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Deubiquitinating Enzymes as Cellular Regulators

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Modification of proteins by the covalent attachment of ubiquitin is a key regulatory mechanism of many cellular processes including protein degradation by the 26S proteasome. Deubiquitination, reversal of this modification, must also regulate the fate and function of ubiquitin-conjugated proteins. Deubiquitinating enzymes catalyze the removal of ubiquitin from ubiquitin-conjugated substrate proteins as well as from its precursor proteins. Deubiquitinating enzymes occupy the largest family of enzymes in the ubiquitin system, implying their diverse function in regulation of the ubiquitin-mediated pathways. Here we explore the diversity of deubiquitinating enzymes and their emerging roles as cellular regulators.

Key words: deubiquitinating enzyme, JAMM, otubain, ubiquitin C-terminal hydrolase, ubiquitin-specific processing protease.

Ubiquitin (Ub) is a well-conserved 76 amino acid polypeptide that is present in all eukaryotes. Many cellular processes are controlled by the Ub post-translational modification to target proteins, including protein degradation, cell-cycle control, stress response, DNA repair, immune response, signal transduction, transcriptional regulation, endocytosis, and vesicle trafficking (*1*). Ub conjugates are formed through an isopeptide bond between its C-terminus Gly and the ε-amino group of Lys of the target protein. Ubs by themselves or in conjugation to proteins are also ligated to additional Ub molecules to form branched poly-Ub chains. Attachment of Ub to proteins is catalyzed by the action of Ub-activating enzyme E1, Ub-conjugating enzyme E2, and Ub ligase E3. The fate of ubiquitinated proteins in part depends on the length and linkage type of the Ub chain. In general, substrates with four or more Ub moieties linked via Lys29 or Lys48 are targeted for degradation by the 26S proteasome (*2*). Rather than marking for degradation, Lys63-linked ubiquitination is involved in regulation of endocytosis (*3*), mitochondrial inheritance (*4*), ribosome function (*5*), post-replicative DNA repair (*6*), and kinase activation (*7*). Attachment of a single Ub moiety functions in endocytosis of a number of plasma membrane proteins, protein sorting, and subnuclear trafficking (*8*).

Protein ubiquitination is a reversible process, and it has become increasingly obvious that Ub deconjugation plays important roles in regulating the Ub-dependent pathways (*9*, *10*). Deubiquitinating enzymes (DUBs) catalyze the removal of Ub from Ub-conjugated substrate proteins. DUBs also function in processing of Ub precursors and Ub adducts (*11*). Ubs are produced as Ub-ribosomal fusion proteins, such as Ub-C-terminal extension peptides of 52 and 80 amino acids (Ub-CEP52 and Ub-CEP80) (*12*) and as a linear poly-Ub fusion in a head-to-tail array (*13*). Such Ub precursor proteins are processed by DUBs to generate free Ub monomers. Ub adducts with small nucleophiles that may be produced by side reactions with cellular thiols and amines must also be hydrolyzed by DUBs. Some DUBs appear to perform 'editing' function that controls the fidelity of the conjugation process, thus preventing inappropriate degradation of cellular proteins. These catalytic functions of DUBs are summarized in Fig. 1. Here we extend our previous review (*9*) with the recent important findings on diversity of DUBs and their role as cellular regulators.

Diversity and classification of DUBs

Since the first deubiquitinating activity that cleaves off Ub from histone H2A was characterized (*14*), a vast number of DUBs have been identified using diverse methods, such as screening for cleavage of Ub-fusion peptides (*15*) or specific probe for DUBs (*16*, *17*) and genome sequencing (*18*). Until recently, DUBs have been categorized as cysteine proteases based on their sensitivity to inhibition by thiol reagents, such as *N*-ethylmaleimide and Ub-aldehyde (*9*, *10*). These classical DUBs fall into two distinct families: Ub C-terminal hydrolases (UCHs) and Ub-specific processing proteases (UBPs). Yeast has 1 UCH and 16 UBPs, which were identified by virtue of the conserved catalytic domains. The *Drosophila* genome project has identified 4 UCHs and 19 UBPs (*19*). The human genome project has led to identification of nearly 100 potential DUBs, of which more than 80% belong to the UBP family (*9*, *10*). In Arabidopsis, 27 distinct UBP sequences have been identified through database search (*20*). In most organisms, the member of the UBP family outnumbers that of the UCH family. Importantly, two new DUB families have recently been introduced: the otubain and JAMM families, both of which do not show any sequence homology to the UCH or UBP family.

