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## Proteasome interaction with ubiquitinated substrates: from mechanisms to therapies

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### Abstract

The 26S proteasome is responsible for regulated proteolysis in eukaryotic cells. Its substrates are diverse in structure, function, sequence length, and amino acid composition, and are targeted to the proteasome by post-translational modification with ubiquitin. Ubiquitination occurs through a complex enzymatic cascade and can also signal for other cellular events, unrelated to proteasome-catalyzed degradation. Like other post-translational protein modifications, ubiquitination is reversible, with ubiquitin chain hydrolysis catalyzed by the action of deubiquitinating enzymes (DUBs), ~90 of which exist in humans and allow for temporal events as well as dynamic ubiquitin-chain remodeling. DUBs have been known for decades to be an integral part of the proteasome, as deubiquitination is coupled to substrate unfolding and translocation into the internal degradation chamber. Moreover, the proteasome also binds several ubiquitinating enzymes as well as shuttle factors that recruit ubiquitinated substrates. The role of this intricate machinery and how ubiquitinated substrates interact with proteasomes remains an area of active investigation. Here, we review what has been learned about the mechanisms used by the proteasome to bind ubiquitinated substrates, substrate shuttle factors, ubiquitination machinery, and DUBs. We also discuss many open questions that require further study or the development of innovative approaches to be answered. Finally, we address the promise of expanded therapeutic targeting that could benefit from such new discoveries.

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## Introduction

The timely and regulated removal of damaged and obsolete proteins is essential to the health of eukaryotic cells. There is an immense cellular investment in this process, which is mediated by the Ubiquitin-Proteasome System (UPS). Approximately 1000 enzymes interact with each other and additional components to form multi-step enzymatic cascades that convert hundreds of proteins (either obsolete, damaged, or misfolded) into substrates for the 26S proteasome, a 2.5 MDa proteolytic machine. At the center of the 26S proteasome is the cylindrical 20S Core Particle (CP) that contains the proteolytic active sites and is capped on either end by a highly dynamic 19S Regulatory Particle (RP), consisting of base and lid sub-assemblies (Fig. 1A). The base sub-assembly includes three intrinsic ubiquitin receptors (Rpn1, Rpn10, and Rpn13), the scaffolding protein Rpn2, and six AAA+ ATPase subunits (Rpt1-Rpt6) that form a heterohexameric ring-shaped motor. The lid sub-assembly contains the essential DUB Rpn11/poh1 and several structural Rpn subunits. To be efficiently recognized and degraded by the 26S proteasome, substrates require a polyubiquitin tag for binding to the proteasomal ubiquitin receptors and an unstructured initiation region for subsequent engagement by the ATPase motor [1–3], which mechanically unfolds and translocates the substrate polypeptides into the CP for degradation (Fig. 1B). Prior to this translocation, ubiquitin modifications must be removed from substrates by the deubiquitinating activity of Rpn11. The Rpt subunits of the base terminate in a 3-amino acid HbYX (hydrophobic-tyrosine-any amino acid) motif [4] and landmark cryo-electron microscopy (cryo-EM) studies from 2012 [5–7] showed that these C-termini dock into hydrophobic pockets on the apical face of the CP, trigger the opening of the CP gate, and thus allow substrate translocation into the degradation chamber (Fig. 1C). These and subsequent studies [8–19] came to reveal the proteasome in distinct states that differ in the conformation of the RP, the alignment of the central processing channel, the position of Rpn11 near the substrate entrance of the proteasome, and the extent of CP gate opening.

Central to the UPS is the small 76 amino acid protein ubiquitin, whose C-terminal glycine can be attached through an isopeptide bond to primary amines, namely the N-terminus (M1) or lysine (K) side chains, in protein substrates or other ubiquitin moieties to form chains. These ubiquitin chains show different topology, depending on whether the subsequent ubiquitin is linked to the N-terminus (M1) or any of the seven surface-dispersed lysines, K6, K11, K27, K29, K33, K48, and K63, of the previous moiety (Fig. 2A). There is an intrinsic polarity to ubiquitin chains, defined by the proximal end where the C-terminal G76 is either conjugated to a protein substrate or unanchored, and the distal end at the other side. Sequential ubiquitin addition during chain formation can occur by using only one of the eight linkage sites in each ubiquitin, leading to homotypic chains, or involve multiple linkage sites to yield heterotypic or branched chains (Fig. 2B). The nature of ubiquitin chains attached to a substrate, referred to as the ubiquitin code [20], is interpreted by the

ubiquitin receptors, some of which are linkage-specific, empowering this post-translational modification with the cellular fate of the ubiquitinated protein [21–23].

The configuration of ubiquitin chains is defined by the enzymatic cascade involved in ubiquitination. For example, the anaphase-promoting complex/cyclosome (APC/C) catalyzes formation of heterotypic K11/K48 branched ubiquitin chains, which bind to the proteasome and stimulate substrate degradation [24, 25]. Ubiquitination begins with an E1 ubiquitin-activating enzyme charging ubiquitin in an ATP-dependent manner for thioester transfer to an E2 ubiquitin-conjugating enzyme; in humans, there are two E1 and ~40 E2 enzymes. Aided by scaffolding E3 ubiquitin ligase enzymes, E2 enzymes can modify substrates directly or, alternatively, attach ubiquitin to a catalytic cysteine of the E3 for relayed transfer to a substrate. There are ~600 E3 enzymes in humans as well as ~90 deubiquitinating enzymes (DUBs) that reverse or modulate the ligase effects by ubiquitin chain hydrolysis. Greater than 25% of DUBs bind proteins involved in ubiquitination [26], suggesting that DUBs may rescue ubiquitination machinery from auto-ubiquitination and/or promote dynamic chain remodeling. The proteasome relies on its Rpn11 DUB activity to hydrolyze ubiquitin chains from substrates as they are translocated into the RP.

With its well-recognized  $\beta$ -grasp fold (Fig. 2A), ubiquitin defines a superfamily of ubiquitin-like (UBL) proteins, a subset of which is also covalently attached to protein substrates by analogous enzymatic cascades. There is significant crosstalk between various UBLs and their modifiers, as well as other signaling pathways, including phosphorylation [27, 28]. Thus, in addition to the ubiquitin code, the allosteric amplitude of weak interactions involving protein substrates and other modifications appear to play important roles in determining the signaling outcome of ubiquitination [29, 30]. Several multi-domain proteins bind to different sites of the proteasome RP by using embedded UBL domains, including the DUB Usp14/Ubp6 [31, 32], the E3 ligase parkin [33], the shuttle factors that deliver ubiquitinated substrates to the proteasome [32, 34–39], and co-chaperone proteins [40–42]. The ubiquitin fold is pervasive in the UPS and adapted for multiple purposes. Moreover, interactions with UBL domains can modulate proteasome activity [43–45] through mechanisms that are still not entirely clear.

Here, we review the current understanding of how the 26S proteasome interacts with ubiquitinated substrates via its ubiquitin receptors, the proteasomal AAA+ motor, and the essential deubiquitinase. We also discuss proteasomal interactions with ubiquitination machineries, DUBs, and substrate shuttle factors as well as the many challenges that remain to be addressed for developing a complete mechanistic understanding of substrate processing at the proteasome.

