

Polyubiquitin Binding and Disassembly By Deubiquitinating Enzymes

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1. Introduction

Ubiquitin (Ub) is a highly conserved protein of 76 amino acids that is covalently linked to target proteins altering their localization, function, or stability.^{1–3} Proteins can be modified with a large number of different isoforms of ubiquitin, and these different ubiquitins are thought to signal different outcomes. The question of how these different forms of ubiquitin are recognized is central to understanding the specificity of various types of ubiquitination.^{4–6}

Ubiquitin acts as a signal by being conjugated to proteins through three sequential steps. In the first step, ubiquitin is activated by the ATP-dependent formation of a thiolester bond between the C-terminus of ubiquitin and the active site cysteine of an ubiquitin activating enzyme or E1. The second

step involves the transfer of the ubiquitin molecule from the E1 to the active site cysteine of an ubiquitin-conjugating enzyme or E2. Finally, the ubiquitin is transferred to a lysine residue of the target protein in a reaction catalyzed by an ubiquitin ligase or E3. This last step occurs in a substrate-specific manner and it is highly regulated.^{7–9}

Several rounds of ubiquitination can occur on ubiquitin itself, leading to the formation of a polyubiquitin chain. Any of seven lysines, or the amino terminus, of ubiquitin can be used to polymerize ubiquitin, and so there are a huge number of differently linked polyubiquitin signals that can be formed. Chains can be linked by the same lysine on each ubiquitin (K29, K48, K63, etc.) to yield homogeneous chains, or can utilize different lysines on some ubiquitins to yield heterogeneous chains. In the latter case, the lysine used can vary from ubiquitin to ubiquitin, or chains can be formed that are branched at a single ubiquitin by linking two ubiquitins to two different lysines at the branch point. It is commonly assumed that different polyubiquitin chains are associated with different cellular fates. Receptors are thought to recognize the different ubiquitin modifications (mono- and polyubiquitin) attached to the target proteins and to mediate the different signaling outcomes.^{4,10} These receptors have ubiquitin binding domains that interact with ubiquitin or polyubiquitin, and may also have domains that can interact with the modified target proteins or other macromolecules.

Like most posttranslational modifications, ubiquitination is reversible,¹¹ and its removal is carried out by enzymes collectively known as deubiquitinating enzymes (DUBs).¹² DUBs are proteases that have been implicated in a wide variety biological processes.^{12,13} They are responsible for the removal of ubiquitin or polyubiquitin from target proteins, the processing of ubiquitin precursors, and the disassembly of unanchored polyubiquitin (a polyubiquitin chain not attached to another protein) that is either synthesized de novo, or released by the action of other DUBs. Thus, like the cellular targeting receptors they recognize the different forms of ubiquitin and polyubiquitin. For instance, the tumor suppressor CYLD acts exclusively on K63-linked chains,¹⁴ yeast OTU1 prefers long K48-linked chains,¹⁵ and USP5 cleaves both linkages.¹⁶ Nearly 100 DUBs in five different protein families are encoded by the human genome. Several DUBs have been shown to bind or process polyubiquitin or polyubiquitinated substrates in vivo, and many DUBs have been shown to cleave polyubiquitin in vitro.

This review will discuss the specificity of ubiquitin and polyubiquitin binding by DUBs. The DUBs discussed will be limited to those where binding and specificity have been directly demonstrated, either through structure determination or direct binding and catalytic studies. It will focus on the current body of knowledge regarding structure of ubiquitin binding domains of DUBs and the mechanisms by which

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Dr. Keith Wilkinson majored in chemistry at Albion College in Albion, Michigan, graduating in 1972. He received his Ph.D. in biological chemistry from the University of Michigan in 1977 working with Charles H. Williams, Jr., on the catalytic mechanism of *Escherichia coli* lipamide dehydrogenase. He next went on to do postdoctoral studies with Nobel Prize winner Irwin A. Rose at the Institute for Cancer Research in Philadelphia. While there he continued studies on mechanistic enzymology, studying hexokinase. Toward the end of his stay in Philadelphia he began to work on the ATP- and ubiquitin-dependent proteolysis system and was the first to show that the small protein required for ATP-dependent proteolysis was ubiquitin. In 1981 he came to Emory University as Assistant Professor of Biochemistry and began his work on deubiquitinating enzymes. He pioneered this field with the development of assays and the cloning of the first mammalian enzyme. His research has been supported by grants from the NIH, NSF, and the pharmaceutical industry, as well as a Career Development Award from the NIH. He has served as a member of the Biochemistry and the Membrane Biology and Protein Processing NIH study sections and is an associate editor of the *Journal of Biological Chemistry*. He is currently Professor and Vice Chair of Biochemistry and Director of the Graduate Division of Biological and Biomedical Sciences.

these DUBs recognize and selectively disassemble different polyubiquitin chains. In addition to clarifying the mechanisms of chain recognition by DUBs, the conclusions gleaned from these proteins may well serve as a model for the recognition of these chains by other receptors.

2. The Polyubiquitin Modification

Ubiquitin adopts a β -grasp fold that consists of a central five-stranded β -sheet wrapping one α -helix and a short $_3$ -helix.¹⁷ The C terminus of ubiquitin, involved in the

formation of the isopeptide bond with target proteins or between ubiquitins, protrudes from the body of ubiquitin. The form of ubiquitin that becomes attached to the target protein (linkage and length) appears to ultimately determine the signaling outcome.^{10,18–20} Monoubiquitination, the conjugation of one ubiquitin to a target protein, acts as a signal that has been implicated in histone regulation, DNA repair, endocytic trafficking, and virus budding.^{18,21,22} Polyubiquitination, the conjugation of polyubiquitin to a target protein, results from the subsequent conjugation of ubiquitin monomers to any of the seven lysine residues of ubiquitin (K6, K11, K27, K29, K33, K48, or K63) or to M1 of ubiquitin.^{4,5,10,3,23–25} Conjugation of differently linked isoforms of polyubiquitin to proteins are thought to target the modified proteins to different fates.^{4,5,10} The various signaling outcomes probably result from the recognition by receptors of the different three-dimensional structures adopted by the different polyubiquitin isoforms. Ultimately these structures depend on which lysine residue is utilized in the polyubiquitin chain formation.^{26–31}

The best understood isoforms of polyubiquitin are the K48- and K63-linked chains. K48-linked polyubiquitin is usually, although not always,³² involved in proteasomal degradation, while K63-linked chains act as nonproteolytic signals in intracellular processes including endocytosis, activation of kinases in the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, DNA repair, autophagy, and ribosome function.^{20,33–37} Structural studies aimed at understanding the differences between various chains have largely focused on K48- and K63-linked polyubiquitin since they can be synthesized *in vitro* in large quantities. The structure of these two types of chains reveals that they adopt different conformations in solution.

NMR and X-ray crystallographic studies show that K48-linked chains can adopt at least two different conformations, demonstrating that these chains are flexible.^{26,27,29,31,38} In the “closed” conformation the L8–I44–V70 hydrophobic patch, implicated in the binding to most ubiquitin binding domains (UBDs)^{39,40} including those present on the proteasome,⁴¹ forms intrachain contacts between adjacent ubiquitin units causing the patch to be sequestered at the ubiquitin/ubiquitin interface in the chains (Figure 1A). In the “open” conformation, the hydrophobic patch of ubiquitin is solvent exposed. This would allow the hydrophobic patch to directly participate in the interaction with UBDs (Figure 1B). Although K48-linked polyubiquitin can adopt two distinct conformations in solution, little is known about the predominant form adopted when the chains are bound to polyubiquitin binding proteins. NMR studies show that a UBD, the ubiquitin associated domain (UBA) of Rad23, preferentially binds K48-linked polyubiquitin by interacting with surfaces on both ubiquitins that surround the isopeptide bond, thereby stabilizing the open conformation.^{30,42} The closed conformation has been proposed to occur only at the distal end of the chain since K48 of the distal ubiquitin in the closed conformation is not accessible for isopeptide linkage to another ubiquitin. Thus, the steric constraints of the observed structure prevent the closed conformation from being accommodated in the interior ubiquitins of a chain.^{29,38}

K63-linked chains predominantly form an extended conformation that, like the open conformation observed in K48-linked polyubiquitin, has the I44 hydrophobic patch exposed to the solvent (Figure 1C).²⁸ In this conformation there is no direct contact between the hydrophobic surfaces on adjacent ubiquitin monomers in diubiquitin. However, it is

