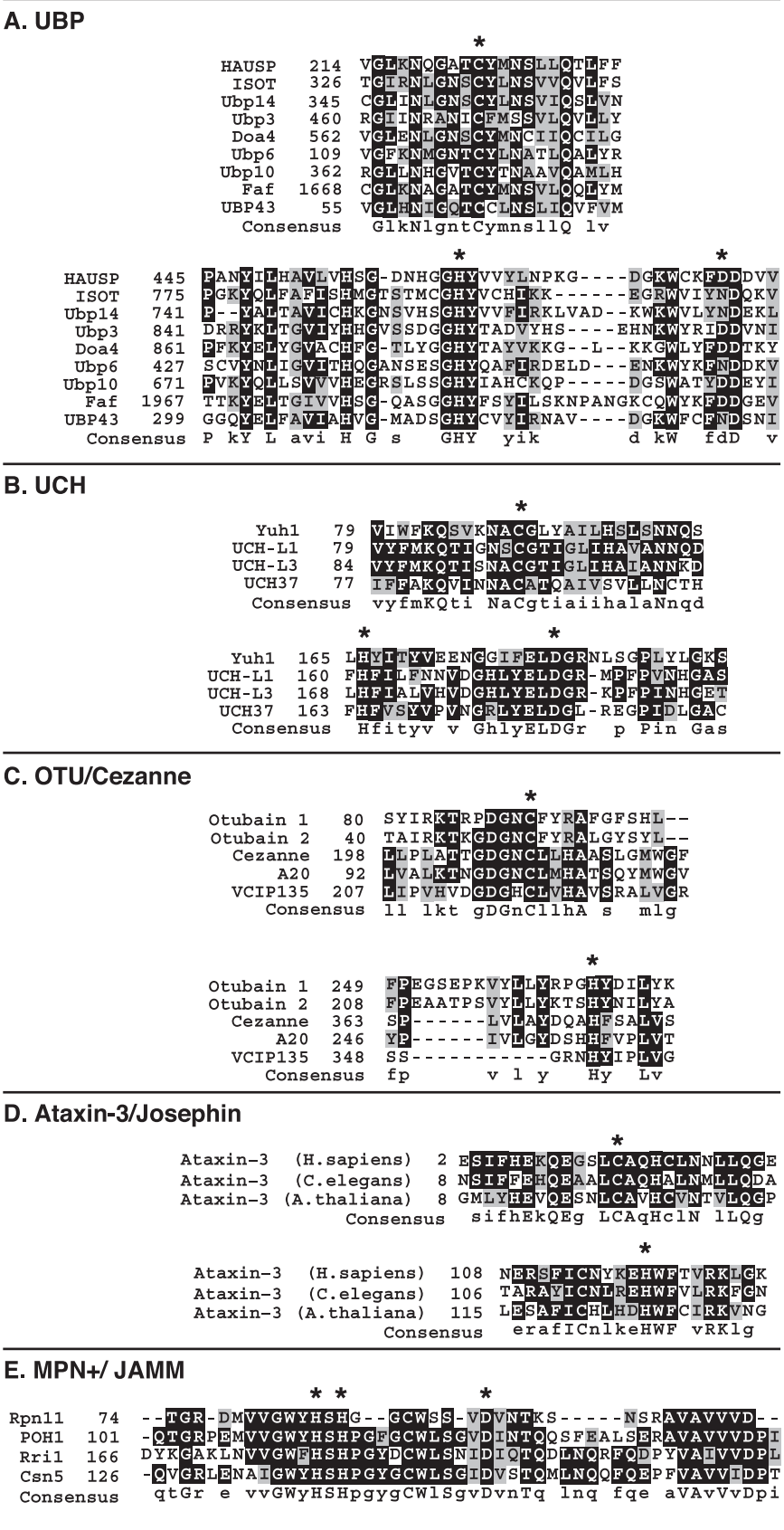
2. Classes of DUBs

The DUBs are a large group of enzymes that specifically cleave ubiquitin-linked molecules after the terminal carbonyl of the last residue of ubiquitin (Gly76) [11]. If the ubiquitin-linked molecule is a protein, the linkage is generally an amide bond. Ubiquitin is always synthesized in an inactive precursor form with a C-terminal extension beyond the terminal ubiquitin glycine. The amide bond that must be hydrolyzed in this case is of the standard peptide variety. When ubiquitin is attached posttranslationally to a protein, it is usually to a lysine ε-amino group, resulting in a distinct amide or "isopeptide" bond. Activated ubiquitin is also susceptible to attack by small intracellular nucleophiles. Some, such as glutathione and polyamines, are of considerable abundance, so DUBs are essential for preventing all of the cellular ubiquitin from being rapidly titrated by these compounds. The precise division of labor between various DUBs within the cell for cleaving this wide range of ubiquitin conjugates is not well understood. Many DUBs can hydrolyze different kinds of chemical bonds, although not necessarily with equal efficiency. For instance, many members of this family of enzymes can cleave ester, peptide and isopeptide bonds to ubiquitin at high rates [12].

2.1. The UBP and UCH subfamilies

The DUBs fall into at least five distinct subfamilies based on their sequence similarities and likely mechanisms of action (Fig. 2). Four of the subfamilies are specialized types of cysteine proteases, while the fifth group is a novel type of

Fig. 2. Amino acid sequences of the conserved motifs surrounding catalytically active amino acid residues (marked by asterisks) in five known classes of DUBs. (A) The catalytic triad of UBPs in the sequence context of the Cys (top) and His boxes (bottom). (B) The catalytic triad of UCHs. (C) Catalytic cysteine and histidine residues of the OTU family of DUBs. (D) Predicted catalytic cysteine and histidine residues of the ataxin- 3/Josephin family of DUBs. (E) The central part of the MPN+/JAMM motif found in the Rpn11/POH1 proteasome subunit and the related COP9/signalosome subunits. The essential histidines and asparate involved in Zn^{2+} coordination and catalysis are highlighted. An absolutely conserved glutamate in this group of enzymes that is upstream of the sequences shown is not depicted. Active site residues in UBP, UCH, OTU and MPN+/JAMM families of DUBs were identified based on biochemical and structural studies. SwissProt Database entries shown are: Yuh1 (*S. cerevisiae*; SW: P35127); UCH-L1 (human; SW: P09936); UCH-L3 (human; SW: P15374); UCH37 (bovine; SW: Q9XSJ0); HAUSP (human; SW: Q93009); ISOT (human; SW: P45974); Ubp14 (*S. cerevisiae*; SW: P38237); Ubp3 (*S. cerevisiae*; SW: Q01477); Doa4 (*S. cerevisiae*; SW: P32571); Ubp6 (*S. cerevisiae*; SW: P43593); Ubp10 (*S. cerevisiae*; SW: P53874); Faf (*D. melanogaster*; SW: P55824); UBP43 (mouse; SW: Q9WTV6); otubain 1 (human; SW: Q96FW1); otubain 2 (human; SW: Q96DC9); Cezanne (human; SW: Q9NQ53); A20 (human; SW: P21580); VCIP135 (rat; SW: Q8CF97); ataxin-3 (human; SW: P54252); ataxin- 3 (*C. elegans*; SW: O17850); ataxin- 3 (*A. thaliana*; SW: Q9M391); Rpn11 (*S. cerevisiae*; SW: P43588); POH1 (human; SW: O00487); Rri1 (*S. cerevisiae*; SW: Q12468); and Csn5 (human; EP: AAH01187).


A. UBP
HAUSP 214 VGLKNQGATCYMNSLLQTLFF
ISOT 326 TGIRNLGNSCYLNSVVQVLFS
Ubp14 345 CGLINLGNSCYLNSVIQSLVN
Ubp3 460 RGIINRANICFMSSVLQVLLY
Doa4 562 VGLENLGNSCYMNCIIQCILG
Ubp6 109 VGFKNMGNTCYLNATLQALYR
Ubp10 362 RGLLNHGVTCYTNAAVQAMLH
Faf 1668 CGLKNAGATCYMNSVLQQLYM
UBP43 55 VGLHNIGQTCCLNSLIQVFVM
Consensus GlkNlgntCymnsllQ lv
HAUSP 445 PANYILHAVLVHSG-DNHGGHYVVYLNPKG----DGKWCKFDDDVV
ISOT 775 PGKYQLFAFISHMGTSTMCGHYVCHIKK------EGRWVIYNDQKV
Ubp14 741 P--YALTAVICHKGNSVHSGHYVVFIRKLVAD--KWKWVLYNDEKL
Ubp3 841 DRRYKLTGVIYHHGVSSDGGHYTADVYHS----EHNKWYRIDDVNI
Doa4 861 PFKYELYGVACHFG-TLYGGHYTAYVKKG--L--KKGWLYFDDTKY
Ubp6 427 SCVYNLIGVITHQGANSESGHYQAFIRDELD---ENKWYKFNDDKV
Ubp10 671 PVKYQLLSVVVHEGRSLSSGHYIAHCKQP-----DGSWATYDDEYI
Faf 1967 TTKYELTGIVVHSG-QASGGHYFSYILSKNPANGKCQWYKFDDGEV
UBP43 299 GGQYELFAVIAHVG-MADSGHYCVYIRNAV----DGKWFCFNDSNI
Consensus P kY L avi H G s GHY yik d kW fdD v
B. UCH
Yuh1 79 VIWFKQSVKNACGLYAILHSLSNNQS
UCH-L1 79 VYFMKQTIGNSCGTIGLIHAVANNQD
UCH-L3 84 VYFMKQTISNACGTIGLIHALANNKD
UCH37 77 IFFAKQVINNACATQAIVSVLLNCTH
Consensus vyfmKQti NaCgtiaiihalaNnqd
Yuh1 165 LHYITYVEENGGIFELDGRNLSGPLYLGKS
UCH-L1 160 FHFILFNNVDGHLYELDGR-MPFPVNHGAS
UCH-L3 168 LHFIALVHVDGHLYELDGR-KPFPINHGET
UCH37 163 FHFVSYVPVNGRLYELDGL-REGPIDLGAC
Consensus Hfityv v GhlyELDGr p Pin Gas
C. OTU/Cezanne
Otubain 1 80 SYIRKTRPDGNCFYRAFGFSHL--
Otubain 2 40 TAIRKTKGDGNCFYRALGYSYL--
Cezanne 198 LLPLATTGDGNCLLHAASLGMWGF
A20 92 LVALKTNGDGNCLMHATSQYMWGV
VCIP135 207 LIPVHVDGDGHCLVHAVSRALVGR
Consensus ll lkt gDGnCllhA s mlg
Otubain 1 249 FPEGSEPKVYLLYRPGHYDILYK
Otubain 2 208 FPEAATPSVYLLYKTSHYNILYA
Cezanne 363 SP------LVLAYDQAHFSALVS
A20 246 YP------IVLGYDSHHFVPLVT
VCIP135 348 SS----------GRNHYIPLVG
Consensus fp v l y Hy Lv
D. Ataxin-3/Josephin
Ataxin-3 (H.sapiens) 2 ESIFHEKQEGSLCAQHCLNNLLQGE
Ataxin-3 (C.elegans) 8 NSIFFEHQEAALCAQHALNMLLQDA
Ataxin-3 (A.thaliana) 8 GMLYHEVQESNLCAVHCVNTVLQGP
Consensus sifhEkQEg LCAqHclN lLQg
Ataxin-3 (H.sapiens) 108 NERSFICNYKEHWFTVRKLGK
Ataxin-3 (C.elegans) 106 TARAYICNLREHWFVLRKFGN
Ataxin-3 (A.thaliana) 115 LESAFICHLHDHWFCIRKVNG
Consensus erafICnlkeHWF vRKlg
E. MPN+/ JAMM
Rpn11 74 --TGR-DMVVGWYHSHG--GCWSS-VDVNTKS-----NSRAVAVVVD--
POH1 101 -QTGRPEMVVGWYHSHPGFGCWLSGVDINTQQSFEALSERAVAVVVDPI
Rri1 166 DYKGAKLNVVGWFHSHPGYDCWLSNIDIQTQDLNQRFQDPYVAIVVDPL
Csn5 126 -QVGRLENAIGWYHSHPGYGCWLSGIDVSTQMLNQQFQEPFVAVVIDPT
Consensus qtGr e vvGWyHSHpgygCWlSgvDvnTq lnq fqe aVAvVvDpi