## Ubiquitin binding at the proteasome

The proteasome has three established ubiquitin receptors that function in the recruitment of ubiquitinated proteins, namely, Rpn1 [32], Rpn10 [46], and Rpn13 [35, 47]. These three receptors are structurally diverse and use different binding modes to recognize a hydrophobic patch on ubiquitin that contains L8, I44, and V70, and is bound by most ubiquitin receptor proteins (Fig. 2A, [48]). A study of scRpn1 (*S. cerevisiae* Rpn1)

demonstrated this receptor to use an interface within its toroid structure (toroid 1, T1) comprised of three outer helices to bind two ubiquitin moieties [32] (Fig. 3A). The Rpn1 toroid contains an additional recognition site (toroid 2, T2) for the ubiquitin fold at the N-terminal end of deubiquitinating enzyme Ubp6/Usp14 [32] (Fig. 3A), but whether the T1 and T2 sites act independently or are allosterically coupled is not yet known. hRpn10 (human Rpn10) can also bind to two ubiquitin moieties simultaneously and does so at two distinct ubiquitin interaction motifs (UIMs), each of  $\alpha$ -helical structure and embedded within an intrinsically disordered region [49, 50] (Fig. 3B). hRpn13 binds diubiquitin at a single ubiquitin-binding site, formed by the loops of a pleckstrin-like receptor for ubiquitin (Pru) domain [35, 47, 51] (Fig. 3C). Like Rpn1, Rpn13 also binds to a deubiquitinating enzyme, namely Uch37/UHL5, although in this case, through a separate structural domain [52–54] (Fig. 3C). Proteasomes with these three ubiquitin receptors deleted or impaired failed to bind a ubiquitinated model substrate, and the yeast strain containing these mutated proteasomes was highly sensitive to canavanine, an arginine derivative that promotes protein misfolding [32]. The viability of this strain, however, suggests the presence of other ubiquitin receptor sites at the proteasome. Such cryptic binding sites have been proposed to be within the base ATPase subunit Rpt5 [55], the lid subunit Sem1/Dss1 [56], and Rpn10's globular von Willebrand factor type A (VWA) domain [57]; yet, solid evidence is lacking to substantiate the importance of these regions as ubiquitin receptor sites in the proteasome context. Rpt5 was suggested based on ubiquitin cross-linking, which, however, may have also originated from the crosslinker reacting with ubiquitins bound to other RP subunits nearby, in particular Rpn10's UIMs and Rpn11. Cryo-EM structures have revealed ubiquitin chains interacting with Rpn11 to extend along the Rpt4/Rpt5 coil coiled domain [58, 59]. Sem1's ubiquitin-binding region overlaps with its proteasome interface, and no defect in ubiquitin binding was observed upon Sem1 deletion in yeast [32], indicating this subunit's limited efficacy as a ubiquitin receptor at the proteasome. Rpn10 binds to the proteasome through its N-terminal VWA domain, which is proposed to be ubiquitinated for expulsion from the RP and to also bind ubiquitin non-covalently [57]. Similar to Sem1, future studies are needed to evaluate whether the VWA domain functions as a proteasomal ubiquitin receptor *in vivo*.

The receptor sites contributed by Rpn1 T1, Rpn10 UIMs, and Rpn13 Pru share a preference for K48-linked chains and collectively can interact with all ubiquitin chain types *in vitro* [38]. This combined ability to interact with all chain types is consistent with a quantitative proteomic study in *S. cerevisiae* that found all non-K63 ubiquitin chains to mediate proteasomal degradation *in vivo* [60]. More recently, a role even for K63-linked ubiquitin chains in proteasomal degradation was reported; namely, that they can serve as an initiator signal for the production of K63/K48 branched ubiquitin chains to subsequently trigger degradation [61]. Further evidence for branched chains promoting degradation by the proteasome was provided by a study focused on UHL5, which has poor activity towards homotypic ubiquitin chains [52, 62], but readily hydrolyzes K48 branched chains [63]. *In vitro*, the proteasome is able to efficiently degrade model substrates modified with K63-linked ubiquitin chains [64], illustrating its promiscuity and suggesting that alternative factors make this chain type a poor signal for proteolysis *in vivo* [65].

A striking feature of proteasomal substrate binding is the inherently dynamic nature of interaction between receptors and ubiquitin chains, as revealed by the nuclear magnetic resonance (NMR) spectroscopy analyses of the isolated ubiquitin-binding regions in complex with K48-linked diubiquitin [32, 49, 51]. Differential ubiquitin labeling revealed that all three ubiquitin receptors switch in their interactions between the two moieties of K48-linked diubiquitin. K48-linked ubiquitin chains adopt open conformations [51, 66] in dynamic exchange with a ‘closed’ state that is dominant in solution [51] and has the binding sites for proteasomal receptors occluded [67]. NMR studies showed that hRpn13 induces and maintains the extended, open conformational state for K48-diubiquitin, most likely by dynamically switching its interactions between the two ubiquitin moieties [51]. This effect has the potential to also increase the accessibility of K48-linked ubiquitin moieties to the other proteasome receptor binding sites in Rpn1 and Rpn10.

Individually, none of the known ubiquitin receptor sites at the proteasome has high affinity for a single ubiquitin. Rpn13 exhibits increased, yet still moderate, affinity for K48-linked diubiquitin in part due to interactions with the ubiquitin linker region [35, 39, 51, 68]. NMR experiments indicate that scRpn13 binds similarly to ubiquitin compared to hRpn13, but with weaker affinity and a more loosely folded domain structure [35, 47]. However, it is possible that these differences are attenuated when the proteins are assembled into the proteasome. Each of the two ubiquitin-binding sites in the scRpn1 T1 binds to an individual ubiquitin molecule weakly; however, the two sites act in concert to achieve a moderate affinity of 11  $\mu\text{M}$  for K48-diubiquitin [32]. The two Rpn10 UIMs act similarly, binding at the same time to ubiquitin moieties of K48-diubiquitin to yield an affinity of 8.9  $\mu\text{M}$  [32, 49, 69, 70]. *Schizosaccharomyces pombe* Rpn10 (SpRpn10) and scRpn10 each have only one UIM, but bind to K48-diubiquitin with equivalent affinity compared to hRpn10 [71, 72], perhaps due to interactions with the K48 ubiquitin linker region [71].

The combined actions of Rpn1, Rpn10, and Rpn13 in the context of the proteasome is expected to enhance the affinity for ubiquitinated substrates, while preserving the dynamics observed in the isolated systems. These dynamics are likely critical for enabling the proteasome to bind substrates with varying ubiquitin-chain architectures, allowing multivalent ubiquitin interactions with the three receptors and associated DUBs, and orienting the unstructured initiation regions of substrates in a manner appropriate for engagement by the ATPase motor.

## Substrate shuttle factors for the proteasome

In addition to the direct binding of ubiquitinated substrates to the ubiquitin receptors, ubiquitin-binding shuttle factors, namely Rad23, Dsk2, and Ddi1 family members deliver substrates to the proteasome [32, 34–39, 73, 74]. They interact through their UBL domains with receptor sites on Rpn1, Rpn10, and Rpn13 in a manner similar to ubiquitin, but contain amino acid substitutions that result in higher affinity for certain sites [38, 39, 75, 76]. For example, the UBL of Dsk2’s human orthologs preferentially bind hRpn10’s N-terminal UIM [38, 77], whereas hRad23s’ UBLs prefer the C-terminal UIM [36, 75, 76]. scRad23 favors one of the two scRpn1 T1 ubiquitin-binding sites where it forms additional hydrophobic interactions compared to ubiquitin due to substitution of ubiquitin L8 for phenylalanine

[78]. The identity of the shuttle factor delivering a substrate thus likely influences how the attached ubiquitin chain(s) are presented to the proteasome receptor sites and how a substrate's flexible initiation region accesses the central pore. The hRad23 ortholog hHR23b and Dsk2 ortholog hPLIC1 are consistently observed by mass spectrometry to be associated with proteasomes isolated from cells [79, 80]. Similarly, the UBL of Rad23 from *S. cerevisiae* binds scRpn1 with an affinity of  $64 \pm 25$  nM [32], and how these more strongly binding shuttle factors are released from the proteasome has not yet been revealed.

