Published in final edited form as:

Nat Rev Mol Cell Biol. ; 12(9): 605–620. doi:10.1038/nrm3173.

The Predator becomes the Prey: Regulating the Ubiquitin System by Ubiquitylation and Degradation

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Abstract

Ubiquitylation (also known as ubiquitination) regulates essentially all intracellular processes in eukaryotes through highly specific, and often tightly spatially and temporally regulated, modification of numerous cellular proteins. Although most often associated with proteasomal degradation, ubiquitylation frequently serves non-proteolytic functions. In light of its central roles in cellular regulation, it has not been surprising to find that many of the components of the ubiquitin system itself are regulated by ubiquitylation. This observation has broad implications for pathophysiology.

A large number of cellular proteins are modified by ubiquitin and UBLs (ubiquitin-like proteins). These modifications generate a recognition element *in trans* for different downstream effectors that bind to the conjugated ubiquitin or UBL and affect the fate and/or function of the modified protein. The best-studied result of this modification is the targeting of ubiquitin-tagged proteins for proteasomal degradation. The UPS (ubiquitin-proteasome system) is responsible for the regulated degradation of myriad proteins that control numerous central processes, including, for example, the cell cycle and transcription, as well as protein quality control. In light of the large number of UPS substrates, the high specificity

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About the authors

Allan M. Weissman obtained his B.S. in biochemistry from Stony Brook University, New York, USA, and his M.D. from Albert Einstein College of Medicine, New York. Following training in internal medicine at Washington University, St. Louis, Missouri, USA, he joined the US National Institutes of Health (NIH), where he now is a Laboratory Chief in the National Cancer Institute Frederick, Maryland, USA. His group within the Laboratory of Protein Dynamics and Signaling focuses on the physiological and pathological roles of the ubiquitin-proteasome system and on understanding structure-function relationships for ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s).

Nitzan Shabek is a post-doctoral fellow in the laboratory of Aaron Ciechanover in the Faculty of Medicine at the Technion in Haifa, Israel. There, he continues the studies that he initiated when he was a graduate student, also with Aaron Ciechanover, on the mechanisms involved in degradation of ubiquitin by the ubiquitin system.

Aaron Ciechanover was born in Haifa, Israel in 1947. He obtained his M.Sc. in biochemistry and his M.D. from the Hebrew University in Jerusalem, Israel, and his D.Sc. from the Technion in Haifa. As a graduate student with Avram Herskho, and in collaboration with Irwin Rose, they discovered that the covalent attachment of ubiquitin to a target protein signals it for degradation, and deciphered the mechanism of conjugation. His laboratory currently focuses on the mechanisms that underlie the degradation of the ubiquitin system's own components, and on the decoding of the polyubiquitin proteolytic signal. For the discovery of the ubiquitin proteolytic system, he was awarded – along with Avram Herskho and Irwin Rose – the 2004 Nobel Prize in Chemistry.

of their recognition, and the complex processes regulated, it was not surprising to find that the system comprises ~1,000 components (~5% of the genome), and that aberrations in its function underlie the mechanism of many diseases, including malignancies, inflammatory disorders and neurodegeneration.

The structure of the UPS is hierarchical. Activated ubiquitin is transferred through a cascade from the one predominant ubiquitin-activating enzyme (E1) to one of several of the 30–40 mammalian ubiquitin carrier proteins (ubiquitin-conjugating enzymes (UBCs); E2s), to the substrate, which is bound specifically to one of hundreds of different ubiquitin-protein ligases (E3s). This process is repeated to generate polyubiquitin chains, which are signals that target conjugated proteins for degradation by the 26S proteasome. After proteasome targeting, ubiquitin is recycled by DUBs (deubiquitylating enzymes), some of which also function to oppose the action of E3s (Figure 1).

The UPS is highly regulated at multiple levels. An emerging concept is that the specificity-conferring components of the UPS, the E3 ligases, as well as other components of the system, can be regulated by ubiquitylation (known also as ubiquitination, the term coined by the discoverers of this modification as related to proteolysis⁰) and degradation, either mediated by themselves (self- or auto-ubiquitylation), or by heterologous ligases. However, there are also clear examples where self-ubiquitylation of E3s serves not as a degradation signal, but rather to alter the function of these proteins. This review focuses on examples of both of these concepts, as well as the regulated degradation of ubiquitin and the proteasome.

DEGRADATION OF UBIQUITIN

The ability of the UPS to carry out its numerous functions depends on free ubiquitin. Cellular ubiquitin levels are regulated by synthesis and degradation, as well as by rates of protein ubiquitylation and deubiquitylation. Although ubiquitin levels vary among tissues, developmental stage and with differentiation and metabolic activity (for example, under stress, when proteolysis rates increase, free ubiquitin levels fall)^{1,2}, the ratio of free to protein-conjugated ubiquitin is approximately 1:1 (REFS 3,4). As ubiquitin is physically stable and remains properly folded and active after exposure to extreme conditions^{5,6}, it was thought to be resistant to catabolism. However, ubiquitin has a finite half-life, and its degradation and steady state level reflects overall protein turnover^{3,7,8}. Ubiquitin can be also depleted when it is not properly removed and recycled from targeted degraded substrates (REF. 9 and below).

Ubiquitin can be degraded by the 26S proteasome in three ways: as a monomer; along with its conjugated substrate through a 'piggyback' mechanism; and as a linear fusion protein with a short C-terminal tail, or as part of an isopeptide conjugate with a short peptide^{1,10,11} (N.S. and A.C. unpublished observations) (Figure 2A).

Degradation of monomeric ubiquitin—Ubiquitin is relatively stable and has a half-life of ~10 hours^{1,3,7,8,10}. Its degradation requires ATP and is mostly proteasome-mediated^{1,3,8}. Since monomeric ubiquitin binds to the proteasome but does not have a long enough 'tail' to allow it to be 'pulled' into the 20S catalytic particle (CP), it itself has to be ubiquitylated in order to be degraded. This provides the tagged ubiquitin with sufficient freedom of movement to allow for entry into the proteasomal CP. Ubiquitylation is catalyzed by a specific E2, E2-25K (also known as UBE2K), which catalyzes E3-independent 'canonical' proteasome-targeting K48-linked chains on monomeric ubiquitin¹², and by the HECT (Homologous to the E6-AP C-Terminus) domain E3 TRIP12 (ThyRoid hormone-Interacting Protein 12), which can also ubiquitylate ubiquitin and target it for degradation¹³. The relative importance of these two modes of ubiquitylation of ubiquitin (E3-independent and -

dependent) to the degradation of monomeric ubiquitin, and the overall contribution of the degradation of monomeric ubiquitin to its catabolism are not known.

Intriguingly, although ubiquitin is ubiquitylated to become a target substrate, it has also been shown that ubiquitin can exist as ‘unanchored’ free polyubiquitin chains. Although the physiological role(s) of the low steady state levels of these chains remains elusive, several functions have been attributed to them, including involvement in NF- κ B signaling¹⁴, and serving as an easily accessible source of monomeric ubiquitin when disassembled by DUBs¹⁵.

Degradation of ubiquitin with its target substrate—Ubiquitin can be degraded in a piggyback mechanism, along with its target substrate. The accelerated degradation of cellular proteins that occurs with various forms of cellular stress, probably owing to misfolding or other forms of protein damage, is accompanied by accelerated degradation of ubiquitin^{1,3,8}. Also, deletion of proteasome-associated DUBs, such as yeast Ubp6 (USP14 in mammals) and UCH37 — which presumably functions with the proteasome subunit RPN11 to remove ubiquitin chains prior to entry of the substrate into the 20S CP — accelerates substrate degradation and results in depletion of ubiquitin^{9,16–19}. Interestingly, depletion of ubiquitin results in transcriptional upregulation of Ubp6 (REF. 20), suggesting an autoregulatory feedback loop. Also, Ubp6 acts non-catalytically to slow protein degradation by decreasing flux of proteins through the proteasome, possibly to allow efficient removal and recycling of ubiquitin from the chains tagged to the substrate before its entry into the 20S CP to be degraded²¹. Using a reconstituted cell free system, it has been shown that addition of bona fide substrates of the UPS enhances proteasome-dependent degradation of ubiquitin, when preceded by conjugation of the degraded ubiquitin to the substrates. This finding provides direct evidence in support of the piggyback mechanism¹⁰. It is assumed that part of the chain, probably the proximal part, which is closer to the substrate, is probably degraded with the substrate, whereas the other moieties, probably the ones that are more distal to the substrate, are ‘rescued’ and recycled.