zinc-dependent metalloprotease. The largest and most diverse of these subfamilies is the UBP or *ub*iquitin-specific processing *p*rotease group. These cysteine proteases contain two short but well-conserved motifs, named the Cys and His boxes, which include all the catalytic triad residues as well as other residues in the active site pocket (Fig. 2A). The second ubiquitin-specific cysteine protease subfamily is made up of the UCHs or *u*biquitin *c*arboxy-terminal *h*ydrolases (Fig. 2B). They are generally small proteins, which were originally identified by their ability to hydrolyze small amides and esters at the C-terminus of ubiquitin.

2.2. The OTU-related proteases

The remaining three known subfamilies of DUBs have been discovered only recently. Based on a bioinformatics analysis, a novel family of cysteine proteases, the OTU (*o*varian *tu*mor)-related proteases, had been predicted (Fig. 2C) [13]. These proteins display structural similarity in a presumed catalytic core domain containing conserved Cys, His and Asp residues thought to comprise the proteolytic catalytic triad. The OTU protease family includes members in which the OTU-related motif is actually part of a UBP family protein, providing the first clue for a connection with the ubiquitin system.

Recent experiments have demonstrated that OTU domain-containing proteins do indeed have DUB activity, which requires the conserved cysteine residue in the OTU domain. In one report, a negative regulator of NF-κB, a 100-kDa cytoplasmic protein called Cezanne, was shown to have DUB activity in vitro [14]. Cezanne can hydrolyze linear polyubiquitin translation products and isopeptide-linked polyubiquitin chains. It is also active against ubiquitin–protein conjugates. Overexpression of Cezanne prevents accumulation of ubiquitinated proteins in HeLa cells treated with the proteasome inhibitor MG132. Cezanne is structurally similar to protein A20, which, like Cezanne, has a capacity to suppress NF-κB signaling [15]. A20 is also a DUB, which is active against both Lys48- and Lys63-linked ubiquitin oligomers [16,17]. Interestingly, in addition to the OTU domain, A20 has a zinc finger domain that acts as a ubiquitin–protein ligase (see below). Another OTU protease, VCIP135, which is essential for reassembly of mitotic Golgi fragments, also possesses deubiquitinating activity against polyubiquitin chains [18]. Finally, two additional members of the OTU family, otubain 1 and 2, can cleave ubiquitin from either a ubiquitin–GFP fusion protein or a tetraubiquitin fusion [19]. Intriguingly, otubain 1 regulates T cell anergy via an interaction with the ubiquitin–protein ligase GRAIL [20]. Taking together, these data strongly suggest that other members of the OTU family may function as DUBs.

2.3. Ataxin-3 and the Josephin domain

The fourth DUB family, for which ataxin-3 is the only demonstrated member to date, is characterized by a domain called the Josephin domain (Fig. 2D) [21,22]. Ataxin-3 is mutated in the neurodegenerative polyglutamine expansion disease called spinocerebellar ataxia type 3. Ataxin-3 has the typical properties of DUBs: the enzyme disassembles ubiquitin–lysozyme conjugates, cleaves ubiquitin-7-amido-4-methylcoumarin (ubiquitin-AMC), and binds to the DUB inhibitor ubiquitin aldehyde (Ubal) [21]. The Josephin domain, which is found in over 30 predicted proteins, most of unknown function, includes segments that show weak similarity to the His and Cys boxes of UBPs and UCHs, suggesting that this region of Ataxin-3 and its relatives will also assume the papain-like protease fold that characterizes these other cysteine proteases.

2.4. JAMM/MPN+ proteases

The last subfamily of DUBs is represented by a subunit of the proteasome, Rpn11/POH1 (see Fig. 9), which has features of a metalloprotease specific for protein-linked ubiquitin. The Rpn11 sequence bears a distinct motif that is a subtype of the MPN motif (Fig. 2E). The MPN domain is found in an even broader group of proteins, including another proteasome subunit. The sequence variation typified by Rpn11 has been called the MPN+ or the JAMM motif to distinguish it from the broader class [23,24]. Very recently, another protein with the MPN+/JAMM motif, AMSH (*a*ssociated *m*olecule with the *SH*3 domain of STAM), was found to have deubiquitinating activity as well [25]. This metalloprotease motif includes two absolutely conserved His residues and an Asp residue that together coordinate a zinc ion important for proteolytic activity. These residues are essential for the function of the Rpn11 subunit when integrated into the proteasome [23,24,26]. Another conserved residue, Glu48, is thought to serve as a general acid–base catalyst, and this residue was found by mutagenesis to be critical for activity of the signalosome subunit Csn5 ([27] and below).

3. Structural insights into DUB mechanism and specificity

Structural data are now available for representative members of the UCH, UBP, MPN+/JAMM and OTU classes of DUBs (Figs. 3–8) [27–32]. Importantly, for the UCH and UBP families, X-ray crystal structures were solved for both the free enzyme and the enzyme in a covalent complex with ubiquitin (for the UCH analysis, the free and complexed UCH were from different species, but these isozymes are very similar in structure and sequence). The C-terminal carboxylate of ubiquitin can be reduced to an aldehyde (Ubal), and when the active site cysteinyl group of the DUB attacks this moiety, a relatively stable hemithioacetal intermediate is trapped. This allowed isolation of sufficient quantities of the DUB–Ubal complexes for crystallization.

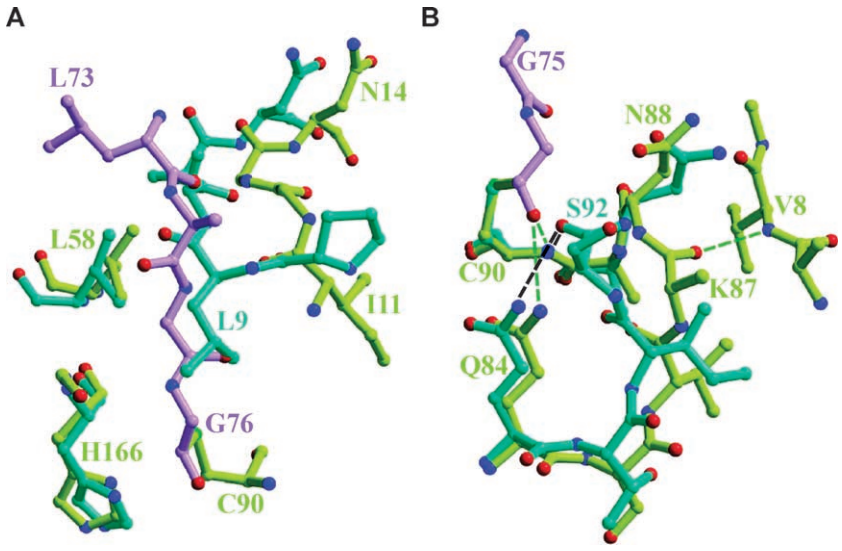


Fig. 3. Ubiquitin binding causes conformational changes at the UCH active site. (A) In the UCH-L3 apoenzyme (blue-green), the active site is not accessible due to occlusion by Leu9 of the space that is taken by Gly75 of ubiquitin in the Yuh1 (yellow-green) complex with ubiquitin aldehyde (Ubal) (magenta). Ubal binding results in ~4-Å displacement of the equivalent residue Ile11 of Yuh1. (B) The oxyanion hole of UCH-L3 is blocked by the backbone carbonyl oxygen of Ser92. In the Yuh1–Ubal complex, the Ser92 equivalent Lys87 residue is rotated ~180°, displacing the carbonyl oxygen ~5 Å relative to its position in the UCH-L3 structure. Reprinted with permission from Refs. [28,29].