Binding of shuttle factor UBL domains stimulates the ATP-hydrolysis and proteolytic activities of the proteasome [45], thus further linking binding at substrate receptor sites with induction of proteasome activity. It is therefore possible that proteasomes found to have hPLIC1 or hHR23b present are in activated states. It is not yet clear whether multiple shuttle factors are typically bound simultaneously to a particular proteasome; however, their ubiquitin-associated domains (UBAs) can bind a common substrate [81] and their UBL preferences for different receptor sites [32, 34–39, 73–76] make delivery of a substrate through multiple receptors a possibility.

hPLIC and hHR23 not only bind ubiquitin and ubiquitinated proteins, but also each other through UBL/UBA interactions [82], which for hHR23a occur intramolecularly as well [83]. These UBL/UBA proteins can therefore induce liquid-liquid phase separation [84, 85]. Under hyperosmotic stress, phase separated nuclear foci are formed that contain proteasomes and p97 [84]. Formation of these proteolytic compartments is triggered by multivalent interactions of hHR23b's two UBA domains [84], which simultaneously bind ubiquitin moieties [69]. These studies highlight the multi-faceted functional roles of proteasome shuttle factors, but how these and their many other activities are integrated is not yet known.

## Challenges to studying ubiquitin-chain binding to proteasome receptors

The intrinsic flexibility of proteasomal receptors and the dynamics of their ubiquitin interactions are likely paramount to substrate processing. A technical consequence of these inherent and functionally critical dynamics is the intricacy of studying them with any of the modern structural biology tools. Ubiquitin receptors and ubiquitin chains bound to the proteasome have been at best poorly resolved by cryoEM. Only hRpn1 has been observed with a bound ubiquitin [58], whereas Rpn13 is missing entirely from cryo-EM density maps of the human proteasome and has been visualized only at low resolution in the context of the *S. cerevisiae* proteasome [5, 7, 10, 15, 17, 86]. hRpn13 binds an intrinsically disordered 14 amino acid C-terminal extension of hRpn2 (Fig. 3C), which enables motion relative to the rest of hRpn2 and the proteasome [62, 68, 87], and likely aids in substrate capture, but challenges observation by cryoEM.

The C-terminal ubiquitin-binding region of Rpn10 is highly dynamic and missing from all cryo-EM density maps, which is unsurprising given that it is preceded by a flexible linker (Fig. 3B). Currently, solving structures by single particle cryo-EM relies on the detection of thousands of particles that are trapped in different orientations, yet a common state – a requirement unachievable for highly dynamic molecules. The applicability of NMR to the



intact proteasome, on the other hand, is limited by signal decay due to the slow tumbling of such a large complex [88]. Moreover, the RP has refracted crystallization, presumably due to its dynamic structural heterogeneity, whereas the more rigid CP has been crystallized many times [89]. An interesting thought experiment is to consider the relevance of a structure for the proteasome RP in a substrate-engaged, trapped state, with its receptors rigidly bound to a ubiquitin chain. Such a sample may be more amenable to crystallization or cryoEM study, but how well it would represent functionally relevant states of the proteasome as a highly dynamic proteolytic machine remains unknown.

*In vitro* biochemical and single molecule experiments with model substrates have provided mechanistic insights that are complementary to the results obtained from structural studies. The proteasome architecture based on structural analyses suggests that two distinct ubiquitin-chain pathways exist, one that extends along an hRpn10/hRpn13 axis and another that is directed across hRpn1 (Fig. 3D). This model is supported by single molecule studies demonstrating more efficient degradation at the proteasome for a model substrate with equivalent number of ubiquitin moieties, but distributed as two separate diubiquitins rather than a single tetraubiquitin chain [90]. A more recent study demonstrated that supernumerary ubiquitin chains can promote degradation of otherwise poor substrates [91], suggesting an advantage in proteasomal degradation for substrates with multiple ubiquitin chains. This study also identified substrate unfolding as the rate-limiting step of degradation occurring on the timescale of seconds [91], which provides insights into the time that the receptors may need to hold on to ubiquitinated substrates. The time of receptor interactions required for successful degradation depends on how stably a substrate's unstructured initiation region engages with the ATPase motor to prevent substrate release during repeated unfolding attempts, and when, relative to the rate-limiting unfolding, an affinity-conferring ubiquitin chain is removed from the substrate by the Rpn11 DUB.

### Substrate engagement and translocation by the ATPase motor

Ubiquitin-chain binding to proteasomal receptors increases the residency time of a substrate at the RP, such that an internal unstructured region or flexible tail of the substrate can passively enter the central processing pore and engage with the ring-shaped AAA+ ATPase motor of the base sub-assembly for ATP-hydrolysis driven translocation and unfolding (Fig. 1B). The AAA+ motor of the proteasome consists of six distinct ATPase subunits in the clockwise order Rpt1, Rpt2, Rpt6, Rpt3, Rpt4, and Rpt5 (Figs. 1C, 4A) [92]. All Rpts have the same domain architecture, which features an N-terminal coil, followed by an oligosaccharide-binding fold (OB-fold) domain and C-terminal AAA+ ATPase domain that in the hexamer assemble into an N-terminal domain ring (N-ring) and an ATPase motor ring, respectively (Fig. 4A). The nucleotide-binding site in the ATPase domain of each Rpt is located at the interface with the clockwise neighboring subunit, allowing coordinated ATP hydrolysis and conformational changes in the hexamer. Motor interactions with the substrate polypeptide are primarily mediated by the so-called pore-1 loops that project from each AAA+ domain into the central channel of the ATPase ring and contain an Aromatic-Nonpolar-Gly motif [93–97]. The highly conserved aromatic residues in this motif, Tyr in scRpts and Tyr or Phe in hRpts, together with the preceding Lys or Met, form pockets

to accommodate substrate side chains and directly contact the polypeptide backbone for mechanical pulling during translocation [58, 98].

Biochemical studies have found that proteasomal substrate degradation depends on a flexible initiation region or terminal tail of at least 25 amino acids [3, 91], a length required to reach through the N-ring and into the ATPase ring for engagement by the pore-1 loops [98]. The initiation region also needs some degree of sequence complexity, as stable engagement relies on sufficient grip of the pore-1 loops to prevent substrate escape out of the pore. Substrates with tails that are too short or low in complexity have been shown to be poorly engaged or degraded more slowly [91, 99, 100]. The redundancy of the ubiquitin receptors and the dynamic nature of their ubiquitin binding likely play important roles in properly positioning the substrate for tail insertion and engagement with the pore-1 loops.

Not all proteasome substrates in the cell contain unstructured regions that are long enough for engagement [101], and while most of these proteins may get prepared for proteasomal degradation through ubiquitin-dependent unfolding by Cdc48/p97 [102, 103], a recent study found that ubiquitin attachment itself can destabilize folded domains and induce transient unfolding sufficient for proteasome engagement [104]. Disordered initiation regions can also be obstructed by attached ubiquitin chains that prevent substrate engagement with the translocation machinery prior to deubiquitination [91]. If the obstructing chain is the only ubiquitin modification on a substrate and thus providing the affinity for proteasomal receptors, its removal leads to substrate dissociation and escape from degradation. These findings highlight that individual processing steps at the proteasome need to be strictly coordinated, and substrate engagement has to precede ubiquitin chain removal for efficient degradation, which is accomplished through the direct coupling of mechanical translocation by the ATPase motor and deubiquitination by Rpn11 (see below).

