Leading Edge
Review

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A Genomic and Functional Inventory of Deubiquitinating Enzymes

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Posttranslational modification of proteins by the small molecule ubiquitin is a key regulatory event, and the enzymes catalyzing these modifications have been the focus of many studies. Deubiquitinating enzymes, which mediate the removal and processing of ubiquitin, may be functionally as important but are less well understood. Here, we present an inventory of the deubiquitinating enzymes encoded in the human genome. In addition, we review the literature concerning these enzymes, with particular emphasis on their function, specificity, and the regulation of their activity.

Introduction

Over the last few decades, protein modification by ubiquitin (Ub) and ubiquitin-like (Ubl) molecules has emerged as a critical regulatory process in virtually all aspects of cell biology. Indeed, the 2004 Nobel Prize in Physiology or Medicine was awarded for the discovery of Ub-mediated proteolysis.

More than a dozen different Ub and Ubl modifications have been described, and up to 20% of yeast proteins are conjugated to Ub under standard culture conditions (Peng et al., 2003; Welchman et al., 2005). In yeast, potentially all seven conserved lysines of Ub itself (K6, 11, 27, 29, 33, 48, and 63) are used as branching sites for the generation of Ub polymers.

The topic of ubiquitination, the proteins involved, and their functions in various pathways and signaling networks has been well reviewed (see Hershko and Ciechanover [1998]; Pickart and Eddins [2004]). Here, we discuss the enzymes that remove Ub from polypeptides. These deubiquitinating enzymes (DUBs) play key regulatory roles in a multitude of processes from hereditary cancer to neurodegeneration. Despite the importance of DUBs, our knowledge of their mode of regulation and substrate specificity is surprisingly scant. A detailed annotation of individual family members of this enzyme group is an important step toward elucidating the molecular functions of DUBs in health and disease. To this end, we provide a comprehensive overview of putative DUBs encoded in the human genome. In addition, we discuss the lacunae in our understanding of these enzymes by drawing on examples from yeast and higher eukaryotes.

In our attempt to classify these enzymes, we have made some arbitrary decisions as to which genes to include or exclude as potential DUBs. Therefore, we present three caveats to this list. First, we cannot exclude the fact that proteins or protein families not included in this overview can remove Ub from polypeptides. For instance, a recent in silico effort to predict new Ub signaling components suggested a previously undetected family of Ub peptidases (Iyer et al., 2004). Second, protein domain prediction based on gene transcripts depends on consensus sequences. Thus, divergent but true family members can be missed due to low homology scores. Finally, we wish to emphasize that it is unlikely that all predicted DUBs are truly specific for Ub: some will display additional activity or exclusive activity toward Ubl molecules.

DUBs Are Proteases

DUBs belong to the superfamily of proteases, of which an estimated 561 members are present in the human genome (Puente and Lopez-Otin, 2004). Based on the mechanism of catalysis, proteases are divided into five classes—aspartic, metallo, serine, threonine, and cysteine proteases—and further subdivided based on phylogeny.

Two classes of proteases (cysteine and metallo) contain DUBs, although most DUBs are cysteine proteases. By definition, the enzymatic activity of cysteine proteases relies on the thiol group of a cysteine in the active site. Deprotonation of this cysteine is assisted by an adjacent histidine, which is polarized by an aspartate residue. These three residues make up the catalytic triad. During catalysis, the cysteine performs a nucleophilic attack on the carbonyl of the scissile peptide bond, which, in the case of DUBs, is between the target and Ub. The intermediate, which contains an oxyanion, is stabilized in the so-called oxyanion hole. This oxyanion hole is generally provided by a glutamine, glutamate, or asparagine residue and the main chain of the catalytic cysteine. The result of the reaction is release of the target protein and formation of a covalent intermediate with the Ub moiety. Reaction of this intermedi-

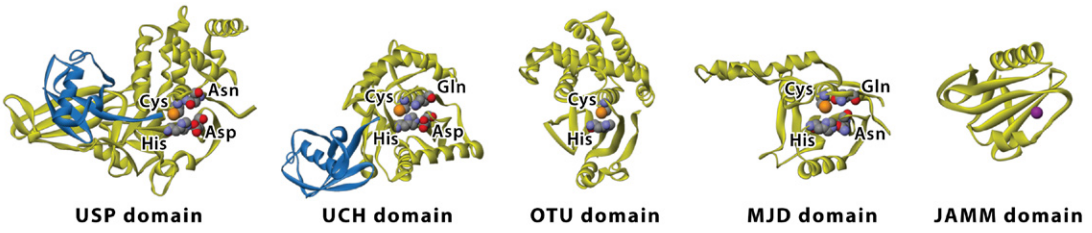

Figure 1. Structures of the Catalytic Domains of the Five Subclasses of Ub-Specific Proteases (Yellow) with Ub (Blue)
Structures show the remarkable variability in secondary structure between the DUB classes. Catalytic centers are shown as Van der Waals spheres (carbon, gray; nitrogen, blue; oxygen, red; sulfur, orange; zinc, purple) and have been aligned for easy comparison. The OTU domain of OTU2 lacks the conserved Asp in the catalytic center and the Asn/Glu/Gln that is normally used to stabilize the oxyanion hole in these proteases. For detailed structural information see Amerik and Hochstrasser (2004). Protein Databank (PDB) codes: USP7, 1nbf; UCH-L3, 1xd3; OTU2,1tff; Ataxin-3, 1yzb; JAMM, 1r5x.

ate with a water molecule results in the release of the free enzyme and Ub.

In contrast to cysteine proteases, metalloproteases generally use a Zn^{2+} bound polarized water molecule to generate a noncovalent intermediate with the substrate. The metal atom is primarily stabilized by an aspartate and two histidine residues (Ambroggio et al., 2004). The intermediate is further broken down by proton transfer from a water molecule causing the release of the DUB.

The Human DUB Genes

The cysteine protease DUBs can be further organized into four subclasses based on their Ub-protease domains: ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase (UCH), Otubain protease (OTU), and Machado-Joseph disease protease (MJD). All DUBs that are metalloproteases have a Ub protease domain called JAMM (JAB1/MPN/Mov34 metalloenzyme). The structures of the catalytic domains of the different subclasses of DUBs reveal an impressive diversity in secondary structure (Figure 1).

We used the ENSEMBL human genome database (v32, July 2005) to retrieve all putative DUBs from the human genome by selecting genes whose transcripts encode one of the five Ub protease domains. Our search identified all known DUBs except two DUBs with OTU domains (*Otubain-1* and *Otubain-2*). This analysis indicated that the human genome encodes approximately 95 putative DUBs, including many that have not been previously reported. These can be broken down into 58 USP, 4 UCH, 5 MJD, 14 OTU, and 14 JAMM domain-containing genes, many of which are associated with multiple transcripts (see Table S1 in the Supplemental Data available with this article online). For six unnamed genes, we have submitted gene names to the HUGO gene nomenclature committee (HGNC) (Table S1). To determine whether the putative DUB genes are expressed, we searched NCBI human-expressed sequence tag (EST) databases for transcripts corresponding to the predicted protein sequence. We obtained further evidence for expression of a number of genes with relatively low numbers of ESTs from additional sources (such as SAGE and UniGene). For five predicted DUBs, we could not find any convincing data supporting transcription.