The crystallographic results revealed several interesting common features between the UCH and UBP subfamilies. First, the catalytic core structures of both the UCH and UBP enzymes match very closely to that of the classical cysteine proteases such as papain; indeed, these segments of the UCH and UBP proteins are nearly indistinguishable in their three-dimensional folds despite their lack of obvious sequence similarity (Fig. 5). The conformations of the catalytic triad residues are also superimposable. A second remarkable feature of the UCH and UBP enzymes is that the active sites in their respective free forms are not in catalytically competent conformations (Figs. 3 and 4). Both appear to undergo a ubiquitin-induced rearrangement that either eliminates steric obstructions in the active site cleft or brings the catalytic residues into their proper relative positions. The advantage of ubiquitin-dependent enzyme activation is that these enzymes will be proteolytically inert against most cellular proteins, and specificity can be fine-tuned toward either ubiquitin or particular UBLs (see below).

In the UCH enzymes, the active site cysteine lies at the bottom of narrow groove in the surface of the protein [28,29].

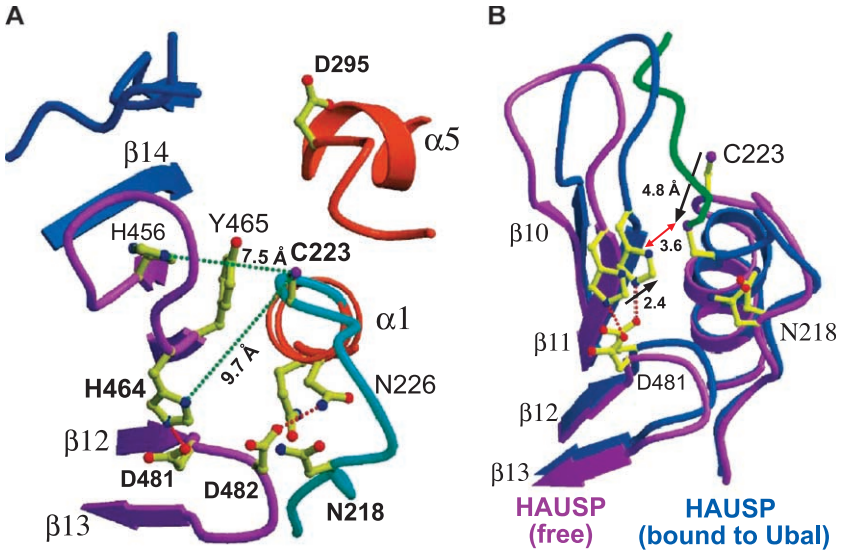


Fig. 4. Rearrangement of the UBP active site upon ubiquitin binding. (A) The active site residues in the free form of the HAUSP UBP enzyme are misaligned. The 9.7-Å distance between catalytic residues Cys223 and His464 makes a productive interaction impossible. The Cys and His boxes (see Fig. 2B) are shown in cyan and magenta, respectively. (B) Ubal binding (green) induces localized conformational changes in HAUSP that shift Cys223 and His464 toward each other. This closes the gap between the imidazole $N^{\delta 1}$ of His464 and the sulfur atom of Cys223 to 3.6 Å. Reprinted with permission from Ref. [30].

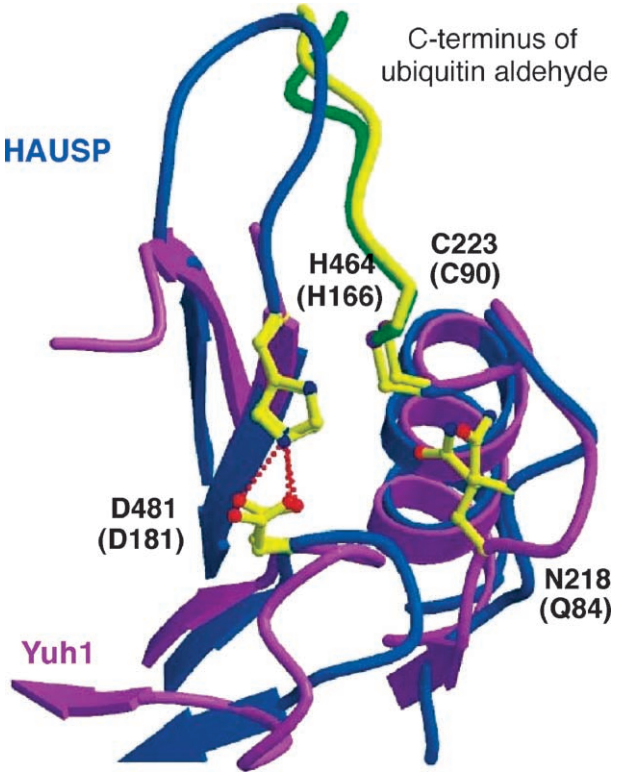


Fig. 5. Structural superimposition of the catalytic cores of the Yuh1 (magenta) and HAUSP (blue) enzymes when both are bound to Ubal. The catalytic triad residues and residues that form the oxyanion hole are depicted. Reprinted with permission from Ref. [30].

Ubiquitin (and most UBLs) terminates with a pair of glycines, and it appears that residues with larger side chains could not be accommodated in this groove, providing a strong constraint on the substrate selectivity of UCHs. When not bound to ubiquitin, the aliphatic side chain of a specific UCH residue occupies part of the groove (and the oxyanion hole is occluded by part of the UCH backbone) (Fig. 3). In addition to these mechanisms for enhancing substrate selection, there are extensive and highly specific binding interactions between the UCH and ubiquitin. The interface between ubiquitin and the yeast UCH Yuh1 buries approximately 2500 Å^2 of solvent-accessible surface area. The contacts include some 20 hydrogen bonds and numerous van der Waals interactions. A number of these interactions, including a salt bridge between Arg72 of ubiquitin and an invariant Asp (residue 35 in Yuh1), were shown by kinetic and mutational studies to enhance UCH specificity for ubiquitin [29].

Many members of the UCH family can only cleave small adducts or unfolded polypeptides from the C-terminus of ubiquitin. This restriction appears to be due largely to a peptide segment that arcs directly over the active site. The active site crossover loop, which is disordered in the free enzyme, becomes ordered upon contact with ubiquitin. To be cleaved from ubiquitin, the substrate moiety must pass through this loop, and even in its maximally open state, the loop diameter is no greater than 15 Å. This is much smaller than the majority of folded proteins, explaining why most UCHs cannot act on ubiquitin–protein conjugates.

Crystal structures of the core domain of a human UBP, the herpesvirus-associated ubiquitin-specific protease (HAUSP), have been solved for both its free form and when covalently complexed with Ubal (Fig. 4) [30]. The HAUSP core folds into three major globular domains, with the active site of HAUSP located in a deep cleft between two of them. Based on sequence conservation, the three-domain architecture of the HAUSP core seems to be conserved in other members of the UBP family. As was found with the UCH enzyme Yuh1, ubiquitin makes extensive contacts with the UBP, resulting in the burial of ~3600 Å^2 of solvent accessible surface area.

The identities of the His and Asp/Asn residues in the UBPs that were expected to comprise part of a catalytic triad were not known with certainty prior to the work of Hu et al. [30]. The HAUSP–ubiquitin cocrystal shows that in addition to Cys223, which is the active site nucleophile, His464 and Asp481 are the remaining triad residues; the latter two residues are both part of the His box. Interestingly, in the free form of HAUSP, the catalytic triad is misaligned. The relevant atoms of Cys223 and His464 are 9.7 Å apart, which is too far for a productive interaction (Fig. 4A). Binding of Ubal dramatically changes the structure of the catalytic cleft relative to the free form of the enzyme. A 4.8-Å shift of Cys223 and a 2.4-Å shift of His464 shorten the distance between the side chains to 3.6 Å, which is close to hydrogen-bonding distance (Fig. 4B).

The structural data shed considerable light on the mechanism and exquisite substrate specificity of these two major DUB subclasses. In the absence of substrate, the enzymes are in inactive conformations. Extensive and specific contacts between ubiquitin and the DUB cause significant, highly localized structural rearrangements in

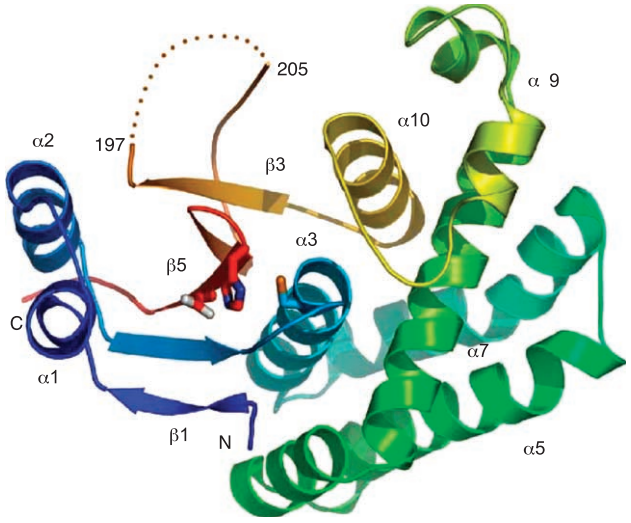


Fig. 6. Overall structure of otubain 2. Color changes from blue (N-terminus) to red (C-terminus). The catalytic triad residues (Cys51, His224 and Asn226) are shown in a stick representation. Selected elements of the secondary structure are labeled. Reprinted with permission from Ref. [32].