UCH (Ub C-terminal hydrolase) family. UCHs are composed of relatively small size proteins of 20–30 kDa, with a few exceptions (see below). UCHs have a core catalytic domain (~230 amino acids) that is structurally defined by the presence of a catalytic triad consisting of positionally conserved Cys, His, and Asp residues (*21*). *In vitro*, UCHs can remove peptides and small molecules

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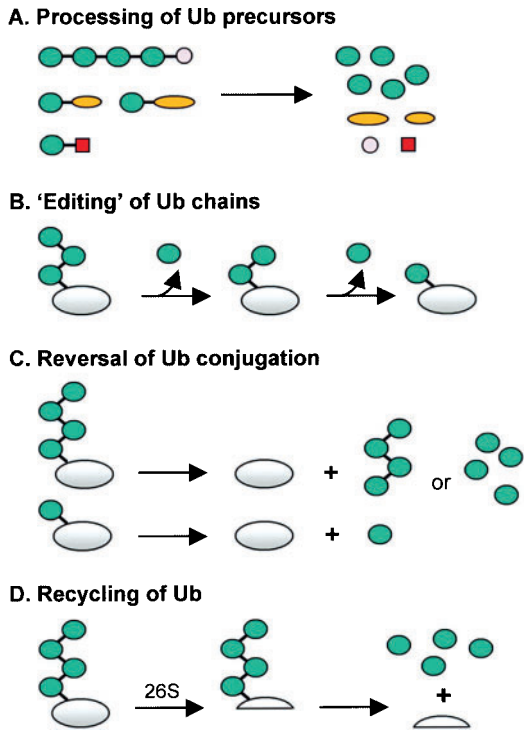

Fig. 1. **Catalytic functions of DUBs.** (A) Processing of Ub precursors by DUBs (preferentially by UCHs) generates free Ub monomers. Ubs are colored with green, CEP52 and CEP80 as orange, a single amino acid as pink, and Ub adducts as red. (B) UCH37 may function in 'editing' of Ub from distal ends of poly-Ub chains conjugated to protein substrates. (C) Reversal of Ub conjugation could occur before or after binding of the ubiquitinated proteins to the proteasomes to rescue them from proteolysis or facilitate their degradation. In this process, DUBs may cleave the isopeptide bond of poly-Ub chain proximal to target proteins (*e.g.*, Rpn11) or hydrolyze poly-Ub chains into Ub monomers (*e.g.*, Ubp6 and Doa4). Reversal of mono-ubiquitination (*e.g.*, by UbpM) is involved in regulation of cellular pathways other than the control of the Ub-proteasome pathway. (D) The isopeptide bonds in proteolytic remnants are cleaved by UBPs (*e.g.*, Doa4) for regenerating free Ub monomers.

from the C-terminus of Ub, some of which are attached non-specifically by the reaction with activated Ub during its conjugation cascade (*22*). However, most of them could not release Ub from Ub-protein conjugates or disassemble poly-Ub chains. Thus, UCHs seem to play a role in elimination of small adducts from Ub and in generation of free monomeric Ub from its precursors. However, certain UCHs (*e.g.*, UCH37: see below) can release Ub from the distal end of poly-Ub chains, thus also functioning in 'editing' of polyubiquitinated proteins.

UBP (Ub-specific processing protease) family. UBPs have a core catalytic domain of ~350 amino acids. Unlike the highly conserved UCHs, the UBP family exhibit strong homology only in two regions that surround the catalytic Cys and His residues, so called Cys box (~19 amino acids) and His box (60–90 amino acids). They vary in size from 50 to 300 kDa with a variety of N-terminal extensions, occasional C-terminal extensions, and insertions in the catalytic domains. The functions of these divergent sequences remain poorly understood, although the extensions and insertions have been suggested to function in substrate recognition, subcellular localization of the enzymes, and protein-protein interactions. In addition to processing of Ub precursors and salvage of trapped Ubs, UBPs are responsible for removing Ub from polyubiquitinated proteins and for disassembly of free poly-Ub chains. Large family members of UBPs have been identified in a variety of organisms, which appear to regulate a diverse set of biological processes. As the human has the largest member of the UBP family, the Human Genome Organization (HUGO) Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature) has proposed a systematic nomenclature for the enzymes using the abbreviation USP for ubiquitin-specific protease (*23*). Numbering is essentially arbitrary, and at least 29 USPs have been assigned to date.

Otubain (OTU-domain Ub-aldehyde-binding protein) family. Balakirev *et al.* (*24*) and Evans *et al.* (*25*) have isolated a new DUB family that has no sequence homology to known DUBs, but which belongs to the OTU (ovarian tumor) superfamily of proteins. The OTU superfamily comprises a group of putative cysteine proteases that are homologous to the *ovarian tumor* gene product of *Drosophila* (*26*). Approximately 100 identified OTU family members include proteins from eukaryotes, virus, and pathogenic bacteria. Two members of this family, called otubain-1 and otubain-2, have been isolated from HeLa cells by Ub-aldehyde affinity purification (*24*). Of these, otubain-1 cleaves the isopeptide bond in poly-Ub chain, but not the peptide bond in Ub-GFP fusion protein. Otubain-2 is also capable of cleaving poly-Ub chains, but only when the five amino acids from its C-terminus were deleted, suggesting that the C-terminal region might function in regulation of the enzyme activity. Cezanne (cellular zinc finger anti-NF-κB) that also belongs to the OTU family has been shown to be capable of cleaving the isopeptide bond in poly-Ub chains (*25*). These proteins contain the conserved Cys, His, and Asp residues that define putative catalytic triad of cysteine protease (*27*), but do not show any sequence homology to the UCH or UBP family. Thus, the OTU family member may constitute a new DUB family carrying Ub-aldehyde sensitive Cys active site.