Next, we generated sequence alignments to ensure conservation of the catalytic residues and made an inventory of DUBs reported to display Ub protease activity. This indicated that of the 90 putative DUBs that are expressed, 11 are unlikely to display Ub-protease activity. Together, these data indicate that humans express approximately 79 putative DUBs that are functional (Table S1).

To investigate sequence homology between the various putative DUBs, we used two strategies. We used CLANS (Cluster analysis of sequences) software to visualize pairwise all-against-all sequence BLAST matches (Frickey and Lupas, 2004). As expected, very few positive BLAST results were found between the five subclasses, whereas the members within the subclasses clustered together (Figure 2A). This analysis revealed that within the subclasses some relatively divergent members are present (for example, Otubain-1 and Otubain-2, USP55, and CYLD). Similar results were obtained using the CLUSTAL alignment algorithm (Figure 2B).

The Five DUB Subclasses

Three-dimensional structures of DUB catalytic domains from all subclasses, some of them in complex with Ub derivatives, have been solved (Figure 1). These studies reveal intriguing similarities and differences between the four cysteine protease subclasses. This topic, including the structure of a JAMM domain, has recently been extensively reviewed by Amerik and Hochstrasser (2004). Here, we comment on the new structural features of the DUB subclasses.

Ubiquitin C-Terminal Hydrolases (UCHs)

The human UCH subclass of DUBs consists of four proteins that share close homology in their catalytic domains. Structural and biochemical studies have indicated that the UCH subclass of DUBs prefers to cleave relatively small protein substrates (up to 20–30 amino acids) from Ub (Amerik and Hochstrasser, 2004). This size limit is thought to be imposed by a loop that partially occludes the active site of these enzymes. However, recent biochemical and structural studies show that certain large substrates can nevertheless be accommodated (Misaghi et al., 2005).

Although UCHs were the first described DUBs, their specific functions remain poorly understood. UCHs are thought to mainly act in the recycling of Ub when Ub is

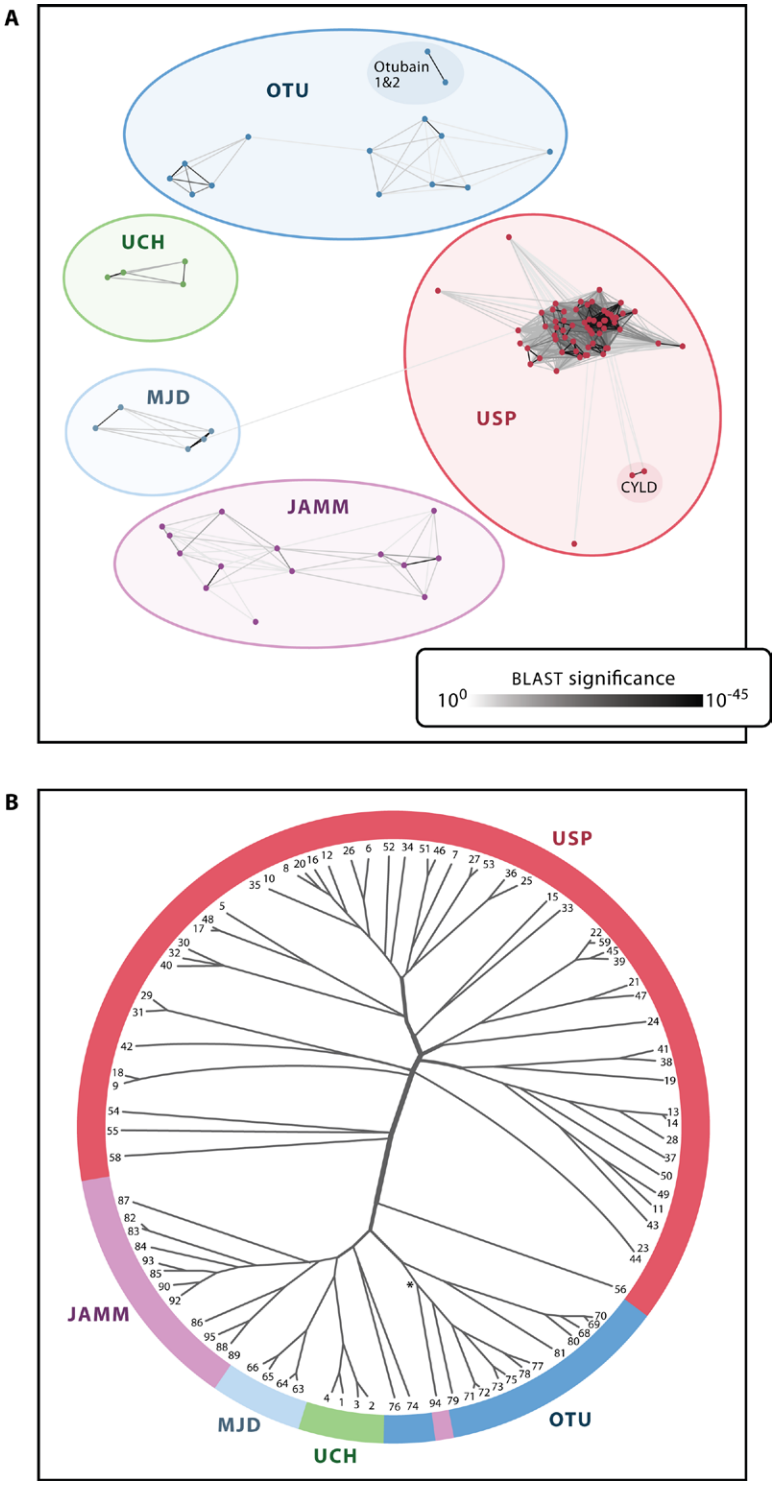

Figure 2. Phylogenetic Map of Human DUBs

(A) Graphic two-dimensional representation of sequence similarities between all Ub protease domains of DUBs using CLANS software. CLANS performs all-against-all BLAST searches and uses the significant high-scoring segment pairs (HSPs) to draw a three-dimensional graph represented here in two dimensions. Each node represents a Ub protease domain and each edge (line) represents a significant HSP (edges are shaded according to p value). DUB subclasses are highlighted in the graph. The start and end positions of the DUB Ub-protease domains, as defined by Interpro, were used to generate the protein sequences. Proteins with a partial or short and misaligning DUB domain were excluded from the analysis.