An interesting question is whether this piggyback mode of degradation serves a mechanistic role and is a consequence of the need to degrade the substrate, or whether it is a by-product of the proteolytic process. It is possible that part of the ubiquitin chain must always be present on the substrate to secure its binding to the proteasome, and its premature removal can result in detachment of the substrate before complete entry into the proteasome. It has been reported recently that the initial, relatively weak and reversible association of the conjugated substrate with the proteasome, which is mediated by the ubiquitin chain, is followed by a tighter association between substrate and the proteasome that requires ATP hydrolysis and a loosely folded domain on the protein, but appears to be ubiquitin-independent²². Thus, according to this study, it is possible that, mechanistically, it is not necessary to degrade part of the ubiquitin chain along with the tagged substrate, and it can be completely rescued and/or detached while the substrate, which is now bound in a tighter association that does not require ubiquitin, is degraded. However, as experimental evidence suggests that part of the chain is degraded along with the substrate¹⁰, it is possible that the movement of the substrate into the CP can be more rapid than the hydrolytic activity of proteasomal DUBs, and therefore part of the chain cannot be rescued. Alternatively, the chain is required throughout most of the proteolytic process, cannot be released earlier and is, therefore, partially degraded. Additional detailed mechanistic studies will be necessary in order to dissect the role of the polyubiquitin chains once the substrate has reached the interior of the CP and degradation has been initiated. Besides DUBs, other proteins have recently been found to be involved in regulating ubiquitin homeostasis. These include RFU1 (Regulator of Free Ubiquitin chains 1), which inhibits the DUB Doa4 and probably controls the equilibrium between monomeric ubiquitin and free ubiquitin chains¹⁵.

Degradation of ubiquitin fused or conjugated to short peptides—Several studies have demonstrated that ubiquitin with a C-terminal extension that is longer than 20 amino acids is degraded efficiently by the proteasome, and that the degradation is not dependent on further ubiquitylation^{1,10,11}; however, ubiquitin with a shorter extension is stable and not degraded. Similar findings have been shown using ubiquitin synthetically conjugated with an isopeptide bond to short peptides (N.S. and A.C., unpublished observations)²³. The *in vivo* sources of these ubiquitin-bound peptides (which were synthetically generated as model substrates in the studies described) are unknown, and they have not been isolated from cells. They could be intermediates of proteasomal degradation of larger cellular substrates in which the ubiquitin moiety is bound to either an internal Lys residue of the substrate via an isopeptide bond, or to its N-terminal residue via a linear peptide bond²⁴. Such ubiquitin conjugates can also be products of the ubiquitylation of peptides that might either have their own cellular function or are derived from larger proteins via different processing or destructive processes. Thus, to be removed, these peptides must undergo ubiquitylation to be targeted for proteasomal degradation. Although short peptides to which ubiquitin is bound either linearly or via an isopeptide bond have not been isolated and/or characterized in mammalian cells, evidence for their existence was found in yeast cells lacking Doa4 (REF. 25), which is a DUB implicated in endocytosis and vacuolar degradation²⁶.

An important implication of the efficient degradation of C-terminally tailed ubiquitin is that it defines the two minimal requirements for proteasomal recognition and degradation of intact substrates: a proteasomal-binding domain (ubiquitin) and an unstructured tail that is long enough (>20 residues) to allow the molecule to physically extend through the 19S regulatory particle (RP) into the CP²⁷ (Figure 2Ac). The requirement for an unstructured tail has been demonstrated for several model substrates. Whether this concept holds true in cells for polyubiquitylated and normally folded cellular proteins awaits determination. For such proteins, an important problem is determining the mechanism (or mechanisms) that unfolds a segment to allow entry of the substrate into the CP. It is possible that ubiquitylation destabilizes the protein and results in an unfolded segment. Alternatively, a chaperone-assisted mechanism could be involved. Also, there could be a role for the ATPases in the base of the 19S subunit in this process.

A naturally occurring pathological variant of ubiquitin is UBB⁺¹, which is ubiquitin that has been extended by a 19 residue C-terminal tail. UBB⁺¹ is synthesized due to a dinucleotide deletion during transcription and is implicated in the pathogenesis of an early onset form of Alzheimer's disease and other neurological and conformational disorders²⁸. UBB⁺¹ cannot be activated by E1 and therefore is not conjugated to other proteins, but it has been shown to inhibit proteasome function^{10,29}. Its toxicity is probably due to two mechanisms: it binds to the proteasome but it is resistant to degradation owing to the short 19 residue tail; and it can be ubiquitylated, which creates UBB⁺¹-anchored ubiquitin chains that are resistant to DUBs. The high affinity of the ubiquitylated UBB⁺¹ with its non-degradable ubiquitin chains for the 26 proteasome makes it an efficient inhibitor of the proteasome^{10,29}. Experimental extension of the tail of UBB⁺¹ by a single residue renders the protein susceptible to proteasomal degradation without further ubiquitylation¹⁰. The finding that tailed ubiquitin is degraded efficiently by the proteasome raises the possibility that polyubiquitylation is required to increase the affinity of large substrates to the proteasome to render proteolysis more efficient, but for short substrates, a single moiety or short oligoubiquitin chains may be sufficient when an adequate unstructured domain is also present.

Regulating UPS Enzymes by Ubiquitylation

Specificity in ubiquitylation is determined largely by E3s, of which there are over 600 in humans. These function together with subsets of E2s. Given the exquisite regulation of ubiquitylation and its ability to alter both the fate and function of substrates, it is not surprising that E3s might be regulated by targeting themselves for ubiquitylation (regulatory self-ubiquitylation) (Figure 2B). Self-ubiquitylation is a general property of E3s *in vitro*³⁰ and is often used to confirm that proteins are active ligases, and to assess functionally significant interactions with specific E2s. There are now a number of examples of documented self-ubiquitylation in cells (see Table 1 for examples).

The canonical result of ligase self-ubiquitylation is auto-regulation or targeting for self-destruction, with the main role of the ligase in most cases being to target heterologous substrates. The complexity of E3 regulation is further accentuated by increasing examples in which an E3 that may or may not self-ubiquitylate, is subject to ubiquitylation by another E3 (regulation by heterologous E3s; Figure 2B), with self- and heterologous ubiquitylation of the same ligase signaling for either the same or different outcomes. Where different outcomes occur, self-ubiquitylation has been shown to serve regulatory, non-proteolytic functions, whereas the heterologous modification targets for degradation (discussed below; Table 1).

The complex relationship between the multisubunit APC/C (Anaphase Promoting Complex/Cyclosome) and the SCF (Skp1-Cullin-F-box) families of E3s throughout the cell cycle provides the ultimate example of both self- and heterologous regulation of E3s by ubiquitylation and proteasomal degradation, in this case to ensure regulated cell proliferation and genomic integrity (Box 1). Similarly, DUBs and E2s are also subject to ubiquitylation with evidence, at least in one case, suggesting E3-independent self-ubiquitylation of an E2 (REF. 31) (Table 1 and Box 1). There are also examples where enzymes involved in UBL modification of proteins are targets for ubiquitylation or vice versa. The most extensively-studied UBL-mediated modification of E3s, and the one with the widest range of known consequences, is the activating modification of the cullin subunits of the CRL (cullin-RING ligase) superfamily of E3s by the UBL Nedd8 (Table 1). Moreover, an increasing number of proteins that include ubiquitin receptor domains (reviewed in REF. 32) are also ubiquitylation substrates.

Box 1

Cross-regulation of ubiquitin ligase complexes during the cell cycle

The APC/C (Anaphase Promoting Complex/Cyclosome) is central to cell cycle regulation. The 13 subunit APC/C includes either of two substrate recognition elements with distinct specificities. APC/C^{CDC20} and APC/C^{CDH1} are tightly regulated to ensure appropriate cell cycle-dependent degradation of substrates (reviewed in REF. 101). The cullin-containing SCF (Skp1-Cul1-F-box proteins) E3 complexes are also essential for cell cycle progression, with the levels (Skp2) and activities towards specific substrates (β -TrCP) of SCF E3s regulated during the cell cycle. APC/C and SCF E3s are cross-regulated to ensure correct cell cycle progression (see the figure, APC components – red, SCF components – blue). In late G2 and early M-phase the APC/C is inactivated by the spindle assembly checkpoint (SAC; also known as the mitotic checkpoint complex (MCC)) pending alignment of chromosomes along the mitotic spindle (1). Once this occurs, APC/C^{Cdc20} is activated and targets substrates essential for completion of mitosis (reviewed in REF.101) (2). After mitosis APC/C^{Cdc20} is inactivated (reviewed in REF. 102) through replacement of Cdc20 by Cdh1, which, once dephosphorylated, can associate with the APC/C (3). This dephosphorylation is an indirect effect of APC/