JAMM (Jab1/Pad1/MPN-domain metallo-enzyme) family. In 1993, Hershko and co-workers identified an ATP-dependent, Ub-aldehyde insensitive deubiquitinating activity associated with the 26S proteasome from reticulocyte lysates (*28*). Verma *et al.* (*29*) and Yao and Cohen (*30*) have explored this activity and suggested that a motif within the MPN (Mpr1, Pad1 N-terminal) domain of Rpn11 in the proteasome lid complex is responsible for the activity. The MPN domain is also present in COP9 signalosome (CSN) and eIF3 complex. Interestingly, some eukaryotic and all prokaryotic MPN domains have a number of polar residues that are conserved in a highly coordinated fashion in a subset of MPN domains. This $EX_nHXHX_{10}D$ motif, referred to as JAMM (*29*) or MPN+ (*31*), is frequently found in the active site of enzymes or as the coordinating ligands in metal-binding proteins. Furthermore, the deubiquitinating activity of Rpn11 is insensitive to classical DUB inhibitors, such as Ub-aldehyde, but is destroyed by the Zn^{2+}-specific chelator TPEN [*N,N,N′,N′*-tetrakis(2-pyridylmethyl)-ethylenediamine] (*30*) as well as by other metal chelators, such as *o*-phenanthroline (*29*). Thus, Rpn11 with other eukaryotic pro-

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teins containing the JAMM motif may constitute a new DUB family that has a metal-binding site.

Crystal structure of DUBs

To date, three crystal structures of DUBs have been determined: UCH-L3 and Yuh1 by Johnston *et al*. (*32*, *33*) and HAUSP by Hu *et al*. (*34*). UCH-L3 and Yuh1 show structural similarities with the papain-like cysteine proteases, particularly in the region including the active site catalytic triad. The oxyanion hole in the enzymes accommodates the negative potential on the substrate carbonyl oxygen during the first step in catalysis. However, a remarkable topological difference from papain is a crossover loop (20 residues in UCH-L3 and 21 in Yuh1) that lies directly over the active site. Presence of the active site-crossover loop may function to restrict the size and nature of substrates. Only efficiently processed substrates are allowed to pass through this loop, consistent with the observations that most UCHs preferentially act on small Ub adducts and short extensions in Ub precursor proteins.

Hu *et al*. (*34*) have revealed the crystal structure for the 40-kDa catalytic core domain (residues 208–560) of HAUSP alone and in complex with Ub-aldehyde. The HAUSP catalytic core domain is composed of three domains: Fingers, Palm, and Thumb. The general conservation of the residues that comprise the secondary structural elements in the three domains suggests the conservation of the Fingers-Palm-Thumb architecture among other UBP proteins. The highly conserved Cys and His boxes are positioned on the opposite sides of the catalytic cleft created by the Palm and Thumb scaffold. This open-cleft structure of the HAUSP catalytic domain and the three-domain architecture are suitable for deubiquitination of large substrates, such as polyubiquitinated proteins and free poly-Ub chains, which are cleaved by UBPs but unlikely by UCHs. However, the HAUSP catalytic core by itself may be insufficient for recognition of ubiquitinated substrates *in vivo*. Interestingly, the C-terminal segment of p53 (residues 357–382) contains five of the six putative ubiquitination sites. It is possible that the N-terminal domain of HAUSP recognizes the ubiquitinated region of p53, thus recruiting it to the HAUSP catalytic core domain. Likewise, the highly divergent N- and/or C-terminal extensions of UBPs may contribute to the recruitment of specific substrates to the catalytic domains of the enzymes.

Biological functions

DUBs are involved in regulation of Ub-dependent pathway by direct or indirect association with the protesomes. DUBs are also implicated in numerous biologically important pathways, including cell growth and differentiation, development, oncogenesis, neuronal diseases, and chromosome structure and transcriptional regulation.

Regulation of proteasome function. Various DUBs are associated with the 26S proteasome and regulate its function. Cohen and coworkers (*35*, *36*) have demonstrated that UCH37, an intrinsic subunit of the 19S regulatory complex (also called PA700) of the 26S proteasome, is involved in 'editing' of ubiquitinated substrates according to the length of poly-Ub chains rather than the structure of the target protein themselves. By disassembling the degradation signal from the distal end of Lys48-linked poly-Ub chains, UCH37 could selectively rescue poorly ubiquitinated or slowly degraded Ub-conjugated proteins from proteolysis. It may also enhance degradation by facilitating release of polyubiquitinated substrates from its initial binding site in the 19S regulatory complex for translocation to the proteolytic core of the 20S proteasome. Holzl *et al*. (*37*) have identified a homolog of UCH37, p37A from the 19S regulatory complex of *Drosophila*. Fission yeast also contains a homolog of UCH37, Uch2p, of which the C-terminal extension is required for the association with the 26S proteasome (*38*). However, its physiological function remains unknown since *uch2*$^+$ is not essential for cell growth**.**