FRET (Förster Resonance Energy Transfer)-based biochemical analyses combined with cryo-EM structures indicated that substrate engagement by the pore-1 loops triggers a large conformational change of the entire RP, leading to the coaxial alignment of the N-ring, the ATPase ring, and CP, and thus facilitating substrate transfer into the degradation chamber [8, 91] (Fig. 4B). In this engaged state, the AAA+ motor domains and their pore-1 loops form a spiral staircase arrangement around the substrate, a feature that was first shown for the 26S proteasome [8] and subsequently described for numerous other translocating AAA+ protein remodelers [105]. Two recent studies have revealed high-resolution cryo-EM structures of the substrate-bound *S. cerevisiae* and human proteasomes in apparently consecutive states of ATP hydrolysis and substrate translocation [58, 98]. These snapshots suggest that hydrolysis events progress counterclockwise around the ATPase ring and drive substrate translocation by a hand-over-hand mechanism. In this mechanism, 4 or 5 ATP-bound subunits are in contact with the substrate polypeptide, as the post-hydrolysis subunit disengages from the substrate at the bottom of the spiral staircase to move up and, after ATP binding, re-engage at the top; a process that leads to a downward translocation step of two amino acids [58, 98, 105].



## Substrate interactions and conformational states of the proteasome

Deep classification of large-scale single-particle cryo-EM data revealed multiple distinct conformations of the proteasome that can be broadly separated into a substrate-free “apo” state (termed s1 for the *S. cerevisiae* proteasome) and several engaged states (termed s2, s3, s4, s5 and s6), representing the AAA+ motor at various stages of the ATPase cycle and with different registers of the Rpt staircase [5–15, 17–19]. In the apo state, the N-ring, ATPase ring, and CP are offset from a coaxial alignment, leading to a narrow, discontinuous substrate-processing channel (Fig. 4B). Rpn11 is located above the N-ring but shifted laterally relative to the central pore, opening up the accessibility to the flexible initiation regions of substrates that are tethered to ubiquitin receptors (Fig. 4C). Substrate insertion and engagement by the pore-1 loops triggers a major conformational change of the entire RP that aligns the N-ring, ATPase ring, and CP, opens the CP gate, and induces distinct spiral-staircase arrangements of Rpt subunits around the substrate. This conformational change also rotates the lid relative to the base sub-assembly, such that Rpn11 moves to a position directly above the entrance to the processing channel, leaving only a small gap to the N-ring for the already engaged substrate to be pulled through [8, 58, 91, 98]. In this engaged state, Rpn11 is thus ideally positioned to capture and remove ubiquitin modifications from translocating polypeptides, but sterically blocks the pore entrance for newly arriving substrates. Efficient substrate insertion into the central channel and engagement by the ATPase motor therefore seems to depend on the apo state of the proteasome, whereas processive translocation, unfolding, co-translocational deubiquitination, and substrate transfer into the CP are facilitated by the engaged conformational states. This model is supported by mutational studies, showing that the disruption of apo-state specific lid-base interactions shifts the conformational equilibrium towards the engaged states despite the absence of substrate and results in degradation defects that originate from inhibited substrate engagement [19]. Furthermore, FRET-based biochemical analyses indicate that the substrate-induced conformational switch represents a crucial kinetic gateway to select for substrates that contain appropriate ubiquitin modifications and unstructured initiation regions, mechanistically coupling these requirements to the commitment to degradation [91].

Binding of ubiquitin or UBLs to proteasomal receptors or the DUB Usp14/Ubp6 also appears to influence the conformational equilibrium of the proteasome, as various functional studies revealed a consequent increase in CP gate opening, proteolysis, and ATP-hydrolysis activities [45, 106–109]. However, cryo-EM analyses of the yeast proteasome in the presence of K48-linked tetraubiquitin chains showed only very minor differences compared to the conformational states in the absence of ubiquitin [110]. Thus, additional structural and mechanistic studies are needed to investigate how ubiquitin-chain binding affects the conformational dynamics of the proteasome.

Important *in vivo* evidence regarding the distribution of proteasome states and the conformational switch upon substrate engagement is provided by *in situ* cryo-electron tomography studies, which revealed that the majority of proteasomes adopt the apo state in intact neurons [111] as well as in the cytosol and nucleus of *Chlamydomonas reinhardtii* [112, 113]. Introduction of poly-GA containing protein aggregates into neurons results in the recruitment of proteasomes and their enrichment in the engaged states, suggesting that these

proteasomes attempt to process and stall on the poly-GA protein aggregates or switch their conformation in response to binding the ubiquitinated aggregates [114].

## DUBs at the proteasome

Two independent cryoEM studies of yeast [98] and human [58] proteasome in trapped states bound to model substrates with conjugated K63-linked ubiquitin chains revealed density for the substrate threaded through the center of the ATPase ring and for ubiquitin bound to Rpn11. Rpn11 removes ubiquitin chains en bloc from substrates [115] in a co-translocational manner, with its cleavage activity enhanced by substrate translocation of the ATPase ring [16]. Interaction between ubiquitin and Rpn11 was first observed in a crystal structure of the isolated Rpn11-Rpn8 heterodimer from yeast complexed with monoubiquitin, which revealed that the Rpn11 Insert-1 region transitions from an inhibitory loop across the catalytic groove in the absence of ubiquitin to a  $\beta$ -hairpin that forms a three-stranded  $\beta$ -sheet with the C-terminus of ubiquitin, thereby stabilizing it for isopeptide cleavage [16]. This loop-to-hairpin transition of the Insert-1 region was found to be rate-limiting for ubiquitin cleavage, which explains how mechanical substrate translocation and pulling attached ubiquitin moieties into the Rpn11 catalytic groove can significantly accelerate deubiquitination. Interaction between ubiquitin and hRpn11 is similarly observed by cryoEM for human proteasome complexed with M1-linked hexa-ubiquitin that is not conjugated to a substrate [59]. Thus, hRpn11 is fully accessible and binds ubiquitin at this location independent of a protein substrate interacting with the ATPase ring.

Not known, however, is the role of hRpn10 in the recruitment of ubiquitin chains to this location. The VWA domain that docks Rpn10 to the proteasome is well-resolved by cryoEM [5–7]. In the apo state, it binds the lid with no observable contacts to the base, yet in the engaged state of the proteasome, a rotation of  $\sim 30^\circ$  causes the VWA domain to interact with the N-terminal coiled coil of Rpt4/Rpt5 (Fig. 4C). This interaction is apparently important for proteasome integrity, as loss of the Rpn10 VWA domain destabilizes lid-base association in both human [116] and yeast [117] proteasomes. The location of the VWA domain is nearby to where ubiquitin binds on hRpn11, however, the hRpn10 portion beyond the VWA, including the UIMs, is not visible in cryoEM density maps of the proteasome [11, 12, 18, 58, 118, 119]. CryoEM proteasome structures show the unanchored M1-linked chains [59] as well as substrate-conjugated K63-linked chains [58] extending beyond Rpn11 and along the Rpt4/Rpt5 coiled. It is therefore likely that hRpn10 contributes to ubiquitin-binding at this location, either through its UIM or VWA domain. This contribution, however, may be less important for substrate-attached ubiquitin chains, as the mechanical translocation of a substrate into the central pore is expected to pull the attached ubiquitin into the Rpn11 active site.

In addition to the essential Rpn11, the DUBs hUsp14 (scUbp6) and hUCHL5/hUCH37 transiently bind to the proteasome. scUbp6 binds via its UBL domain to the Rpn1 T2 site in the base (Fig. 3A and 3D) [12, 32, 107, 120]. Ubp6's DUB activity is sensitive to the conformational state of the proteasome and stimulated when its catalytic USP domain contacts the coaxially aligned N-ring and ATPase ring in the engaged states of the proteasome [31, 107, 120]. Consistently, ubiquitin-bound Ubp6 stabilizes the engaged

state of the proteasome and can therefore prevent subsequent substrate engagement by blocking the apo conformational state required for the insertion of a substrate's flexible region into the AAA+ motor ring [107, 120]. Although the detailed mechanism is unknown, Ubp6 has been proposed to remove supernumerary ubiquitin chains from proteasomal substrates [121]. hUCL5 binds to Rpn13 [52–54] and, similar to Usp14, its DUB activity is stimulated through this interaction with the proteasome [52–54, 122–124]. hUCL5 has been proposed to edit ubiquitin chains on substrates or ubiquitinated proteasomal subunits, and to cleave ubiquitin chains bound to proteasome receptors [125–127]. Both Usp14 and UCL5 stimulate the ATPase and peptidase activity of the proteasome [128–130], yet the detailed underlying mechanisms and how these effects contribute to substrate degradation remain unclear.