C^{Cdc20} , which targets cyclin A for degradation, thereby inactivating the kinase Cdk1, which is critical for Cdh1 phosphorylation (reviewed in REF.101). APC/C^{Cdh1} further inactivates APC/C^{Cdc20} by targeting Cdc20 for polyubiquitylation (Ubn) and degradation beginning in telophase and continuing in G1 (4). To prevent premature G1 to S progression, the Cyclin E-Cdk2 complex is kept inactive by the G1-S CDK inhibitor p27^{KIP}. A key E3 for p27^{KIP} is, SCF^{Skp2} (REFS 103-105). To maintain p27^{KIP} levels, Skp2 is targeted by APC/C^{Cdh1} during G1 (REFS 106,107) (5). As G1 ends, APC/C^{Cdh1} is inactivated through multiple mechanisms, including targeting of the E2 UbcH10 (known also as E2C or E2X)¹⁰⁸ for degradation by APC/C^{Cdh1} (6), and ubiquitylation of Cdh1 by both APC/C^{Cdh1} (REF. 109) and an unidentified SCF E3 ligase¹¹⁰ (7,8). This allows accumulation of SCF^{Skp2} and p27 degradation, which facilitates transition into S phase (9). Synthesis of the APC/C ‘pseudosubstrate’ EMI1 initiates in late G1. EMI1 binds APC/C^{Cdh1} during late G1 and S, contributing to APC/C^{Cdh1} inactivation (REFS 111-113, reviewed in REF. 114) (10). In some cells, EMI1 similarly inhibits APC/C^{Cdc20} (11). In early M, EMI1 is phosphorylated by Cdk and PLK1 leading to its targeting for degradation by SCF^{β-TrCP} (REFS.115-118) (12). Until prometaphase, the SAC/MCC is the primary means by which APC/C^{Cdc20} is held in check (1).

Self-ubiquitylation in regulating the tumor suppressor p53—Regulatory self-ubiquitylation (Figure 2B) is illustrated by considering the control of the p53. This protein, known as the guardian of the genome, is normally maintained at low levels (largely due to the UPS) to allow normal cell growth and proliferation; however, under conditions of genotoxic stress its degradation is suppressed through a variety of mechanisms, leading to upregulation of genes that result in either growth arrest or apoptosis. In this regard mutation or loss of p53 expression is a common feature of many malignancies (reviewed in REF.33).

Although a number of E3s have been suggested to be involved in p53 ubiquitylation, Mdm2 (Mouse Double Minutes 2; often referred to as Hdm2 in humans) is of unquestionable importance. Mdm2 is a RING finger E3 that can form homodimers through its RING finger and binds directly to p53, which exists primarily as a homotetramer. Mdm2 targets multiple C-terminal lysines of p53 for ubiquitylation (reviewed in REF. 34). Mdm2 undergoes self-ubiquitylation *in vitro*^{35,36} and targets itself and p53 for RING finger-dependent degradation *in vivo*³⁵. The closely related protein, MdmX (Mdm4; HdmX/Hdm4 in humans), has little propensity to form homodimers but rather forms heterodimers with Mdm2 through an extended surface centered on their RING fingers³⁷. Although the MdmX RING finger lacks ligase activity, the heterodimer is an active E3 that is generally believed to be the predominant cellular ligase for p53 (Ref. 38, reviewed in REFS 33,34). Mdmx can also directly bind p53 in a fashion similar to Mdm2. Notably, MdmX lacks the nuclear export

and nuclear and nucleolar localization signals that are characteristic of Mdm2. MdmX can also be ubiquitylated and targeted for degradation by Mdm2, presumably in the context of Mdm2-MdmX heterodimers (reviewed in REFS 33,34). Consistent with this, expression of MdmX decreases ubiquitylation and degradation of Mdm2, presumably by competing with Mdm2 in the formation of dimers and thereby decreasing the capacity of one Mdm2 molecule to ubiquitylate another. There is evidence that the Mdm2:MdmX ratio plays important roles in p53 ubiquitylation and degradation. MdmX enhances the activity of Mdm2 towards p53 (REFS 39, 40). However, MdmX has also been associated with stabilization of p53 (REF. 41). This role is attributed to the attenuation of nuclear export of p53 by Mdm2, which is believed to lead to degradation of p53 in the cytoplasm. Thus, while it is critical for issues of p53 stability/degradation and localization that Mdm2 and MdmX be maintained in a proper stoichiometric relationship, exactly how the relationship affects p53 function remains an area of intense investigation. An additional level of complexity in the relationship between p53, Mdm2 and MdmX is illustrated by the function of the DUB USP7/HAUSP. This DUB, which was first shown to deubiquitylate p53, has as its primary activity, under non-stressed conditions, the deubiquitylation and stabilization of Mdm2 and MdmX^{42,43}. However, in response to genotoxic stress, phosphorylation of Mdm2 and MdmX leads to decreased HAUSP association and to their destabilization, which further contributes to p53 activation by ATM and downstream kinases^{44,45}.

Heterologous and self-ubiquitylation in regulating transmembrane signaling—

Ubiquitylation plays important roles in the regulation of transporters, receptors and associated components of signaling pathways at the plasma membrane and in early endosomes. In general, this modification downregulates these molecules by targeting them for lysosomal/vacuolar degradation (reviewed in REF. 46). Prominent among E3s acting in these pathways are members of the Nedd4 (Neural precursor cell Expressed Developmentally Down-regulated protein 4) family of HECT E3s and the Cbl (Casitas B-lineage Lymphoma) family of RING finger E3s.

The Nedd4 family (yeast ortholog is Rsp5) has nine members; prominent among these are the functionally distinct Nedd4-1 and Nedd4-2 (also known as Nedd4L) isoforms and Itch. Nedd4 family members are generally characterized by an N-terminal C2 domain, 2 to 4 WW domains and a C-terminal conserved HECT ubiquitin ligase domain. C2 domains serve as sites for membrane localization by binding to phospholipids and to proteins. Most known substrate interactions occur through the Trp-based WW domains. Like SH3 domains, these generally recognize Pro-containing 'PY' motifs (reviewed in REF. 47). However, these labels belie the combinatorial complexity between various WW domains and PY motifs⁴⁸.

The Cbl family of E3 ligases was characterized consequent to the discovery of v-Cbl as the product of the transforming gene of the murine Cas NS-1 virus (reviewed in REF. 49). Mutations in Cbl that result in loss of its RING finger-dependent E3 activity, including v-Cbl, can function as dominant negatives, resulting in sustained signaling. Such mutations can therefore be oncogenic, as evidenced by the association between such Cbl mutations and myeloid leukemias (reviewed in REF. 50). There are three mammalian Cbl proteins: Cbl (also known as c-Cbl), Cbl-b and Cbl-c (also known as Cbl-3). Each has an N-terminal tyrosine kinase binding (TKB) domain, a conserved RING finger and a Pro-rich region. Both the TKB domain and the Pro-rich region mediate numerous protein interactions⁵¹. Cbl proteins are recruited through TKB domains to active signaling complexes, most notably to transmembrane RTKs (Receptor Tyrosine Kinases) and receptors linked to tyrosine kinases. They target these receptors and associated components for ubiquitylation, leading to their lysosomal targeting and degradation. Thus, Cbls serve as potential tumor suppressors by attenuating receptor-mediated mitogenic signaling (reviewed in REF. 49). As part of this process Cbl proteins undergo self-ubiquitylation, leading to their degradation and thereby

regulating their own levels (Figure 3A - left). The degradation of Cbl proteins is dependent on their interactions with RTKs (and vice versa) and on Cbl E3 activity. Degradation of activated receptors, as well as associated signaling molecules and recruited Cbl, is blocked by either lysosome or proteasome inhibitors, suggesting coordinated degradation of the signaling complex⁵². Whether this means of Cbl degradation occurs in lysosomes, proteasomes or both is unclear.

Another mechanism by which Cbl proteins can be targeted for degradation is by interacting with the Nedd4 family of HECT domain E3s, mediated through interactions between the Pro-containing regions of Cbls and the WW domains of Nedd4 family members. Nedd4-1 and Itch interact with and target all three Cbls for ubiquitylation and degradation (Figure 3A - right). This Cbl ubiquitylation leads to proteasomal degradation and is dependent on an intact HECT domain, but independent of a functional RING finger⁵³. Thus, Cbl proteins are bona fide Nedd4 and Itch family substrates. The consequences of this interaction include delayed downregulation of activated EGFR (Epidermal Growth Factor Receptor) and consequently sustained MAP (Mitogen Activated Protein) kinase activation⁵³. The relationship between the two classes of E3s is asymmetrical; there is little evidence that Nedd4 family members are targeted by Cbls. Such asymmetry is a recurrent theme in many of the examples in subsequent sections.