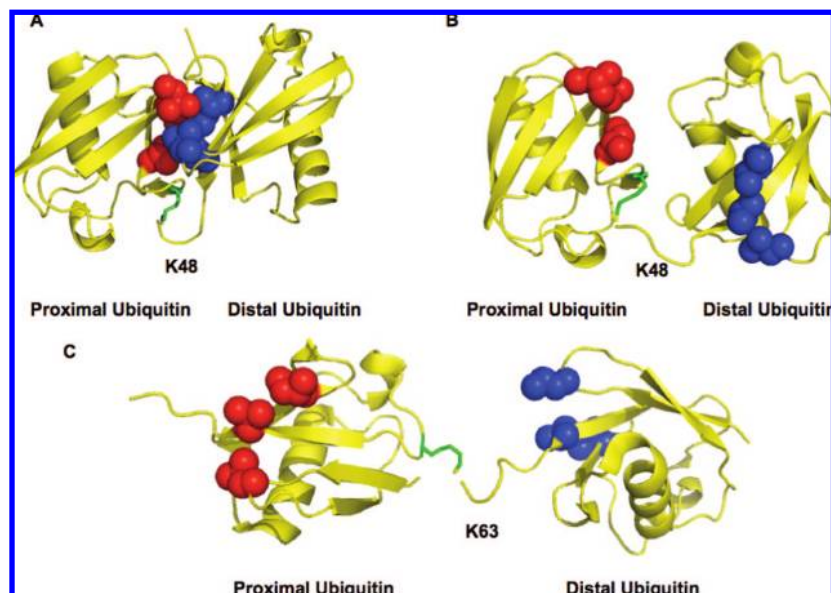


Figure 1. The structures of K48 and K63-linked diubiquitin. (A) Closed conformation of K48-linked diubiquitin. (B) Open conformation of K48-linked diubiquitin. (C) Extended conformation of K63-linked diubiquitin. The ubiquitin moieties in the chain are in yellow. The hydrophobic patch in the distal and proximal ubiquitins is shown in blue and red respectively. K48 or K63 of ubiquitin that participated in the isopeptide bond is in green. The protein data bank codes are 1AAR, 1TBE, and 2JF5 for A, B, and C, respectively.

possible that longer K63-linked chains could adopt a more compact conformation through hydrophobic contacts between nonadjacent ubiquitins in the chain.¹⁰

In contrast to our detailed understanding of the role and three-dimensional structures of K48 and K63-linked polyubiquitin, much less is known about other polyubiquitin isoforms. For instance, both K29 and K11-linked polyubiquitin have been implicated in proteasome-dependent protein degradation,^{43,44} but no structures are available to compare to those of the K48- or K63-linked chains.

The above conclusions are derived from studies on homogeneous polyubiquitin (ubiquitin chains linked through only a single lysine residue). However, heterogeneous polyubiquitin chains linked through multiple lysines can also be synthesized *in vitro*,^{45,46} and have been detected *in vivo* using mass spectrometric analyses.²⁴ Heterogeneous chains contain mixed linkages using different lysines to link monomers in a chain. Heterogeneous chains can also be branched, using two different lysines of a single ubiquitin at the branch point to attach two distal ubiquitins. It is not known how frequently these types of chains are formed *in vivo*, nor what physiological roles they might serve.⁴⁶ In the ubiquitin fusion degradation (UFD) pathway, it is thought that heterogeneous chains linked through K29 and K48 are utilized to target proteins for proteasomal degradation.⁴³ These early studies did not demonstrate conclusively that the chains attached to the target protein were heterogeneous, but instead relied on mutagenesis of the lysine residues of ubiquitin to show both K29 and K48 were required. Indeed, a more recent study, which employed mass spectrometry analysis, showed that some protein substrates modified with branched heterogeneous chains are resistant to proteasomal degradation, and that branched polyubiquitin is poorly disassembled by the proteasome-associated DUBs *in vitro*.⁴⁶

How are the different forms of polyubiquitin recognized by receptors and DUBs? Studies aimed at understanding recognition have focused predominantly on monoubiquitin derivatives and K48- or K63-linked polyubiquitin. Although some DUBs exhibit little polyubiquitin isoform selectivity,⁴⁷ some have been shown to be specific for one chain isoform

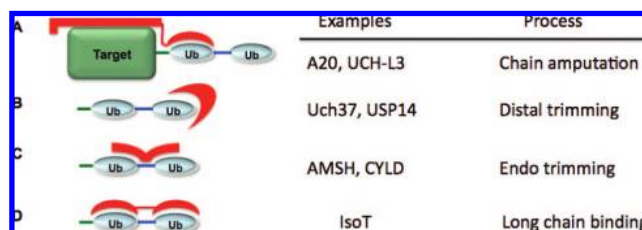


Figure 2. Possible mechanisms of polyubiquitin recognition by DUBs. (A) DUBs can interact with both the target protein and polyubiquitin. (B) DUBs can recognize the distal ubiquitin of the chain. (C) DUBs could bind simultaneously to two ubiquitins by interacting with surfaces on both ubiquitins that surround the isopeptide bond. (D) Finally, a DUB can recognize polyubiquitin through the use of multiple ubiquitin binding domains. Polyubiquitin is shown in blue, the target protein is in green, and the polyubiquitin receptor is in red.

over another.^{14,48–51} The chain specificity of DUBs can be intrinsic to their catalytic core domains¹⁴ or can be mediated by additional domains, including UBDs. At least 16 UBDs have been described, including: UBA, UIM, MIU, DUIM, CUE, GAT, NZF, A20 ZnF, UBZ, Ubc, UEV, UBM, GLUE, Jab1/MPN, and PFU. Multiple surfaces on ubiquitin interact with these UBDs, but most often a hydrophobic patch consisting of L8, I44, and V70. The affinities of these isolated UBDs with monoubiquitin are commonly weak, with $K_d > 100 \mu\text{M}$. Affinity can be increased by avidity, commonly by polymerizing ubiquitin or by utilizing multiple UBDs in a single enzyme or receptor. The observed ubiquitin binding affinity varies widely for DUBs. Yeast OTU1 shows no binding up to 2 mM ubiquitin, UCH-L3 binds ubiquitin with an affinity of 100–500 μM and USP5 binds with a K_d of 50 nM. Polyubiquitin affinity is usually higher and USP5 binds K48-linked tetraubiquitin with a dissociation constant of less than 1 nM.¹⁶

This discussion suggests at least four mechanisms for recognition of polyubiquitin. First, DUBs could interact with both the target protein and the proximal end of polyubiquitin (Figure 2A). A second mechanism would involve the binding of the distal ubiquitin in the chain by the DUB (Figure 2B).

This specificity could be enforced by binding to a surface of ubiquitin that is only exposed in the closed conformation or to a surface that would be blocked by a more distal ubiquitin (Figure 2B). A third mechanism would involve the recognition of surfaces on both ubiquitins that surround the isopeptide bond (Figure 2C). These surfaces will vary depending on the type of linkage utilized. Finally, a fourth mechanism by which a DUB can recognize polyubiquitin involves binding to multiple ubiquitin binding domains (Figure 2D). In this mechanism, the flexibility of the polyubiquitin receptor itself would be necessary if the DUB were to bind different isoforms of polyubiquitin. Thus, the constraints on the relative orientation of the ubiquitin binding domains could be a major determinant of specificity.

The consequences of these different modes of interaction could determine the enzymatic specificity. For instance, Model 2A would be ideal for the specific amputation of an intact polyubiquitin chain. Model 2B could result in distal chain trimming, while model 2C could lead to endocleavage of polyubiquitin chains or specific cleavage at the branch points of heterogeneously linked chains. Finally, model 2D would be ideal for recognizing longer polyubiquitin chains. In the following sections we will discuss examples of how ubiquitin and polyubiquitin recognition is achieved by the different DUB families.

3. DUB Families

DUBs constitute one of the larger classes of enzymes in the ubiquitin system. A recent bioinformatics study suggested that humans have approximately 100 different DUBs.¹³ The catalytic core domains of DUBs are responsible for the recognition and proper active site positioning of ubiquitin containing the scissile bond. Therefore, these active sites contain ubiquitin-interacting surfaces. In addition to the catalytic domains, DUBs have N- and C-terminal extensions that modulate their substrate specificity, or cellular localization.¹² Some extensions include UBDs, ubiquitin-like domains, and other protein–protein interaction domains.¹³

Eukaryotic genomes encode five families of DUBs; four are cysteine proteases and one is a zinc-dependent metalloprotease.^{12,13} The cysteine protease families include the ubiquitin specific processing proteases (USP or UBP in yeast), the ubiquitin C-terminal hydrolases (UCH), ovarian tumor related proteases (OTU), and the Josephin/Machado-Joseph disease proteases (MJD). The zinc metalloprotease DUBs contain a JAB1/MPN/Mov34 metalloenzyme (JAMM) domain.