(B) Unrooted dendrogram of the DUBs using Clustal software. Clustal generates a multiple sequence alignment file based on pairwise alignments. From this information a phylogenetic tree can be constructed. The robustness of the phylogenetic relations can be assessed by "bootstrapping," a mathematical technique that introduces noise in the alignment and measures how often the phylogenetic relationships reproduce. An asterisk indicates a bootstrapping percentage <10% (lowest branch only). The numbers correspond to the genes in Table S1.

inappropriately conjugated to intracellular nucleophiles (for example, glutathione, polyamines). They also may be involved in the processing of newly synthesized Ub, which is translated either as a polyubiquitin precursor or fused to ribosomal protein precursors. However, other DUBs also display in vitro activity toward linear Ub fusions, suggesting that processing of newly synthesized Ub is performed by multiple DUBs.

Some studies suggest a role for UCHs in specific Ub-regulated processes. Mutations in UCH-L1 (a UCH specifically expressed in neurons) that reduce its DUB activity have been described in two siblings with Parkinson's disease (PD), and a polymorphism in this gene has been linked to reduced PD risk (Leroy et al., 1998; Liu et al., 2002). However, not all studies have found a strict relationship between UCH-L1 activity and PD. Furthermore, although mice that have a mutation in *Uch-L1* exhibit neurodegeneration, they do not display PD-like symptoms (Saigoh et al., 1999).

Ubiquitin-Specific Proteases (USPs)

The USP subclass represents the bulk of the DUBs encoded by the human genome. As the number of Ub E3 ligases (the third factor in the ubiquitination cascade that determines target specificity) increased during evolution, so did the number of USPs, suggesting an intimate relationship between the two resulting in their coevolution (Semple, 2003).

The catalytic domain of USPs contains two short and well-conserved motifs, called Cys and His boxes, which include the residues critical for catalysis. However, the size of the complete domain varies from approximately 300 to 800 amino acids due to the large unrelated sequences that

Table 1. Mammalian DUBs and Their Reported Functions

Name	Substrate(s)	Process	Remarks	References
UCHs				
UCH-L1	Unknown	Parkinson's disease	Homodimer has E3 activity; mutant mice display ataxia	Leroy et al. (1998); Liu et al. (2002); Saigoh et al. (1999)
BAP1	Unknown	Unknown	Binds to BRCA1	Jensen et al. (1998)
UCH-L5	Unknown	Ubiquitin editing, TGF-β signaling?	Binds to proteasome	Lam et al. (1997); Wicks et al. (2005)
USPs				
CYLD	TRAF2/6, NEMO	NF-κB and JNK signaling	Familial tumor suppressor (cylindromatosis)	Brummelkamp et al. (2003); Kovalenko et al. (2003); Trompouki et al. (2003); Reiley et al. (2004)
USP1	FANCD2	DNA repair		Nijman et al. (2005)
USP2	Fatty acid synthase	Androgen signaling	Circadian-regulated	Graner et al. (2004); Oishi et al. (2003)
USP4	Unknown	Unknown	Transforming activity	Gupta et al. (1994)
USP5	Unknown	Unknown	Binds K29 chains, binds ISG15 and Ub	Hemelaar et al. (2004); Russell and Wilkinson (2004)
USP6	Unknown	Putative oncogene, actin remodeling	Transforming activity; rearrangements and fusions found in cancer	Masuda-Robens et al. (2003); Oliveira et al. (2005); Paulding et al. (2003)
USP7	HDM2, p53, H2B	p53 signaling, Polycomb silencing	Binds herpes virus protein Vmw110	Cummins et al. (2004); Everett et al. (1997); Li et al. (2004); van der Knaap et al. (2005)
USP8	NRDP1	Endocytosis	Oncogenic fusion with p85-PI3K	Janssen et al. (1998); Kato et al. (2000); Wu et al. (2004)
USP9X	β-catenin, epsins, AF-6	Wnt-, Notch signaling, endocytosis		Murray et al. (2004); Overstreet et al. (2004)
USP9Y	Unknown	Spermatogenesis	Mutants associated with azoospermia	Sun et al. (1999)
USP11	BRCA2	DNA repair?	Interacts with RanBPM	Ideguchi et al. (2002); Schoenfeld et al. (2004)
USP14	Unknown	Synapse function	Mutant mice develop ataxia; binds to proteasome	Borodovsky et al. (2001); Wilson et al. (2002)
USP15	RBX1	COP9 signalosome		Hetfeld et al. (2005)
USP16	H2A?	Chromosome condensation?		Mimnaugh et al. (2001)
USP18	Unknown	JAK-STAT signaling, immunity, brain function	ISG15-specific; mRNA is induced by IFN-α and IFN-β	Malakhova et al. (2003); Ritchie et al. (2004); Ritchie et al. (2002)
USP20	DIO2?	Thyroid hormone metabolism, hypoxia signaling	Interacts with pVHL	Curcio-Morelli et al. (2003); Li et al. (2002b)
USP21	Unknown	Unknown	Cleaves Ub and NEDD8 but not SUMO	Gong et al. (2000)
USP26	Unknown	Spermatogenesis	mUsp26 is testis specific	Paduch et al. (2005); Stouffs et al. (2005); Wang et al. (2001)
USP33	HIF1-α DIO2?	Hypoxia signaling	Interacts with pVHL	Curcio-Morelli et al. (2003); Li et al. (2002b); Li et al. (2005)

Table 1. Mammalian DUBs and Their Reported Functions (continued)

MJDs				
Ataxin-3	Unknown	MJD disease	Sequence has CAG repeats	Burnett et al. (2003); Scheel et al. (2003)
OTUs				
A20	RIP	NF-κB signaling	Also E3 ligase	Wertz et al. (2004)
VCIP135	Unknown	Golgi disassembly		Wang et al. (2004)
JAMMs				
POH1	Unknown	Proteasome		Verma et al. (2002); Yao and Cohen (2002)
AMSH	EGFR?	Endocytosis		McCullough et al. (2004)
CSN5	Cullins	CSN function	Mainly NEDD8 as substrate	Cope et al. (2002); Groisman et al. (2003)
BRCC36	Unknown	G2/M checkpoint signaling	Enhances BRCA1/BARD1 E3 ligase activity	Dong et al. (2003)

Listed are DUBs that have been linked to specific pathways, processes, and substrates (based on published studies).

are interspersed between the two motifs, which may serve a regulatory function (Figure 3).

Upon closer examination of the catalytic domains of USPs, we noted that a subset (USP16, USP30, USP39, USP45, and USP52) lack catalytic residues previously thought to be critical for protease activity (Figure S1). USP30 and USP16 lack only the aspartate in the catalytic triad but retain enzymatic activity against a model substrate (Table S1 and M.P.A.L.-V. and T.K.S., unpublished data). This indicates that, as is the case for the Otubain-2 protein, USP30 and USP16 may use a different residue to stabilize the active site histidine. Additional structural information about USPs may shed light on this issue.