The relationship between these ligase families has been confirmed in helper T cells from *Nedd4*^{-/-} embryos. These T cells have decreased activation, as assessed by IL-2 production and increased Cbl-b levels, which inhibits signaling downstream of the TCR (T Cell Antigen Receptor). In addition, siRNA knockdown of Cbl-b increases IL-2 levels⁵⁴. These observations are in accord with evidence implicating Cbl-b as a negative regulator of TCR signaling (reviewed in REF.49,55).

Some insights into the physical and functional relationship between members of these families come from studies on Itch and Cbl. Itch is a substrate for JNK (Jun N-terminal Kinase)⁵⁶, which is activated by a variety of stimuli, including signaling through the EGFR. Itch phosphorylation results in a conformational change that activates Itch as determined by enhanced self-ubiquitylation⁵⁶. This phosphorylation also facilitates binding of Cbl and other substrates⁵⁷. This suggests a positive feedback loop in which EGFR signaling can be prolonged by downregulation of Cbl by activated Itch. A further level of regulation arises from the JNK-dependent binding of WW domains of Itch to the DUB USP9x/FAM (mammalian counterpart of *Drosophila* Fat-Facets). At a minimum, this interaction decreases ubiquitylation of Itch and therefore increases its availability^{57,58}. Whether this DUB diminishes Itch-mediated ubiquitylation of Cbl or increases Cbl ubiquitylation by increasing active Itch remains unanswered. As multiple WW Nedd4 family members can target different Cbl proteins, it is now of great interest to determine the means by which specificity in interactions between the multiple members of these families is achieved *in vivo*. Non-mutually exclusive possibilities include: differential expression of family members, post-translational modifications that regulate interactions and spatial juxtaposition. It will also be important to understand the relative roles of self-ubiquitylation and Nedd4 family-mediated ubiquitylation in the dynamic regulation of Cbl levels and how this relates to the oncogenic potential of Cbl mutations in different tissues.

Heterologous and self-ubiquitylation at the endoplasmic reticulum—

Ubiquitylation at the ER (endoplasmic reticulum) leading to proteasomal degradation is a way of disposing of misfolded proteins and unassembled protein subunits, as well as proteins of the secretory pathway that require tight regulation. ERAD (ER-Associated Degradation) represents a critical homeostatic and regulatory set of processes. Failure to adequately degrade proteins results in ER stress, which in turn triggers a complex cellular

response known as the UPR (unfolded protein response) (reviewed in REF. 59). gp78 is a pro-metastatic mammalian polytopic ERAD E3⁶⁰ that targets substrates by means that are not limited to direct interactions. Recognition of already ubiquitylated proteins may occur via the ubiquitin binding CUE domain of gp78⁶¹, or proteins may be targeted through co-localization to microdomains of the ER where the degradation and ER dislocation machinery are concentrated⁶². If the levels of gp78 are too high, critical regulatory proteins such as Insig-1 (insulin-induced gene 1; a regulator of cholesterol metabolism) and KAI1/CD82 (a metastasis suppressor) may be inappropriately degraded^{63,64}. Presumably as a means to avoid such targeting, gp78 self-ubiquitylates, leading to proteasomal degradation in a manner that is dependent on its RING finger, its CUE domain and its unusual binding site for its cognate E2 (Ube2g2/MmUbc7) known as the G2BR (Ube2g2 Binding Region), which allosterically activates gp78 by increasing the affinity of Ube2g2 for gp78 (Figure 3B). This leads to both enhanced self-ubiquitylation and degradation of substrates^{65,66}. Other mammalian and yeast ERAD E3s have also been shown to undergo self-ubiquitylation (Table 1).

In addition to targeting itself for degradation, gp78 is also targeted for ubiquitylation and proteasomal degradation by another mammalian polytopic ERAD E3, Hrd1 (also known as synoviolin (SYVN or SYNO) in mice)^{67,68}. This targeting is unidirectional (Figure 3B) and, unlike the self-ubiquitylation of gp78, is independent of the ligase activity of gp78. Importantly, the targeting of gp78 for degradation is not just a function of acute manipulation, as gp78 in MEFs from *Syvn*^{-/-} mice is stabilized⁶⁷. Interestingly, co-immunoprecipitation of gp78 with Hrd1 is abrogated by mutation of the gp78 CUE domain⁶⁸, which binds ubiquitin⁶⁵. Whether this CUE domain-mediated interaction contributes to gp78 being targeted for degradation by Hrd1, or simply reflects the intermolecular 'glue' provided by domains such as CUE that bind ubiquitin chains remains to be determined. The significance of this targeting is underscored by the finding that the alteration of gp78 levels by Hrd1 impacts gp78 substrates but not those of Hrd1 (REF. 67).

Unlike gp78, endogenous Hrd1 is a relatively stable protein. Studies on its similarly stable yeast counterpart Hrd1p (also known as Der3p)⁶⁹, which has a critical role in targeting 3-hydroxyl-3-methylglutaryl (HMG) CoA reductase and thereby regulating sterol synthesis, have led to important insights into the determinants of stability for Hrd1p. In particular, the stability of this yeast RING finger E3 was found to be dependent on its stoichiometric relationship with its polytopic binding partner Hrd3p, with loss of Hrd3p leading to Hrd1p degradation⁶⁹; recently a similar role has been shown for Sel1L, which is a mammalian ortholog of Hrd3p⁷⁰. The degradation of Hrd1p is dependent on a third transmembrane component of the HRD1 ligase complex USA1p⁷¹, which is necessary for its normal function in ERAD^{71,72}. USA1p serves as part of the scaffold for oligomerization of the HRD1 complex⁷². USA1p and particularly its N-terminal ubiquitin-like domain (UBD) is required for the self-ubiquitylation and proteasomal degradation of Hrd1p⁷¹. Further, consistent with a role in oligomerization, this USA1p-dependent self-ubiquitylation of Hrd1p occurs *in trans* (i.e. one Hrd1p molecule ubiquitylates another)⁷¹. Thus, these yeast genetic studies illustrate two important concepts: first that other interacting molecules can play a critical role in self-ubiquitylation of ligases leading to degradation (see also apoptosis section below), and second that self-ubiquitylation can clearly occur between two molecules of the same ligase.

Heterologous and self-ubiquitylation in gene regulation—E3s can both self-ubiquitylate and be ubiquitylated by heterologous ligases, and the self-ubiquitylation can have functions other than targeting the ligases for proteasomal degradation. One such example is the RING1B component of PRC1 (Polycomb Repressive Complex 1), which self-ubiquitylates to increase its activity. The Polycomb Group (PcG) complex regulates

repression of transcription during *Drosophila* development via post-translational modifications of nucleosomal histones. There are two PRCs, PRC1 and PRC2. The human PRC1 contains, among other proteins, two RING finger-containing proteins, RING1B and BMI1 (B lymphoma Mo-MLV Insertion). PRC2 contains EZH2 (Enhancer of Zeste 2), which is a histone methyl transferase. The concerted histone-modifying activities of the two complexes silences gene expression. Initially, PRC2 methylates Lys27 on histone H3 (H3K27), leading to the recruitment of PRC1 (Ref.73). Following binding to methylated H3K27, RING1B catalyzes monoubiquitylation of histone H2A on Lys119. Like MdmX, BMI1 lacks intrinsic E3 activity; however, when it dimerizes with RING1B through their RING fingers, it enhances the ligase activity of RING1B towards histone H2A⁷⁴.

Besides catalyzing ubiquitylation of histone H2A, RING1B mediates its own polyubiquitylation. However, the self-ubiquitylation does not target RING1B for degradation, as mutation in the RING finger that abolishes self-ubiquitylation does not affect its proteasomal degradation⁷⁴. It seems that the self-generated ubiquitin chains do not target RING1B for degradation because, rather than forming canonical homogenous K48 or other proteasome-targeting polyubiquitin chains, RING1B generates atypical, mixed and multiply branched ('forked'; in which one ubiquitin moiety can be modified by several ubiquitins anchored to different Lys residues) K6-, K27-, and K48-linked polyubiquitin chains⁷⁴. These forked chains are probably not degraded by the proteasome⁷⁵. Strikingly, this unique modification stimulates the RING1B histone H2A monoubiquitylating activity, thus providing a novel mechanism for ligase activation through this 'non-canonical' self-ubiquitylation. This finding raised the hypothesis that RING1B must be targeted for degradation by a heterologous ligase. E6-associated protein (E6-AP; also known as UBE3A) was identified as one ligase that can target RING1B⁷⁶. It generates K48-linked chains on RING1B, which targets the protein for degradation⁷⁶. The self-ubiquitylation of RING1B and the modification by E6-AP both tag the same Lys residues on RING1B, suggesting a high level of regulation of activation versus degradation of RING1B. Both types of chains are disassembled by USP7, which stabilizes RING1B and resets its activity (the regulation of RING1B by ubiquitylation is depicted in Figure 4A). The elevated levels of RING1B and ubiquitylated H2A observed in Purkinje neurons (and other tissues) from E6-AP deficient mice⁷⁶, could play a role in aberrant transcriptional repression. This may have implications for the pathogenesis of Angelman Syndrome, a neurodevelopmental disorder caused by E6-AP deficiency.

