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Functions of the 19S Complex in Proteasomal Degradation

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Abstract

The 26S proteasome degrades ubiquitylated proteins. It consists of the 20S proteasome and the PA700/19S complex. PA700 plays essential roles in processing ubiquitylated substrates; it can bind, deubiquitylate, and unfold ubiquitylated proteins, which then translocate into the proteolytic chamber of the 20S proteasome for degradation. Here, we summarize the current knowledge of PA700-mediated substrate binding and deubiquitylation, and provide models to explain how substrate binding and deubiquitylation could regulate proteasomal degradation. We also discuss the features and potential therapeutic uses of the two recently identified small molecule inhibitors of the proteasome-residing deubiquitylating enzymes.

Keywords

deubiquitylating enzyme; proteasome; ubiquitin; polyubiquitin chain; deubiquitylation and protein degradation

The 26S proteasome and degradation of ubiquitylated proteins

The 26S proteasome is composed of two large subcomplexes: the 28-subunit 20S proteasome and the 19-subunit PA700 complex (also called the 19S complex or 19S regulatory particle), which have a collective molecular weight of approximately 2.5 mDa [1,2]. The 26S proteasome is the protein processing machine that degrades ubiquitylated proteins in eukaryotes (Box 1). The PA700/19S complex possesses the ability to bind, deubiquitylate and unfold ubiquitylated proteins, which prepares the substrate proteins for translocation into the 20S proteasome for degradation. Several recent studies depicted subnanometer-level electron microscopic (EM) structures of the 26S proteasome isolated from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or human cells [3–6] (Figure 1a). The overall topologies of the 26S proteasomes from these species share remarkable similarity and several new structural features of the PA700/19S complex were unveiled. For example, the catalytic domain of the deubiquitylating enzyme (DUB) Rpn11 is positioned right above the pore formed by six ATPases (Figure 1b). The two integral ubiquitin (Ub) receptors of the 26S proteasome, S5a/Rpn10 and Adrm1/Rpn13 (in this review, human orthologs are listed before yeast homologs), sit on opposite sides of Rpn11 (Figure 1b). Rpn2, the largest proteasomal subunit, interacts with several non-ATPase subunits (Figure 1a), presumably stabilizing the PA700/19S complex. The 20S proteasome has a barrel-

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shaped structure consisting of four hetero-heptameric rings [7], in which the central chamber, formed by the β subunits, houses peptidase activities [7]. To gain access to the proteolytic sites, protein substrates have to pass through the substrate translocation channel, which consists of the two rings formed by six ATPases of the PA700/19S complex, and the α chamber formed by α subunits of the 20S proteasome. The substrate translocation channel can only accommodate unfolded polypeptides [8,9], thus substrate unfolding, performed by the ATPases, is a prerequisite for degradation of ubiquitylated folded proteins.

26S proteasome-mediated protein degradation is powered by ATP hydrolysis [10,11], which likely coordinates with substrate binding, deubiquitylation, unfolding, translocation and peptide hydrolysis [12]. In the past few years, great progress has been made in understanding how substrates are recognized and deubiquitylated. Here, we summarize these new findings and provide potential models to explain the versatile routes for substrate recognition and the double-edged effect of substrate deubiquitylation in regulating proteasomal degradation. In addition, we discuss the features of two recently identified small molecules that target the proteasome-residing DUBs and regulate proteasomal degradation.