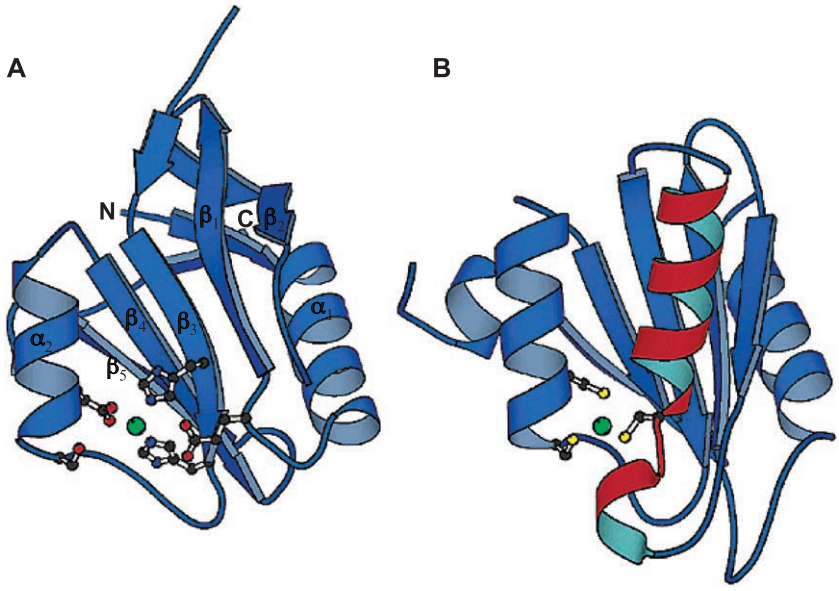


Fig. 7. Overall structures of MPN+/JAMM protein AF2198 (A) and cytidine deaminase (B). The residues that coordinate zinc in both proteins are represented as balls and sticks. The zinc ions are shown as green spheres. The additional α helix in deaminase is colored in red. See Refs. [31 and 35] for details. Reprinted with permission from Ref. [31].

the DUB, which switches the enzyme into an active configuration.

A crystal structure of human otubain 2, a member of the OTU family of DUBs, has been described recently [32]. It includes a five-stranded β-sheet placed between two helical domains. The active site of otubain 2 is formed by the helix α3 and the loop that connects strands 4 and 5 of the β-sheet (Fig. 6). The catalytic triad of otubain 2 appears to be amino acid residues Cys51, His224 and Asn226. Despite the absence of significant sequence similarity between the UBPs, UCHs and otubain 2, their active sites display almost identical geometries. An even more distantly related cysteine protease that acts on the Ubl called SUMO, rather than on ubiquitin (see later), was also found to have this classical cysteine protease active site configuration [33,34], indicating that all four types of enzyme utilize a similar catalytic mechanism and one closely related to the heavily studied papain family of proteases.

Phylogenetic analysis had suggested that Asp48 of otubain 2 would be the third member of the catalytic triad [13], but the 8-Å distance between Asp48 and the catalytically active His224 is too long for productive interaction, assuming the enzyme is in an active state. Instead, a critical hydrogen bond appears to be formed between His224 and Asn226, and substitution of Asn226 to Ala inhibits otubain 2 enzyme activity. Curiously, however, Asn226 is not conserved in all the OTU-related proteases. In the A20 enzyme, for example, a valine is found at this position (Fig. 2C). An OTU protease–Ubal cocrystal structure(s) should help resolve this issue.

Unlike HAUSP, the active site residues in otubain 2 appear to be positioned in a productive form even in the absence of bound substrate molecule. Nevertheless, free otubain 2 may be in a self-inhibited state because of the loop that precedes the active site helix α3. This loop has a conformation that spatially restricts the active site.

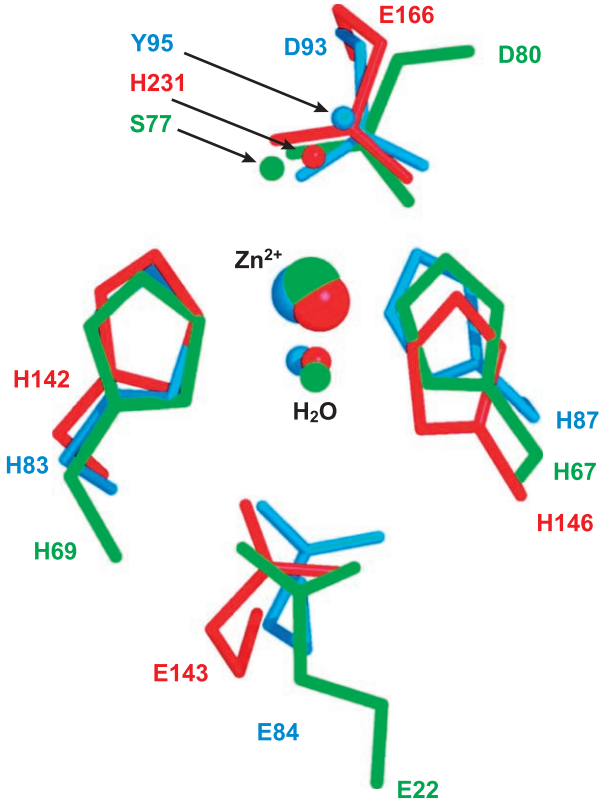


Fig. 8. Structural superimposition of active site residues in *Streptomyces caespitosus* zinc endoprotease (blue), thermolysin (red) and AF2198 (green). Reprinted with permission from Ref. [27].

Although no structural data are available yet for Rpn11/POH1, crystal structures of the MPN+/JAMM domain protein AF2198 from *Archaeoglobolus fulgidus* have been solved recently by two groups (Fig. 7A) [27,31]. Two conserved His residues and an Asp residue that coordinate a zinc ion are located in the third strand of a central β sheet and in a flanking α helix, respectively. A conserved Ser residue located between the active site histidines forms a hydrogen bond with a Glu residue expected to function in general acid–base catalysis in the MPN+/JAMM enzymes.

Interestingly, despite very low sequence identity, the overall structure of AF2198 is similar to cytidine deaminase, which catalyzes conversion of cytidine to uridine (Fig. 7B). The catalytic mechanism of this enzyme is well characterized [35]. Cytidine deaminase is a metalloenzyme that utilizes a zinc ion in catalysis. The ion polarizes a water molecule that then attacks a carbon atom in the cytidine pyrimidine ring. This nucleophilic attack results in formation of an unstable tetrahedral intermediate that in turn rapidly collapses, leading to the reaction products. The cytidine deaminase and AF2198 structures are readily superimposed in the main β sheet and two of the flanking α helices, suggesting that the two proteins are evolutionary related. Zinc ions are located in the same environment in the tertiary structures. Hence, it is likely that the MPN+/JAMM proteases uses a cytidine deaminase-type mechanism for isopeptide bond hydrolysis.

The arrangement of the AF2198 zinc cofactors is also very similar to that found in the thermolysin family of proteolytic enzymes despite a complete lack of structural similarity between the two groups of enzymes (Fig. 8) [27]. In addition, Glu22 in AF2198 seems to be a functionally equivalent to the catalytic glutamate in thermolysin. Indeed, mutation of the corresponding residue in the Csn5 subunit of the COP9/signalosome (CSN) (see below) compromises proteolytic activity of that complex.

Unexpectedly, the AF2198 structure apparently lacks elements that could serve as peptide-binding sites, unlike most other proteases. AF2198, like Rpn11/POH1, might therefore only be active when the protease is incorporated into a larger heteromeric complex. In the case of Rpn11, neighboring 19S subunits might participate in ubiquitin–protein conjugate binding. The ATP-dependent Rpn11 DUB activity, like that of other DUBs, might also be subject to strong conformational controls.

4. Biochemical activities of the DUBs

In this section, we discuss some of the basic biochemical functions of various DUBs. Because a major consequence of protein ubiquitination is the targeting of the modified protein for degradation, deubiquitination can have major effects on protein half-life and steady-state level. At present, much of our knowledge about the in vivo activities of DUBs relates to their effects on protein metabolic stability.

4.1. Processing of ubiquitin precursors

As noted earlier, all ubiquitin genes encode C-terminally extended forms of ubiquitin. The ubiquitin precursors are either fusions with certain ribosomal proteins or head-to-tail-linked ubiquitin multimers that also have an additional amino acid following the last ubiquitin monomer [36]. Proper processing of these precursors is essential for the generation of conjugation-competent ubiquitin. Many of the DUBs are able to cleave the peptide bond linking ubiquitin to various C-terminal peptide extensions, at least in vitro, and no DUB in yeast is essential for viability (other than Rpn11) [37]. These data suggest that ubiquitin precursor processing is performed by multiple DUBs, although it is likely that some precursor-cleaving DUBs are more efficient (or simply more abundant) than others. Processing is extremely rapid in vivo and can occur cotranslationally [38]. Why all ubiquitin proteins (and most Ubls) are synthesized in precursor form is not actually known. A strain that encodes only mature ubiquitin appears to be fully viable [39]. It is possible that precursor processing provides a quality control step: if a DUB cannot bind and cleave an aberrantly synthesized or folded version of ubiquitin precursor, the ubiquitin will not be able to enter the active cellular pool. A requirement for such a checkpoint might not be detected by the simple laboratory growth assays used in the aforementioned study.

4.2. Editing or rescue of ubiquitin conjugates

Degradation of proteins is an irreversible process, so specificity in the ubiquitin system must be maintained at a very high level. DUBs contribute in two known ways to this exquisite specificity. First, some ubiquitinated proteins can be selectively deubiquitinated by certain DUBs. Discussion of substrate-specific deubiquitination is reserved for the next section. The second mechanism is a more general editing role for DUBs in reversing the modification of a wide range of proteins. There might be a general susceptibility of many or most ubiquitinated proteins to the action of a variety of DUBs in vivo. Increases in the extent of ubiquitination of a particular protein could then be achieved by enhancing its specific rate of ligation to ubiquitin, by sequestering it from cellular DUBs, and/or by inhibiting DUB activity. In general, the dynamic nature of ubiquitin–protein conjugates provides a timing control for ubiquitin-dependent events: the modified protein will have only a limited time to effect a particular process, e.g., binding to the proteasome, before a DUB returns the protein to its deubiquitinated state.