Heterologous and self-ubiquitylation in apoptosis—The UPS plays major mechanistic roles in regulating apoptosis (reviewed in REFS 77, 78). Critical regulatory proteins involved in cell death pathways are modified and degraded by the ubiquitin system. These include, for example, the pro-survival protein Mcl-1 and the pro-apoptotic proteins Bak and Bax. Key proteins involved in related pathways are also regulated by the UPS and include, among others, p53, cell cycle regulators, DNA damage response pathways components and transcription factors such as NF- κ B. Inhibitors of apoptosis proteins (IAPs), which are critical negative regulators of cell death, are RING finger ubiquitin ligases⁷⁹. Among these are XIAP (X chromosome-linked IAP), cIAP1 (cellular IAP1) and cIAP2, which suppress cell death by inactivating pro-apoptotic regulators such as caspases. As regulation of IAPs has important roles in regulating cell death (and survival), it is not surprising that they are regulated by several mechanisms, including self-ubiquitylation to regulate ligase degradation and activity, as well as regulation by heterologous ligases^{80–82}. Thus, following an apoptotic stimulus, the pro-apoptotic protein Reaper — and possibly also Hid (Head Involution Defective) and Grim, which are members of the RHG family of proteins, induces accelerated self-ubiquitylation and rapid degradation of Diap1 (*Drosophila* IAP1). Reaper binds to Diap1 via a short IAP-Binding Motif (IBM) at its N-terminus, a binding that probably facilitates the transfer of a ubiquitin moiety from the E2 to the ligase

(REF. 81, reviewed in REF. 83). By contrast, the basal self-ubiquitylation catalyzed by the enzyme in the absence of Reaper appears to generate non-canonical K63-linked chains that do not target the ligase for degradation, but rather decrease its activity towards heterologous substrates, such as the caspase Dronc⁸². This suggests yet another novel role for self-ubiquitylation in attenuating ligase activity, which may occur under basal conditions (the regulation of Diap1 is depicted in Figure 4B). Under basal conditions it appears that Diap2 ubiquitylates Diap1, targeting it for degradation⁸².

Besides Diap2, Morgue, which has an F-box domain and an E2-like domain that lacks the active site Cys, also targets Diap1 for degradation, probably acting as an SCF E3 (REF. 84). In mammalian cells, cIAP1 and XIAP are specifically ubiquitylated and degraded following induction of apoptosis in thymocytes by glucocorticoids or etoposide. The IAPs catalyze their own ubiquitylation, which requires their RING domain. The self-ubiquitylation and degradation of IAPs may be a key event in the apoptotic program⁷⁹. Similar to the *Drosophila* RHG family members, in mammalian cells Smac3, and possibly also second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and ARTS (Apoptosis-Related protein in the TGF- β Signaling pathway), stimulate the autoubiquitylating activity, leading to accelerated degradation of XIAP following apoptotic stimuli⁸⁵. Unlike, however, the RHG family member proteins, the mammalian proteins are mitochondrial, and exit the mitochondria in response to the apoptotic stimulus. Also, unlike Diap1, it is not known whether self-ubiquitylation of mammalian IAPs leads also to the synthesis of non-canonical chains that serve non-proteolytic functions.

Because of the need for tight regulation, IAPs are also controlled by heterologous ubiquitylation. For example, XIAP1 is regulated by cIAP1 (REF. 86)(for the regulation of cIAP and XIAP, see also Figure 4C). The targeting of IAPs by one another in both *Drosophila* and mammalian cells appears to establish regulatory loops within apoptotic pathways that fine tune survival and death signals. XIAP is also regulated by the ubiquitin ligase SIAH1 (Seven In Absentia Homolog 1), a reaction that also appears to be mediated by ARTS, which binds to both proteins and appears therefore to facilitate efficient ubiquitylation⁸⁷.

THE 26S PROTEASOME

The highly abundant ~2 MDa 26S proteasome is the proteolytic arm of the UPS. It is made of two sub-complexes, the 19S regulatory particle (RP) and the 20S catalytic particle (CP), and in many cases two RPs cap either end of a CP. The CP is made of two β rings that contain the catalytic sites, each is made of seven subunits (β_{1-7}) flanked on both sides by two α -rings, also made of seven subunits each. Thus, the structure of the 20S CP is $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The RP includes a 'base' and a 'lid'. The base is composed of a hexameric ring of ATPases that function to unfold the substrate and open the gate of the interlacing N-terminal segments of the α subunits, thus allowing entry of the unfolded substrate into the catalytic chamber. The lid is involved mostly in specific recognition of the ubiquitin signal (reviewed in REF. 88) (for structure of the 26S complex see Figure 5A). Because of its complex structure, numerous targets, and the need for rapid adaptation to various pathophysiological conditions, this multi-catalytic enzyme complex is stable and not regulated by degradation. Rather, it is primarily regulated by compositional variation.

Some of the integral 20S proteolytic subunits can be replaced in an inducible and tissue-specific manner that alters proteolytic specificities and adapts it to changing needs, most notably immune challenges. In addition to the 19S cap, other proteins and complexes, such as PA28, bind to the end of the 20S cylinder and activate it by opening the 'gate'. Furthermore, proteasome-associated DUBs and E3s can remodel substrate-anchored

polyubiquitin chains, which may modulate their susceptibility for degradation. Other proteins, such as the chaperone Ecm29, stabilize the association between different sub-complexes of the 26S proteasome (reviewed in REF. 88). Consistent with its longevity, the proteasome appears to be degraded by the lysosome, probably through microautophagy⁸⁹.

Recent studies reported the specific ubiquitylation of distinct subunits of the proteasome; however, these modifications appear to serve non-proteolytic functions. Monoubiquitylation of RPN10 regulates the ability of this subunit to bind substrates by sterically inhibiting its UIM (ubiquitin-interacting motif)⁹⁰. Ubiquitylation by the RING finger E3 Not4 is essential to the integrity and function of the 26S proteasome, probably by affecting the function of Ecm29, such that, in the absence of the ligase, Ecm29 is ubiquitylated and degraded. A ubiquitylation target(s) of Not4 has not been identified; thus, the underlying mechanism of its action is unknown⁹¹. It is possible that Not4 targets the Ecm29 ligase, and in the absence of Not4, the ligase targets Ecm29. Cyclin-dependent kinase-associated protein 1 (Cks1) plays a role in transcriptional activation that is independent from its role in regulating the cell cycle⁹². This requires the Cks1 ubiquitin-binding domain, which allows it to bind to the proteasome via its ubiquitylated subunits. Cks1 can probably bind to other ubiquitylated complexes, thus displaying a broad array of transcription-regulating activities.

Several studies suggest that selective degradation of critical components of the 26S proteasome, or induced dissociation of sub-complexes, are involved in the regulation of its activity. Treatment of hippocampal neurons with the neurotransmitter NMDA (N-Methyl-D-Aspartate) leads to dissociation of the 26S to the 19S cap and the 20S core, and to proteasomal degradation of the 19S (REF. 93). The mechanism that underlies the NMDA effect is not known. It is possible that since there is also a decrease in ubiquitin conjugates following NMDA treatment (see below), the effect on the proteasome is indirect, and proteasome levels decrease when there are fewer substrates to degrade. Interestingly, it was reported that binding of polyubiquitylated substrates to the 19S RP activates proteasomal activity. This probably occurs by inducing conformational changes in the 20S CP that stabilize the gate opening of the α subunits and thereby facilitate channeling of substrates into the 20S and their access to its active sites. Although it has not been shown experimentally, this crosstalk between the 19S RP and 20S CP, and the stabilization of protrusion of the N-termini of the α subunits into the 19S RP, may contribute to the strengthening and stabilization of the association between the two sub-complexes^{94,95}. Also, since the proteasome is involved in the endocytosis of glutamate receptor (for which NMDA is a ligand), the effect of NMDA on the proteasome may serve to potentiate the excitatory influence of the transmitter by inhibiting receptor endocytosis and subsequent degradation. It is assumed that the 19S dissociates into individual subunits prior to its degradation, although evidence for their ubiquitylation is lacking. Along with the 19S, two of its associated E3s, E6-AP and HUWE1 (HECT, UBA, and WWE domain-containing protein 1), are also degraded in response to NMDA⁹³. It is possible that the destruction of the proteasome-associated ligases suppresses conjugation and degradation, and stabilizes a subset of proteins required for synaptic activity during NMDA excitation.