Substrate recognition on the 26S proteasome

Ub recognition subunits

The two integral Ub receptors of the 26S proteasome, S5a/Rpn10 and Adrm1/Rpn13, are located on the periphery of the PA700/19S complex (Figure 1b). Neither of them is essential for maintaining the structural integrity of the 26S proteasome [13]. Human S5a and yeast Rpn10 both bind the 26S proteasome through their N-terminal regions, and bind polyubiquitin (polyUb) chains through at least one C-terminal Ub interacting motif (UIM) domain [14-16]. However, S5a has two UIMs, named UIM1 and UIM2, whereas Rpn10 contains only UIM1. Human Adrm1 and its yeast ortholog Rpn13 both contain a pleckstrin-like receptor for Ub (Pru) domain that binds Ub chains [13,17]. However, Adrm1 has a unique C-terminal region that is absent from Rpn13 [18-23]. Interestingly, this C-terminal region was found to bind and activate the DUB Uch37 [20,22,23], which is absent in budding yeast. The Ub receptors might function differently in targeting proteins for degradation in mammals and yeast. For instance, knockout of the *S5a* homolog of mouse (*mS5a*) or expressing *mS5a* lacking the UIM domains (*mS5a Δ UIM Δ UIM*) caused embryonic lethality, and mice expressing *mS5a Δ UIM Δ UIM* showed impaired proteasomal degradation [24]. Thus, S5a might play a critical role for degradation of some proteins in mammals. This is supported by data suggesting that S5a could be the major docking site for substrates of endoplasmic reticulum-associated protein degradation [25]. By contrast, deletion of *RPN10* or *RPN13* had a less dramatic effect on proteasomal degradation in yeast [13], likely because their function can be compensated by several proteasome-associating Ub shuttling proteins, including Rad23, Ddi1 and Dsk2 [1,26]. These Ub shuttling proteins contain both Ub-like and Ub-associated domains, which serve to bind the 26S proteasome and ubiquitylated proteins, respectively. Thus, they are able to shuttle ubiquitylated substrates for proteasomal degradation [1,26].

Mechanism of Ub recognition

Several models are plausible for substrate recognition by the two integral Ub receptors of the 26S proteasome. In one model, a conjugated Ub chain is tethered to the proteasome by binding either of the two Ub receptors (Figure 2a (i) and (ii)). S5a prefers binding to polyUb chains [14,15], likely by utilizing its two UIMs to simultaneously bind two Ub moieties in a Ub chain [16,27]. The Pru domain in Adrm1/Rpn13 was found to bind di-ubiquitin (diUb) and longer Ub chains with high affinity [13,17]. In addition, both S5a/Rpn10 and Adrm1 can

also bind monoUb with weak affinity, which could allow the 26S proteasome to recruit proteins with a Ub-like domain [13,28], such as Ub shuttling proteins, and recognize some monoubiquitylated proteins for degradation [29-33]. In this regard, S5a was recently found to mediate degradation of monoubiquitylated proteins in human cells [29]. In another model of Ub recognition by the proteasome, the two Ub receptors could bind a conjugated Ub chain coordinately (Figure 2b). A structural study has determined that purified S5a and Rpn13 could concurrently bind K48-linked diUb [27], suggesting that S5a/Rpn10 and Adrm1/Rpn13 might coordinate to bind polyUb chains. On the proteasome, S5a/Rpn10 and Adrm1/Rpn13 are separated by approximately 90 Å [4,34]. A four-ubiquitin chain (tetraUb) is expected to fit into the distance between the two Ub receptors [4,34], which might explain why tetraUb is the minimal chain length with high proteasomal binding affinity [35]. In a third model, substrates conjugated with multiple short Ub chains could be recognized by one (Figure 2c (i)) or both of the two Ub receptors, each binding a Ub chain (Figure 2c (ii)). In support of this, it was recently found that multiple monoUb or short Ub chains are sufficient to target some proteins for proteasomal degradation in mammalian cells [30,31].

Proteasomal substrates have diverse ubiquitylation features, including variable chain linkages, lengths and quantities, therefore, recognition modes other than those described are plausible, especially when considering Ub shuttling proteins [1,26]. The multiple Ub receptors on the 26S proteasome enable versatile routes for substrate binding, which is presumably necessary for the 26S proteasome to handle a large quantity of differentially ubiquitylated proteins in cells. Currently, little structural information is known about how the 26S proteasome binds polyUb chains. The unambiguously assigned subunit organization of the PA700/19S complex [3-6], including Ub receptors, offers an opportunity to employ single particle cryo EM to determine the structures of the 26S proteasome bound to various polyUb chains.