## Ubiquitination machinery at the proteasome

As new functional roles continue to be discovered for the shuttle factors, multiple E3 ligases have been reported to associate with the proteasome as well [31, 79, 116, 131, 132]. It is possible that these ligases are recruited to ubiquitinate proteasome components [126, 132, 133]. For UBE3C, the alternative role of remodeling substrate-attached ubiquitin chains at the proteasome has been proposed [134]. UBE3C was the first E3 ubiquitin ligase reported to physically interact with the proteasome [134, 135], but its binding location has yet to be elucidated, and its recruitment to the proteasome appears to be assisted by structurally impaired substrates [133].

Recently, the UBE3A/E6AP AZUL (amino-terminal *zinc-binding domain* of ubiquitin E3a ligase) was found to bind with 12 nM affinity to a site in the C-terminal flexible region of hRpn10, named RAZUL (Rpn10 AZUL-binding domain) [116]. Although intrinsically disordered on its own, RAZUL adopts a helical structure to form a 4-helix bundle with the E6AP AZUL domain (Fig. 3B) [116]. Despite the presence of ~600 cellular E3 ligases, knockdown of UBE3A by RNAi leads to a reduction of ubiquitinated proteins at the proteasome [116]. Whether this observable reduction is caused by a global effect on all substrates or a specific subset of substrates particularly reliant on UBE3A remains to be elucidated. This finding suggests that UBE3A may belong to a subset of E3 ligases that are prioritized for the proteasome, such as by physically co-localizing substrate ubiquitination and proteasomal degradation.

UBE3A isoform 3 depends on binding to hRpn10 for its nuclear localization [136], although it is not clear whether this interaction occurs with isolated hRpn10 outside of the 26S proteasome context. In the nucleus, UBE3A was found to co-localize with proteasomes within phase separated liquid droplets induced by hyperosmotic stress [84]. UBE3A also appears to have a regulatory role in the formation of these proteolytic compartments, which decrease in abundance by ~30% upon UBE3A knockout [84]. This effect may be caused by reduced substrate ubiquitination, as hHR23b and ubiquitinated proteins are required for proteasome foci formation [84].

UBA3A is at the forefront of multiple diseases. It is hijacked by human papilloma virus (HPV) oncoprotein E6 to trigger p53 degradation, contributing to cervical cancer [137–

139]. Furthermore, it appears to be a driver of metastatic prostate cancer [140, 141], loss-of-function mutations are linked to Angelman syndrome [142–144], and elevated gene dosage is correlated with autism spectrum disorders [145]. The recent findings of UBE3A having a dedicated, high affinity binding site at the proteasome, and its involvement in proteasome foci formation spur new research directions towards understanding the role of UBE3A in these diseases.

Huwe1, which also ubiquitinates p53 [146–148], has been found at the proteasome [132], although its mode of interaction remains unclear. Similarly, the N-end rule E3 Ubr4 [149] binds to the proteasome through an unknown mechanism [132]. The E3 ligase parkin is recruited to the proteasome by its UBL domain binding to the Rpn13 Pru domain [33]. Mutations in parkin and its activating protein kinase PINK1 cause defects in mitophagy, the process by which damaged mitochondria are removed from cells, and lead to autosomal-recessive juvenile parkinsonism [150, 151]. The clearance of parkin substrates upon mitochondrial membrane depolarization is reported to be delayed following Rpn13 knockdown [33], suggesting that interaction with the proteasome through Rpn13 plays a role in parkin-mediated protein degradation.

## Proteasome as a therapeutic target

Capping the aforementioned advancements in the current understanding of proteasome biology is the breakthrough application of PROTAC (proteolysis targeting chimera) strategies to combat various diseases [152]. Whereas conventional pharmaceutical approaches target and inactivate proteins of interest, PROTACs use a bifunctional architecture to bridge proteins of interest with an E3 ubiquitin ligase for ubiquitination and in turn degradation by the proteasome. This mechanism mimics the activity described for HPV E6-induced ubiquitination of p53 by UBE3A. An outstanding question is whether HPV E6 targets UBE3A among the ~600 E3s because it can directly bind the proteasome and, by extension, whether this strategy of targeting a proteasome-binding E3 would be advantageous as a PROTAC approach. Critical to assess is also why PROTAC approaches lead to rapid degradation of some target proteins, but fail for many others. One aspect to consider is whether ubiquitination of a particular substrate will be sufficient for proteolysis by the proteasome. Some targets may lack appropriate intrinsically unstructured initiation regions, and it is not clear how pervasively the ubiquitin-dependent protein unfoldase Cdc48/p97/VCP can assist by preparing such ubiquitinated proteins of interest for proteasomal engagement and degradation.

Currently, less than ten E3s have been exploited as PROTACs. High affinity binders for the Cullin RING E3 ligase substrate recognition subunits cereblon (CRBN) and von Hippel-Lindau (VHL) tumor suppressor protein have allowed these two E3 complexes to be exploited effectively for the PROTAC technology [153–156]. The success of these PROTACs will likely drive expansion to other E3 ligase complexes, adding new modes of control over protein lifetimes. Alternative targeting strategies are also emerging, including autophagy-targeting chimeras (AUTACs) that use a guanine derivative as a degradation tag [157]. Endogenous 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) modification (S-guanylation) of bacteria causes subsequent K63-linked ubiquitination and

triggers clearance by ubiquitin-mediated selective autophagic [158, 159]. By exploiting this mechanism, AUTACs have been demonstrated to promote targeted mitochondrial turnover via mitophagy and improve mitochondrial activity in Down syndrome-derived fibroblast cells through preferential degradation of impaired mitochondria [157].

The first application of proteasome biology to medicine was the inhibition of the CP, which is an established treatment for hematological cancers, reviewed in [160]. In an effort to develop alternative proteasome inhibitors, a class of chalcone derivatives, including RA190, were identified to bind to hRpn13, induce apoptosis in cancer cell lines, and restrict growth of tumor xenografts [161, 162]. An independent study further identified an hRpn13-binding peptoid ligand that similarly induces death of multiple myeloma cells [163]. Each of these hRpn13-targeting approaches were found to be synergistic with inhibition of the proteasome CP [163, 164]. RA190-induced accumulation of ubiquitinated proteins at the proteasome is lost upon deletion of hRpn13 [62] or its Pru domain [165]. Loss of cell viability however by either of these two hRpn13-targeting compounds does not seem to require hRpn13 according to hRpn13-knockdown experiments [166, 167] and a cell line with a defective hRpn13 Pru domain [165], although conflicting data on this matter exists [164]. These compounds are able to interact with other cellular components, which likely contribute to the induction of apoptosis [166, 167]. Nonetheless, these findings collectively suggest that hRpn13 is a promising therapeutic target. RA190 was fused to the CRBN binding ligand thalidomide to induce its degradation, but the benefit in this case was found to be modest [168]. Of higher interest may be the development of a PROTAC that targets proteins to hRpn13 at the proteasome and thus substitutes for ubiquitin in the delivery of substrates to the degradation machine.