In another study it was reported that activation of apoptosis results in caspase-mediated cleavage of the proteasomal subunits S6' (Rpt5), S5a (Rpn10), and S2 (Rpn1), resulting in proteasome inactivation⁹⁶. As a result, pro-apoptotic proteins, such as Smac that are targeted by the UPS are stabilized, which is assumed to facilitate the execution of the apoptotic program. Interestingly, in myotubes, caspase-3-mediated cleavage of Rpt2 and Rpt6 increases proteasomal activity. This appears to be a specific feed-forward mechanism that accelerates proteolysis in muscle during catabolic states⁹⁷. Oxidative stress has been shown to induce disassembly of the proteasome to its sub-complexes⁹⁸. It was suggested that this dissociation protects cells by enabling the released 20S CP to degrade the oxidized proteins

that are generated under these conditions, which bypasses the need for ubiquitylation, as the 20S can degrade proteins in a ubiquitin-independent manner *in vitro*. It is unclear, however, whether the 20S can degrade cellular substrates *in vivo*, as several strong lines of evidence suggest that even unfolded, oxidized and otherwise damaged proteins are degraded in the cell via a ubiquitin-dependent mechanism. Thus, in one study it was demonstrated that degradation of damaged cellular proteins exposed to heat, cadmium or paraquat, required the E2s Ubc4 and Ubc5, the proteasomal subunit RPN10, and the CDC48-Ufd1-NPL4 complex⁹⁹. Also, the absolute requirement for ATP for all types of protein degradation suggests a need for ubiquitylation, which is ATP-dependent, and/or the 26S complex, which requires ATP for its assembly and function. By contrast, degradation by the 20S CP is energy-independent. An independent study that supports the notion that the 20S proteasome is inactive in cells was described in yeast, when during the stationary phase, the 26S proteasome similarly dissociates¹⁰⁰. In this case, the released 20S is inactive, as the N-termini of the α chains at either end of the CP remain interlaced, thus the entry gate to the CP is closed. This study suggests that dissociation is essential to slow proteolysis to maintain viability during nutrient shortage in the stationary phase. Regulation of the 26S proteasome by association-dissociation of its sub-complexes and degradation of its different subunits is described in Figure 5B.

PERSPECTIVE

Ubiquitin, ubiquitin-conjugating enzymes and ubiquitin-protein ligases are not exempt from the powerful regulation of protein fate and function that is conferred by the UPS, and it is likely that individual proteasome components are also targeted. Deubiquitinating enzymes are similarly targeted for ubiquitylation, although here the role of ubiquitylation appears to generally not result in proteasomal degradation.

In considering the degradation of ubiquitin, a number of critical questions remain. Perhaps foremost among these is why is ubiquitin degraded. In many ways, one can view this polypeptide as a reversible modifier that should not be consumed as part of the major process that it facilitates — proteasomal degradation. Yet, it is degraded. Is it simply degraded as bystander or is its degradation integral to the mechanism by which ubiquitylated substrates are fed to the 26S proteasome? Beyond this, although we know free cellular ubiquitin levels vary with different conditions, we have little insight into how degrading ubiquitin might play a part in cellular homeostasis or might benefit the organism during development, stress and in other settings.

The regulation of E2 and E3s by ubiquitin is a fascinating area where we are clearly just beginning to scratch the surface. We now understand that the self-ubiquitylation that is stimulated in the test tube is more often a reflection of what can occur *in vivo* than we may have originally perceived. But what cellular factors determine whether any particular E3 will target itself or another E3 for ubiquitylation? And among the types of ubiquitin modifications that can occur through this process, what determines the type of linkage generated and thus the fate of the protein? Although E3s can clearly target one another among the examples discussed herein, Nedd4s-Cbls, Hrd1-gp78, E6-AP-RING1B, CIAP1-XIAP1 and Diap2-Diap1, this apparently occurs in a unidirectional manner. Why this vectorial ubiquitylation occurs is not intuitively obvious as, in many cases, ubiquitylation occurs as a result of juxtaposition of the ligase and any of a number of different lysines or other acceptors on a substrate either naturally or through artificial targeting. One possible explanation is that acceptor residues are somehow exposed on one ligase and not the other when the two associate, although there are certainly a number of other possibilities. Regardless, of the mechanism of association, the basis for these particular asymmetrical

relationships between E3s represents new physiological and mechanistic questions to add to the yet unanswered questions about the functions and mechanics of ubiquitylation.

Finally, and related to the previous point, with phosphorylation there exist multiple examples of kinase cascades. In the examples cited herein we deal at the most with two ligases. We should consider the possibility that within the dense cellular milieu, linear or pyramidal cascades of ubiquitylation of E2s and E3s are taking place (Figure 2Bc). These probably include bidirectional ubiquitylation of ligases with the dynamics of ubiquitylation regulated in part by specific DUBs. As with the ubiquitin system in general, should such cascades exist, they are, in all probability, exquisitely regulated in a temporal-spatial manner.

Acknowledgments

Space constraints do not allow us to cite many of the studies in this evolving, yet already prolific research area, and we apologize for that. Research in the laboratory of A.M.W. is supported by the National Institutes of Health, National Cancer Institute, Center for Cancer Research. Research in the laboratory of A.C. is supported by grants from the Dr. Miriam and Sheldon Adelson Foundation for Medical Research (AMRF), the Israel Science Foundation (ISF), the German-Israeli Foundation for Research and Scientific Development (GIF), the Deutsch-Israelische Projektkooperation (DIP), and Rubicon - the European Union (EU) Network of Excellence studying the *Role of Ubiquitin and Ubiquitin-like Modifiers in Cellular Regulation*. A.C. is an Israel Cancer Research Fund (ICRF) USA Professor.

GLOSSARY

UNFOLDED PROTEIN RESPONSE (UPR)

A cellular response that is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum (ER) and that results in the transcriptional upregulation of ER chaperones and degradative enzymes and a general inhibition of protein synthesis

MICROAUTOPHAGY

Formation of vacuoles containing a small portion of the cytosol that is digested by the lysosomal enzymes following the destruction/dissolution of the surrounding membrane. The process occurs under basal metabolic conditions, and unlike stress-induced macroautophagy, the vacuoles are small, and their generation does not involve formation of new membrane and engulfment and digestion of membrane-limited organelles

CATALYTIC (CORE) PARTICLE

The 20S core particle of the 26S proteasome. It is made of four rings two external α rings that are made each of seven distinct subunits (which are identical between the rings), and two adjacent β rings, made also of seven distinct subunits (which are also identical between the rings). Three of the seven β subunits are proteases with distinct cleaving activities

REGULATORY PARTICLE (RP)

The 19S complex of the 26S proteasome consists of two sub-complexes – the base and the lid, which are linked together. The base contains the ATPases that are involved in unfolding of the substrate and in opening the entry to the catalytic 20S sub-complex. The lid contains the polyubiquitin chain-recognizing subunits. The RP also includes DUB(s) that recycle ubiquitin

CANONICAL UBIQUITIN CHAINS

Lys48-based chains that are well-characterized as being proteasome targeting signals

HELPER T CELLS

T cells that functions as an inducer of the effector cells for humoral and cell-mediated immunity. These cells recognize and bind to antigen

UBIQUITYLATION

Known also (and originally described) as ubiquitination. This is the multi-step process leading to the modification of a protein with single ubiquitin molecules or chains of ubiquitin

DEUBIQUITYLATING ENZYME

Known also as deubiquitinating enzyme or ubiquitin protease. These enzymes play multiple roles including in processing ubiquitin precursors, in disassembling and trimming ubiquitin chains and in specifically antagonizing the activity of ubiquitin-protein ligase in general or towards specific substrates

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Online ‘at-a-glance’ summary

- Monomeric ubiquitin is relatively stable, however it appears to be degraded by the proteasome following its own ubiquitylation mediated by the TRIP12 ligase. Ubiquitin is also degraded via two other mechanisms: along with the target substrate as part of the polyubiquitin chain attached to it, and along with a peptide attached either linearly or in an isopeptide bond to its C-terminal Gly residue.
- Ubiquitin-protein ligases (E3s) are largely responsible for conferring substrate specificity to the ubiquitin-proteasome system (UPS). An increasing number of these are being shown to be subject to selfubiquitylation (autoubiquitylation), ubiquitylated by heterologous ligases or both. In some cases both selfubiquitylation and ubiquitylation by heterologous ligases lead to degradation of the protein. In other cases self-ubiquitylation can regulate the cellular function of the ligase while ubiquitylation by a heterologous E3 results in degradation of the target ligase.
- Other components of the ubiquitin proteasome system including ubiquitin-carrier enzymes (E2s) and deubiquitylating enzymes are also subject to ubiquitylation.
- Components of the ubiquitin system are also subject to modification by other ubiquitin-like protein modifiers.
- The 26S proteasome is a stable, long-lived complex, and is probably degraded via microautophagy. As part of the response to some specific cellular signals, such as oxidative stress, starvation, and stimulation of the N-methyl-D-aspartate (NMDA) receptor, it is disassembled into its two subcomplexes, the 19S regulatory particle (RP) and the 20S catalytic (or core) particle (CP). The RP is probably dissembled into its individual subunits, which are degraded by the proteasome following ubiquitylation. Caspase-mediated cleavage of specific 19S subunits has also been shown to regulate proteasomal activity under certain conditions.
- The effect of disassembly of the 26S proteasome on the 20S complex has remained unclear: in some cases it was shown to inhibit its activity, to avoid damage of uncontrolled degradation, whereas in others it has been shown to be stimulate activity and to efficiently remove excess damaged proteins.