USP39 (also known as SAD1) does not contain the conserved catalytic cysteine or histidine and does not cleave a model substrate in vitro, indicating that USP39 is not a bona fide DUB (M.P.A.L.-V. and T.K.S., unpublished data). However, USP39 plays a critical role in spliceosome maturation in both yeast and human cells, and many of the other residues within the catalytic domain are conserved (Lygerou et al., 1999; Makarova et al., 2001). Therefore, it is tempting to speculate that USP39 can still interact with Ub. An analogous situation exists in Ub conjugation. Here, a Ub interaction motif known as UEV (ubiquitin-conjugating enzyme variant) strongly resembles the catalytic domain of E2s (the second enzyme in the ubiquitination cascade) but lacks activity (Hicke et al., 2005). In keeping with this nomenclature, these catalytically inactive USP domains are hereby referred to as USPV (ubiquitin-specific protease variant). The functions of these variants with respect to Ub await further investigation.

Machado-Joseph Disease Protein Domain Proteases (MJDs)

A bioinformatics search for other classes of Ub proteases identified Ataxin-3 and a number of Ataxin-3-like proteins (Scheel et al., 2003). Experiments in vitro confirmed that wild-type Ataxin-3, but not a mutant with the active site cysteine mutated, could deubiquitinate a model substrate (Burnett et al., 2003). Sequence similarity between the catalytic domain of Ataxin-3 and other DUBs is low (Figure 2A), but recent NMR structures show that the overall arrangement of the catalytic triad is conserved (Figure 1; Mao et al., 2005; Nicastro et al., 2005).

Instability of a CAG nucleotide repeat in the *Ataxin-3* gene leads to a hereditary neurological condition known as spinocerebellar ataxia type-3 or Machado-Joseph disease (OMIM 607047). Like other polyglutamine neurodegenerative disease-associated genes, expansion of the CAG repeat in *Ataxin-3* leads to protein misfolding, resulting in aggregation and cellular toxicity. Some experimental evidence indicates that the normal function of Ataxin-3 involves transcriptional regulation, but whether its DUB activity plays a role in this process remains unclear (Li et al., 2002a). In evolutionary terms, MJDs likely represent a relatively late addition to the Ub system, as no homologs have been identified in yeast. However, protease activity of the other family members has not yet been demonstrated, and their biological functions remain unknown.

Ovarian Tumor Proteases (OTUs)

A bioinformatics approach also led to the identification of the Ovarian Tumor (OTU) subclass of Ub proteases (Makarova et al., 2000). The *otu* gene is involved in the development of the *Drosophila melanogaster* ovary where it may regulate the localization and translation of certain RNA transcripts (Goodrich et al., 2004; Steinhauer et al., 1989). Using the *Drosophila otu* gene and its homologs as a starting point, Makarova and colleagues (2000) found sequence similarity between these genes and those encoding viral cysteine proteases. A recently solved OTU structure shows that, unlike other cysteine protease DUBs, the catalytic triad is incomplete and is stabilized by a new method involving a hydrogen bonding network (Nanao et al., 2004).