It is significant that efficient binding of polyubiquitinated proteins to the proteasome generally requires polymers of at least four ubiquitins [40,41]. If a DUB

preferred to remove single ubiquitin moieties from the end of the chain most distal to the substrate, it could in principle act as an editing or proofreading enzyme for proteasome targeting. Specifically, substrates with longer ubiquitin chains could maintain proteasome-binding competence longer than those with only short chains (near the limit of four ubiquitins), so inappropriately ubiquitinated substrates, which may generally have shorter chains, could be rescued from proteolysis. A logical place for such an editing DUB to work is on the proteasome itself. Indeed, Lam et al. [10,42] identified a UCH in purified bovine PA700 complexes (the regulatory subcomplex of the proteasome) that shows distal-to-proximal ubiquitin chain disassembly activity. This ubiquitin isopeptidase, named UCH37, is an intrinsic subunit of the bovine 19S/PA700 complex. No ortholog of UCH37 has been identified in the yeast *S. cerevisiae*.

4.3. Coupling of protein deubiquitination and degradation by the proteasome

Another important function of deubiquitination is to release ubiquitin chains from ubiquitin–protein conjugates once they have been targeted to the proteasome and are committed to degradation, perhaps by an initial unfolding of the substrate. Failure to detach polyubiquitin either could lead to inappropriate degradation of the ubiquitin tag along with the substrate or could interfere with entry of substrate into the narrow opening leading to the central proteolytic chamber of the proteasome. Several DUBs other than UCH37 are associated with or built into the 26S proteasome regulatory particle (Fig. 9). These are Doa4, Ubp6/USP14, and Rpn11/POH1. The yeast Doa4 protein, a UBP, is found in substoichiometric amounts in proteasome preparations, and while genetic data suggest a function for Doa4 action on at least a subset of proteasomes, there are no definitive biochemical data supporting this hypothesis [43,44]. Instead, Doa4 probably functions primarily in membrane protein trafficking (see below).

Ubp6, on the other hand, is found in nearly stoichiometric amounts in the proteasome regulatory particle, and its ubiquitin hydrolase activity is strongly stimulated (~300-fold) by proteasome binding [45,46]. Ubp6 directly associates with the 19S regulatory particle via a ubiquitin-like domain at its N-terminus. Deletion of the *UBP6* gene from yeast leads to mild phenotypic abnormalities commonly associated with mutants of the ubiquitin–proteasome pathway [37,46]. The mutant cells display reduced levels of free ubiquitin and ubiquitin–protein conjugates and cannot efficiently degrade the highly sensitive reporter substrate ubiquitin-P-β-galactosidase. The drop in ubiquitin levels in *ubp6* cells is due to its abnormally rapid degradation, presumably by the proteasome. In fact, the defects of these cells are strongly suppressed by supplementation of ubiquitin. Moreover, deletion of *UBP6* has little effect on the

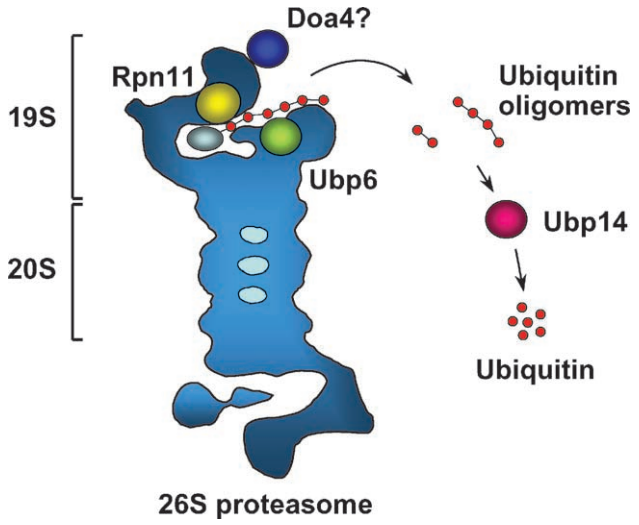


Fig. 9. Deubiquitination at the yeast 26S proteasome. The Rpn11/POH1 subunit of the lid is believed to cleave the isopeptide bond between the protein substrate and the ubiquitin chain in an ATP-dependent manner. Ubp6 physically interacts with the base subunit Rpn1 and may act on substrates modified with the long ubiquitin oligomers. Doa4 can associate with the 26S proteasome and may be involved in the degradation of a subset of ubiquitin–proteasome substrates, although its primary function is probably at the late endosome (Fig. 10). Ubp14/Iso T works downstream of the proteasome-associated DUBs and is responsible for disassembly of unanchored ubiquitin oligomers.

degradation of several other substrates of the 26S proteasome. These data suggest that Ubp6 participates in an ancillary or partially redundant fashion in proteasome function. Perhaps a subset of ubiquitinated proteasome-bound substrates are more efficiently deubiquitinated by Ubp6 than by Rpn11 or other DUBs, and degradation of these proteins can proceed slowly with concomitant unfolded and degradation of the attached ubiquitin.

Compelling data identifying the key proteasome DUB responsible for release of polyubiquitin from proteasome-targeted proteolytic substrates was recently reported [23,26]. This protein, Rpn11/POH1 (see earlier sections), is probably responsible for the Ubal-insensitive, ATP-dependent ubiquitin isopeptidase activity reported for the proteasome over a decade ago [47]. The DUB activity of the Rpn11 metalloprotease, unlike any other DUB, is essential for viability of yeast and is critical for ubiquitin-dependent proteolysis of proteasomal substrates both in vitro and in vivo. In the context of the 26S proteasome, the deubiquitinating activity of Rpn11 is fully ATP-dependent in vitro. This likely reflects a coupling of Rpn11 isopeptidase activity with the activity of the ATPase subunits in the base of the proteasome regulatory particle. These ATPases appear to unfold and help translocate unfolded protein substrates into the 20S proteasome core. The ATP dependence of Rpn11 activity suggests that deubiquitination is obligatorily associated with degradation, which might help prevent premature release of substrate protein from the proteasome. An important question that remains to be addressed is whether ubiq-

uitin-chain release by Rpn11 precedes the initiation of degradation or can occur later. The answer might be substrate-dependent and may be related to the finding that the proteasome can sometimes release intact domains from certain ubiquitinated substrates, such as the p105 NF-κB precursor or the yeast transcription factor Spt23, rather than destroying them completely [48–50].

4.4. Disassembly of ubiquitin oligomers

Most eukaryotes contain significant levels not only of free ubiquitin but also of unanchored polyubiquitin chains [51]. These chains can be generated de novo by endogenous ubiquitin-ligating enzymes or through release from polyubiquitinated substrates by DUBs. Accumulation of excessive levels of these ubiquitin chains, however, can inhibit ubiquitin-dependent processes, particularly proteasomal proteolysis [52]. Surprisingly, a single DUB is responsible for the bulk of unanchored ubiquitin chain disassembly in vivo, at least in yeast. This DUB, called Ubp14 in yeast and isopeptidase T in mammals, is among the enzymologically best characterized DUBs [53,54]. In vitro, isopeptidase T acts preferentially, if not exclusively, on unanchored ubiquitin chains. In direct contrast to the proteasome-bound UCH37 enzyme, isopeptidase T disassembles ubiquitin oligomers from the free "proximal" end, i.e., the end with an unattached Gly76 carboxyl group. Isopeptidase T is very sensitive to any modification of the C-terminus of the proximal ubiquitin in the chain; for example, deletion of the last two glycine residues in this ubiquitin makes the ubiquitin oligomer completely refractory to the action of the isopeptidase. Similar properties were reported for yeast Ubp14 [52]. Thus, Ubp14/isopeptidase T cannot act on polyubiquitinated protein substrates; instead, the ubiquitin chain must first be released from substrates by another DUB.

It is worth pointing out here that in addition to cleaving the Lys48-linked polyubiquitin chains associated with proteasomal targeting, isopeptidase T can also cleave, albeit less efficiently, ubiquitin polymers in head-to-tail peptide linkage, such as occurs in the polyubiquitin precursor [54,55]. In this light, it is also likely that isopeptidase T can disassemble other types of ubiquitin polymers such as those with Lys29 and Lys63 linkages. There might be specific DUBs that can cleave these alternative linkages more efficiently than can isopeptidase T, or, more generally, DUBs that specifically process particular ubiquitin–ubiquitin linkages.

4.5. Deubiquitination and membrane protein trafficking

As alluded to earlier, there is a second type of ubiquitin-mediated protein degradation in which proteins are targeted to the vacuole/lysosome rather than to the proteasome. The substrates in this pathway are membrane proteins, most commonly cell surface proteins that are endocytosed and directed to the vacuole by ubiquitin attachment to their cytosolic domains [6,7,56]. Monoubiquitination of protein at the cell surface appears to be important for the internalization step of endocytosis, but subsequent trafficking steps can also depend on this modification. Internalization of some substrates is enhanced by their attachment to short ubiquitin chains in which ubiquitin monomers are linked via Lys63 of ubiquitin. The best characterized ubiquitin-dependent step following endocytosis is protein sorting during the maturation of the late endosome into a multivesicular body (MVB). Not only endocytosed proteins but also biosynthetic cargo moving from the *trans*-Golgi to the vacuole are sorted here (the vacuolar-protein sorting or VPS pathway) [57]. The MVB forms by involution and vesiculation of the endosome's delimiting membrane. Proteins that are destined for the vacuolar interior, such as cell surface proteins targeted for proteolysis, must sort into these involuting membrane regions, and this requires that they be (mono)ubiquitinated.