Doa4 in yeast is required for the rapid degradation of protein substrates of the Ub-proteasome pathway. In *doa4* cells, small polyubiquitinated peptide remnants accumulate with reduction of Ub levels. This enzyme interacts physically and substoichiometrically with the 26S proteasome (*39*). Thus, Doa4 appears to promote proteolysis through removal of Ub from proteolytic intermediates on the proteasome before or after initiation of substrate breakdown. Ubp6 in yeast also physically interacts with the purified proteasomes, and this interaction markedly increases the deubiquitinating activity of Ubp6 (*40*). This association is mediated by the interaction between the N-terminal Ub-like domain of Ubp6 and Rpn1 in the base of the 19S regulatory complex. Ubp6 mutants exhibit accelerated turnover of Ub, suggesting that deubiquitination events catalyzed by Ubp6 prevent translocation of Ub to the proteolytic core and rescue Ub from degradation. USP14, a mammalian homolog of Ubp6, has also been found to bind the 26S proteasome upon specific labeling of the Cys active site of the enzyme with Ub-vinylsulfone (*17*).

Although the entity of Rpn11 as a proteasome-associated DUB was revealed a decade ago, its unique and intricate function has been explored recently. Unlike the classical DUB families, it has the metal-binding JAMM motif conferring the sensitivity to metal chelators but not to thiol-blocking agents (*29*, *30*). Single amino acid substitutions in the JAMM motif cause slow growth, sensitivity to temperature and amino acid analogs, and defects in general proteasome-dependent proteolysis (*31*). Mutations of the conserved Cys residue in Rpn11, which is a corresponsive active site of classical DUBs but is not a part of JAMM motif, shows no effect on cell growth, consistent with the finding that the deubiquitinating activity is not sensitive to thiol reagents. In contrast, replacement of two predicted active site His residues by Ala in the JAMM motif of Rpn11 causes cell death and stabilizes Ub-pathway substrates. Therefore, it has been proposed that Rpn11 couples between substrate deubiquitination and degradation by releasing the attached poly-Ub chain, allowing full translocation of the substrate polypeptide to the proteolytic core. However, it remains unknown whether recombinant Rpn11 or the lid of the 19S regulatory complex alone has deubiquitination activity. So it is conceivable that other proteasomal subunits are required for the deubiquitinating activity of Rpn11.

Cell growth and differentiation. UBP41 is the smallest functional UBP identified to date (*41*), and has a

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virtually identical catalytic domain with a set of UBPs (UBP46, UBP52, and UBP66), which has distinct N- and/or C-terminal extensions (*42*). Overexpression of UBP41 has been shown to elicit all features of apoptosis in human cells, including DNA fragmentation and caspase-3 activation (*43*). Since UBP41 overexpression also causes deubiquitination of a broad range of proteins, it was expected to strongly interfere with the Ub-proteasome system. However, UBP41 overexpression does not stabilize the cell cycle proteins p21, p27, and cyclin B, which are known to be the substrates of the proteasome (*1*). Treatment with a proteasome inhibitor MG132 results in cell cycle arrest in G(2)/M, and subsequently in moderate apoptosis. But UBP41 overexpression does not arrest cells in G(2)/M. Therefore, it has been suggested that UBP41 shows a proapoptotic activity by stabilization of specific substrates that may be related with the activation of apoptotic pathway. UBP41 seems to also play a role in bone formation (*44*). The osteotropic agents, such as PTH (parathyroid hormone), PTHrP (parathyroid hormone-related peptide), and PGE_2, which increase intracellular cAMP level, promote bone formation in both animals and humans (*45*). Expression of UBP41 mRNA is up-regulated by the exposure of metaphyseal and diaphyseal bones as well as of cultured osteoblasts with these agents but not with PTH analogs that do not increase cAMP level. Thus, UBP41 may also contribute to bone homeostasis possibly via the PKA/cAMP pathway (*44*).

UBP45 and UBP69 in rat have an identical C-terminal region spanning the highly conserved Cys and His boxes, but with distinct N-terminal extensions. Park *et al*. (*46*) have demonstrated that UBP45 and UBP69 play an antagonistic role in regulation of myogenesis. Stable expression of UBP69 promotes both myoblast fusion and accumulation of muscle specific proteins, whereas that of UBP45 blocks both of the myogenic process. Both enzymes cleave off Ub from polyubiquitinated proteins *in vivo* as well as *in vitro*. Thus, the distinct N-terminal extensions of UBP45 and UBP69 may differentiate their substrate specificity or subcellular localization and in turn their function in negative and positive regulation of muscle cell differentiation. It has also been reported that expression of UBP45 and UBP69 (also called UBP-t1 and UBP-t2, respectively) in testis is developmentally regulated, suggesting that the enzymes could also function in spermatocyte differentiation (*47*).

Cytokines regulate cell growth and differentiation by inducing expression of specific target genes. D'Andrea and coworkers (*48–50*) have isolated three members of the murine DUB subfamily (DUB-1, DUB-2, and DUB-2A) that are rapidly induced by different cytokines in various hematopoietic cells. DUB-1 is induced by interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas DUB-2 expression is specifically regulated by interleukin-2. These proteins are highly related throughout their primary sequences except for a hypervariable region at their C-termini, suggesting that the C-terminal region may confer the substrate specificity of each enzyme. Therefore, it has been proposed that different hematopoietic cytokines induce expression of specific DUB genes and each induced enzyme regulates the degradation or the ubiquitination state of an unknown regulatory factor(s), resulting in a cytokine-specific growth response.