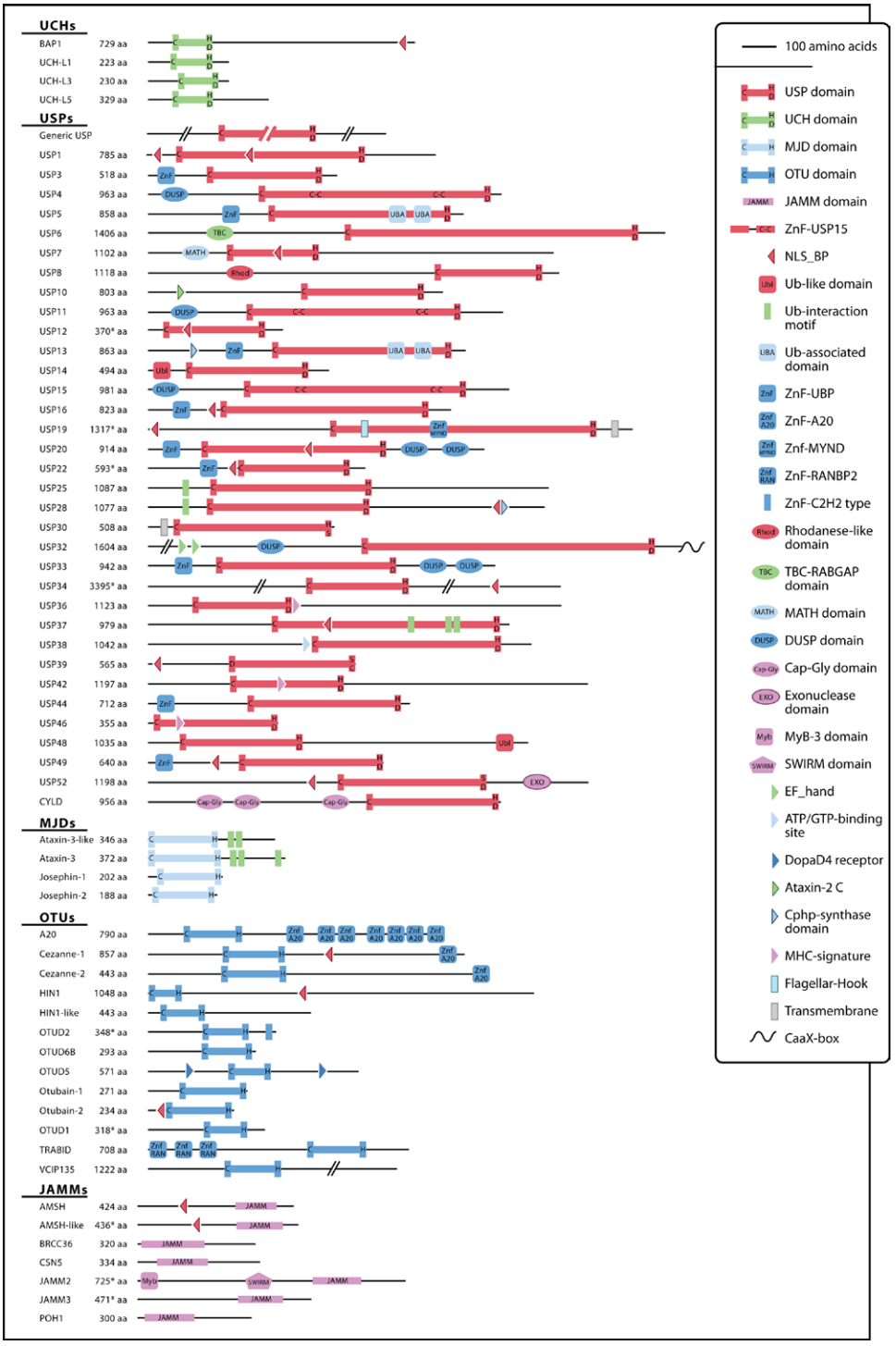

Figure 3. Comparison of the Domain Structures of Putative DUBs

For each primary DUB transcript (the transcript associated with the HUGO or RefSeq ID), we retrieved information concerning domain architecture and signal motifs using ENSEMBL, SMART (simple modular architecture research tool; http://smart.embl-heidelberg.de/), Pfam (protein families database; http://www.sanger.ac.uk/Software/Pfam/), and PROSITE databases. The USPs without additional domains are indicated as "generic USP." Only JAMM and MJD domain proteins with predicted catalytic activity are shown. An asterisk indicates that the ENSEMBL-predicted translational start site is uncertain. Proteins and domains are plotted on an approximate scale. Select abbreviations: ZnF, zinc finger; NLS_BP, bipartite nuclear localization signal; MATH, meprin and TRAF homology; DUSP, domain in ubiquitin-specific proteases. For additional information concerning the indicated domains visit http://www.ebi.ac.uk/interpro/.

Otubain-1 and Otubain-2 were the first two OTU proteins found to display in vitro DUB activity (Balakirev et al., 2003). Shortly thereafter, Cezanne, another OTU-domain containing protein, was found to interact with poly-Ub in a yeast two-hybrid assay and to contain DUB activity in vitro, suggesting that this is a general OTU feature (Evans et al., 2003). However, for most OTU proteases, their physiological role in vivo, including their putative role as DUBs, remains to be investigated.

JAMM Motif Proteases

The JAMM domain is found in all three major kingdoms of life (bacteria, archaea, and eukarya). However, bacteria do not contain Ub protease activity, and an analogous Ub-like conjugation system has not yet been identified in prokaryotes. This suggests that JAMM domains have adopted new protease functions during evolution and indicates that at least some of the human JAMM proteases may be involved in more than Ub (or Ubl) processing. Indeed, recent work has identified the protein product of the *Mycobacterium tuberculosis* gene *mec*$^+$ as a JAMM domain peptidase involved in cysteine biosynthesis by cleaving cysteine from a peptide intermediate (Burns et al., 2005).

Sequence alignment of the JAMM-domain proteins revealed that seven of the 14 members have at least one amino acid change in the conserved Zn^{2+} ion-stabilizing residues, indicating that they may not be functional proteases. Three family members (POH1, CSN5, and AMSH) will be briefly discussed in the section concerning DUB function.

DUB Specificity

Accumulating evidence indicates that most DUBs regulate a limited number of proteins and pathways, suggesting that they target specific substrates (Table 1). In the case of DUBs, specificity can refer to either the Ub or Ubl moiety itself (substrate specificity) or the target protein to which the moiety is conjugated (target specificity). In reality, it may not be possible to separate these types of specificities. It is likely that in many cases a combinatorial mechanism relying on recognition of both the target and the attached moiety determines overall DUB specificity. Additional mechanisms, such as protein localization and interactions with binding partners, may further contribute to in vivo specificity. In the following sections, we will discuss current insights into DUB specificity.

Substrate Specificity: Ubiquitin Polymers

Protein ubiquitination comes in many different flavors that serve distinct functions (Welchman et al., 2005). Whereas poly-Ub chains linked through the lysine residues of Ub at position 48 (K48) target proteins for proteasomal degradation, the attachment of a single Ub moiety (monoubiquitination) appears to regulate subcellular localization and recruitment of Ub binding proteins. Besides mono- and K48-linked polyubiquitination, other poly-Ub branches using alternative lysines on Ub have been described. The relevance and function of most of these different types of polymers is currently unknown, but future studies may reveal unique regulatory roles. A notable exception is K63 polyubiquitination, which has been described for a number of mammalian proteins, including RIP, NEMO and TRAFs (Sun and Chen, 2004). These signaling molecules are involved in activation of NF-κB signaling, a pathway involved in inflammation, apoptosis, and tumorigenesis. As in the case of mono-Ub, K63 polyubiquitination is required for the activation of downstream molecules, like kinases, or recruitment of other proteins. K63-linked Ub molecules differ remarkably from K48 chains in their three-dimensional structure, which probably accounts for their distinct functions (Varadan et al., 2004). Furthermore, this suggests that certain DUBs may act on specific Ub branches. Indeed, the yeast DUB Ubp2 prefers K63 over K48-linked Ub chains as a substrate (Kee et al., 2005). Conversely, examples of DUBs cleaving K48 but not K63-linked Ub polymers include USP8 and USP14 (Hu et al., 2005; McCullough et al., 2004). Another DUB, UCH-L5 can cleave various types of branches but does not display activity toward linear Ub dimers. Other examples further support the notion that DUBs cleave poly-Ub variants with varying efficiency, at least in vivo. One such example is the protein product of the Cylindromatosis tumor-suppressor gene (CYLD), a DUB involved in inhibiting NF-κB signaling (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). CYLD cleaves linear Ub fusions in vitro; yet in vivo it appears to be specific for non-K48-linked Ub chains. The basis for the specificity observed in vivo remains unclear, but phylogenetic analysis indicates that the catalytic domain of CYLD is relatively divergent from other DUBs, as indicated by its unique protease family identifier, C67 (Table S1 and Figure 2). This information leads us to speculate that the architecture of the enzymatic cleft contributes to its specificity. The OTU-type DUB A20 is another potent inhibitor of NF-κB signaling (Wertz et al., 2004). In vitro, A20 can cleave both K48- and K63-linked Ub polymers with similar efficiency. Yet in vivo, A20 deubiquitinates K63 but not K48 polyubiquitinated RIP (the protein we mentioned previously that is involved in NF-κB activation). However, given that A20 also contains (K48) E3 ligase activity toward RIP, it is not clear if the apparent K63 DUB activity in vivo is due to true substrate preference or simply due to its ability to catalyze the addition of K48-linked Ub polymers.