Despite the low sequence similarity between the cysteine protease DUB families, and their different overall structure, the catalytic cores that contain the active site residues closely resemble the classical cysteine protease papain.¹² This core contains the papain-like C–H-strand and loop.^{12,15,16,50,52–58} The active site C and H are located on the α -helix and the β -strand, respectively. Often, the D/N of the catalytic triad is located in the loop; however, its precise location is variable. The structure of at least one member of each type of DUB family has been solved, in some cases in the presence of monoubiquitin-based inhibitor and in one case in the presence of a polyubiquitin chain isoform. The catalytic core domains of most DUBs recognize monoubiquitin, and in at least one case, polyubiquitin.^{15,48,53,56,59} The structure of the catalytic core domains (both unbound and bound to ubiquitin based inhibitors) reveals that most must undergo active site rearrangements to productively bind substrate and

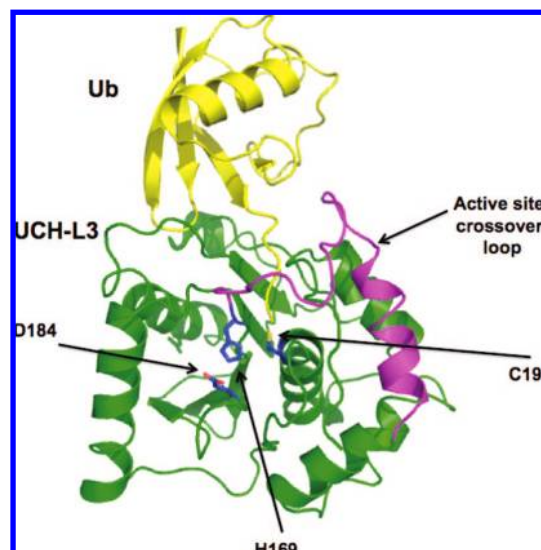


Figure 3. Structure of a UCH family DUB bound to Ub-VME, UCH-L3. Ub-VME is in yellow, and UCH-L3 is in green. The active site loop is in magenta. The active site residues are shown in blue. The protein data bank identification code is 1XD3.

catalyze its hydrolysis. In the following section we will review the structures of each type of DUB domain and how these structures provide insights into ubiquitin recognition and catalysis.

3.1. The UCH Family

The ubiquitin C-terminal hydrolase (UCH) family of DUBs was the first to be identified.^{60,61} In humans, there are four UCH domain-containing DUBs, while in yeast there is only one, Yuh1.^{62–64} Early studies suggested that UCH family DUBs preferentially cleave small or flexible leaving groups, and, given this substrate preference, this family of DUBs was proposed to act predominantly in the recycling of ubiquitin that has become inappropriately conjugated to intracellular nucleophiles.⁶⁵ However, the *in vivo* substrates for UCHs have not been clearly established,^{65,66} and some UCH family DUBs can disassemble polyubiquitin- and ubiquitin-protein conjugates. For example, UCH37 disassembles polyubiquitin chains,⁶⁷ UCH-L3 has been implicated in reversing the ubiquitination of the epithelial sodium channel,⁶⁸ and the *Drosophila* UCH can act on ubiquitinated protein.^{69,70} Another proposed role for this class of DUBs is the cotranslational processing of ubiquitin precursors. Ubiquitin is always synthesized as a fusion protein that must be processed and the pro-proteins consist of either a single copy of ubiquitin with a C-terminal ribosomal protein or a polyubiquitin precursors that has an additional amino acid following the last ubiquitin monomer.^{71–73}

The structures of two human UCH domain DUBs (UCH-L1 and UCH-L3) and that of yeast Yuh1 have been solved, the latter two in the presence of an ubiquitin-based inhibitor (Figure 3).^{55,56,63,74} The UCH domain consists of a six- or seven-stranded antiparallel β -sheet surrounded by eight α -helices.⁶³ The catalytic triad is located at the bottom of a pocket in the surface of the protein. This pocket is wide enough to accommodate the diglycine motif of the ubiquitin C terminus but too narrow to accommodate residues with larger side chains. UCH-L3 and Yuh1 were cocrystallized in the presence of the inhibitors ubiquitin vinyl methyl ester (Ub-VME), and ubiquitin aldehyde (Ubal), respectively.^{55,63} Ub-VME is an irreversible inhibitor that forms a thioether

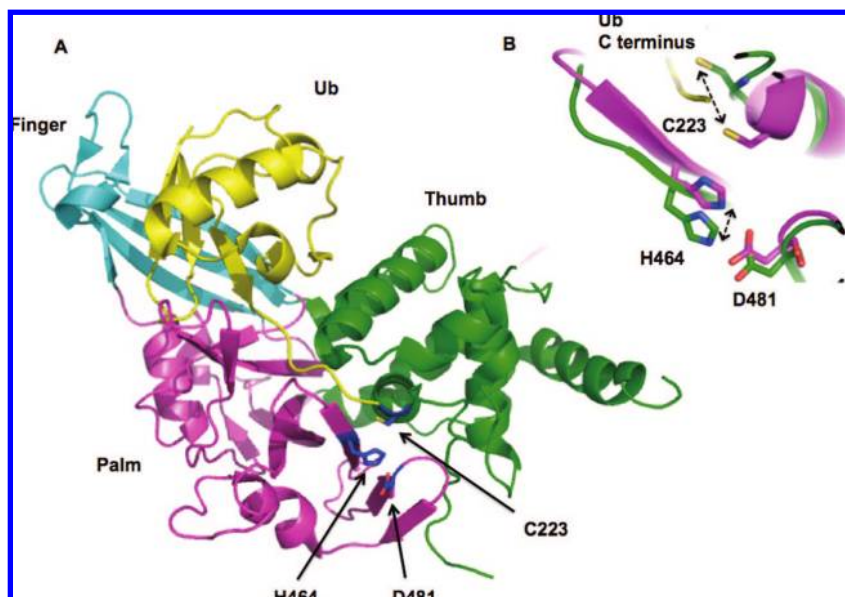


Figure 4. Structure of a UBP/USP family DUB, USP7. (A) Structure of Usp7 bound to Ubal. Ubal is in yellow. The three domains that make up the USP domain are in cyan (Finger), green (Thumb), and magenta (Palm). The active site residues are shown in blue. (B) Conformational rearrangement of the active site residues upon binding of Ubal to Usp7. Ubal unbound and bound Usp7 are in green and magenta, respectively. The ubiquitin C terminus is in yellow. The protein data bank identification codes for the unbound and bound structure of Usp7 are 1NB8 and 1NBF, respectively.

bond with the active site cysteine.⁷⁵ Ubal is a reversible inhibitor that forms a thiohemiacetal with the active site cysteine mimicking the tetrahedral reaction intermediate.⁷⁵ In both cocrystals contacts between the UCH domains and ubiquitin are made with the C-terminus and the first N-terminal loop of ubiquitin.

The structures of UCH-L3 and the Ub-VME adduct reveal that the active site undergoes significant conformational changes upon binding to ubiquitin. In the unliganded structure, a loop that must cross over the active site could not be resolved, suggesting that it is disordered.⁵⁶ In the UCH-L3·Ub-VME complex, the disordered loop becomes stabilized into a α -helix followed by an S-shaped loop that crosses over the active site (Figure 3).⁵⁵ This conformation is also seen in Yuh1 despite the low sequence identity (approximately 30%), suggesting that other UCHs may undergo a similar conformational change.

The structure of the unliganded UCH-L1, a DUB implicated in Parkinson's disease and neuronal function, indicates that the active sites cysteine and histidine are not in a productive conformation (unlike in the unliganded UCH-L3 structure).⁷⁴ The residues are 8.2 Å apart suggesting that a conformational change must occur upon substrate binding to place these two residues in close proximity for catalysis. An additional constraint on catalysis is that in the unliganded UCH-L1 the active site loop covers the active site. For an ubiquitinated substrate to enter the active site the leaving group must be less than 10 Å in diameter (the distance between the loop and the active site cleft) or the loop must be displaced as is observed in UCH-L3.

Thus, purified UCH isozymes bind to monoubiquitin and rapidly cleave small⁷⁰ or disordered domains by model 2A where large folded domains are not well tolerated. There is no evidence that purified UCH isozymes can cleave polyubiquitin at a significant rate, probably because the jutadistal ubiquitin is tightly folded and prevents access of the scissile bond to the active site cleft. UCH37 can cleave polyubiquitin when bound to the proteasome, but that reaction may require

the additional action of proteasome components to possibly unfold the polyubiquitin chain. Other purified UCHs can cleave ubiquitin fusion proteins very slowly and may require the partial unfolding of the leaving group by conformational flexibility or the action of other accessory proteins. The identification and structural studies of a complex between UCH-L1 and an *in vivo* substrate should clarify the specificity and the mechanism of activation of this class of enzymes.