Since substrate deubiquitination is an essential step for proteasomal degradation, additional proteasome inhibitors have been developed by targeting the zinc-dependent metalloprotease Rpn11. Two small-molecule inhibitors of Rpn11 were discovered by screening a library of metal binding pharmacophores; 8-thioquinoline (8TQ,  $IC_{50}$  ~2.5  $\mu$ M) and capzimin (a 8TQ derivative,  $IC_{50}$  ~400 nM) [169], which also block proliferation of cancer cells in culture. Additional Rpn11 inhibitors have been described to prevent substrate deubiquitination by acting as zinc chelators, like SOP11 ( $IC_{50}$  ~1.3  $\mu$ M) and thiolutin. SOP11 was identified via high throughput screening by using a novel proteasome degradation assay, and was found to stabilize some proteasomal substrates, induce the unfolded protein response, and trigger apoptosis [170]. Thiolutin is a disulfide-containing antibiotic and antiangiogenic compound produced by *Streptomyces* that, in its reduced form, inhibits Rpn11 [171]. SOP11 and reduced thiolutin were found to also inhibit other JAMM metalloproteases, suggesting that additional optimization may be required for improved Rpn11 specificity. A more extensive review of these inhibitors can be found in [172, 173].

## Perspectives

Over the past decade, an arsenal of modern scientific technologies has been applied to the UPS, unveiling the proteasome as a sophisticated machine that tightly couples structure and molecular motion to functional activity. It is clear that the UPS relies on many additional layers of regulation and mechanistic action beyond the current understanding,

which most likely represents a bare-bones and incomplete comprehension. For example, the role of the many known post-translational modifications to proteasome subunits remain undefined. It has been postulated that the presence of shuttle factors and even intrinsic subunits at the proteasome is influenced by ubiquitination [57, 132], sumoylation [174], and phosphorylation [175, 176]. Nonetheless, what has emerged is a strong appreciation of the dependency of functional activity on dynamic motions, including redistribution of the relative population of conformational states upon binding of substrate or effectors. How this redistribution is implemented remains to be discovered, but single molecule experiments are likely to provide important insights by revealing whether the relative population of distinct proteasome states is defined by longer residency time of the more populated states, shorter times in lesser populated states, and/or changes to activation barriers that affect switching between such states. Additional factors will likely influence how these dynamic relationships play out in a crowded cellular context. Over the next decade, it will be critical to define these aspects of biological function, which will also serve as a catalyst of new emergent technology, just as the need for structural information drove forward technological advancements in cryoelectron microscopy, x-ray crystallography, and NMR spectroscopy. Such interplay between dynamics and function is sure to apply to many other biological systems.

We focused in this article on events that occur after proteasome assembly, beginning with the dynamic binding of ubiquitinated substrates, which occurs at multiple, weak affinity receptor sites that can collectively impart the affinity needed to hold substrates at the proteasome without rigidly locking them into a fixed orientation. The ubiquitin-binding UBA domains of shuttle factors expand this activity, but how all of these binding sites are organized and whether multiple shuttle factors bind to the RP at the same time is not yet known, and further structural studies will be required to answer these questions. Based on current knowledge, it is reasonable to propose that dynamic ubiquitin interactions with various binding sites enables the stochastic motion of the substrate required for its unstructured initiation region to engage with the ATPase motor. Engagement of the substrate's initiation region with the ATPase motor then drives the conformational switch that facilitates the mechanical translocation and co-translocational deubiquitination needed for substrate entry into the degradation chamber. Given the presence of additional DUBs and E3 ligases at the proteasome, it is likely that ubiquitin chains are remodeled, but future experiments are needed to test this model and assess to what extent proteasome subunits themselves are ubiquitinated (or modified in other ways) as part of the protein degradation cycle.

We have been limited in what we can cover in this article, omitting discussion of proteasome assembly as an important area of research that requires further investigation. We have also focused on the RP-capped 26S proteasome, although there are other ATP-independent regulators capping the proteasome CP. The function of these regulators and whether they act in concert with the RP remains to be elucidated. For example, it is still unclear what fraction of proteasomes exist in hybrid states with different regulators capping each end of the CP. Long-range allosteric interactions have been uncovered between regulators and the CP [177–180], as well as evidence that this long-range allostery does not extend to opposite ends of the CP, suggesting that the CP is capable of being simultaneously sensitive to allosteric



signaling at both ends [181]. A greater understanding of these long-range interactions and in particular how they occur between the RP and CP will likely emerge in the next decade and provide a more complete understanding of the functional roles performed by the many CP-binding factors.

The proteasome is an impressive biomachine, the scrutiny of which provides foundational information on how cells apply structural and dynamic properties to execute mechano-chemical events. Yet its detailed study is also motivated by the already existing and growing therapeutic impact of the UPS. PROTAC-mediated turnover of target proteins provides revolutionary opportunity in precision medicine. That this approach mimics viral strategies in interacting with eukaryotic hosts, an evolutionary relationship that spans ~2.5 billion years, is perhaps the most compelling reason to invest heavily in this approach. Finding ways to expand PROTAC targeting to include a larger plethora of E3 ligases will open avenues for precise substrate targeting in specific tissues. The UPS is thus likely to spur myriad new medicines over the next decade directed towards the improvement of human health.

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## Abbreviations

<b>8-nitro-cGMP</b>	8-nitroguanosine 3',5'-cyclic monophosphate
<b>8TQ</b>	8-thioquinoline
<b>AAA+</b>	ATPases Associated with various cellular Activities
<b>APC/C</b>	anaphase-promoting complex/cyclosome
<b>AUTACs</b>	autophagy-targeting chimeras
<b>AZUL</b>	amino-terminal zinc-binding domain of ubiquitin E3a ligase
<b>CP</b>	core particle
<b>CRBN</b>	cereblon
<b>cryo-EM</b>	cryo-electron microscopy
<b>DUB</b>	deubiquitinating enzyme
<b>HbYX</b>	hydrophobic-tyrosine-any amino acid
<b>HPV</b>	human papilloma virus
<b>JAMM</b>	Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+)

<b>NMR</b>	nuclear magnetic resonance
<b>PROTAC</b>	proteolysis targeting chimera
<b>Pru</b>	pleckstrin-like receptor for ubiquitin
<b>RAZUL</b>	Rpn10 AZUL-binding domain
<b>RP</b>	regulatory particle
<b>Rpn</b>	Regulatory particle non-ATPase
<b>Rpt</b>	Regulatory particle triphosphatase
<b>UBA</b>	ubiquitin-associated
<b>UBL</b>	ubiquitin-like
<b>UIM</b>	ubiquitin interaction motif
<b>UPS</b>	ubiquitin proteasome system
<b>VHL</b>	von Hippel-Lindau
<b>VWA</b>	von Willebrand factor type A