The role of disordered regions in substrate recognition

Bioinformatics studies suggest that large disordered regions are present in the majority of proteins in higher eukaryotes [36]. The presence of a disordered region in a ubiquitylated substrate promotes its degradation by the 26S proteasome [37-39]. Functionally, the disordered regions directly bind the 26S proteasome [38] and initiate substrate translocation [37,38,40]. Presumably, binding of the disordered region and binding of the conjugated Ub/polyUb coordinate to mediate substrate recognition (Figure 2). The high proteasomal binding affinity of polyUb chains would target substrates to the 26S proteasome, then a disordered region in the substrate would 'seek' and bind to the ATPase(s) to prepare for translocation. Strikingly, a recent study has shown that a certain distance between the polyUb chain and the disordered region must be maintained, because too short or too great a distance could not support degradation [41]. This might reflect the organization of the binding sites for both polyUb chains and disordered regions on the 26S proteasome. The requirement of both a polyUb chain and a disordered region to target a substrate makes sense in regard to coupling substrate binding with unfolding and translocation. Addressing several key issues would further validate this model. For instance, it is important to identify the proteasomal subunit(s) that directly binds disordered regions of substrates, and to identify the features of disordered regions that mediate the interaction, because not all disordered regions have the ability to bind the 26S proteasome [38]. After the initial targeting, the substrate engages with the substrate translocation channel, and deubiquitylation is required for efficient degradation.

Substrate deubiquitylation in proteasomal degradation

The 26S proteasome-residing DUBs

The human 26S proteasome has three DUBs, Rpn11, Usp14 and Uch37, which belong to different DUB families (Box 1). Rpn11 is a JAMM domain-containing metalloprotease. Usp14 belongs to the USP family. Uch37 is one of the four members in the UCH family. Rpn11 is an integral subunit of the 26S proteasome [3,4], Usp14 and Uch37 are proteasome-associating proteins. The positions of Usp14 and Uch37 were not revealed in the recently determined EM structures of the 26S proteasome [3-6]. Rather, their positions were inferred through their interactions: Usp14/Ubp6 was found to bind Rpn1 [42], and Uch37 could bind both Adrm1 and S5a/Rpn10 [20,22,23,43]. The DUB activities of Rpn11, Usp14/Ubp6 and Uch37 are activated when they assemble into or associate with the 26S proteasome, although the activating mechanisms are still mysterious.

Functionally, *RPN11*, *USP14* and *UCH37* were found to be important for development and cell viability. For instance, deletion of *RPN11* or substitutions of some conserved residues in the putative catalytic site of Rpn11 caused lethality in yeast [44-46]. Mice bearing a loss-of-function mutation in *USP14* developed severe tremors by 2-3 weeks of age, followed by hindlimb paralysis and death by 6-10 weeks of age [47]. This phenotype might relate to the depletion of free Ub [48], because more substrate-conjugated Ub is degraded, rather than recycled [49]. Deletion of *UCH37* resulted in prenatal lethality in mice, which was associated with severe defects in embryonic brain development [50]. It is not known whether all of these phenotypes relate to their DUB activities on the 26S proteasome, because these proteins could play proteolysis-independent functions as well [49,51-55]. However, the DUB activities of Rpn11, Usp14 and Uch37 clearly play critical roles in regulating proteasomal degradation.