3.2. The USP/UBP Family

The USP family is the largest and most diverse family of DUBs. There are 16 UBPs in yeast, and more than 50 USPs in humans.^{12,13,76} The USP catalytic core domain is approximately 350 amino acids long. In contrast to the UCH family, USPs generally cleave larger leaving groups from the C terminus of ubiquitin.¹³ The USP family contains two well-conserved sequences, the Cys box and His box, which contain the active site residues that form the catalytic triad. Recently, the structures of six different USP domains have been described.^{14,48,53,77,78} Despite the low sequence similarity, the USP domain fold is highly conserved. Three well-defined domains (termed Thumb, Finger, and Palm) form a structure that resembles a right-hand (Figure 4A).

The Thumb is predominantly alpha helical and contains the Cys Box, a motif that includes the active site cysteine. The Palm is composed of beta strands supported by alpha helices and contains the remaining active site residues that form the catalytic triad: a His and an Asp or Asn residue. The junction between the Thumb and the Palm domains forms a cleft that accommodates the C-terminal tail of ubiquitin, and the active site residues involved in catalysis. The K48 side chain of ubiquitin aldehyde bound to the catalytic site is oriented toward the Thumb domain and is only partially solvent exposed. In contrast the side chain of K63 is entirely solvent exposed, which would allow the enzymes to tolerate an ubiquitin distal to the ubiquitin bound at the USP domain. If this conformation is catalytically

relevant it would suggest that K48 linked chains might be disassembled from the distal end while K63-linked chains may also be cleaved internally.

The Finger domain is composed of four β -strands and in USP8 and USP2 it contains a CXXCXnCXXC motif that chelates one zinc.^{77,78} Although four of the solved structures lack this motif, the overall fold of the Finger domain is maintained. The CXXCXnCXXC motif is lacking in nine of the 54 putative human USPs, suggesting that the zinc binding ability is dispensable for the integrity of the Finger domain fold.⁷⁷ The role of the Finger domain is to serve as a scaffold that contacts the globular body of ubiquitin, and the zinc-chelating site does not appear to be involved in catalysis.⁷⁷ Interestingly, in the USP domain of CYLD the Finger domain is significantly smaller due to the shortening of the β -strands.¹⁴ CYLD also differs from the other USP domains by the insertion of a Zn binding domains that closely resembles a B-box. This insertion occurs between β 9 and β 10 in the Palm domain. The B-box is not required for deubiquitinating activity, but instead appears to be important for the cytoplasmic localization of CYLD.¹⁴

Multiple studies have demonstrated that the catalytic activity of USP DUBs is regulated by substrate- or scaffold-induced conformational changes.^{48,53,78} The structures of two USP family DUBs (both in the presence and absence of Ubal) reveal at least two different modes of catalytic activation.^{48,53} The first structure corresponds to USP7 (also known as HAUSP, Herpes associated USP), a DUB that preferentially deubiquitinates MDM2 (murine double minute 2), the ubiquitin ligase for the tumor suppressor p53, as well as p53 itself.⁵³ In the unliganded form of USP7, the catalytic triad is misaligned. The active site cysteine is approximately 10 Å away from the active site histidine, too far for catalysis to occur (Figure 4B). Upon binding to the inhibitor, Ubal, a major conformational change occurs in the catalytic core domain causing the active site cysteine and histidine to be positioned within hydrogen bond distance from one another, rendering the enzyme catalytically competent.

The structure of USP14 in the presence and absence of Ubal reveals a different type of activation mechanism.⁴⁸ USP14 is a proteasome-associated DUB that helps remove polyubiquitin from protein substrates that are being degraded by the proteasome⁴⁸ (see section 4.1). Binding of USP14 or its yeast ortholog, UBP6, to the proteasome activates the catalytic activity of the DUB through an unknown mechanism.⁷⁹ However, comparison of the structures of the free and Ubal-bound USP domain of USP14 suggests a mechanism for the activation.⁴⁸ Unlike USP7, the catalytic triad of the free USP14 is productively aligned, indicating that the active site is catalytically competent. However, the binding groove that accommodates the C-terminal tail of ubiquitin is blocked by two surface loops that undergo significant conformational changes upon ubiquitin binding. It is proposed that the movement of this loop out of the catalytic cleft could also occur upon binding to the proteasome, resulting in the observed activation of the enzyme.⁷⁹

A third type of conformational change is thought to occur in USP8 (also known as UBPY), a DUB that is involved in removing ubiquitin from endocytosed substrates such as the epidermal growth factor⁷⁸ (see section 4.4.1). In the structure of the unliganded USP domain of USP8, the tip of the Finger domain is positioned inward toward the Palm resulting in a closed conformation that leaves insufficient room for an ubiquitin molecule. Unlike the USP7, USP14, or UBP6 USP

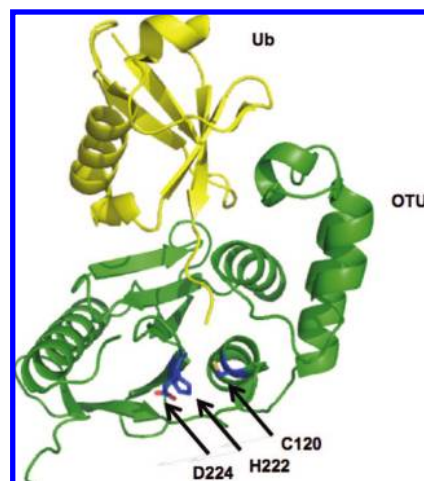


Figure 5. Structure of yeast OTU1 bound to Ub-Br3. OTU1 is shown in green. Ub-Br3 is shown in yellow. The active site residues are shown in blue. The protein data bank identification code is 3BY4.

domains, USP8 and USP2 have a unique α -helix juxtaposed to the fingers and this helix may be involved in stabilizing the closed conformation observed in USP8 in the absence of ubiquitin.^{77,78} Although there is no structure available of a USP8–ubiquitin complex, the closely related USP domain of USP2 has been solved in the presence of ubiquitin.⁷⁷ In the USP2–ubiquitin complex the Finger domain is displaced outward to adopt the conformation observed in the other USP domains. It is possible that upon target protein binding, the Finger domain of USP8 moves to the position observed in the other USPs, allowing the activation of the enzyme and subsequent binding to ubiquitin.

3.3. The OTU Family

The ovarian tumor gene is involved in the development of *Drosophila melanogaster* ovaries.^{80,81} A bioinformatics study using the OTU gene and its homologues found a family of genes encoding viral, eukaryotic, and pathogenic bacterial cysteine proteases.⁸² Recent studies have demonstrated that OTU-related proteases have deubiquitinating activity and the crystal structures of four different DUBs containing OTU domains have been recently described:^{50,52,83–85} otubain-2, a protein of unknown biological⁵² function; otubain-1, a DUB that regulates the E3 ligase GRAIL (Gene Related to Anergy In Lymphocytes) and which functions in the induction of CD4 T cell anergy; A20, a negative regulator of the NF- κ B pathway; and OTU1, a yeast DUB that was shown to interact with the AAA-ATPase (ATPases Associated with diverse cellular Activities) Cdc48.^{50,52,86–88} In all four OTU domain DUBs the core domain is formed by a five-stranded β -sheet sandwiched between two helical domains (Figure 5). The size of the helical domains varies among OTU DUBs.^{15,50,89} Only OTU1 has been crystallized in the presence of a monoubiquitin based active site inhibitor, Ub-Br₃.¹⁵ The active site cysteine and histidine are properly positioned in all four OTU DUBs. However, the identity of the third residue of the catalytic triad is unclear and has been suggested to be Asp224 in OTU1, Asn226 in human Otubain 2,⁵² or D70 in A20.⁵⁰ D70 is located on α -helix 3 and is positioned 4.4 Å away from the putative active site histidine residue, suggesting that if D70 forms part of the catalytic triad, a conformational change must during catalysis occur to position D70 within H-bonding distance of the histidine residue.