In at least some cases, domains outside the catalytic domain may contribute to Ub chain specificity (Lin et al., 2001). Splice variants of USP2 and a mutant containing only the core catalytic domain of this DUB cleave both linear Ub fusions and K48-linked Ub polymers. However, their relative efficiency varies considerably. The core domain prefers linear fusions, but full-length USP2b was most efficient in cleaving K48-linked Ub. Although USP2 does not contain an additional, known Ub-interaction motif, it is conceivable that sequences outside the catalytic domain contribute to selection and positioning of specific Ub chains. In fact, Ub-interaction motifs found in some E2 ligases have been implicated in determining linkage specificity. Indeed, it was recently described that a previously unnoticed Ub-interacting Zinc finger domain in USP15 is needed for disassem-

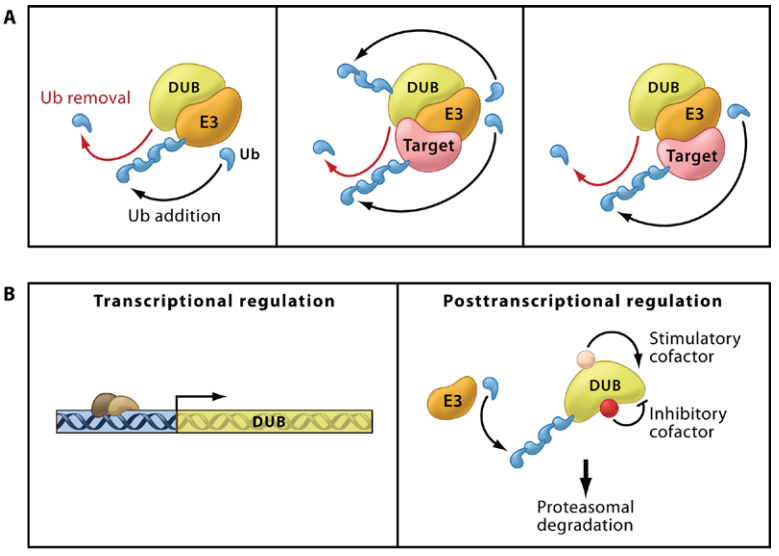

Figure 4. DUB Specificity and Regulation

(A) DUB/E3 interactions. DUBs and E3 are often found in a complex together. These interactions, which occur between USP7 and HDM2, for example, serve to reverse E3-mediated autoubiquitination (left panel) or allow the E3 to regulate the target and its DUB simultaneously as in the case of USP20 and pVHL (middle panel). Alternatively, DUB/E3 interactions confer specificity to the DUB, as in the case of Ubp2 and Rsp5 (right panel). E3 Ub-ligase and Ub-protease activity is indicated with black arrows and red arrows, respectively. Ub conjugated to E2 is not shown for clarity.

(B) DUB activity is regulated at various levels, including transcription (left panel), degradation, and binding to stimulatory or inhibitory cofactors (right panel). The exact mechanism whereby these cofactors regulate DUB activity is unknown but may occur at multiple levels (for example, phosphorylation, subcellular localization), stimulating conformational changes or conferring specificity.

bling branched Ub polymers but not for cleavage of a linear Ub-GFP fusion (Hetfeld et al., 2005). Another recent study showed that addition of a UBA domain that recognizes K48-Ub chains to USP5 skewed its substrate preference toward this type of Ub polymer (Raasi et al., 2005). Similarly, other Ub binding domains frequently encountered in DUBs, like UIM and ZnF-UBP (also called PAZ) may also contribute to Ub chain selection (Figure 3).

Substrate Specificity: Ubiquitin and Ubiquitin-like Molecules

The vast majority of putative DUBs tested so far display Ub protease activity in vitro (Table S1). Nonetheless, some predicted DUBs may be active toward Ubl moieties. Therefore, when considering DUB specificity, we wish to extend our discussion to both Ub and Ubl moieties. USP21 and UCH-L3 cleave both Ub and the Ubl molecule NEDD8. USP18 has been proposed to specifically cleave another Ubl, ISG15 (Gong et al., 2000; Malakhov et al., 2002; Wada et al., 1998). Furthermore, although some circumstantial evidence indicates that CSN5 may contain Ub protease activity, its main proteolytic target is thought to be the Ubl NEDD8 (Groisman et al., 2003; Verma et al., 2002; Yao and Cohen, 2002). For other Ubl molecules, distinct proteases have been identified. For instance, newly synthesized Ubl Atg8 is processed by a distinct protease (Apg4) of which five family members are found in the genome (Kirisako et al., 2000). Likewise, protease activity toward SUMO (small ubiquitin-like modifier) has thus far been restricted to the SENP family of cysteine proteases, of which seven genes are present in the human genome. However, within this family proteolytic activity is not limited to SUMO; SENP8 (also known as DEN1) is a NEDD8-specific protease (Gan-Erdene et al., 2003; Wu et al., 2003).

Our current knowledge of the motifs or residues in these proteases that are responsible for distinguishing Ub from Ubl moieties is limited. Clearly, more in vitro and in vivo analysis of DUBs and Ubl proteases, including structural information, is required.

Target Specificity and DUB/E3 Interactions

The recognition of targets by DUBs may be directed by sequences and motifs outside the conserved catalytic core. For instance, one of the Cap-Gly domains of CYLD mediates its interaction with NEMO, a potential CYLD substrate (Figure 3; Saito et al., 2004). However, like most enzyme/substrate interactions, DUB/target interactions are expected to be weak and transient in nature, making the identification of in vivo targets frustrating. A more stable complex between a DUB and its target may occur in the case when proteins are inappropriately K48 polyubiquitinated (Figure 4A, left panel). These proteins need to be continuously deubiquitinated to protect them from unwanted degradation. Autoubiquitination by ring finger-type E3 ligases is a frequently observed phenomenon resulting from nonspecific ubiquitination of proximal lysines (careless gunplay). For instance, the E3 ligase NRDP1 stimulates its own turnover as well as a number of cellular targets. The DUB USP8 associates with NRDP1 resulting in its deubiquitination and stabilization, suggesting that interaction with a DUB may simply serve to antagonize this self-inflicted degradation (Wu et al., 2004). Similarly, the interaction of USP7 with HDM2 and USP15 with Rbx1 results in the stabilization of these E3 ligases (Canning et al., 2004; Cummins et al., 2004; Hetfeld et al., 2005; Li et al., 2004). Interestingly, USP7 was also found to stabilize the herpes virus E3 ligase ICP0, indicating that viruses can hijack cellular DUBs to stabilize viral proteins. The importance for controlling Ub dynamics in the herpes virus life cycle is further underscored by the recent finding that the *herpesviridae* contains a distinct class of cysteine protease DUBs without known mammalian homologs (Kattenhorn et al., 2005).

Not all DUB/E3 interactions strictly serve to regulate E3 ligase stability. The E3 tumor suppressor protein pVHL regulates the stability of HIF1 transcription factors that are important regulators of angiogenesis. USP33 interacts with this E3 (Li et al., 2002b) and appears to regulate HIF1

stability by deubiquitination (Li et al., 2005). This suggests that, in this case, interaction of the DUB with the E3 allows the E3 ligase to differentially regulate the primary proteasomal target (HIF1) as well as its deconjugating enzyme (Figure 4A, middle panel).