UBPY accumulates upon growth stimulation of starved human fibroblasts, and its levels decrease in response to growth arrest induced by cell-cell contact (*51*). Inhibition of UBPY accumulation by anti-sense microinjection prevents the cells from entering S-phase in response to serum stimulation. Thus, UBPY (also called USP8) appears to be involved in cell-growth control. USP21 has also been implicated in regulation of cell growth, since its overexpression has a profound growth inhibitory effect on cultured cells (*52*). Significantly, USP21 has dual specificity for both Nedd8 and Ub. Nedd8 is a Ub-like protein that is known to facilitate the proteasome-mediated degradation of cell cycle-related proteins upon conjugation to cullins, a component of E3 Ub ligase (*53*).

Development. *Drosophila* fat facets (Faf) is a DUB required in a cell communication pathway during eye development to limit the number of photoreceptors in each facet to eight (*54*). The liquid facets (*lqf*) locus encodes epsin, a vertebrate protein associated with the clathrin endocytotic complex (*55*). Chen *et al*. (*56*) have demonstrated that Faf deubiquitinates Lqf, and prevents its degradation by the Ub-proteasome pathway. Thus, Faf and Lqf appear to facilitate endocytosis and function in common cells to generate an inhibitory signal that prevents ectopic photoreceptor determination. DiAntonio *et al*. (*57*) have demonstrated that neuronal overexpression of Faf leads to synaptic outgrowth and impairs synaptic transmission. They also have shown the genetic interactions between Faf and Highwire, a negative regulator of synaptic growth that has structural homology (a RING-H2 finger) to a family of E3 Ub ligase (*58*). Thus, synaptic development may be controlled by the balance between positive and negative regulation of Lqf ubiquitination.

Fam is the mouse homolog of *Drosophila* Faf, and shows substrate-specificities to AF-6 and β-catenin (*59*, *60*). AF-6 is a component of tight junctions and adherens, and participates in cell-cell adhesion regulation downstream of Ras during development (*61*). β-Catenin plays a dual role in development: it serves as a cell junction protein that links the actin cytoskeleton to cell-cell adhesion (*62*) and as a nuclear protein that modulates transcription in response to the Wnt signaling pathway (*63*). The catalytic domain of Fam specifically binds to both AF-6 and β-catenin *in vivo* as well as *in vitro*. AF-6 is ubiquitinated in cultured cells and the catalytic domain of Fam can deubiquitinate it. β-Catenin is stabilized by overexpression of the C-terminal half of Fam. In mouse embryo, Fam is expressed at all stages of pre-implantation development from ovulation to implantation (*64*). Depletion of Fam by exposure of two-cell embryos to Fam-specific antisense results in a decrease in cleavage rate and an inhibition of cell adhesive events, concomitant with the loss of β-catenin and mis-localization of AF-6 at the apical surface of blastomeres. Thus, Fam appears to be involved in pre-implantation mouse embryo development. The human homolog of Faf, called DFFRX/Y (USP9X/Y), has also been isolated (*65*). However, it has not yet been determined whether DFFRX/Y shows deubiquitinating activity.

Dictyostelium UbpA has sequence similarity with yeast Ubp14 and human isopeptidase T, which disassem-

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bles free poly-Ub chains (*66*). Yeast *ubp14* cells show a defect in proteolysis, due to accumulation of excess poly-Ub chains that competitively inhibit the binding of polyubiquitinated substrates to the proteasomes (*67*). UbpA-deficient *Dictyostelium* grows normally and shows normal responses to starvation, but fails to develop fruiting bodies even under conditions that the mutant cells were treated with pulses of cAMP, which allow cell aggregation and expression of cAMP-induced genes. Thus, UbpA appears to play a role in specific developmental transitions in *Dictyostelium* by catalyzing disassembly of poly-Ub chains and thus facilitating the degradation of specific proteins via the Ub-proteasome pathway. Like UbpA, *At*UBP14 in *Arabidopsis thaliana* has sequence similarity with yeast Ubp14 (*68*). However, unlike *Dictyostelium* UbpA and yeast Ubp14, *At*UBP14 is essential in *Arabidopsis*. T-DNA insertion mutations in the single gene encoding *At*UBP14 cause an embryonic lethal phenotype, with the homozygous embryos arresting at the globular stage. The arrested seeds have substantially increased levels of poly-Ub chains, indicating that *At*UBP14 also functions in disassembly of free poly-Ub chains. Thus, *At*UBP14 and the Ub-proteasome pathway seem to play an important role in early embryo development in *Arabidopsis*.