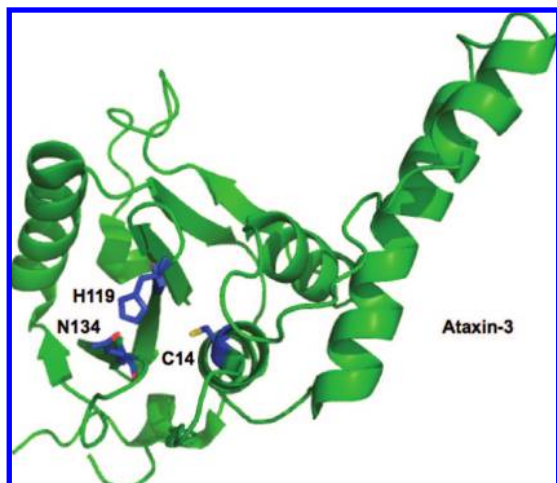


Figure 6. Structure of a Josephin domain family DUB, ataxin-3. Ataxin-3 is shown in green. The active site residues are shown in blue. The protein data bank identification code is 1YZB.

This change in conformation would be reminiscent of the reorganization of the active site residues of USP7 upon binding of Ubal. In the otubain-2 crystal structure, the active site residues appear to be positioned in a catalytically productive conformation; however, a disordered loop, and six residues preceding the disordered loop are oriented in a position that would clash with the bound ubiquitin.⁵² In the structure of OTU1 reacted with the active site probe Ub-Br₃, this loop becomes ordered into a β -strand that contacts the globular body of ubiquitin.¹⁵ This suggests that otubain-2 may exist in a self-inhibited state, and that upon substrate binding the loop may adopt the conformation observed in the OTU1·Ub-Br₃ adduct.

3.4. The Josephin/MJD Family

Like the OTU family, the Josephin domain family of DUBs was discovered through a bioinformatics approach⁹⁰ that identified the Josephin domain in Ataxin-3 and at least 30 other proteins as putative DUBs. Biochemical studies confirmed that Ataxin-3 had DUB activity and mutation of the active site cysteine rendered the protein inactive against a model substrate.⁹¹ Furthermore, DUB activity has been detected for other Josephin domain containing proteins.⁹²

Ataxin-3 is mutated in spinocerebellar ataxia type 3, also known as Machado-Joseph disease.⁹¹ This disease is an autosomal dominant neurodegenerative disorder caused by the expansion of a polyglutamine stretch in the Ataxin-3 gene, leading to protein misfolding, aggregation and cellular toxicity. Ataxin-3 binds both K48- and K63-linked chains; however, it selectively hydrolyzes long K63-linked chains (albeit, extremely slowly).⁹³ The N-terminal UIM domains mediate Ub binding and mutation of these domains abolishes chain specificity. A model is suggested whereby the UIM domains bind to polyubiquitin but only K63 linkages can be positioned for cleavage. However, it must be noted that this cleavage is so slow that it raises the possibility that a cofactor or proper cellular location may enhance the enzymatic activity of Ataxin-3.

The structure of the Josephin domain of Ataxin-3 has been solved by NMR methods in its unliganded form (Figure 6).^{54,58,94} The overall fold of the Ataxin-3 Josephin domain resembles that of the UCH family of DUBs. Based on the structural similarity to the UCH domain, and on NMR

chemical shift mapping, a binding surface for ubiquitin has been proposed.^{54,58} The Josephin domain is predicted to interact with the C terminus of ubiquitin, the first N-terminal loop of ubiquitin, and the C-terminal half of ubiquitin's α -helix. Residues located in α 3, the loop between α 3 and α 4, and the C-terminal portion of α 2 of the Josephin domain are the most perturbed upon ubiquitin binding. This binding mode would predict discrimination against K6 and K29 linked polyubiquitin although this has not been tested. A structure of the Josephin domain with polyubiquitin, ubiquitin, or a ubiquitin-based inhibitor may confirm these interactions.

3.5. The JAMM Family

The JAMM domain is found in both prokaryotes and eukaryotes, although bacteria are assumed to lack ubiquitin and an ubiquitin-like conjugation system. Three eukaryotic JAMM domains have DUB activity,^{49,95–97} suggesting that JAMM domains may have evolved to have deubiquitinating activity only in higher organisms. These three JAMM domain DUBs are Rpn11 in yeast (POH1 in humans),^{95–97} a subunit of the proteasome that cleaves ubiquitin chains from substrate proteins that are being degraded by the proteasome; CSN5, a subunit of the COP9 signalosome⁹⁸ which cleaves Nedd8 (Neural Precursor Cell Expressed, Developmentally Down-regulated 8, a ubiquitin-like protein) conjugates; and AMSH (associated molecule with the SH3 domain of STAM) a deubiquitinating enzyme involved in endocytosis.⁴⁹

The crystal structure of the JAMM domain of a protein from *Archaeoglobobolus fulgidus*, AF2198, was the first structure of a JAMM domain to be determined^{99,100} and the structure of three other JAMM-motif proteins were recently solved: Prp8 (pre-mRNA processing factor 8),^{101,102} Mov34 (a metalloprotein subunit of the proteasome);¹⁰³ and AMSH-LP (AMSH-like protein).⁵⁹ Prp8 and Mov34 proteins do not bind zinc and are not expected to have DUB activity. However, the JAMM motif of Prp8 has ubiquitin binding activity suggesting that a subset of these domains may act as ubiquitin binding domains.^{104,105} AMSH-LP (Associated Molecule with the SH3 of STAM Like Protein) is a DUB involved in endocytosis that binds both ubiquitin subunits of diubiquitin using the JAMM domain and two AMSH-family specific sequences (see below).

The overall structure of the JAMM domain core resembles that of cytidine deaminase.¹⁰⁰ The catalytic zinc residue is chelated by a histidine and an aspartic acid residue located in β 3 and α 2 of the domain, respectively. Because of the structural similarity with cytidine deaminase, the JAMM domain has been proposed to use a similar mechanism in the hydrolysis of the isopeptide bond in ubiquitin or ubiquitin-like protein conjugates. In cytidine deaminase, the zinc ion activates a water molecule to form a hydroxide ion that makes a nucleophilic attack on the C4 carbon in the pyrimidine ring.¹⁰⁶ In the JAMM domain DUBs, the nucleophilic attack would occur in the carbonyl carbon of the isopeptide bond. In both cases the result is the formation of a tetrahedral intermediate that subsequently collapses releasing ammonia in the deamination reaction or the target protein in the isopeptide hydrolysis reaction. Both proteins also contain a glutamic acid residue that can function as a proton donor or acceptor during the deamination or isopeptide hydrolysis reaction. Consistent with this hypothesis, mutation of the homologous glutamic acid residue in CSN5 causes a defect in activity.⁹⁷

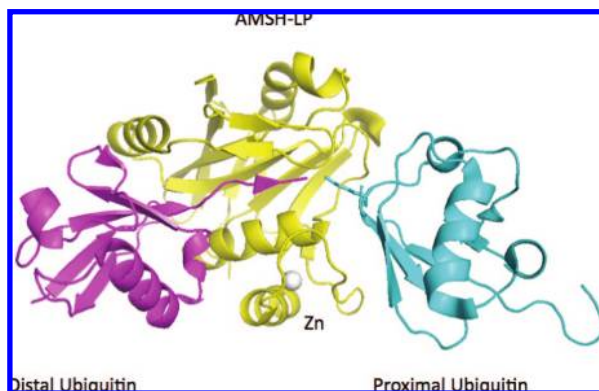


Figure 7. Structure of a JAMM domain (yellow) from the human AMSH-like protein complexed to K63-linked diubiquitin⁵⁹ (cyan and magenta). The structure consists of the JAMM domain core surrounding the zinc atom (gray) and two AMSH-specific inserts that interact with the proximal (cyan) and distal (magenta) domains of diubiquitin. The protein data bank identification code is 2ZNV.

The core JAMM domain of AMSH-LP resembles the structure of the JAMM domain from AF2198, and coordinates an active site zinc ion through two His residues, one Asp, and a water molecule, which is hydrogen bonded to a Glu residue⁵⁹ (Figure 7). However, the JAMM domain of AMSH-LP differs from AF2198 by the presence of two amino acid insertions, Ins-1 and Ins-2, and an additional zinc-binding motif. The second zinc ion is chelated by two His and one Cys residues from Ins-2 and a His residue from the core domain. The structure of the JAMM domain of an AMSH-LP has also been solved in complex with K63-linked polyubiquitin⁵⁹ (see below) providing for the first time a molecular description of the catalytic core domain of a DUB bound to polyubiquitin (Figure 7).

4. Mechanisms of Polyubiquitin Recognition and Disassembly by DUBs

Recent studies aimed at understanding how DUBs cleave and recognize polyubiquitin chains have shown that these enzymes can process polyubiquitin through various mechanisms. DUBs have been shown to cleave polyubiquitin chains from the proximal end, the distal end, and the interior of the polyubiquitin chain through an endocleavage activity. In the following section we will discuss mechanisms utilized by DUBs that disassemble polyubiquitin.