Rpn11-mediated degradation-coupled deubiquitylation

Rpn11 is the only DUB identified so far that, when incorporated into the 26S proteasome, catalyzes Ub chain amputation to release the whole Ub chain from the substrate [44]. Two elegant studies have demonstrated that Rpn11-catalyzed deubiquitylation couples with degradation and requires ATP hydrolysis [44,45], although Rpn11 itself does not bind or hydrolyze ATP. The JAMM domain of Rpn11 is located right above the pore formed by the ATPases (Figure 1b). This unique localization might provide a potential mechanistic explanation for the degradation-coupled deubiquitylation event. As discussed earlier, both a polyUb chain and a disordered region in the substrate are recognized by the 26S proteasome [38,39,41]. A disordered region of the substrate likely interacts with the ATPase(s), and then diffuses into the substrate translocation channel by passing through the ring formed by the ATPases (Figure 3a). The inner, channel-facing surfaces of the ATPases have hydrophobic residues that could recognize other hydrophobic residues within substrates [56]. Perhaps the substrate is thus tightly 'grabbed' and engaged for subsequent unfolding and translocation, at which point the binding affinity provided by a polyUb chain becomes dispensable. In support of this, a disordered region of the substrate was found to contribute to the tight binding of a polyubiquitylated protein to the 26S proteasome after initial binding of a polyUb chain [39]. As degradation goes on, the conjugated Ub and/or Ub chain could move along with the substrate toward the translocation entrance, where Rpn11-mediated Ub chain amputation would release the conjugated Ub and/or Ub chain from the substrate (Figure 3a (i) and (ii)). Chain amputation would facilitate substrate unfolding and translocation because Ub is an extremely stable protein and the proteasome may not unfold Ub efficiently. Moreover, recycling Ub is important to maintain Ub homeostasis in cells. This model predicts that Ub chain amputation occurs after the substrate engages with the ATPases for unfolding and translocation, explaining its ATP hydrolysis-dependence. Certainly, another

simple model to explain why deubiquitylation is ATP hydrolysis-dependent is that the DUB activity of Rpn11 relies on a proteasome conformation that is induced by ATP hydrolysis. Future investigation is necessary to elucidate how Rpn11 is activated and why its activity couples with ATP hydrolysis. In contrast to Rpn11-mediated Ub chain amputation, Uch37 and Usp14/Ubp6 trim polyUb chains into monoUb and short Ub chains [49,57], a process that is likely not coupled with degradation.

Ub chain trimming can promote or inhibit proteasomal degradation

It was originally suggested that Ub chain trimming rescued some ubiquitylated proteins from the 26S proteasome by editing the conjugated Ub chain when it was not long enough to support strong binding [57]. Later studies have found that trimming chains too rapidly inhibited proteasome-mediated degradation [49,58,59]. In agreement with these results, inhibition or depletion of the Ub chain trimming enzymes on the proteasome promoted proteasomal degradation *in vitro* [49,57-60]. Thus, Ub chain trimming could inhibit proteasomal degradation. In other reports, Ub chain trimming was found to promote proteasomal degradation. For instance, depletion of Ubp6 was found to impair degradation of Ub-Pro- β -galactosidase in budding yeast [42]. In mammalian cells, RNAi-mediated knockdown of Uch37 and Usp14 in combination caused substantial accumulation of ubiquitylated proteins in cells, indicating impairment of proteasomal degradation [61]. Knockdown of Uch37 was recently found to inhibit degradation of I κ B α and inducible nitric oxide synthase in several cell lines [62]. Also, inhibition of both Uch37 and Usp14 by a reversible inhibitor, Ub aldehyde, abolished efficient degradation of polyubiquitylated proteins *in vitro* [12,63]. Thus, Ub chain trimming could promote or inhibit proteasomal degradation, likely depending on whether the rate of Ub chain trimming can coordinate with actions of substrate unfolding and translocation (discussed below).