Because ubiquitin is long-lived in vivo, it must be recovered from these involuting membrane proteins prior to complete vesiculation (assuming this process is irreversible). The yeast DUB implicated in this process is Doa4 (Fig. 10) [9,58]. A genetic screen for spontaneous suppressors of a *doa4* mutation revealed that many of the phenotypic abnormalities of the mutant could be suppressed by inactivation of factors required for MVB maturation. These so-called class E *vps* mutants fail to vesiculate the late endosome, resulting in the accumulation of large, flattened cisternae called the class E compartment. Many of these class E Vps factors can be trapped at the surface of the class E compartment when a specific ATPase, Vps4, is inactivated [59]. Strikingly, the Doa4 enzyme can also be trapped at this site in the *vps4* mutant, suggesting that the late endosome is the site of action of Doa4 in this pathway [58]. Doa4 localization to the endosome can be blocked by elimination of certain other class E factors. These class E factors were

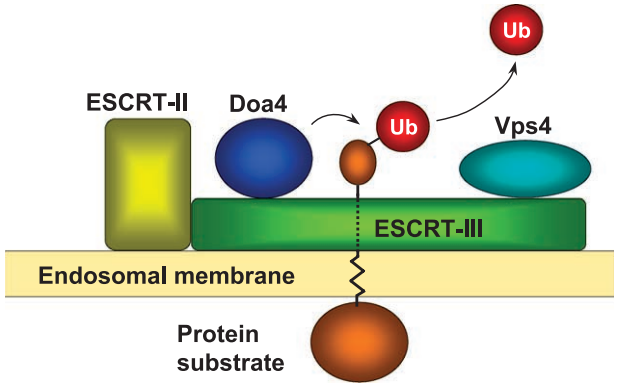


Fig. 10. Deubiquitination at the late endosome. Doa4 functions at the cytosolic face of the late endosome membrane and requires the ESCRT III protein complex for recruitment and/or stable association with the endosome. The Vps4 ATPase is thought to disassemble and release the ESCRT-III factors and Doa4 from the endosome surface.

subsequently shown to form a large assembly called ESCRT III at the endosome surface (Fig. 10) [60]. This suggests that ESCRT III may help recruit Doa4 to the late endosome and direct it toward ubiquitinated membrane proteins after they have been committed to inclusion in the involuting membrane. In support of these interpretations, uracil permease, a plasma membrane protein that undergoes ubiquitin-dependent endocytosis and targeting to the vacuole, accumulates in ubiquitinated form in the vacuoles of *doa4 pep4* mutant cells, which are deficient for vacuolar proteolysis [61]. Inactivation of the class E Vps protein Vps27 in these cells restores deubiquitination of the permease. These observations suggest that by blocking involution of the late endosome membrane, the *vps* mutants allow another DUB(s) to access proteins that are normally only deubiquitinated efficiently by Doa4.

Interestingly, the human DUB most closely related to Doa4 is the growth-regulated UBPY protein [62]. This enzyme interacts with the Hrs-binding protein (Hbp) in vitro [63]. Hrs and Hbp are both involved in endocytic trafficking in mammalian cells. Moreover, the yeast class E Vps proteins Vps27 and Hse1 appear to be the functional homologs of Hrs and Hbp, respectively [64]. These data suggest that UBPY may be the human counterpart of Doa4 and may act in the regulation of endosomal protein sorting as well.

5. Substrate-specific deubiquitination

Not surprisingly, early analyses of DUBs focused primarily on those enzymes that act in general processes such as recycling ubiquitin from proteasome-targeted substrates and breaking down ubiquitin oligomers, as described in the previous section. It was therefore initially not obvious to what extent substrate-specific deubiquitination would be physiologically important. That it would be a significant factor in ubiquitin pathway regulation was largely an inference from the fact that eukaryotic genomes encode so many potential DUBs, and it seemed unlikely that all would be required for simple recycling and processing reactions. Several recent examples from disparate model systems indicate that specific DUBs can indeed target specific proteins and that these deubiquitination events can have a major physiological impact.

5.1. Deubiquitination and eye development in Drosophila

The first example of a substrate-specific deubiquitination pathway in vivo came from studies of the *Drosophila fat facets* (*faf*) gene, which encodes a UBP and has clear orthologs in vertebrates [65]. The most noticeable abnormality of flies with *faf* mutations is a defect in eye development. Specifically, a *faf* mutant eye has a greater than normal number of photoreceptors in each ommatidium, which is the result of the loss of function of *faf* in neighboring, non-photoreceptor cells [66]. In addition, *faf* mutations have a maternal effect phenotype; embryos from homozygous faf^{-}/faf^{-} mothers are unable to reach the syncytial blastoderm stage. The deubiquitinating activity of Faf is critical for its function in *Drosophila* as alleles encoding catalytically inactive Faf fail to complement a *faf* null mutation.

Significantly, the *faf* mutant phenotype is strongly suppressed by the introduction of mutant alleles of a 20S proteasome subunit [65]. This suggests that Faf limits the extent of ubiquitination and, thereby, the degradation of one or more regulators of eye development. A subsequent screen for dominant enhancers of a weak *faf* eye phenotype led to the identification of Liquid facets (Lqf), a protein related to the mammalian endocytic factor called epsin [67,68]. Loss-of-function mutations in *faf* and *lqf* have similar eye phenotypes, and the two genes function in the same cells to generate a signal that prevents differentiation of nearby cells into supernumerary photoreceptors. Strikingly, even a single extra copy of the lqf^{+} gene overcomes a *faf* deficiency. These data are consistent with Lqf being the critical regulator of eye development whose function is positively regulated by Faf.

Biochemical data have confirmed this inference [69]. Wild-type eye imaginal discs contain ~3-fold more Lqf protein than do *faf* mutant discs, a difference that can be eliminated by reintroduction of a faf^{+} transgene into the faf^{-} homozygous mutant. In contrast, a transgene expressing catalytically inactive Faf fails to complement this faf^{-} phenotype. Based on their co-immunoprecipitation from disc extracts, Faf and Lqf physically interact with each other. Moreover, Western blot analysis of extracts prepared from faf^{+} and faf^{-} eye discs showed that Lqf is ubiquitinated in vivo and that Faf is responsible for its deubiquitination. Fischer and colleagues therefore concluded that Faf regulates the levels of Lqf by deubiquitinating it and preventing its degradation by the proteasome. How Lqf levels regulate specific endocytosis events and how these in turn signal the differentiation of neighboring cells in the developing eye remains to be determined.

5.2. HAUSP and p53 stabilization

A feature of many cancers is a defect in the function of the tumor suppressor protein p53, which allows pre-cancerous cells to survive and proliferate, eventually leading to a tumor [70,71]. The p53 tumor suppressor is a short-lived protein. In normal cells, it is polyubiquitinated, usually by the Mdm2 ubiquitin ligase, and degraded by the proteasome [72,73]. Activation of p53, which is largely due to its regulated stabilization, results in cell cycle arrest and other alterations that eventually lead to programmed cell death [71].

Recently Li et al. [74] identified HAUSP (see earlier) as a novel p53-interacting protein. HAUSP can deubiquitinate

p53 both in vivo and in vitro. Overexpression of this UBP in cultured cells rescues p53 from Mdm2-dependent degradation; other ubiquitinated proteolytic substrates are not affected. Importantly, overproduction of catalytically inactive HAUSP has the opposite effect: p53–ubiquitin conjugate levels increase and p53 stability decreases. In support of the idea that p53 deubiquitination by HAUSP is physiologically relevant, the overproduced UBP can strongly inhibit growth of human carcinoma lung cells expressing wild-type p53, but this has no effect on a p53-null carcinoma line. Overexpression of HAUSP also reduces the inhibitory effect of Mdm2 on p53-dependent apoptosis. Taken together, these data suggest that the balance between ubiquitination and deubiquitination of p53 plays a key role in regulating p53 activity in mammalian cells. Whether rates of p53 deubiquitination by HAUSP can be regulated is not known, but it is intriguing that the herpes simplex virus protein ICP0 binds HAUSP and may control HAUSP activity toward particular proteins in vivo [75].

5.3. USP2a rescues prostate cancer cells from apoptosis by stabilizing fatty acid synthase (FAS)

FAS is frequently overexpressed in aggressive tumors, including prostate cancer cells. Human FAS and its yeast homolog are ubiquitinated and degraded by the 26S proteasome [76]. Recent studies have demonstrated that deubiquitination plays an important role in FAS regulation. It has been found that FAS colocalizes and physically interacts with the androgen-regulated DUB USP2a in a LNCaP prostate cancer cell line. This interaction is likely to be physiologically relevant. Transfection of the LNCaP cells with USP2a prevents ubiquitin-dependent degradation of FAS, whereas overexpression of the USP2a-Cys276Ala active site mutant enhances degradation of FAS, suggesting that the catalytically inactive derivative acts in a dominant-negative fashion. The ubiquitination status of endogenous FAS depends on USP2a. Knockdown of USP2a expression by RNA interference leads to accumulation of ubiquitinated forms of FAS and a significant reduction of FAS protein levels, while levels of the FAS mRNA remain unaltered.

As noted above, overexpression of FAS is a hallmark of many aggressive tumors. It appears that FAS protects cancer cells from apoptosis by an as yet unknown mechanism. Either USP2a antisense oligonucleotides or the FAS inhibitor cerulenin induces apoptosis in prostate cancer cells. Moreover, USP2a is massively overexpressed in prostate carcinomas [76]. These correlations suggest that USP2a rescues FAS from degradation and thereby prevents apoptosis of cancer cells.

5.4. Deubiquitination and the regulation of gene silencing

Within eukaryotic chromosomes, there are transcriptionally inactive tracts of chromatin called heterochromatin [77]. A number of proteins that are important for the establishment and maintenance of heterochromatic gene silencing have been identified. The first suggestion that protein deubiquitination by specific DUBs may modulate gene silencing came from studies in yeast. Among the proteins known to contribute to silencing are the products of the *SIR1*, *SIR2*, *SIR3*, and *SIR4* genes [78] and the histones H3 and H4 [79,80]. Using Sir4 protein-based affinity chromatography, several proteins in yeast extracts were identified as potential interacting factors, including the deubiquitinating enzyme Ubp3 [81]. Deletion of the *UBP3* gene leads to a significant increase in transcriptional silencing at telomeres (and at the silent mating-type loci). In contrast, no influence of Ubp3 inactivation on silencing was detected when the reporter gene was located at an internal chromosomal position, indicating that the effect on transcription depends on the chromosomal position of the reporter. The mechanism of Ubp3 action as a negative regulator of silencing has not been pursued. It is noteworthy that a loss-of-function mutation in a DUB from *Drosophila*, D-Ubp-64E, has also been associated with an enhancement in gene silencing (position-effect variegation) in this organism [82].