4.1. Disassembly of Polyubiquitin By Proteasome Associated DUBs

The proteasome is a multiprotein complex formed by two subcomplexes: the 20S core particle and the 19S regulatory particle.^{107–109} The 20S core particle contains the proteolytic sites and consists of four stacked rings, each formed by seven subunits.¹¹⁰ The outer rings are composed of seven different α -subunits, which are implicated in binding the 19S regulatory particle. The two inner rings each consist of seven different β -subunits, three of which (from each set) contain the protease active sites.¹¹¹ Deubiquitination of substrates is necessary for optimal rates of protein degradation and is carried out by the 19S regulatory particle.^{79,95–97,112–115} The 19S regulatory particle includes 19 different subunits, 10 of which form a base that binds the α -subunits of the 20S proteasome. Six proteins of the 10-protein-base are AAA-ATPases that are thought to help unfold and translocate the

substrate into the lumen of the 20S protease. The remaining protein subunits of the 19S regulatory particle form the lid. Some of the subunits of the lid and base are implicated in binding to the polyubiquitin signal.^{116–120} The removal of the polyubiquitin signal from target proteins during protein degradation is carried out by DUBs that are a core component of, or associated with, the 19S regulatory particle.^{79,121,122} Failure to remove the polyubiquitin chain from the substrate protein is thought to impede passage of substrates through the entrance pore of the 20S proteasome. Perhaps for this reason deubiquitination has also been shown to be required for efficient proteolysis. Most eukaryotes contain three proteasome associated deubiquitinating enzymes: POH1 (95–97,123), Usp14,^{48,124–126} and Uch37.^{112–115} The budding yeast *S. cerevisiae*, only has two of these DUBs, Rpn11^{95,96} and Ubp6,^{79,127} the homologues of POH1 and Usp14, respectively. POH1/Rpn11 belongs to the JAMM domain family of DUBs, is a core component of lid of the 19S regulatory particle, and is associated with the proteasome in stoichiometric amounts. POH1/Rpn11 is essential for viability, while the other two proteasomal associated DUBs are dispensable. Mutations in the metal chelating residues of the JAMM domain of POH1 in *Drosophila*¹²⁸ or human cells,¹²⁹ and Rpn11 in yeast are lethal.^{95,96} One group has reported that a mutation at the putative active site in yeast was not lethal but led to defects in substrate degradation.¹³⁰ Furthermore, knockdown of POH1 in human cells results in accumulation of polyubiquitin conjugates and defects in protein degradation, in part due to a defect in proteasome assembly.¹²³ The deubiquitinating activity of POH1/Rpn11 in the proteasome is dependent on ATP hydrolysis, suggesting that deubiquitination may be coupled to protein unfolding during degradation.^{95,96} POH1/Rpn11 deubiquitinating activity can only be detected when bound to the 26S proteasome,⁹⁶ the 19S regulatory particle,⁹⁵ or the lid subcomplex.¹³⁰ Together these data suggest that other proteasomal subunits associated with the lid and POH1/Rpn11 may modulate the DUB activity. POH1/Rpn11 appears to remove polyubiquitin from substrate proteins en bloc, cleaving the isopeptide bond linking the polyubiquitin chain to the substrate protein.⁹⁵ These, and other, data are consistent with a model in which POH1/Rpn11 activity is coupled to protein unfolding as the protein is being degraded by the 20S proteasome. A defect in POH1/Rpn11 deubiquitinating activity would impede translocation of the protein due to steric hindrance by the polyubiquitin chain.

Like POH1, UCH37 is a stoichiometric component on the proteasome that associates with the proteasome through the proteasomal subunit Adrm1, the human homologue of Rpn13.^{113–115,123} Adrm1 interacts with the C-terminus of UCH37 via a C-terminal domain, which is absent in *S. cerevisiae* Rpn13. This interaction not only recruits UCH37 to the proteasome, but it also activates its catalytic activity. However, while the interaction of UCH37 with Adrm1 activates the hydrolysis of the in vitro substrate Ub-AMC, it does not activate the hydrolysis of diubiquitin.¹¹³ When bound to the 19S regulatory particle, UCH37 exhibits approximately 100-fold increased activity for a diubiquitin-based substrate, suggesting proper localization is required for activation. UCH37 has been shown to cleave polyubiquitin from the distal end,⁶⁷ and a recent study has provided insights into how this cleavage may occur when bound to Adrm1.¹²⁰ Adrm1 also acts as a polyubiquitin receptor that interacts with polyubiquitin through a novel ubiquitin binding

domain, the Pru domain.^{119,120} The Pru domain is located in the N-terminus of Adrm1 and mediates both the binding to the Rpn2/S1 subunit of the proteasome and to polyubiquitin. Notably, the Pru domain of Adrm1 interacts with the proximal ubiquitin in diubiquitin,¹²⁰ and this interaction may allow the positioning of the distal ubiquitin in close proximity to UCH37 to facilitate polyubiquitin hydrolysis from the distal end of the chain. Since UCH37 cleaves polyubiquitin chains from the distal end, it has been proposed to have an editing function to prevent the degradation of poorly modified or inappropriate substrates.⁶⁷

Unlike POH1/Rpn11 and UCH37, USP14/Ubp6 does not stably associate with the 26S proteasome; instead its association is reversible and salt-sensitive.^{79,123} Usp14/Ubp6 contains an N-terminal ubiquitin like domain (UBL) that mediates its interaction with the Rpn1 and Rpn2 subunits of the base of the proteasome.⁷⁹ Like UCH37, USP14/Ubp6 activity is significantly increased upon incorporation into the proteasome.^{48,79} Ubp6 is not an essential gene in yeast, but its absence causes an ubiquitin-depletion phenotype.^{79,131} Ubp6 appears to delay the breakdown of proteins by the proteasome since deletion of Ubp6 causes an enhancement in the rate of hydrolysis by the proteasome.¹²⁷ Surprisingly, this inhibitory effect does not depend on its catalytic activity, suggesting a noncatalytic function of Ubp6 in the regulation of proteasomal protein degradation.¹²⁷ Ubp6 was also shown to work in cooperation with a proteasome associated E3, Hul5, to modulate the length of the polyubiquitin chains attached to target proteins.¹²¹ Increased Hul5 activity leads to retention of the polyubiquitinated protein, while deubiquitination by Ubp6 has the opposite effect. In agreement with this function, Usp14, like UCH37, appears to cleave monoubiquitin from the substrate or polyubiquitin chains from the distal end of the chain.⁴⁸ The cooperation of these two activities may be regulated upon changes in the cellular environment, such as lack of nutrients, or throughout the cell cycle.

The unifying theme for all three proteasomal associated DUBs is the requirement for an adapter to interact with their polyubiquitinated substrates. All three interact with polyubiquitinated substrates by virtue of their localization to the proteasome, with the polyubiquitin being presented to the DUB by multiple receptors for polyubiquitin.^{116–120} In addition all three are active only when interacting with the proteasome scaffold, thereby restricting their DUB activity to proteasomal protein degradation.

4.2. Isopeptidase T (Usp5) Recognizes Polyubiquitin Through Its Multiple Ubiquitin Binding Domains

Unanchored polyubiquitin can be formed by the release of polyubiquitin from target proteins by DUBs such as POH1/Rpn11 or by de novo synthesis by the conjugation machinery (possibly for subsequent transfer to a target protein).^{132,133} The levels of unanchored polyubiquitin are tightly regulated in the cell by deubiquitinating enzymes, probably to prevent inhibition of the proteasome and receptors that recognize polyubiquitinated proteins.^{132,134,135} Isopeptidase T (IsoT or USP5/UBP14) is a deubiquitinating enzyme that specifically disassembles unanchored polyubiquitin.⁵¹ IsoT orthologs have been shown to be responsible for the majority of unanchored polyubiquitin disassembly in four different organisms.^{132,134–136} Lack of Ubp14, the yeast

homologue of IsoT, results in the marked accumulation of polyubiquitin, the sensitivity to the arginine analog canavanine, and defects in the degradation of model proteasome substrates.¹³² In *Dictyostelium* deletion causes developmental defects,¹³⁵ and in *Arabidopsis*, deletion is lethal.¹³⁴ Transient knockdown of IsoT in humans cells causes an increase in unanchored chains and defects in the proteolysis of p53. Furthermore, human IsoT can complement the Δ Ubp14 phenotype demonstrating that yeast Ubp14 is the functional homologue of human IsoT.¹³²