Figure 1. Schematic of the UPS

(i) Ubiquitin is activated by the ubiquitin-activating enzyme (E1), which uses ATP to form a high energy labile E1–thiol ester intermediate. (ii) The activated ubiquitin is transferred to a ubiquitin carrier protein (ubiquitin-conjugating enzyme; E2) to generate a similar thiol ester intermediate. (iii)a) Activated ubiquitin can be transferred from the E2 to a HECT (Homologous to the E6-AP C-Terminus) domain ubiquitin protein-ligase (E3) to generate a third labile thiol ester intermediate. It is then transferred to the substrate (S), where, in most cases, a stable isopeptide bond between the activated carboxy terminal Gly76 of ubiquitin and an ϵNH_2 group of an internal lysine residue in the substrate is formed. Additional ubiquitin moieties are then added to generate a polyubiquitin chain. (iii)b) Alternatively, activated ubiquitin can be transferred directly from E2 to an internal lysine residue in a substrate that is bound to a RING finger ubiquitin ligase. Polyubiquitin chains linked through Lys48 of ubiquitin are characteristically associated with proteasomal degradation. However, linkages through several of the other seven lysines in ubiquitin have recently been shown to also target for proteasomal degradation^{119–122}. In some cases, ubiquitin is conjugated to the αNH_2 group of the target substrate (reviewed in REF. 24), and there are a few examples of conjugation to internal substrate Thr, Ser, or Cys residues^{122–128}. Certain substrates are recognized by several ligases that act either under different conditions, sequentially or in parallel, recognizing different motifs in the substrate. Such a mechanism may be necessary to ascertain recognition and degradation of different domains in the substrate. (iv) Degradation of the ubiquitin-conjugated substrate to short peptides by the 26S proteasome. The binding of ubiquitinated proteins to the proteasome can be direct or mediated or enhanced by shuttle proteins (v) Most of the ubiquitin chain is disassembled by deubiquitinating enzymes (DUBs), the rest is probably degraded along with the substrate.

Select substrates are processed rather than completely degraded by the 26S proteasome. This is due, at least in part, to unstructured sequences (for example, GlyAla repeats) that may inhibit entry into the proteasome, although additional mechanisms may be involved.

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Figure 2. Modes of degradation of ubiquitin and ubiquitin-protein ligases

A. Ubiquitin can be degraded in three ways. (Aa) It can be degraded as a monomer following its polyubiquitylation (indicated in light blue). The process is probably slow, as the ubiquitin substrate molecule is short and lacks an unstructured tail that appears to be an important structural characteristic necessary for efficient proteasomal degradation. (Ab) Ubiquitin can be degraded with its conjugated substrate. The proximal part of the polyubiquitin chain is probably degraded along with the substrate, whereas the distal part is recycled by DUBs as free and reusable ubiquitin. (Ac) Ubiquitin with a C-terminal tail unstructured tail that is longer than 20 amino acid residues can be degraded efficiently by the proteasome. **B. Modes of degradation of ubiquitin-protein ligases by ubiquitylation.** (Ba) Degradation of a ligase mediated by self-ubiquitylation. Both RING and HECT domains-containing ligases can catalyze self-ubiquitylation, which generate polyubiquitin chains that target the proteins for proteasomal degradation. Self ubiquitylation is shown as occurring *in cis* (i.e. on the same molecule). However, as many E3s dimerize and may form higher order oligomers, ubiquitylation can also occur *in trans* as discussed in the text for Hrd1p. (Bb) Ubiquitylation of a ligase by a heterologous ligase. Whereas in the described cases of this mode of ubiquitylation the reaction is unidirectional (see Text and Figures 3 and 4), bidirectional ubiquitylation is also a possibility. (Bc) Hierarchical ubiquitylation of ligases. In this hypothetical scheme, one ligase targets several substrate ligases in a hierarchical manner. As has been described for several substrates of the ubiquitin system, one ligase substrate can be targeted by more than a single ligase (middle ligase in lower row and left ligase in middle row). The ligase at the top is targeted by self-ubiquitylation or by one of the ligases that it directly or indirectly regulates.

Figure 3. Targeting of Specific Ligases for Degradation by both Self- and Heterologous Ubiquitylation

A. Regulation of Cbl proteins and effects on signaling. Left hand side: (i) Binding of ligand induces dimerization and cross-phosphorylation of receptor tyrosine kinases (for example, EGFR – represented here) or receptor-associated tyrosine kinases as well as phosphorylation of other substrates, including other components of the signaling complex and Cbl proteins. The receptors together with other components of the signaling complex (not shown) are endocytosed and phosphorylated; Cbl proteins are recruited. (ii) Cbls mediate the ubiquitylation of receptors and associated proteins as well as self-ubiquitylation, resulting in targeting of receptors for lysosomal degradation and the destruction of Cbl molecules – whether this degradation is lysosomal or proteasomal has not been determined with certainty. (iii) The net result is decreased recycling of receptors to the cell surface and downregulation of signaling. Right hand side: (iv) Nedd4 family members target Cbl proteins for proteasomal degradation lowering the cellular levels of these E3s. (v) As a result ubiquitylation of activated receptor tyrosine kinase complexes is decreased. This results in attenuation of receptor down-regulation leading to increased mitogenic signaling. **B. gp78 and ERAD.** (i) gp78 is a polytopic RING finger E3 found in the ER that mediates self-ubiquitylation using the E2, Ube2g2. This activity requires an intact gp78 RING finger, the ubiquitin binding CUE domain (whose function in this process is not yet clear) and a highly specific binding site for Ube2g2 that is distinct from the RING finger⁶⁵. (ii) gp78 can additionally be targeted for ubiquitylation by a heterologous RING finger E3, Hrd1 (human Hrd1)/Synoviolin. This targeting is dependent on the Hrd1/Synoviolin RING finger but is independent of the gp78 RING finger. Both of these modes of degradation lead to proteasomal degradation of gp78 and consequently increased levels of gp78 substrates

including Insig-1, and KAI1 (also known as CD82), which are regulators of cholesterol metabolism and a metastasis suppressor, respectively.

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Figure 4. Regulation of specific ligases

A. Regulation of RING1B by ubiquitylation. (i) Self-ubiquitylation of RING1B generates ‘non-canonical’ - mixed (K6-, K-27-, and K-48-based) and multiply branched polyubiquitin chains that stimulate the monoubiquitinating activity of the ligase towards histone H2A, but do not target it for degradation. (ii) The HECT E3 E6-AP (and possibly an additional ligase) targets RING1B for proteasomal degradation by generating K48-based polyubiquitin chains. Both the self and the E6-AP-catalyzed ubiquitylations target the same lysine residues on RING1B, thus preventing opposing and wasteful activities - destruction of an active and needed enzyme, or untoward activation of an un-needed enzyme. (iii) USP7 de-ubiquitylates both the activating and the proteasome-targeting polyubiquitin chains. Its activity releases free RING1B, thus enabling inactivation, and at the same time stabilization of RING1B without destroying it. **B. Regulation of Diap1.** (i) Self-ubiquitylation of Diap1 generates K63-based polyubiquitin chains that attenuate the ligase activity towards its substrates such as caspases. (ii) Diap2 targets Diap1 for proteasomal degradation by generating K48-based polyubiquitin chains on Diap1. (iii) Following an apoptotic stimulus, Reaper, Hid and

possibly Grim (which are known as the RHG family) induce accelerated self-ubiquitylation of Diap1, generating K48-based chains that target the protein for degradation. (iv) Morgue generates K48-based chains either independently, or in conjunction with the RHG family members. These chains target Diap1 for proteasomal degradation. **C. Regulation of mammalian IAPs.** In mammals both cellular IAP1 (cIAP1) and X chromosome-linked IAP (XIAP) can catalyze their self-ubiquitylation, generating K48-based chains that probably target them for proteasomal degradation. It is not known whether, like *D. melanogaster* IAP1 (Diap1), they can also generate non K48-based chains that serve non-proteolytic functions. Intact cIAP1 (or its RING finger domain alone) was shown to catalyze formation of K48-based chains on XIAP1 that target it for degradation. It is not known whether XIAP and/or another ligase can catalyze a similar reaction, targeting cIAP1 for degradation. The ligase SIAH1 can mediate generation of K48-based chains that target XIAP for degradation. This reaction is mediated by ARTS that probably allows for association of SIAH1 and XIAP. Similar to the RHG family members, Smac3 (and possibly Smac/DIABLO and ARTS) can induce accelerated auto-ubiquitylation of XIAP, which leads to its degradation. Solid arrows mark experimentally established ubiquitylations, broken arrows denote putative ubiquitylations. Ubiquitin-conjugating enzymes (E2s) are not represented graphically in this figure to reduce complexity, but should be assumed to be involved in all ubiquitylation reactions.