Kee and colleagues (2005) recently suggested a third type of DUB/E3 interaction. They postulated that in some cases the DUB may hitch along with the E3 ligase. They showed that the target specificity of the yeast DUB Ubp2 is strictly dependent on the E3 ligase Rsp5, which is responsible for recognition of the substrate (Figure 4A, right panel). A remarkable variation on this theme is the previously mentioned protein A20. Here, E3 ligase and DUB activity reside in the same polypeptide (Wertz et al., 2004).

DUB Function

Gene deletion studies in yeast have indicated that none of the USPs are required for cell growth or viability (Amerik et al., 2000). Nonetheless, USPs and other DUBs in lower and higher eukaryotes including mammals have been implicated in regulating various critical cellular processes in a nonredundant manner. Human DUBs (or their murine homologs) of particular interest that have been linked to defined cellular processes or substrates are listed in Table 1.

The functions of DUBs at the proteasome lid, in endocytosis and regulation of chromatin structure, are reasonably well understood and are therefore discussed in some detail in the following sections. In addition, we will propose some directions for future studies.

DUBs and Proteasome Function

Proteins that must undergo fast and dramatic changes in abundance are often regulated by proteolysis. These proteins are targeted to the proteasome by K48-linked polyubiquitination, where they are degraded. The 26S proteasome consists of two 19S regulatory particles and a 20S cylinder-shaped multiprotein complex possessing the proteolytic activity (Pickart and Cohen, 2004). The 19S subunit restricts access to the interior of the 13 Å cylinder of the proteasome, which is where the catalytic residues for proteolysis are located. Deubiquitination of proteins arriving at the proteasome allows recycling of Ub and is required for protein degradation. In fact, deubiquitination, protein unfolding, translocation into the proteasome, and degradation are intimately linked processes. A number of DUBs from various subclasses have been found in complex with the 19S proteasome regulatory component, including the JAMM protease POH1 (Rpn11 in yeast), UCH-L5, and USP14 (Ubp6 in yeast) (Borodovsky et al., 2001; Lam et al., 1997; Park et al., 1997; Verma et al., 2002). Interestingly, residents of a paralogous multiprotein structure known as the COP9 signalosome are the JAMM protein CSN5 and USP15 (Cope et al., 2002; Zhou et al., 2003). Like the proteasome, the COP9 signalosome has been implicated in a diverse array of biological processes. At least some of these functions can be explained by its ability to inhibit the activity of the cullin family of ubiquitin E3 ligases by CSN5-mediated deneddylation.

The main DUB activity at the proteasome appears to be generated by POH1, since deletion of the gene that encodes this enzyme results in defective proteasomal degradation and is lethal in yeast. The functions of the other DUBs may be partially redundant with POH1, only playing a role in the deubiquitination of specific substrates, or in "Ub editing." The Ub-editing concept was postulated as a mechanism to rescue proteins that have been mistakenly ubiquitinated (as recognized by having short Ub chains) from destruction. The suggested Ub-editing mechanism would remove Ub polymers, starting at the distal end, independently of the substrate moiety (Lam et al., 1997). Although UCH-L5 indeed cleaves Ub chains from the distal end, compelling evidence for an Ub-editing function for UCH-L5 has not yet been provided, and no ortholog of UCH-L5 has been found in *Saccharomyces cerevisiae*.

DUBs and Chromatin Structure

An increasing body of evidence implicates dynamic histone ubiquitination in the regulation of transcription and silencing, and even double-strand-break formation during meiosis (Yamashita et al., 2004). Although most histone proteins can be ubiquitinated, the dynamics of H2B monoubiquitination are best understood. In yeast, deubiquitination of H2B by the DUB Ubp10 is required for telomeric silencing. This in turn allows recruitment of the silencing factor Sir2. (Emre et al., 2005). Interestingly, a second USP Ubp8 has been implicated in regulating H2B. In contrast to Ubp10, deubiquitination of H2B by Ubp8 correlates with transcriptional activation (Gardner et al., 2005; Henry et al., 2003). At least at some sites of active transcription, Ub-H2B levels are high during activation and subsequently decrease in an Ubp8-dependent manner. Importantly, both the ubiquitination and deubiquitination of H2B are necessary for optimal transcription, indicating a requirement for dynamic H2B modification by Ub (Henry et al., 2003).

Similar to yeast Ubp10, *Drosophila* USP7 interacts preferentially with silenced genomic regions, including telomeric domains, where it has been suggested to deubiquitinate H2B and thereby contribute to Polycomb-mediated silencing (van der Knaap et al., 2005). In mammals, USP7 associates with HDM2, an E3 ligase critical for regulating p53 turnover, and thereby inhibits degradation of both HDM2 and p53 (Cummins et al., 2004; Li et al., 2004). Indeed, different levels of USP7 can have opposite outcomes with respect to p53 stability. Intermediate inhibition of USP7 result in increased p53 degradation, whereas complete inhibition of USP7 enhances p53 stability. Interestingly, a recent report has suggested that HDM2 can mediate H2B ubiquitination (Minsky and Oren, 2004). Together, these data suggest an attractive model in which HDM2/p53/USP7 complexes mediate transcriptional repression by regulating H2B ubiquitination.

DUBs and Endocytosis

Monoubiquitination and, at least in yeast, the attachment of a K63-linked Ub dimer, play an important role in endocytosis of receptors and sorting of proteins (Haglund et al., 2003; Hicke and Dunn, 2003). After binding to ligands, receptor tyrosine kinases (RTKs) and adaptor proteins are monoubiquitinated at multiple sites, which triggers their

internalization. The RTKs are subsequently either recycled or transported to lysosomes for destruction. The E3 ligase for many RTKs is the protooncogene Cbl, which can also induce proteasome-dependent degradation by stimulating K48 polyubiquitination (Duan et al., 2004). Repeated addition of Ub or reduced deubiquitination may be the trigger for targeting to the lysosomal compartment, though exactly how ubiquitination determines this decision is unclear. Ub-interacting proteins like Hrs subsequently bind to the monoubiquitinated receptor and recruit protein complexes involved in budding of the endocytic vesicle.

DUBs are implicated in the endocytic pathway at multiple levels and also play important roles in other types of intracellular traffic. In yeast, the DUB Doa4 acts to recycle Ub at the late endosome to rescue Ub from destruction. Inactivation of Doa4 interferes with many Ub-related processes since it results in depletion of free Ub and many of the defects observed on Doa4 mutant cells are restored upon expression of additional Ub (Swaminathan et al., 1999). The closest human relative of Doa4 is USP8, which binds the Hrs binding partner (Hbp) and inhibits EGF receptor (EGFR) endocytosis, suggesting that USP8 may act to regulate endocytic traffic (Kato et al., 2000; Mizuno et al., 2005). Remarkably, a second Hrs interacting protein is AMSH, a JAMM domain DUB (McCullough et al., 2004). Inhibition of AMSH results in the accumulation of endosomal Ub and promotes EGFR endocytosis thereby accelerating EGFR downregulation.