IsoT is one of the best biochemically characterized USP DUBs.^{16,51,57,137–141} Recombinant IsoT disassembles unanchored polyubiquitin from the proximal end to the distal end of the chain in a nonprocessive manner.⁵¹ Modification of the C-terminus of the proximal ubiquitin in the chain leads to a defect in the rate of disassembly of the polyubiquitin chain indicating the presence of a pocket that recognizes the intact C-terminus of the proximal ubiquitin. Kinetic and thermodynamic studies indicate that IsoT has at least three more ubiquitin binding sites for ubiquitin subunits in linear and K48-linked polyubiquitin.^{16,51} Correspondingly, IsoT has four putative ubiquitin binding domains, a ZnF UBP domain, a USP/UBP domain, and two UBA domains.^{16,57} It was recently demonstrated that all four ubiquitin binding domains of IsoT mediate polyubiquitin binding and that each forms a unique site that interacts with one ubiquitin subunit at a time.¹⁶ The ZnF UBP domain is responsible for the recognition of the proximal, or first ubiquitin in the chain and structural studies indicate that it possesses a pocket that specifically recognizes the intact C-terminus of the proximal ubiquitin.⁵⁷ This recognition drives a conformational change that activates the enzymatic cleavage of the (iso)peptide bond to the proximal subunit. The USP/UBP, UBA1, and UBA2 domains recognize the second, third, and fourth ubiquitin subunits, respectively, in both linear and K48-linked polyubiquitin.¹⁶ Linear polyubiquitin is predicted to adopt a conformation analogous to K63-linked polyubiquitin and that differs from K48-linked polyubiquitin.¹⁰ Interestingly, the ZnF UBP and the UBP domain are implicated in the discrimination between linear and K48-linked polyubiquitin based upon the difference in binding affinities for linear and K48-linked diubiquitin.¹⁶ However the UBA domains do not significantly discriminate between K48 and linear linkages.

This is an example of polyubiquitin recognition by the mechanism shown in Figure 2D. All four ubiquitin binding domains participate similarly in the interaction between IsoT and two different polyubiquitin isoforms. Each forms analogous sites for binding the ubiquitin subunits of both linear and K48-linked polyubiquitin. Thus, the domains of IsoT and/or polyubiquitin must be flexible enough for similar interactions to take place with the ubiquitin subunits of chains exhibiting very different solution structures. Regions of about 25 amino acids link the different ubiquitin binding domains to each other, raising the possibility that these regions are flexible enough to allow the recognition of multiple polyubiquitin isoforms by the four ubiquitin binding domains of IsoT.

4.3. Processing of Polyubiquitinated Substrates By DUBs That Modulate the NF- κ B Pathway

NF- κ B transcription factors are known to control diverse cellular processes such as inflammation, immunity, and cell survival.^{142,143} In NF- κ B signaling, K63- or K48-linked polyubiquitin can be attached to effectors of the pathway

leading to different outcomes. K63-linked polyubiquitination mediates protein–protein interactions that lead to activation of the signaling pathway. In contrast, K48-linked polyubiquitination leads to the degradation of effectors of the pathway. Normally the NF- κ B transcription factor is held in the cytoplasm by the inhibitor of NF- κ B (I κ B). Signaling through the tumor necrosis factor α (TNF α), interleukin 1- β (IL-1 β) or Toll-like receptor (TLR) pathways induces the degradation of I κ B allowing the NF- κ B transcription factor to be translocated to the nucleus and activate the transcription of specific genes.^{142,144}

Upon receptor binding, several proteins in the pathway are modified with K63-linked polyubiquitin, and this modification is a prerequisite for the activation of the subsequent kinase cascade.¹⁴² Ligand binding causes the K63-linked polyubiquitination of TRAFs and adapters such as RIP1 (receptor interacting protein 1) leading to the recruitment of a cascade of kinases that ultimately phosphorylate I κ B. Phosphorylated I κ B is recognized by an ubiquitin ligase complex that conjugates K48-linked polyubiquitin to I κ B, resulting in its proteasome-dependent degradation. The degradation of I κ B exposes the nuclear localization signal of NF- κ B, allowing for its nuclear translocation and the subsequent expression of specific genes. Two different DUBs, CYLD and A20, act as negative regulators of the NF- κ B pathway by removing K63-linked polyubiquitin from modified proteins involved in the activation of the pathway.^{86,142,143,145–148}

4.3.1. The USP Domain of CYLD Has Intrinsic Selectivity for K63-linked Polyubiquitin

In humans, loss of the deubiquitinating enzyme CYLD leads to a disfiguring benign cancer called cylindromatosis.¹⁴⁹ CYLD has been shown to deubiquitinate TRAFs (TNF receptor-associated factor) 2 and 6, and NEMO (NF- κ B essential modulator) thereby suppressing NF- κ B signaling. Decreased CYLD expression, or mutations that affect its catalytic activity, lead to sustained NF- κ B signaling due to failure in the deubiquitination of TRAF2, TRAF6, and NEMO.^{146–148} Failure to terminate NF- κ B signaling results in prolonged inflammation and immune system dysfunction. In addition to the modulation of the NF- κ B signaling, CYLD has also been shown to regulate apoptosis in *Drosophila* by positively regulating the c-Jun N-terminal kinase pathway.¹⁵⁰

CYLD is a deubiquitinating enzyme belonging to the USP family that preferentially disassembles K63-linked polyubiquitin, and this chain specificity is inherent in its catalytic core domain.¹⁴ The USP domain of CYLD adopts a fold that contains the Palm and Finger domains observed in other USPs.¹⁴ However, the Finger domain is significantly reduced in size due to shortening of the β -sheet that is the central component of the domain. The USP domain of CYLD contains an insertion of a zinc-binding module that resembles a B-box, but that does not appear to influence catalytic activity. Other remarkable differences correspond to the sizes of loops connecting secondary structure elements. One such loop, connecting β 12 and β 13, is significantly longer in comparison to the equivalent loop in USP7. Truncation of this loop to a size equivalent to that of USP7 leads to reduced activity on K63-linked polyubiquitin without significantly altering the hydrolysis of K48-linked polyubiquitin.¹⁴ This experiment suggests that this loop, which is conserved in all CYLD orthologs, is a determinant of CYLD specificity for K63-linked polyubiquitin. Additional biochemical studies

by Komander et al. show that CYLD acts as an endodeubiquitinase, thereby hydrolyzing internal isopeptide linkages in K63-linked polyubiquitin chains. In contrast, other USP domain DUBs (such as USP14 and UCH37) appear to cleave polyubiquitin processively from the distal end of the chain.⁴⁸ This difference in the mechanism of hydrolysis of the chain may be linked to the Finger domain of CYLD. All USP structures contact the side chains of K48 through the Thumb and K63 through the Finger domains. Given that the USP domain of CYLD has a shorter Finger domain with less obstruction of K63 it may be able to utilize a second binding site to interact with a more distal ubiquitin linked through K63, thereby accounting for the endodeubiquitinase activity of CYLD.¹⁴ Thus, the basis for the specificity of CYLD most closely resembles that of Figure 2C.

4.3.2. Recognition of the Target Protein Determines the Chain Selectivity of A20

Like CYLD, A20 negatively regulates NF- κ B signaling.^{86,145} Mice lacking A20 exhibit severe inflammation, cachexia, and premature death.¹⁵¹ Fibroblasts from these mice are hypersensitive to TNF α and are defective in the downregulation of NF- κ B signaling.¹⁵¹ A20 is a remarkable protein that has two enzymatic activities: ubiquitin ligase and deubiquitinating activities.⁸⁶ A20 can downregulate NF- κ B signaling by removing K63-linked polyubiquitin from TRAF6 and RIP1. Through its E3 ligase activity, A20 also mediates K48-linked polyubiquitination of RIP1, causing its degradation and attenuation of NF- κ B signaling.⁸⁶ The E3 ligase activity of A20 resides in its C-terminal domain, which is composed of seven zinc fingers, while its DUB activity resides in its N-terminal OTU domain. Recently, structural and biochemical studies have revealed new insights into the disassembly of polyubiquitin and polyubiquitinated TRAF6 by the OTU domain of A20.^{50,89} In vitro, A20 has very low activity toward monoubiquitin based substrates and inhibitors, but is more efficient in the disassembly of polyubiquitin. It prefers K48-linked over K63-linked polyubiquitin in vitro, although the opposite was reported in vivo.^{50,89} Li et al. showed that this contradiction may be explained by the manner in which A20 cleaves polyubiquitinated substrates.⁸⁹ Li et al. demonstrated that A20 can efficiently deubiquitinate K63-linked polyubiquitinated TRAF6 in vitro by releasing free chains via cleavage of the isopeptide bond to TRAF6 in a manner similar as POH1. These data suggest that A20 may recognize specific polyubiquitinated substrates, perhaps through its recruitment to the NF- κ B activation complex. This would be an example of polyubiquitin recognition by the model in Figure 2A. Furthermore, Li et al. suggest that A20 deubiquitination of RIP1 may occur prior to A20-dependent K48-linked polyubiquitination. However, it remains to be determined whether A20 also cleaves K63-linked polyubiquitin from RIP1 and what prevents it from disassembling K48-linked polyubiquitinated RIP1.