UbpB in *Dictyostelium* interacts with the F-box/WD40 repeats of MEK kinase (MEKKα) that plays a complex role in regulating cell-type differentiation and spatial patterning (*69*). Cells deficient in either MEKKα or UbpB develop precociously and exhibit abnormal cell type patterning. Loss of UbpB alone results in decreased expression of MEKKα, and increases the abundance of ubiquitinated MEKKα. MEKKα interacts not only with UbpB but also with Ubc, implying that temporally and spatially restricted degradation of the MEKKα is mediated by differential control of ubiquitination and deubiquitination. Thus, UbpB and the Ub-proteasome pathway appear to be involved in the control of developmental timing and spatial patterning in *Dictyostelium*.

UCH/CeUBP130 is an UBP homolog in *Caenorhabditis elegans* (*70*). RNA interference targeted for the enzyme results in cell division defective embryos. Since UCH/CeUBP130 is localized in the sperm and at the microtubule-organizing center (MTOC) during early cleavage, it has been suggested that the enzyme is required for the formation of a functional MTOC in the fertilized embryo of *C. elegans*.

Oncogenesis. The mammalian proto-oncogene *tre-2* encodes a DUB, and the TRE-2 oncoprotein exhibits a transforming activity in 3T3 fibroblasts (*71*). Murine Unp (ubiquitous nuclear protein) gene has originally been identified as a proto-oncogene related to the *tre-17* proto-oncogene (*72*). The mRNA levels of Unph (the human homolog of Unp: USP4) have been reported to increase in small cell tumors and adenocarcinomas of the lung, suggesting a possible causative role for Unph in neoplastic process (*73*). Two human Unp isoforms, of which one form contains an internal 47 amino acid insertion, have also been isolated from adult tissues (*74*). However, it has been reported that the levels of both Unph isoforms are reduced in small cell lung carcinoma cell lines, raising a possibility that the *Unp* genes may serve as tumor suppressor genes. Recently, both human and murine Unps have been shown to interact with the retinoblastoma protein (pRb), suggesting that pRb is a potential cellular substrate of Unps. Human USP15 (*23*), its mouse ortholog (*75*), and rat UBP109 (*76*) also have the pRb interaction motif, and share high sequence similarity with Unps. Since pRb is a target for the Ub-proteasome pathway (*77*), these UBP enzymes may collaborate in cell growth regulation by stabilization of pRb.

BAP1 (BRCA1-associated protein1) is a DUB that contains the N-terminal UCH catalytic domain (~250 amino acids), thus belonging to the UCH family (*78*). It is a nuclear protein that binds the tumor suppressor BRCA1. Germline mutations of BRCA1 pre-dispose women to early-onset of familial breast and ovary cancers (*79*). In cells, loss of BRCA1 function leads to spontaneous chromosomal breakage and sensitivity to DNA damage (*80*). BRCA1 forms a heterodimeric complex with BARD1. Both proteins contain an N-terminal zinc RING-finger domain that confers E3 Ub ligase activity. The BRCA1/BARD1 complex catalyzes the formation of multiple poly-Ub chains on itself, and this auto-ubiquitination markedly potentiates its E3 ligase activity. BAP1 has been reported to enhance BRCA1-mediated inhibition of breast cancer cell growth. However, BAP1 does not appear to function in deubiquitination of the BRCA1/BARD1 complex (*81*). CYLD encoded by a gene for familial cylindromatosis also has sequence homology to the catalytic domain of UCHs (*82*). Familial cylindromatosis is an autosomal dominant predisposition to multiple neoplasms of the skin appendages. However, it has not yet been determined whether CYLD has deubiquitinating activity.

HAUSP (USP7) has originally been identified based on its ability to associate with the herpes simplex virus-type1 immediate-early protein Vmw110 (*83*). The RING finger protein Vmw110 that localizes to promyelocytic leukemia-nuclear bodies (PML-NBs) is a non-specific activator of gene expression and is required for efficient initiation of the viral lytic cycle (*84*). HAUSP is distributed in the nucleus in a microfunctuate pattern, and is dynamically associated with PML-NBs during virus infection (*83*). Expression of Vmw110 in cells causes an increase in the recruitment of HAUSP into PML-NBs, followed by dispersion of PML-NBs. Vmw110 may be involved in stimulation or re-direction of the activity of HAUSP to deubiquitinate and stabilize viral proteins that are destined for degradation by the Ub-proteasome pathway. Recently, Li *et al*. (*85*) have shown that HAUSP is a novel p53-interacting protein by mass spectrometry of affinity-purified p53-associated proteins. HAUSP specifically deubiquitinates and stabilizes p53, resulting in p53-dependent cell growth arrest and apoptosis. These findings reveal an important mechanism by which p53 can be stabilized by direct deubiquitination, and also imply that HAUSP may function as a tumor suppressor *in vivo* through the stabilization of p53.

von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome caused by germline mutations of the VHL gene (*86*). The VHL protein (pVHL) associates with elongin C, elongin B, and cullin-2 to form a complex, termed the VCB-CUL2 complex, which acts as an E3 Ub ligase (*87*). Two novel DUBs that interact pVHL have been identified and named VDU1 and VDU2 (*88*, *89*),

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which show high sequence homology with each other. The VDU1- and VDU2-interacting region in pVHL is located in its β-domain harboring naturally occurring mutations in pVHL disease, and these mutations disrupt the interaction between VDUs and pVHL. In addition, both VDU1 and VDU2 can be ubiquitinated in a pVHL-dependent manner for rapid degradation by the proteasome. These findings imply a possible involvement of VDU1/VDU2 in pVHL-associated tumorigenesis.