Models for explaining the roles of Ub chain trimming in proteolysis

In initial substrate targeting to the 26S proteasome, Ub chain binding likely provides the predominant affinity. To promote degradation, it is necessary to maintain Ub chain binding to the proteasome while the substrate engages with the substrate translocation channel for unfolding and translocation, although partial chain trimming could occur during this process (Figure 3a). In the subsequent degradation (Figure 3a (i)), Ub chain trimming shortens a conjugated Ub chain; the resulting short Ub chain would eventually be released from the Ub receptor, allowing it and the substrate to move towards Rpn11 for amputation. In this case, Ub chain trimming functions to release the conjugated Ub chain from the Ub receptor, which could facilitate substrate translocation. Alternatively (Figure 3a (ii)), a polyUb chain could be amputated by Rpn11 while still binding on the Ub receptor. In this case, Ub chain trimming is necessary to cleave the free polyUb chain that is left on the Ub receptor, which frees up the Ub receptor to permit docking of a new substrate. In support of this, free polyUb chains were found to compete with polyubiquitylated proteins for proteasomal binding, thus inhibiting degradation [35]. Moreover, although a deubiquitylation-resistant tetraUb bound the 26S proteasome comparably to a cleavable tetraUb, it blocked proteasomal degradation more potently [63], indicating that timely removal of Ub chains from the Ub receptors is important for efficient degradation. Thus, to support degradation, during substrate targeting the processes of Ub chain trimming and substrate translocation must coordinate to allow engagement of the substrate with the substrate translocation channel. During the subsequent degradation of the substrate, trimming of the Ub chain would facilitate substrate translocation and vacate the Ub receptors.

If Ub chain trimming outpaces substrate translocation during the initial substrate targeting step, this would decrease substrate binding affinity for the proteasome and cause the release of the substrate without degradation (Figure 3b (i)). If this is the case, reducing the chain

trimming rate might promote degradation. In support of this, proteasomes lacking Uch37 and Usp14, or depleted of Ubp6, efficiently degraded ubiquitylated cyclinB1 *in vitro* [64,65]. In this setting, cyclinB1 was predominantly conjugated with mono or short Ub chains on multiple lysine residues [65]. Presumably, chain trimming caused release of cyclinB1 because monoUb and short Ub chains have low binding affinity to the 26S proteasome. Also, reducing the chain trimming activity was found to promote degradation of K63-linked polyUb chain-conjugated substrates [49,58], likely because K63-linked Ub chains are often rapidly deubiquitylated by the 26S proteasome [58]. However, trimming a chain too slowly could inhibit substrate unfolding and translocation, ultimately clogging the 26S proteasome (Figure 3b (ii)). Overall, the models in Figure 3b explain the inhibitory role of Ub chain trimming in proteolysis.

Features of the proteasome and substrates that affect chain trimming or substrate translocation

As discussed above (Figure 3), the rates of Ub chain trimming, substrate unfolding and translocation, which are likely determined by features of the proteasome and the substrate, could all play a role in regulating proteasomal degradation. Studies have shown that association of Usp14/Ubp6 with the 26S proteasome could be regulated by conditions such as salt concentrations and the protein levels of free Ub [49,61,66], thereby these factors might alter the chain trimming capacity of the 26S proteasome. Another feature that might affect the rate of Ub chain trimming is the chain linkage, length and quantity. The 26S proteasome was found to trim K63-linked polyUb chains 6-fold faster than K48-linked polyUb chains [58], which could result in inefficient degradation of K63 chain-conjugated substrates because of their premature release from the 26S proteasome [58,59]. Interestingly, a recent study has shown that substrate unfolding performed by the proteasome-activating nucleotidase (PAN) from *Methanocaldococcus jannashii* is coupled with peptide hydrolysis [8,56]. This indicates that the amino acid sequences that are resistant to proteasomal degradation might block unfolding of downstream domains in substrates. Indeed, a glycine-rich region can impede proteasomal degradation of p105 [67], although whether the glycine-rich region serves to block substrate unfolding requires further investigation. In addition, the folding status of a substrate could affect the rate at which the proteasome further unfolds and translocates it, which could determine whether the Ub chain trimming process can coordinate with substrate unfolding and translocation. For instance, rapid chain trimming might prevent degradation of proteins that are tightly-folded, but it might support degradation of loosely-folded proteins because this class of substrates can easily translocate and engage with the substrate translocation channel. In support of this, K63-linked polyUb chains, which are rapidly trimmed by the proteasome-residing DUBs, were found to both inhibit and promote protein degradation [58,59,68,69]. Thus, the Ub chain trimming activity of the 26S proteasome is a potential tuner for regulating the capacity of proteasomal degradation. This 'tuning' role is functionally supported by two recently identified small molecule inhibitors of the Ub chain trimming enzymes of the human 26S proteasome, IU1 and b-AP15 [51,70].