Ubp10/Dot4, another yeast DUB, affects silencing in yeast in a manner opposite to that of Ubp3, namely, it is required for full levels of silencing [83]. Ubp10 also appears to interact with Sir4, but as is true of the Ubp3 study, it is not clear whether this is a direct interaction or whether this interaction is key to the silencing defects associated with loss of the Ubp. *UBP10* deletion is associated with a significant reduction of the intracellular concentration of Sir4, which might be due to a failure to deubiquitinate Sir4, although Sir4 ubiquitination has not been reported.

A new perspective on chromatin-mediated gene silencing has emerged recently with the discovery of histone ubiquitination in yeast, which had previously been thought to be absent [84]. In mammalian cells, ubiquitin molecules can be ligated to histones H2A or H2B, and this can represent a significant fraction of the total pool of these histones (up to 10% in the case of H2A). It is now clear that histone H2B monoubiquitination in yeast has a role in regulating chromatin structure and transcription. Histone H2B ubiquitination by the E2 Rad6 and the E3 Bre1 is specifically required for normal position-dependent gene silencing. It appears to do this at least in part by stimulating a series of lysine methylations on another core histone, histone H3 (Refs. [85,86] and references therein). Ubiquitinated histone H2B presumably works by recruiting the methylases responsible for these histone H3 modifications to the nucleosome. The histone H3 methylations are not associated with silenced chromatin but with active regions [85,87]. Their requirement for silencing has therefore been suggested to be a consequence of limiting binding of silencing factors to active genes, allowing these factors to concentrate at methylation-deficient silent loci [85].

Interestingly, a specific DUB, Ubp8, has recently been shown to deubiquitinate ubiquitin–H2B and to regulate gene

transcription [88,89]. Ubp8 is a component of several related histone acetyltransferase (HAT) complexes (SAGA and SLIK). Ubp8 is recruited to sites of transcription together with other HAT components, and its activity is required for full activation of genes at these sites. Thus, at least some genes require both histone H2B ubiquitination and then deubiquitination for transcriptional regulation. These sequential changes somehow help establish specific histone H3 methylation patterns, which are important for proper transcriptional control.

5.5. A20 down-regulation of NF-κB signaling

In unstimulated mammalian cells, the transcription factor NF-κB is prevented from accumulating in the nucleus because it binds to the protein IκB, which continually shuttles it back to the cytoplasm [90]. NF-κB activity is rapidly induced in response to a variety of stimuli, including pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α). Binding of TNF-α to its receptor leads to recruitment of several signal adaptor molecules, among them the *r*eceptor-*i*nteracting *p*rotein (RIP) [91,92]. These proteins coordinate interactions between the activated receptor and IκB kinase (IKK); the latter is responsible for phosphorylating IκB on a specific pair of serines, which triggers IκB modification by Lys48-linked polyubiquitin chains and degradation by the proteasome. NF-κB can then concentrate in the nucleus and activate its target genes. Surprisingly, several proteins responsible for IKK recruitment, including RIP, are also polyubiquitinated, but in these cases the chain is of the Lys63-linked variety [93,94].

A20 is an important negative regulator of NF-κB signaling. A20-deficient mice cannot regulate NF-κB and develop severe inflammation. Studies of embryonic fibroblasts from A20-deficient mice suggest that A20 acts at the level of IKK or upstream of it [15]. A20 contains an N-terminal OTU domain, indicating that this protein may function as a DUB. Recent data have confirmed this assumption. Affinity purified A20 can disassemble both Ly48- and Lys63-linked ubiquitin chains [16,17,95]. A20 physically interacts with RIP, suggesting that ubiquitin–RIP conjugates could be natural substrates for this DUB. Indeed, co-transfection of RIP with increasing doses of the A20 cDNA significantly decreased ubiquitination of RIP, whereas A20 with a C103A active site mutation had no effect. A20 is also active against ubiquitinated RIP in vitro; the active site mutant of A20 could still bind RIP but did not deubiquitinate it.

Among the OTU family members, A20 is unusual in that it also contains a zinc finger domain with ubiquitin–protein ligase (E3) activity [16]. This domain modifies RIP with Lys48 ubiquitin chains, which target RIP for degradation by the proteasome. Remarkably, both the OTU and E3 domains cooperate to down-regulate NF-κB signaling. Cleavage of the Lys63-polyubiquitin chain from RIP by the OTU domain prevents the recruitment and activation of additional factors that transduce the TNF-α signal from RIP to the IKK complex. In addition, Lys63-ubiquitin chain removal from RIP is a prerequisite for the subsequent A20 zinc finger domain-mediated Lys48-poly-ubiquitin addition to RIP and RIP degradation. Destruction of TNF-α receptor-associated RIP ensures that the signaling pathway is fully inactivated.

5.6. CYLD down-regulation of NF-κB signaling

The first human disorder linked to a heritable genetic defect in a DUB was cylindromatosis (turban tumor syndrome) [96]. Familial cylindromatosis is a rare, autosomal dominantly inherited predisposition to tumors of the skin appendages, with most occurring in the head and neck area. Recent studies show that CYLD, the protein that is mutated in familial cylindromatosis, is a DUB of the UBP class that also negatively regulates the NF-κB signaling pathway [97–99].

Although CYLD can cleave Lys48-linked ubiquitin oligomers in vitro, Lys63-linked ubiquitin–protein conjugates appear to be its natural substrates in vivo. Notably, CYLD physically interacts with and deubiquitinates TRAF2, an E3 ubiquitin–protein ligase that modifies itself with Lys63-linked ubiquitin chains. Ubiquitination of TRAF2 helps to recruit and activate IKK, leading to NF-κB activation, as described above. A point mutation in CYLD that abolishes CYLD binding to TRAF2 greatly increases polyubiquitin-TRAF2 levels. Tumorigenicity of CYLD correlates with loss of deubiquitinating activity. Thus, it is likely that a defect in the deubiquitination of CYLD substrates such as TRAF2 underlies the pathology of cylindromatosis.

5.7. DUBs and Parkinson's disease (PD)

PD is a neurological disorder that affects approximately 2% of the population over the age of 65. It was initially thought that PD had no genetic components since family history was difficult to discern. However, more recent studies have identified at least three genes that may be linked to familial forms of the disease. The products of these genes are α-synuclein, the ubiquitin–protein ligase parkin, and UCH-L1 [100,101].

The deubiquitinating enzyme UCH-L1 is extremely abundant in the brain, but its exact roles there are uncertain. In vitro, this enzyme is able to hydrolyze C-terminal ubiquitin esters and amides [102]. In 1998, a point mutation of UCH-L1, I93M, was identified in two siblings with a family history of PD [103]. UCH-L1 may modulate the turnover of α-synuclein, a major fibrillar component of the Lewy bodies that are associated with PD pathogenesis. It was shown that UCH-L1 and α-synuclein colocalize to synaptic vesicles and can be co-immunoprecipitated from mammalian brains. In addition, overexpression of UCH-L1 leads to an accumulation of α-synuclein.

A provocative recent study suggests that this build-up of α-synuclein does not depend on the hydrolytic activity of UCH-L1 because the effect of overproducing the I93M mutant, which has impaired UCH activity, was virtually indistinguishable from that of the wild-type enzyme [104]. This observation might be explained by the existence of a second, previously unrecognized biochemical activity of UCH-L1. Indeed, Liu et al. [104] found that UCH-L1 can form dimers, and this form of the protein seems to act as a novel type of ubiquitin transferase capable of ubiquitinating α-synuclein. Another variant of UCH-L1, the S18Y mutant, is considered a "protective" variant of the enyme associated with reduced risk of PD. This mutant shows reduced ubiquitin ligase activity but not lower UCH activity. Interestingly, UCH-L1 promotes formation primarily of Lys63-linked ubiquitin chains, which do not normally target proteins to the proteasome and could conceivably inhibit degradation of α-synuclein. The exact connection between the apparent UCH-L1 ligase activity and PD pathogenesis remains to be dissected, but this new study's suggestion that the ubiquitin transferase activity of DUBs "runs in reverse" could be biologically significant.

Several other mammalian DUBs have been identified as regulators of the growth and proliferation of cultured cells [11]. We do yet know, however, what the substrates and means of regulation of these DUBs might be. Still, we can anticipate a much broader contribution of DUBs to mammalian regulatory mechanisms than is currently appreciated.

6. Proteases that act on conjugates of UBLs

In addition to ubiquitin, a number of ubiquitin-like modifiers (UBLs) have been identified and are now known to use enzymatic pathways for their attachment to substrates that are very similar to the ubiquitin conjugation system. These UBLs include the Smt3/SUMO-1, Rub1/NEDD8, ISG15, and Atg8 proteins, which, like ubiquitin, are also all synthesized as precursors with C-terminal extensions [105]. Deconjugating enzymes have been found in all four of these systems. We briefly discuss them here because their similarities and differences from the DUBs are instructive both for understanding the basis of substrate specificity among the ubiquitin/UBL-cleaving enzymes and for thinking about how these various modification systems may have evolved.

6.1. SUMO-deconjugating enzymes

The number of known targets for this divergent UBL is growing rapidly. Among them are the nuclear pore proteins RanGAP1 and RanBP2; the promyelocytic leukemia gene product (PML); and IκBα, an inhibitory subunit of the transcription factor NF-κB [106–113]. Despite its weak similarity to ubiquitin (12–18% identity, depending on the species), the three-dimensional structure of SUMO is very close to that of ubiquitin [34].