Neuronal Diseases. Several cases of genetic abnormalities within DUBs have been reported to cause or directly contribute to neuronal disease pathogenesis. UCH-L1 was first linked to Parkinson's disease (PD) by identification of an autosomal dominant point mutation (I93M) in two siblings with a strong family history of PD (*90*). Since I93M mutation reduces the *in vitro* hydrolytic activity of UCH-L1 by ~50%, a partial loss of UCH-L1 hydrolytic function was proposed to cause this form of PD. However, simple loss of hydrolytic activity does not explain the PD phenotype in this family. The mutation is not 100% penetrant in the family, and mice lacking functional UCH-L1 (the gracile axonal dystrophy, GAD mouse) do not develop PD phenotype (*91*). Another polymorphic variant of UCH-L1 (S18Y) that is linked to decreased PD risk has subsequently been found (*92*). Liu *et al*. (*93*) have revealed that UCH-L1 has a novel dimerization-dependent Ub ligase activity. Remarkably, the protective polymorphic variant S18Y shows reduced tendency of dimer formation, thereby having reduced ligase activity, but has comparable hydrolase activity as the wild-type enzyme. These findings are consistent with the observations that the protective effect is dependent on the S18Y allele dosage and that the Ser18 residue is not involved in the normal hydrolytic activity of UCH-L1. Furthermore, the UCH-L1 dimer is capable of generating Lys63-linked ubiquitinated α-synuclein by adding Ubs to mono- and di-ubiquitinated α-synuclein, which is not a good substrate for the proteasome, leading to elevation of cytoplasmic concentration of α-synuclein that could form pathogenic aggregates. Thus, two opposing enzymatic activities of UCH-L1 may play a critical role in regulation of the proteasomal degradation of α-synuclein and thereby in PD susceptibility. In addition to PD, UCH-L1 has been implicated in two other neurodegenerative diseases that may also be triggered by protein aggregation: spinocerebellar ataxia (SCA), in which a UCH mutant is a genetic enhancer of degeneration in SCA transgenic *Drosophila* (*94*), and Huntington's disease, in which the UCH-L1 S18Y polymorphism is linked to age at onset (*95*).

Mice that are homozygous with respect to a mutation in the *ataxia* gene develop severe tremors followed by hindlimb paralysis and death (*96*). Wilson *et al*. (*97*) have demonstrated that the *ataxia* gene encodes USP14. In contrast to other neurodegenerative disorders, Ub-positive protein aggregates are not detectable in the central nerve system of *ataxia* mutant mice, consistent with the finding that USP14 can process short Ub side chains but not poly-Ub chains (*98*). Neuronal cell loss is neither detectable in *ataxia* mutant mice. Instead, marked alterations in synaptic transmission were observed both at the central and peripheral nerve systems, apparently due to abnormalities associated with transmitter release at the neuromuscular junctions. Thus, USP14 appears to play a role in regulating synaptic activity in mammals.

USP25 and USP28, showing high sequence homology with each other, have been identified from human genome (*99*). The mouse homolog of USP25 shows a correlation of expression with proliferative neuroepitherial cells and postmitotic neurons upon *in situ* hybridization in mouse embryonic brains (*100*). Interestingly, the expression level of USP25 is increased by about 2-fold in the brains of Down syndrome patients. Thus, USP25 may be involved in Down syndrome pathogenesis.

Chromatin structure and transcriptional regulation. UbpM in a slime mold is phosphorylated during the onset of mitosis and dephosphorylated during metaphase/anaphase transition. Approximately 5–10% of nuclear core histones H2A and H2B are monoubiquitinated during interphase and completely deubiquitinated during metaphase, which coincides with complete condensation of the mitotic chromatin (*101*). Recombinant UbpM is able to deubiquitinate histone H2A *in vitro*. Wild type UbpM localizes in the cytoplasm, but mutant forms, lacking an active site Cys residue, associate closely with mitotic chromosome and localize in the nucleus. Overexpression of mutant UbpM blocks cell division and eventually leads to apoptosis. Thus, UbpM may be involved in the condensation of mitotic chromosome by acting on histones or other relevant protein substrates critical for chromosome condensation.

Transcriptional silencing in yeast is caused by specialized chromatin structures that repress gene expression. Silencing of a locus requires the assembly on the DNA of a multi-component protein complex including silent information regulators (SIRs). Ubp3 in yeast binds to Sir4, and deletion of *ubp3* results in a marked increase in silencing at telomeres and the *HM* mating type locus (*102*). Dot4, another DUB in yeast, also interacts with Sir4 (*103*). However, loss of Dot4 results in a partial loss of silencing at the same loci and in a decrease in the cellular level of Sir4. Moreover, the defect can be partially suppressed by mutations in a subunit of the 26S proteasomes, suggesting that Dot4 has the ability to prevent Ub-mediated degradation. These opposing functions of Ubp3 and Dot4 may balance the gene expression at the specific loci by regulating the proteasome-dependent degradation of Sir4 and/or other factors that are related with the activity or assembly of the SIR protein complex. In *Drosophila*, the gene dosage of a DUB, called D-Ubp-64E, has been shown to affect position variegation (*104*). Genes inserted in different positions of the *Drosophila* genome show different levels of transcription, and this can be affected by the state of the chromatin at the site of integration.