Targeting the chain trimming enzymes by small molecules

IU1 was identified as an inhibitor of the proteasome-bound Usp14 from a screen of 63,052 small molecules [51]. Inhibition of Usp14 by IU1 is reversible and likely acts on the catalytic site of Usp14 [51]. Treating purified 26S proteasome with IU1 was found to promote degradation of ubiquitylated cyclinB1 and Sic1 *in vitro*. Consistent with this, IU1 promoted degradation of several overexpressed proteins that are critical in the pathogenesis and pathology of neurodegenerative diseases, including Tau, TDP-43 and ATXN3. IU1 only promoted their degradation in *USP14*^{+/+} murine embryonic fibroblasts (MEFs), but not in *USP14*^{-/-} MEFs, suggesting that IU1 functions specifically through Usp14. Moreover, IU1

treatment could reduce the accumulation of menadione-induced oxidized proteins and ameliorate menadione or hydrogen peroxide-induced cell death in HEK293 cells [51]. This is a very exciting finding; it not only supports previous observations that Ub chain trimming could play an inhibitory role in proteolysis, but has also demonstrated that the proteasome degradation capacity can be elevated by a small molecule. Enhancing proteasomal degradation might be an intervention for treating some neurodegenerative diseases, where accumulation and aggregation of toxic proteins in neurons are the culprits for causing cell death. This idea can now be tested *in vivo*, by examining whether IU1 treatment leads to clearance of neurotoxic proteins such as Tau and alpha-synuclein in mouse models of neurodegenerative diseases. If so, we could begin to ask whether disease phenotypes are ameliorated with clearance of neurotoxic proteins.

b-API5 was originally identified in a screen for inhibitors that induce the lysosomal apoptosis pathway [71]. Treating cells with b-API5 was subsequently found to induce a gene expression profile similar to that of several characteristic proteasome inhibitors [70], indicating that b-API5 might block proteasome activity. Indeed, b-API5 could inhibit the deubiquitylating activity of purified PA700/19S and the 26S proteasome *in vitro*, and block an active-site-directed-probe from reacting with the catalytic cysteine residues of Uch37 and Usp14 in purified 26S proteasome and in cells. In cell-based assays, b-API5 had effects that were similar to proteasome inhibitors, including inducing the accumulation of polyubiquitylated proteins; blocking degradation of several characteristic proteasome substrates such as kinase inhibitors; arresting cells at G2/M phase; and upregulating apoptotic markers and reducing cell viability. D'Arcy and colleagues further found that administration of b-API5 significantly reduced growth or onset of tumors in five different mouse models, including severe combined immunodeficiency mice with FaDu squamous carcinoma xenografts; nude mice with HCT-116 colon carcinoma xenografts overexpressing *BCL2*; C57BL/6J mice with Lewis Lung carcinomas; BALB/c mice with orthotopic breast carcinoma; and C57BL/6J mice with C1498 leukemia [70]. Thus, b-API5 can inhibit the Ub chain trimming enzymes Uch37 and Usp14 of the 26S proteasome, functions similarly to proteasome inhibitors when used to treat cells, and has potential uses in cancer therapy.

IU1 and b-API5 have different effects on cellular Ub levels. Treating normal MEF cells with IU1 resulted in little change of ubiquitylated proteins and a mild reduction of free Ub [51], which could be caused by forced degradation of conjugated Ub with substrates [49]. By contrast, incubating b-API5 with HCT-116 cells led to massive accumulation of ubiquitylated proteins and significant depletion of free Ub [70]. Depletion of free Ub might contribute to the inhibitory effect of b-API5 on degradation, as it was found that the protein level of free Ub is important for maintaining effective protein degradation in yeast cells [49,66]. Both studies have clearly shown that IU1 inhibits Usp14, b-API5 inhibits Usp14 and Uch37, and neither inhibitor abolishes the activity of several other tested DUBs. The active-site-directed-probe assay used in the study by D'Arcy et al. is useful for further evaluating whether IU1 and b-API5 inhibit any other DUBs and/or proteases in human cells. Because Ub chain trimming could promote or inhibit degradation, it will be interesting to investigate whether both IU1 and b-API5 have such an effect on the degradation of cellular proteins, which could be evaluated by stable isotope labeling using amino acids in cell culture (SILAC) quantitative proteomics. *In vitro* proteasomal degradation assays, as shown in the study by Lee et al. [51], will also be important for evaluating the effect of IU1 and b-API5 on degradation of proteins with various features including different folding statuses and conjugated with Ub chains of different linkages, lengths and quantities. Nevertheless, the studies by Lee et al. and D'Arcy et al. imply that the Ub chain trimming enzymes of the 26S proteasome are potential targets for drug development.