Figure 5. The 26S proteasome and its regulation by degradation

A. Structure of the 26S proteasome. The 26S proteasome is comprised of two sub-complexes - the 20S core catalytic particle (CP), and the 19S regulatory particle (RP). The substrate is first bound, probably via its polyubiquitin chain(s), to specific subunits in the 19S RP. It is then probably unfolded by ATPases residing in the base of the RP (Rpt ring), and inserted via an open gate in the α -ring of the 20S CP into the proteolytic chamber. The ATPases are probably also involved in opening the interlacing chains that close the gate into the α -ring of the CP, thus allowing entry of the unfolded substrate. The RP contains also DUB(s) that recycle ubiquitin and/or edit the polyubiquitin chain to enhance substrate binding to the RP and to recycle ubiquitin. A RP-associated ubiquitin ligase(s) may also function to adapt the chain length/type for optimal recognition by the RP. Proteolysis of the substrate is mediated by three pairs of proteolytically active β subunits inside the proteolytic chamber, which includes two adjacent β rings. **B. Regulation of assembly and disassembly of the 26S proteasome, and degradation of its subunits.** The entire proteasome or its sub-complexes are probably degraded by the lysosome via microautophagy. Different factors control assembly (ATP, Ecm29, ubiquitylated substrates, and proteasome inhibitors, for example) and disassembly of the proteasome to its RP and CP (for example, NMDA (*N*-methyl-D-aspartate) and different stresses, such as oxidative stress and starvation). The RP can be further disassembled into its individual subunits, which are probably degraded by proteasomes following ubiquitylation. For further reading on the proteasome structure and function, see REF. 88.

Table 1

Regulatory modification of the UPS by ubiquitin and UBL

Substrate	Targeting Enzyme(s)	Comments (substrate)	Refs.
<i>E3s as ubiquitylation targets of E3s</i>			
Mdm2	Self	Primary p53 E3	See text
E6-AP	Self	Targets p53 with HPV E6; notably self- ubiquitylation blocked <i>in vitro</i> by p53 and HPV E6 suggesting substrate-mediated inhibition of E6- AP	129, 130
Cbl E3s	self, Nedd4 and Itch WW domain HECT E3s	Critical E3s in modulating transmembrane signaling	See text
gp78	self, Hrd1/synoviolin	Pro-metastatic ERAD E3, multiple substrates including KAI1, INSIG-1	See text
TEB4	Self	ERAD E3; several substrates including the bile salt export pump and iodothyronine deiodinase; mammalian ortholog of Doa10	131–133
Hrd1p/Der3p	Self	A major ERAD E3 of yeast implicated in degradation of multiple substrates including HMG-CoA Reductase, the limiting enzyme in sterol biosynthesis.	See text
Cdc20	APC/C ^{Cdh1}	Substrate recognition element of APC/C; must be inactivated after M phase	See Box 1
Cdh1	APC/C ^{Cdh1} , SCF ^x	Substrate recognition element of APC/C; must be inactivated in G1 to inactivate APC/C	See Box 1
Skp2	APC ^{Cdh1}	Substrate recognition element of SCF; F-box protein that targets p27 for degradation; Skp2 degradation prevents premature entry into S phase	See Box 1
F-box components of SCF E3s: Cdc4, Grr1	SCF, possibly other E3s.	Degradation regulates free levels of these F-box proteins, perhaps optimizing SCF function	134
RING 1B	Self, E6-AP	PRC1 component, targets H2A for mono- ubiquitylation	See Text
cIAP-1	Self	Targets itself for proteasomal degradation in RING-dependent manner	See text
DIAP-1	Self, DIAP2	<i>D. melanogaster</i> inhibitor of apoptosis	See text
TRAF-2	cIAP-1	Integral to NF- κ B signaling pathways and functions by catalyzing non-proteasome-targeting K63 chains; cIAP-1 together with TNFR-II targets TRAF-2 for proteasomal degradation	135
XIAP	SIAH	The mitochondrial protein ARTS (Apoptosis- Related protein in the TGF- β Signaling pathway), which is a potential tumor suppressor, serves as an obligatory adaptor in this process and is lost in malignancies	See Text
BRCA1 (Breast cancer- Associated 1)	HERC2	BRCA1 mutations or loss implicated in breast and ovarian cancer; degraded in the absence of its binding partner, BARD1; HECT E3 HERC suggested to be responsible for targeting of BRCA1 for degradation in the absence of its binding partner BARD1	136
FANCM	SCF ^{β-TrCP}	Targeted for degradation before M phase, this prevents inappropriate activation of FANCM complex and chromosomal abnormalities	137
RNF8, RNF168	HSV-ICP0	Targeting of these DNA repair E3s facilitates lytic activity of Herpes Simplex Virus	138
MARCH7	self	Implicated in neuronal development and lymphocyte proliferation; deubiquitinated and stabilized by USP9X in the cytosol and by USP7 in the nucleus	139
Ro52	self	Normal function unknown; substrates include phosphorylated IKK β when HTLV-1 Tax is expressed, resulting in monoubiquitylation and downregulation of NF κ B signaling;	140,141

Substrate	Targeting Enzyme(s)	Comments (substrate)	Refs.
		target of autoantibodies in Sjogren's syndrome; deubiquitylated by Usp4 (see DUB table below)	
DUBs as ubiquitylation substrates			
Usp6	Unknown	Oncogenic DUB; monoubiquitylation is dependent on calmodulin binding; function unknown	142
UCH-L1	Unknown	Parkinson's disease-associated DUB; monoubiquitylation serves to inhibit interaction with ubiquitylated potential substrates	143
Ataxin-3	gp78, others?	Implicated in protein quality control including ERAD; implicated in poly-Q disease spino-cerebellar ataxia type 3 or Machado-Joseph disease; ubiquitylation reported to increase catalytic activity and not alter preference for K63 chains; ubiquitylation by gp78 targets for degradation	144,145,146
Usp4 (UnpEL)	Ro52 (see above)	Function unknown	147
Usp7 (HAUSP)	HSV-ICP0; endogenous E3s not established	Targeted for ubiquitylation and proteasomal degradation by ICP0; also reported to be both ubiquitylated and Neddylated in cells not expressing ICP0	148,149
Usp25	Unknown	Contains UBA and UIM important for monoubiquitylation on specific Lys and for function; monoubiquitylation activates DUB activity towards its substrate, MyBPC1; sumoylation of the same Lys has opposing effect	150
E2s as self-ubiquitylation or E3 targets			
Ubc7p	Self	Yeast ERAD E2; functions with Hrd1p and Doa10p; ubiquitylation dependent on intrinsic activity and does not require other E2s; self-ubiquitylation occurs <i>in vitro</i> , whether an E3 is required for ubiquitylation <i>in vivo</i> is unknown	31,151,152
Ubc6p	Doa10p	Yeast ERAD E2; ubiquitylation dependent on both Doa10p and itself	153
UbcH10	APC ^{Cdh1}	Critical E2 in APC/C function; degraded by the E3 as part of the process leading to APC/C inactivation in G1	See Box 1
Cross-targeting between ubiquitination enzymes and UBL enzymes			
Ubc9p	Self	Yeast E2 that mediates SUMO modification; self-sumoylation decreases activity towards some substrates	154
E2-25K (Ube2K)	Ubc9	Sumoylation on N-terminal helix decreases ability to be loaded by ubiquitin E1	155
CRL Cullins	Self (Rbx1 component of CRLs) and DCN4	Modification with Nedd8 critical to activation of CRLs	reviewed in Ref. 156