Others. In addition to the DUBs described above, numerous other DUBs have been identified to function in a variety of important cellular processes. In *Aspergillus nidulans*, the *creB* and *creC* genes are involved in carbon catabolite repression, and mutation of these genes affects expression of many genes under both carbon catabolite repressing and derepressing conditions. The *creB* gene encodes a functional DUB and the *creC* gene encodes a protein that contains five WD40 repeat motifs and a Pro-rich region (*105*, *106*). In addition, CreC prevents the degradation of CreB in the absence of carbon catabolite

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repression (*107*). Thus, the Ub-dependent systems appear to be involved in the process of carbon catabolite repression.

Ub conjugation is involved in the endocytic internalization of plasma membrane proteins, followed by their degradation in the vacuole (*8*). In yeast *pep4* cells deficient for vacuolar protease activities, unubiquitinated Fur4p, the plasma membrane protein uracil permease, is accumulated. However, *pep4* cells lacking Doa4 accumulate ubiquitinated Fur4p (*108*). Thus, Doa4 appears to function in deubiquitination of endocytosed plasma membrane proteins, in addition to its role in Ub homeostasis.

A serotonin-inducible UCH, called Ap-Uch, has been implicated in activation of cAMP-dependent PKA in *Aplysia* by stimulating the proteasome-mediated degradation of its inhibitory subunit (*109*). Activated PKA induces the CREB-mediated transcription cascade, which leads to synthesis of new proteins and growth of new synapse, which produce the continuous presynaptic facilitation necessary for long-term behavioral sensitization. Thus, Ap-Uch-regulated proteolysis appears essential for long-term facilitation.

The murine homolog of UBPY, called mUBPy, has been identified as a protein interacting with Ras nucleotide exchange factor CDC25(Mm) (*110*). CDC25(Mm) is ubiquitinated in cultured cells, and coexpression of mUBPy decreases its ubiquitination. In addition, the half-life of CDC25(Mm) is considerably increased in the presence of mUBPy. Thus, mUBPy seems to play a role in controlling the degradation of CDC25(Mm). USP11 has been isolated as a protein binding to RanBPM (RanGTP-binding protein) that is required for correct nucleation of microtubules (*111*). RanBPM is ubiquitinated in cultured cells, and this ubiquitination is inhibited by USP11. Thus, USP11 may play a role in regulating the degradation of RanBPM. USP10 has been isolated as a protein binding to Ras-GTPase activating protein (G3BP). However, G3BP is not a substrate of USP10, and rather inhibits its ability to disassemble poly-Ub chains (*112*). Thus, the role of USP10 in the Ras-pathway remains unknown.

Conclusion

Ubiquitination is a dynamic and reversible process that regulates cellular pathways, including protein degradation, cell-cycle control, stress response, DNA repair, immune response, signal transduction, transcriptional regulation, endocytosis, and vesicle trafficking. There should be many other Ub-dependent pathways that are also regulated by dynamic ubiquitination and deubiquitination. In addition, Ubs are synthesized as precursors, such as Ub-C-terminal extension peptides and linear poly-Ub fusions. Genome projects have identified nearly 100 potential DUBs from human, 27 from *Arabidopsis*, 21 from *Drosophila*, and 17 from *Saccharomyces cerevisiae*, which constitute the UBP and UCH families. A new DUB family (called otubain family) that has no sequence homology to the UBP or UCH family has been introduced. The otubain family belongs to OTU (ovarian tumor) superfamily of proteins, consisting of ~100 identified OTU members. Furthermore, another DUB family (termed JAMM family) that neither has any sequence homology to UBP or UCH family has been identified. The presence of astoundingly increasing number of DUBs strongly suggests that distinct enzymes have specialized functions. Thus, a major challenge in the research of DUBs is to assign specific physiological roles to each member of the DUB families. Genetic studies have assigned a number of DUBs for their specific physiological roles. However, much less is known about *in vivo* protein substrates of the enzymes, even for those whose functions have been revealed. All ubiquitinated proteins are potential substrates for DUBs, but there should be the specificity for most, if not all, of DUBs toward their ubiquitinated target proteins. The specificity is likely to largely depend on the structure of target proteins. Other factors that influence the enzymatic specificity may include the accessibility of the ubiquitinated proteins to DUBs, the rate at which the ubiquitinated proteins are targeted to subcellular organelles, the length and types of poly-Ub chains conjugated to substrate proteins, and the abundance and localization of both DUBs and target proteins. Thus, the future studies should be directed to identification of the specific substrates for each DUB so as to link the specificity of the enzyme to its physiological function.

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