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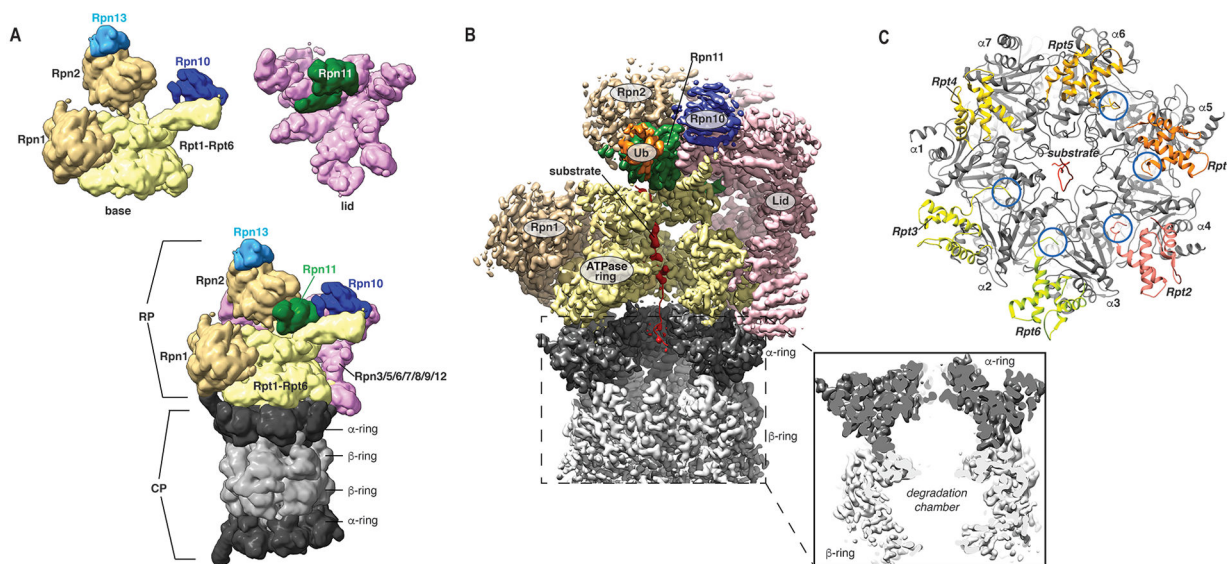
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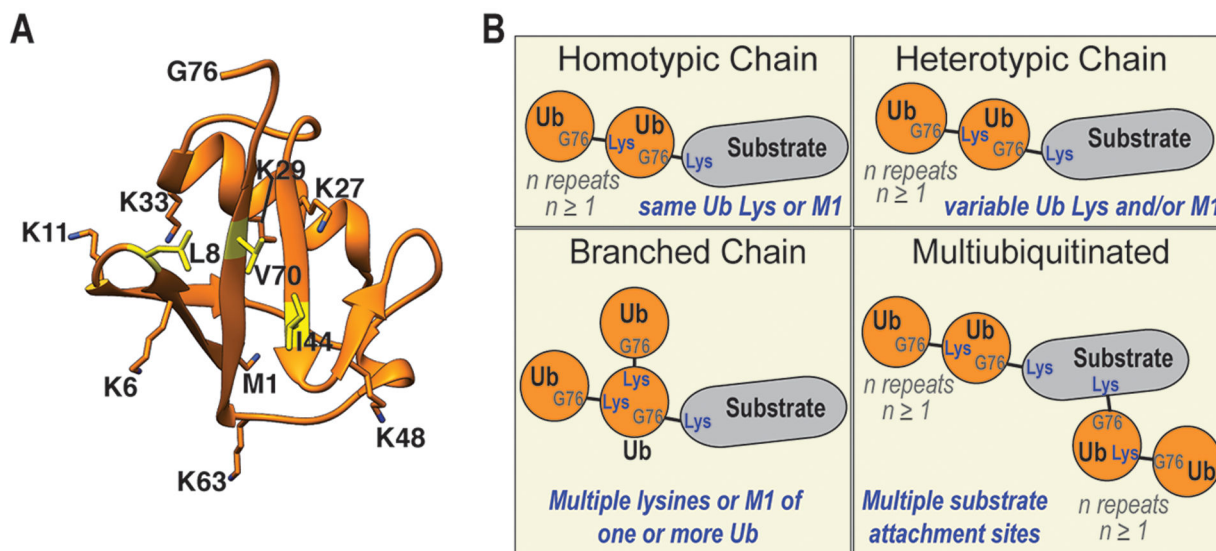


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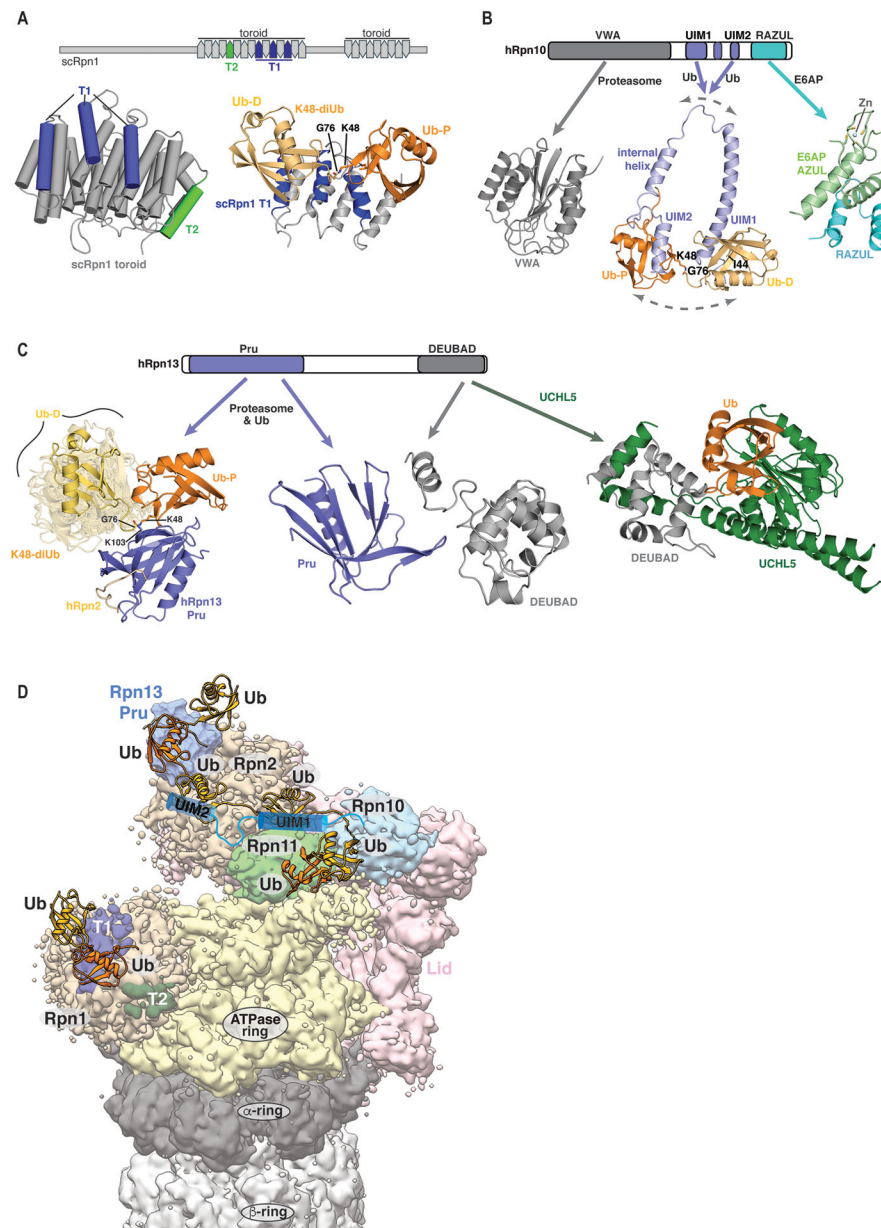


**Fig. 1.** Structure of the 26S proteasome. (A) A cryo-EM reconstruction of 26S proteasome (bottom, EMDB: **1992**) showing the base (upper left) and lid (upper right) sub-complexes and their integration into the RP atop of the CP (bottom image). The DUB Rpn11 (green), together with the components displayed in pink (Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn12, and Sem1), form the lid sub-complex (top right), whereas the remaining RP components Rpn1 (beige), Rpn2 (beige), Rpn10 (indigo), Rpn13 (light blue), and Rpt1-Rpt6 (yellow) from the base sub-complex (top left). (B) Exterior (left) and CP cross-section (lower right) from cryo-EM reconstruction of the substrate-engaged proteasome (EMDB: **9045**; PDB: **6EF3**). The hollow degradation chamber of the CP is apparent in the cross section with the  $\alpha$ -ring and  $\beta$ -rings in dark and light grey respectively. At the RP, a substrate (red) extends through the central channel of the ATPase ring (yellow) with an attached ubiquitin (orange) bound to the DUB Rpn11 (green). Density maps for Rpt5 and two  $\alpha$ -subunits ( $\alpha$ 6 and  $\alpha$ 7) is omitted to show the substrate within the ATPase ring and CP entry. (C) Ribbon diagram of the CP:RP interface depicting the  $\alpha$ -ring (grey), the C-terminal small AAA+ subdomains of Rpt1-6 (spanning red to green coloring), and a substrate (red) at the center of the substrate processing channel. The C-terminal HbYX motifs of Rpts docked into inter-subunit cavities of the CP  $\alpha$ -ring are circled (blue). This figure was generated by using UCSF Chimera [182], UCSF ChimeraX [183], Adobe Illustrator (Adobe), and Adobe Photoshop (Adobe).