4.4. Polyubiquitin Processing By DUBs Involved In Endocytosis

Ubiquitin also acts as a signal that targets membrane proteins to the lysosome for degradation.^{18,21} Monoubiquitination and K63-linked polyubiquitination have both been implicated in this process.^{18,152} Fusion of a single ubiquitin in frame with a cargo protein is sufficient to mediate targeting to the lysosome.¹⁵³ K63-linked polyubiquitin can enhance

the efficiency of this process,¹⁵² perhaps by increasing the affinity for ubiquitin binding domains through avidity. More than 50% of the epidermal growth factor receptor (EGFR),¹⁵⁴ a well-studied endocytic cargo, was shown to be modified with K63-linked polyubiquitin, underscoring the role of this modification in endocytic sorting. When a membrane-associated receptor is ubiquitinated, adaptors that recognize the ubiquitinated cargo are recruited. This in turn results in the recruitment of clathrin and epsin proteins, causing internalization of the receptor through plasma membrane involution.^{155,156} The cargo is released into early endosomes from where it can be either be recycled back to the plasma membrane or enter into the lumen of multivesicular bodies (MVB) that form through involution of the endosomal membrane. These MVBs then fuse with the lysosome (vacuole in yeast) thereby targeting proteins for degradation in the lysosome. Deubiquitinating enzymes have been shown to regulate this process by deubiquitinating the internalized receptor prior to delivery to the lysosome.^{47,49,157–167} In addition, deubiquitination is also required for recycling of ubiquitin prior to lysosomal degradation.¹⁵⁸

4.4.1. USP8/DOA4 Deubiquitinates Endocytic Intermediates

In yeast, deletion of the deubiquitinating enzyme Doa4 leads to a ubiquitin depletion phenotype that can be partially rescued by blocking MVB maturation, suggesting that Doa4 recycles ubiquitin prior to lysosomal degradation.¹⁵⁸ Structurally and functionally, the DUB that most closely resembles Doa4 is USP8 (also called UBPY).¹⁶⁸ Knockdown of USP8 results in the accumulation of ubiquitin at endosomes and decreases the rate of degradation of receptor tyrosine kinases (RTKs) such as the EGFR and the Met receptor, suggesting that it acts primarily in the downregulation of cargo proteins.⁴⁷ However, another group found that knockdown of USP8 results in enhanced downregulation of EGFR.¹⁶⁵ In vitro, USP8 can disassemble K48- and K63-linked polyubiquitin suggesting that it could act in both proteasomal and lysosomal dependent protein degradation. Consistent with a role in proteasomal degradation, knockdown of USP8 leads to a depletion of a member of the MVB sorting machinery, STAM (signal transduction adapter protein). Proteasomal inhibition in USP8 knockdown cells results in the accumulation of ubiquitinated STAM, suggesting that USP8 also regulates the protein level of at least one member of the MVB sorting machinery.

The structure of USP8 reveals the presence of a zinc ribbon at the tip of the Finger domain of the USP active site.⁷⁸ As noted above, this zinc ribbon is conserved in USP2 and some other USP domain DUBs.⁷⁷ In light of the fact that the Finger domain is involved in interactions with K63 of ubiquitin, the zinc-binding module could serve one of two roles. It could act as an additional ubiquitin binding domain that interacts with a second ubiquitin subunit in K63-linked polyubiquitin chains, thereby increasing the affinity for K63-polyubiquitinated cargo proteins.⁷⁸ Alternatively, protein–protein interactions could position the finger domain in such a way as to occlude access to K63 and lead to a shift in specificity toward K48-linked chains found on STAM. Thus, the binding partners of USP8 could toggle the chain specificity depending on the precise substrate and binding partners involved.

4.4.2. The JAMM Domain of AMSH Has Intrinsic Specificity For K63-linked Polyubiquitin

In humans, the membrane bound DUB AMSH has also been shown to regulate endocytosis of receptors.^{49,166,167,169} Knockdown of AMSH results in an increase in the rate of downregulation of EGFR, suggesting that it may act early in the endocytic sorting pathway by recycling receptors before they are committed to degradation by the lysosome.⁴⁹ Like CYLD, AMSH and a paralog, AMSH-LP, have been shown to specifically disassemble K63 linked polyubiquitin.^{49,169} The crystal structure of the catalytic core domain of AMSH-LP bound to K63-linked diubiquitin has provided a molecular description of this specificity.⁵⁹

The structure of the JAMM domain of AMSH-LP has been solved, both bound (Figure 7) and unbound to K63-linked polyubiquitin.⁵⁹ AMSH-LP is 55% identical to AMSH and both cleave K63-linked polyubiquitin specifically.¹⁷⁰ The structure of AMSH-LP bound to K63-linked diubiquitin demonstrates that the recognition of the proximal ubiquitin in diubiquitin determines AMSH-LP linkage selectivity.⁵⁹ The proximal ubiquitin is contacted by residues in Ins-2 and the JAMM core domain. Together these two elements form a surface that interacts with the tripeptide sequence Gln62-Lys63-Glu64 of the proximal ubiquitin, a surface that is unique to K63-linked diubiquitin. Both Ins-1 and the JAMM core domain contact the distal ubiquitin, with hydrophobic and aromatic residue in Ins-1 contacting the hydrophobic patch of the distal ubiquitin. These hydrophobic residues are strictly conserved in POH1/Rnp11, suggesting that this DUB may also recognize ubiquitin through its hydrophobic patch. In addition to interactions with the body of ubiquitin, the core JAMM domain and Ins-1 interact with the C-terminal tail of the distal ubiquitin, and the residues that mediate this interaction are also conserved in POH1/Rnp11. In contrast, the residues implicated in the recognition of the proximal ubiquitin and the zinc coordinating motif in Ins-2 are not conserved in POH1/Rnp11.⁵⁹ This zinc binding motif was shown to be required for hydrolysis of K63-linked diubiquitin by stabilizing the recognition of the proximal ubiquitin.⁵⁹

Thus, the structure of the AMSH-LP domain bound to K63-linked polyubiquitin reveals one mode by which DUBs discriminate between polyubiquitin isoforms. This involves recognition of sequences at the linkage site specific for a particular polyubiquitin isoform and represents an example of the model in Figure 2C.

5. Conclusions

Most studies of DUB specificity have focused on the processing of K48-linked, K63-linked, and linear polyubiquitin. Very little is known regarding the recognition and processing of other types of polyubiquitin. Specificity must be achieved by taking advantage of the structural differences between different polyubiquitin chains, and our understanding will remain incomplete until we learn more about the conformations of free and bound polyubiquitin chains. To date only the structure of a single polyubiquitin specific DUB, AMSH-LP, has been solved bound to polyubiquitin. It remains to be seen how other DUBs, in particular those belonging to the cysteine protease families, can recognize polyubiquitin at the molecular level.

Although new insights into the processing of polyubiquitin or polyubiquitinated substrates by DUBs have emerged in the past decade, studies regarding polyubiquitin recognition

by DUBs or other polyubiquitin receptors are still in the early stages. We have discussed several examples and they suggest at least four modes of recognition that can determine cleavage specificity. These include recognition of the target protein and the proximal ubiquitin leading to chain amputation, recognition of the distal ubiquitin resulting in chain trimming, recognition of specific surfaces on both ubiquitins flanking the isopeptide bond as a means to distinguish different linkages, and the use of multiple ubiquitin binding domains to recognize longer polyubiquitin chains. Each will lend specificity to the process, and a given DUB may employ more than one of these recognition modes to achieve unique specificity. Further, we may anticipate a large number of individual mechanisms may be required to distinguish between the very large number of possible polyubiquitin structures.

Many questions still remain regarding how DUBs recognize and discriminate between different types of polyubiquitin at the molecular level. How specific are DUBs? Can all polyubiquitin signals be disassembled? How do localization and protein partners of DUBs regulate specificity? How does transcriptional and proteolytic control of DUBs contribute to specificity? It is likely that a combination of structural, genetic, and physiological studies will be needed to elucidate these questions. Given that the cellular functions of most DUBs still remain unknown, determining which type of ubiquitin modification they process, and how this recognition is achieved may provide new understanding regarding their physiological functions.

6. References

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