Concluding remarks

The PA700/19S complex plays an essential role in recognition and processing ubiquitylated proteins for proteolysis. PA700/19S recognizes a substrate *via* two binding elements: a conjugated Ub chain and a disordered region. Two subunits of PA700/19S and several proteasome-associating proteins have the capacity to bind Ub chains, offering versatile routes for binding substrates that can be differentially ubiquitylated. After the initial targeting, substrates are unfolded and translocated into the proteolytic chamber for proteolysis; the conjugated Ub chain is released from the substrate by the DUBs of the proteasome. Rpn11 catalyzes degradation-coupled deubiquitylation, which prevents unnecessary unfolding and degradation of Ub. Uch37 and Usp14/Ubp6 catalyze trimming of a Ub chain into monoUb and/or short Ub chains, which can both promote and inhibit proteasomal degradation depending on whether the process of Ub chain trimming can coordinate with the actions of substrate translocation and unfolding. Thus, the Ub chain trimming activity is a potentially tunable factor for regulating the proteasome degradation capacity, which is clearly evidenced by the two recently identified chain trimming enzyme inhibitors IU1 and b-AP15 [51,70].

Although progress has been made in understanding proteasome-mediated substrate recognition and deubiquitination, much is still unknown. Some of the important questions have been discussed above. Based on the studies by Lee et al. [51] and D'Arcy et al. [70], the Ub chain trimming enzymes of the 26S proteasome are potential drug targets for regulating proteasomal degradation. Thus, understanding how Ub chain trimming works on the proteasome is critical. Several key questions remain to be answered: how are the DUB activities of Uch37 and Usp14 activated on the proteasome? Do Uch37 and Usp14 exhibit Ub chain linkage or length specificity? Do Uch37 and Usp14 coordinate to mediate Ub chain trimming on the proteasome? Does one of the chain trimming enzymes play a major role in mediating Ub chain trimming on the proteasome? And, how are the activities of Uch37 and Usp14 regulated in cells?

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Box 1. Ubiquitylation and deubiquitylation of proteins

Ubiquitylation is a posttranslational modification in eukaryotes. Ubiquitin (Ub), a 76 amino acid protein, is covalently attached to proteins through a cascade of enzymatic reactions. In this process, Ub is first activated by a Ub activating enzyme (E1), then transferred to a Ub conjugating enzyme (E2); Ub-charged-E2 then interacts with one of the Ub ligases (E3), of which there are more than 600 hundred in human cells [72,73]. Each E3 directly interacts with specific substrates. The really interesting new gene (RING) family of E3s promotes the direct transfer of Ub from E2s to substrates, whereas the homologues to the E6AP C-terminus (HECT) family of E3s first accepts Ub from E2s and then transfers it to substrates. Ub is attached to a substrate by covalently bonding the C-terminal glycine residue of Ub with, most often, the ϵ -amino group of a lysine residue within the substrate. A single Ub or chains of Ub can be conjugated on a substrate. A polyUb chain can be built progressively on a substrate [74] or a preformed Ub chain on E2 can be transferred *en bloc* to a substrate [75].