Yet more DUBs are implicated in controlling endocytosis. In *Drosophila*, Fat facets (Faf; the homolog of human USP9X), deubiquitinates Liquid facets (Lqf), resulting in enhanced Lqf activity (Overstreet et al., 2004). Lqf and Faf play a role in *Drosophila* eye development by enhancing the internalization of a receptor implicated in cell patterning, called Delta. In humans, Lqf homologs are known as epsins, adaptor molecules involved in the initial steps of endocytosis.

Ubiquitination and deubiquitination appears to be a common theme in vesicle dynamics; monoubiquitination plays a critical role in budding of some viruses. Additionally, VCIP135, a OTU, has been implicated in Golgi assembly after mitosis (Wang et al., 2004).

Other Potential Roles for DUBs

Indications for the function of DUBs may come from various sources, including genetic screens in model organisms, interactome data, and domain- and signaling-motif predictions. Genetic screens in model organisms like the worm *Caenorhabditis elegans* and the fruit fly *Drosophila* are pointing at new roles for DUBs in various pathways. For instance, screens in *C. elegans* for modulators of RNA interference or longevity suggest an involvement of a USP and a UCH (Hamilton et al., 2005; Kim et al., 2005). In addition, a protein with both a USP and OTU domain (Duo-2) has recently been implicated in synapse function (Sieburth et al., 2005). These studies further solidify the broad involvement of the Ub conjugation/deconjugation system in biological processes and will certainly spark research into the functions of the human DUB orthologs.

Data derived from large-scale human protein-protein interaction experiments has implicated a number of USPs in several signaling cascades such as the TGF-β and NF-κB pathways. For instance, USP45 binds specifically to the phosphorylated TGF-β receptor in a mammalian two-hybrid (Barrios-Rodiles et al., 2005). Similarly, USP11 and USP9 interact with the NF-κB transcription factors RelB and p100, respectively (Bouwmeester et al., 2004). Although the significance of these interactions remains to be determined, these findings suggest that DUBs may play a regulatory role in these pathways.

Hints to the function of DUBs may be obtained from additional domains and signal motifs present in the primary amino acid sequence of these enzymes (Figure 3). For instance, USP7 might be involved in specific signaling pathways as it contains a MATH (meprin and TRAF homology) domain. These domains are found in members of the TRAF family of ring finger E3 ligases which mediates signaling via TNF receptors. Similarly, the JAMM2 protein might play a role in transcription and chromatin remodeling, as it contains domains (SWIRM and Myb DNA binding motif) implicated in these processes.

Regulation of DUBs

In contrast to many proteases, such as caspases that are translated as inactive precursors, DUBs are generally produced as active enzymes. Structural analysis has pointed out that the catalytic triad of UCHs and USPs only assume the active confirmation when bound to Ub, thereby preventing spurious protease activity against other substrates. In addition, various studies show that a diverse array of mechanisms regulates DUB activity and additional ones are likely to be discovered.

In the case of at least two JAMM domain proteins (POH1 and CSN5), it appears that incorporation into higher-order protein structures (the 19S proteasome and COP9 signalosome, respectively) is required for peptidase activity (Cope et al., 2002; Verma et al., 2002). Similarly, accessibility of the enzymatic cleft of USP14 appears to be regulated by activity of the 26S proteasome, its resident complex (Borodovsky et al., 2001).

Bre5, a cofactor for the yeast DUB Ubp3, is largely responsible for its in vivo activity toward Sec23, a protein involved in anterograde transport between the endoplasmic reticulum and the Golgi compartment (Cohen et al., 2003). Bre5 does not bind directly to Sec23, suggesting that the interaction between Bre5 and Ubp3 regulates Ubp3 activity. Surprisingly, the human homolog of Bre5, G3BP1, inhibits the activity of USP10, at least in vitro, indicating that cofactors can either restrict or enhance protease activity (Figure 4B, right panel; Soncini et al., 2001). A USP7 cofactor called GMPS that strongly augments USP7 activity was recently identified in *Drosophila* (van der Knaap et al., 2005). In addition, USP7 is regulated during apoptosis by cleavage by caspases. This cleavage presumably inactivates USP7 (Vugmeyster et al., 2002).

DUBs have frequently been found to be degraded by the proteasome, indicating that their abundance is an

important regulatory mechanism (Figure 4B, right panel). Moreover, some DUBs have been reported to be transcriptionally regulated (Figure 4B, left panel), sometimes in a cell-cycle-regulated manner (for example, USP1) or as part of a negative feedback loop (such as CYLD; Jono et al., 2004; Nijman et al., 2005).

In another case, inhibitory phosphorylation of CYLD after TNF-α stimulation is required for the accumulation of one of its proposed substrates, K63-ubiquitinated TRAF2. Interestingly, this event does not appear to modulate the affinity of CYLD for TRAF2, suggesting that phosphorylation may directly regulate CYLD activity by an unknown mechanism (Reiley et al., 2005).

Concluding Remarks

A large number of studies over the last decade have uncovered an unanticipated diversity of protein regulation by Ub and Ubl molecules. Nature has utilized the versatility of Ub in almost any conceivable way. Strikingly, the ubiquitin conjugation/deconjugation system outcompetes the protein phosphorylation system in terms of diversity and complexity. Although the reversal of ubiquitination by DUBs has been firmly established as a critical regulatory mechanism, we are only beginning to uncover the different mechanisms that control the activity of these enzymes.

Remarkably, the Ub E3 ligases greatly outnumber the DUBs encoded in the human genome. This is in contrast to tyrosine kinases and phosphatases, which are roughly equal in number. One possible explanation is that we have not yet identified all DUBs or their associated cofactors that may determine specificity. Indeed, Serine/Threonine kinases outnumber Serine/Threonine phosphatases, but a large variety of cofactors provide additional specificity to these phosphatases. It is also possible that many DUBs have poor substrate specificity and regulate on average up to 10 times more substrates than the average E3 ligase. However, most DUBs studied thus far appear to regulate a small number of targets. Another more likely explanation for the excess of E3 ligases could be that only a fraction of the targets that are ubiquitinated are regulated by specific deubiquitination. For destruction mediated by K48 Ub polymers, we would predict that many proteins are not deubiquitinated prior to arrival at the proteasome. Unless of course when you have made a mistake, why recycle a protein that you have decided to throw away? Possibly, only proteins that require extremely tight regulation, such as p53, require additional regulation by deubiquitination. Indeed, other types of Ub-based modifications, like K63-linked polymers or monoubiquitin require DUBs to "reset" the protein to its unmodified state and are thus more likely to be critically regulated by DUBs. Undoubtedly, future studies aided by detailed genomic annotation, structural information, and other new tools and methods to characterize this intriguing protein family will result in the demystification of these proteases.

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.cell.com/cgi/content/full/123/5/773/DC1/.

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