The first *u*biquitin-*l*ike protein-specific *p*rotease (ULP) responsible for cleavage of SUMO from substrates, Ulp1, was identified in the yeast *S. cerevisiae* [33]. Ulp1 showed no activity against ubiquitin-linked substrates. Based on sequence similarity to Ulp1, the yeast genome encodes two such enzymes, Ulp1 and Ulp2/Smt4, while most other eukaryotes, including humans, have a greater number of paralogs [114]. Ulp1 and Ulp2 are able to cleave SUMO from both peptide- and isopeptide-linked protein conjugates [33,115]. Surprisingly, the ULPs display no obvious sequence similarity to any of the DUBs. Instead, these cysteine proteases are distantly related in sequence to the processing proteases of adenoviruses and proteins found in other DNA viruses and in eubacteria [33,116,117]. Nevertheless, as noted earlier, the catalytic core structure of the ULPs is close to that of the classical cysteine proteases [34]. Therefore, the ULPs appear to have arisen from a distinct lineage of cysteine proteases that branched off long ago from the classical cysteine protease lineage that led eventually to the UCHs and UBPs. The complex between Ulp1 and SUMO shows an extensive interface and many SUMO contacts that would not be possible with ubiquitin, accounting in principle for the ability of Ulp1 to distinguish between these two modifiers [34].

6.2. NEDD8/Rub1-cleaving enzymes

The NEDD8/Rub1 (RUB) protein is much closer to ubiquitin in sequence (~58% identity) than is SUMO, but the enzymes responsible for cleaving RUB from its biosynthetic precursor and from posttranslationally modified substrates are just beginning to be identified. Surprisingly, in yeast the RUB precursor-processing enzyme is none other than Yuh1 (see earlier) [118]. Loss of the Yuh1 enzyme prevents RUB from getting conjugated to protein substrates, a defect that can be completely bypassed by genetically removing the sequence encoding the C-terminal extension of the RUB precursor. Thus, Yuh1 can process both ubiquitin- and RUB-linked substrates. This dual specificity has also been observed in vitro with the mammalian UCH-L3; by contrast, UCH-L1 could not process a C-terminally extended RUB protein [119]. Dual specificity for ubiquitin and RUB might not be limited to UCH isozymes. Overexpression in mammalian cells of a UBP called USP21 could deplete high molecular mass conjugates of both ubiquitin and RUB [120]. However, whether this was a direct effect of the overproduced enzyme on both types of conjugates was not demonstrated.

Until very recently, the only known targets for RUB modification were the cullin proteins, most or all of which are subunits of SCF and SCF-related ubiquitin ligases, and the tumor suppressor protein p53 [121–123]. The SCFs are multisubunit complexes with a minimal catalytic core composed of a cullin subunit and a RING finger protein.

Many short-lived regulatory proteins are ubiquitinated by SCFs and subsequently degraded by the 26S proteasome. These include IκBα, β-catenin, and the cyclin-dependent kinase inhibitor Sic1 [124]. RUB modification of the SCF cullin CUL1 stimulates the ligase activity of the SCF complex, possibly by stimulating E2–ubiquitin binding [121,122]. Unexpectedly, a major RUB-cleaving activity has been traced to the CSN, a 500-kDa protein complex originally identified from its role in photomorphogenesis in plants [125,126]. The Csn5 subunit of the CSN is the key catalytic component of this isopeptidase activity, and it bears the MPN+/JAMM metalloprotease sequence signature also seen in the Rpn11/POH1 proteasome subunit that acts on ubiquitin conjugates (Fig. 2E) [127].

As with Rpn11, Csn5 is only active when in a larger complex. Interestingly, all the subunits of the CSN are related to subunits in a subcomplex of the proteasome called the lid, suggesting a close evolutionary relationship between the two particles [128]. It is noteworthy that the SCF ubiquitin ligases can bind to both proteasomes and to the CSN [126]. Perhaps the CSN evolved from what had originally been (and possibly still could be) an alternative proteasome lid complex, but developed a distinct specificity for RUB-cullins that evolved from its ability to bind SCFs.

Identification of another RUB-specific protease, named DEN1/NEDP1/SENP8, has been reported recently [129,130]. Like CSN, DEN1 may play an important role in controlling cullin proteins modification by RUBs. DEN1 deconjugates NEDD8-modified cullins in vitro and in vivo. The enzyme is also active against NEDD8–p53 conjugates. Xirodimas et al. [123] have found that p53 is modified by NEDD8 in vivo and that this modification can inhibit transcription activity of the tumor suppressor. Remarkably, NEDD8–p53 conjugates were not detected in cells transfected with DEN1, suggesting DEN1 involvement in p53 regulation. As is true for UCH-L3, the DEN1 enzyme is also active against ubiquitin derivatives. However, DEN1 cleaves RUB-AMC 6×10^4 times faster than the corresponding ubiquitin-linked substrate. Surprisingly, DEN1, which is a member of the ULP family, has no detectable activity against SUMO-AMC. Sequence comparisons reveal that several residues thought to be involved in SUMO binding in ULP-family enzymes that cleave SUMO substrates are not conserved in DEN1.

6.3. ISG15–protein cleavage by UBP43

The first UBL discovered was the product of the interferon-stimulated gene 15 (*ISG15*) [131,132]. ISG15, which contains two ubiquitin-like domains and cross-reacts with antiserum raised against ubiquitin, is conjugated to multiple proteins. More recently, the first ISG15-specific protease was identified; the enzyme, UBP43 (USP18), belongs to the UBP sequence family, but in vitro it has no activity against ubiquitin [133]. This is reminiscent of the metalloprotease group of ubiquitin/UBL-cleaving enzymes, which include members that are specific to either ubiquitin or a particular UBL (RUB). It will be of great interest to determine the molecular basis for the ability of different UBPs to distinguish between ubiquitin and the closely related ISG15 protein.

UBP43 is most strongly expressed in brain ependymal cells, which maintain the blood–brain barrier. Deletion of the *UBP43* gene in mice leads to a dramatic increase in ISG15 conjugates in both ependymal cells and in general brain tissue [134]. *UBP43*$^{-/-}$ knockout mice have a reduced life expectancy and display neurological abnormalities. Specifically, the brains of these mice are characterized by necrosis of the ependymal cells, resulting in hydrocephalus, an abnormal accumulation of fluid in the brain ventricles. Interestingly, ISG15 precursor is still processed normally in these mice, indicating the existence of at least one additional ISG15-processing enzyme in this organism. Purification of an ISG15 precursor-processing activity has been reported, but the identity of the enzyme responsible is not yet known [135].

6.4. The Atg8 Ubl is cleaved from phospholipids by a novel hydrolase

Among the most divergent UBLs known, Atg8 is the UBL lacks detectable sequence similarity to ubiquitin but nonetheless displays the ubiquitin superfold [136]. Conjugation-competent Atg8 is required for a starvation-induced process known as autophagy, wherein portions of cytosol are encapsulated in a double-membrane structure called the autophagosome. The autophagosome then fuses with the vacuole, resulting in the hydrolysis of its cytosolic contents. The exact mechanistic contribution of Atg8 to autophagosome formation remains to be worked out, but a unique feature of this UBL is that it is ligated to a lipid, phosphatidylethanolamine (PE), rather than to a protein. Moreover, this attachment needs to be reversible, at least in yeast, for autophagosome formation to proceed normally.

The enzyme responsible for Atg8 cleavage from PE, as well as the processing of the Atg8 precursor, is Atg4 [136]. Atg4 is a cysteine protease, but by primary sequence, it is not related to any known DUB or ULP or, for that matter, to any other known protease. Structural studies on Atg4 should reveal whether or not this enzyme is a distant member of the classical cysteine protease family or derives from a distinct class of proteases.

In summary, the analysis of UBLs has uncovered a number of UBL-specific hydrolases, and these contribute in crucial ways to the physiology of these protein modification systems. While many of the UBL-specific proteases belong to sequence classes that include ubiquitin-cleaving proteases (and in some cases have dual specificities), the enzymes that act on the highly divergent SUMO and Atg8 modifiers do not. SUMO proteases, however, also belong to the classical cysteine protease superfamily. The MPN+/JAMM proteases are metalloproteases rather than cysteine proteases, but they also divide into multiple

subfamilies, including the ones specific for ubiquitin or for RUB. It seems likely that gene duplication and divergence both occurred before the emergence of eukaryotes and more recently have generated the array of proteases that account for the striking dynamics of ubiquitin and most UBL conjugates. Atg4, on the other hand, may represent a unique type of protease distinct in its evolution from any that can act on ubiquitin.

7. Concluding remarks

The study of DUBs is still in its early days [11,137]. Important regulatory functions for DUBs have been predicted for quite some time, and examples are now slowly being uncovered. That DUBs can act as negative regulators of proteolysis by counteracting against the action of the ubiquitination machinery on specific substrates has been clearly demonstrated recently with several DUBs. More examples of this type can be anticipated. DUBs can also modulate nonproteolytic ubiquitin-dependent processes such as membrane protein trafficking decisions and certain signal transduction mechanisms.

It is important that protein deubiquitination occurs at the right place and the right time. For instance, deubiquitination of a membrane protein that is destined for the vacuolar interior must occur after its ubiquitin-dependent commitment to the involuting membrane of the MVB but before it becomes inaccessible to DUBs such as Doa4. Similarly, substrates targeted to the proteasome by a polyubiquitin chain must generally be committed to unfolding or degradation prior to removal of the polyubiquitin signal by Rpn11 or other DUBs. Gaining an understanding of the spatial and temporal controls over these deubiquitination events will be among the major challenges for the field over the next few years. It seems safe to say at this point that the physiological importance of rapid and specific removal of ubiquitin from proteins and other molecules is now well established. What remains to be determined is how commonly and under what circumstances deubiquitination is used as a point of regulation in the ubiquitin system and by what molecular mechanisms substrate-specific deubiquitination is achieved.

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