Chains of Ub can be built by conjugating any of seven internal lysine residues (K6, 11, 27, 29, 33, 48 and 63) of Ub to the C-terminal glycine residue of the next Ub. In addition, linear Ub chains are formed by conjugating the C terminus of Ub to the α -amino group of the previous Ub. Different Ub chain linkages can result in different signals. For example, K6-, 11-, 27-, 29-, 33- and 48-linked Ub chains are signals for proteasomal degradation [76], whereas K63-linked and linear Ub chains mostly play non-proteolytic functions [77]. In addition, monoubiquitylation has been shown to mediate endocytosis, lysosomal degradation and cellular localization [78].

Protein ubiquitylation is reversible and removal of this modification is carried out by deubiquitylating enzymes (DUBs) [79,80]. There are approximately 100 DUBs in human cells, which are divided into six families: the Ub-specific proteases (USP), the Ub C-terminal hydrolases (UCH), the ovarian tumor (OTU) proteases, the Machado-Josephine domain (MJD) proteases, the monocyte chemotactic protein-induced protein (MCPPI) proteases and the JAB1/MPN/Mov34 (JAMM) metalloproteases. The JAMM family of DUBs uses zinc during catalysis, whereas other DUBs are cysteine proteases. The activities of DUBs allow the cell to produce monomeric Ub, recycle Ub from chains, and reverse the signaling resulting from ubiquitylation [79,80].

Figure 1. Subunit organization of the yeast 26S proteasome

(a) Subnanometer cryoelectron microscopy reconstruction of the yeast 26S proteasome. The subunits of the 19S complex are colored with the 20S proteasome in grey. (b) Side and top views of the 19S complex. Figures were adopted from reference 3 with permission. The ubiquitin receptors S5a/Rpn10 and Adrm1/Rpn13 and the deubiquitylating enzymes Rpn11, Uch37 and Usp14/Ubp6 are particularly relevant to this review. Uch37 and Usp14/Ubp6 are not present in the shown 26S proteasome structure.

Figure 2. Simplified models for recognition of ubiquitylated proteins by the two integral ubiquitin receptors of the mammalian 26S proteasome

For substrates conjugated with a polyUb chain (a and b), the polyUb chain could bind S5a (a-i), Adrm1 (a-ii) or both (b). For substrates conjugated with multiple short Ub chains (c), the substrate could be recognized through one Ub chain binding to one of the receptors; as an example, binding of one Ub chain to Adrm1 is shown in (c-i), or S5a and Adrm1 could each bind one Ub chain (c-ii). In addition to binding of a polyUb chain or polyUb chains, an unstructured region (broken white line) in a substrate might interact with the ATPase(s), thereby bringing the substrate close to the entrance of the substrate translocation channel.

Figure 3. Models for Ub chain amputation and trimming in regulating proteasomal degradation
 In these models, Adrm1 and Uch37 are used as examples for polyUb chain binding and trimming, respectively. In productive degradation (a), a polyUb chain provides initial binding affinity for the proteasome while the substrate engages with the substrate translocation channel, meanwhile partial Ub chain trimming could occur (left). In one circumstance (i), the Ub chain could be trimmed and then released from the Ub receptor before encountering Rpn11. Accompanying substrate degradation, the remaining conjugated Ub or Ub chain would move along with the substrate towards the substrate translocation channel and finally be released from the substrate by Rpn11-mediated chain amputation. Alternatively (ii), the Ub chain could be amputated by Rpn11 before dissociating from the

Ub receptor. Subsequent Ub chain trimming cleaves the free Ub chain, which vacates the Ub receptor for new substrate binding. In both a (i) and (ii), Rpn11-catalyzed chain amputation would prevent unfolding and degradation of Ub, which can facilitate substrate unfolding and translocation. In nonproductive degradation (b), if a conjugated polyUb chain is trimmed before the substrate engages with the substrate translocation channel, the substrate could be released from the proteasome without degradation (i). Conversely, trimming the chain too slowly could restrict the substrate from being further unfolded and/or translocated even when a substrate has already engaged with the substrate translocation channel (ii). In this circumstance, prolonged tethering of a polyUb chain on the Ub receptor could clog the 26S proteasome.