**Fig. 2.**

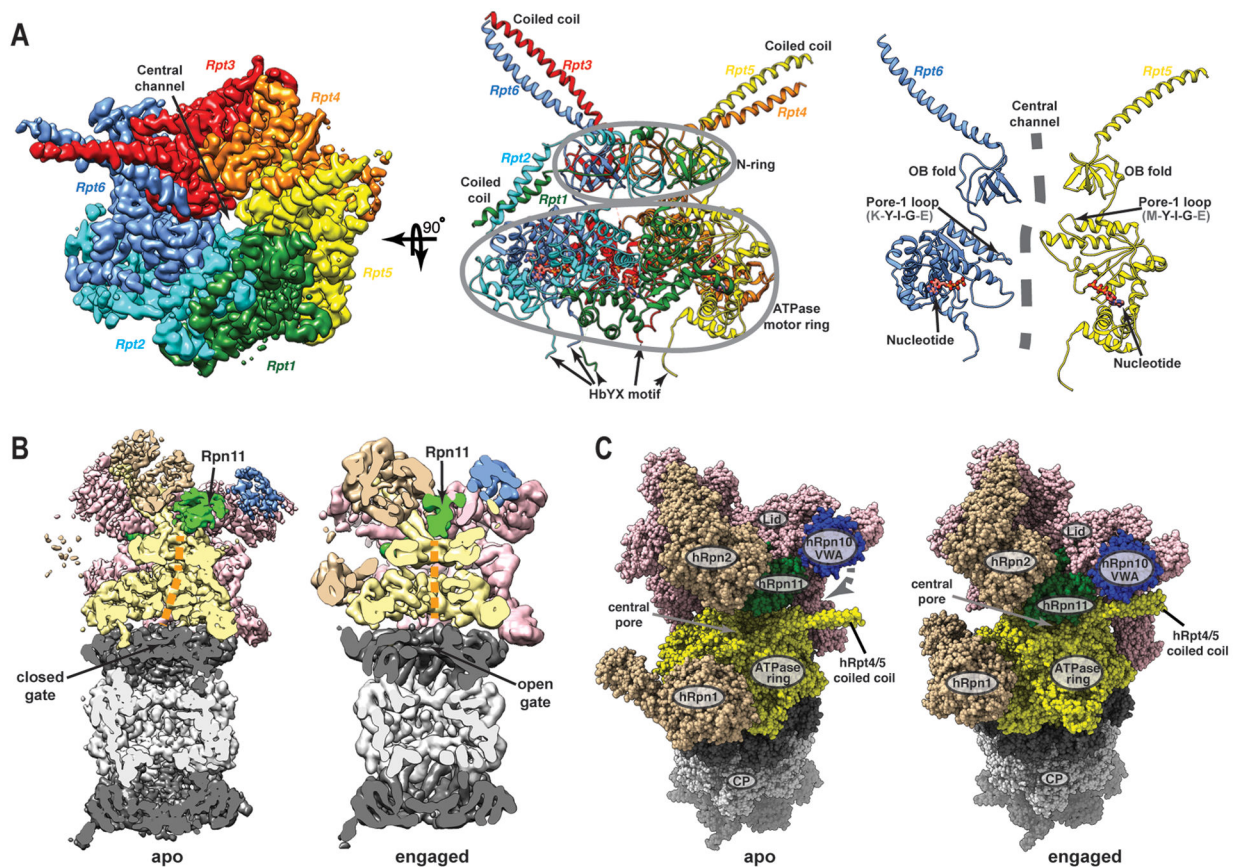
The many forms of ubiquitin modifications. (A) Ribbon diagram of ubiquitin (PDB: **1D3Z**) highlighting functional sites, including the C-terminal glycine (G76) that is conjugated to substrate proteins or other ubiquitin molecules; lysine residues (K6, K11, K27, K29, K33, K48, and K63) and the N-terminus (M1), which are used to form ubiquitin chains; and the hydrophobic amino acids L8, I44, and V70 (yellow) that are typically used to bind receptors. Nitrogen is displayed in blue. (B) Depiction of ubiquitin chain types, with homotypic chains containing only one linkage type, heterotypic chains with mixed linkages, and branched or forked chains, in which one or more ubiquitin moieties have multiple ubiquitin molecules conjugated. In multiubiquitinated states more than one ubiquitin or ubiquitin chain is attached to a substrate. This figure was generated by using PyMOL (PyMOL Molecular Graphics System, <http://www.pymol.org>), Adobe Illustrator (Adobe), and Adobe Photoshop (Adobe).



**Fig. 3.** Structure and functional domains of proteasome substrate receptors. (A) Domain layout of scRpn1 (top) illustrating the ubiquitin-binding T1 site (navy) and the T2 site (green), which binds the UBL domain of the DUB Ubp6. The scRpn1 toroid structure is displayed on the lower left, highlighting the T1 and T2 sites (PDB: **4CR2**). An expanded ribbon diagram of K48-linked diubiquitin bound to the T1 site is shown on the lower right (PDB: **2N3V**). In (A) and (B) the proximal (dark orange) and distal (light orange) ubiquitin moieties are displayed with the K48 linkage site in stick representation (oxygen, red; nitrogen, blue). (B) Domain layout for hRpn10 (top) illustrating the proteasome-binding VWA domain (grey), ubiquitin-binding UIMs and interhelical region (blue), and UBE3A-binding RAZUL domain (cyan). Ribbon diagrams of the VWA domain (lower left), a snapshot of the dynamic

UIM region bound to K48-linked diubiquitin with dashed arrows symbolizing flexibility (middle), and the RAZUL:UBE3A AZUL complex (lower right) are displayed. In the AZUL domain, Zn is shown as a blue sphere with coordinating cysteine sulfur atoms in yellow. PDB **2X5N**, **2KDE**, and **6U19** were used to generate this figure. (C) Domain layout of hRpn13 (top) highlighting the ubiquitin and proteasome binding Pru domain (blue) and UCHL5-binding DEUBAD domain (grey). When free of a binding partner, the two domains interact, as demonstrated in the central ribbon diagram. This structural image has omitted the interdomain region, which is intrinsically disordered. The structure of the Pru domain bound to an extended, dynamic form of K48-linked diubiquitin and 14-amino acid C-terminal region of Rpn2 (gold) is displayed on the lower left. Proximal ubiquitin (orange) is shown bound to the Pru domain loops. The dynamic distal ubiquitin (yellow, eight conformers displayed) exhibits limited order that is defined by interactions at the inter-ubiquitin linker region. A hydrogen bond between the G76 of the distal ubiquitin and K103 of the Pru domain contributes to hRpn13's preference for K48-linked ubiquitins. The structure of the hRpn13 DEUBAD domain complexed with UCHL5 (green) and a suicide ubiquitin variant (orange) is displayed on the lower right. The DEUBAD domain splits to wrap around UCHL5. PDB **6UYI**, **2KR0**, and **4WLR** were used to generate this figure. (D) Hypothetical model of the ubiquitin-bound 26S proteasome, highlighting the ubiquitin receptors Rpn1 (beige) with T1 (navy) and T2 (green) sites, Rpn10 with the VWA domain (cyan) and UIMs (blue cartoon), and Rpn13's Pru domain (blue). The ATPase ring, lid (except for Rpn11), Rpn2, Rpn11, and CP are colored yellow, pink, beige, grey, and light green, respectively. Ubiquitin moieties are displayed as yellow or orange ribbon diagrams with the linkage site rendered in stick representation (oxygen, red; nitrogen, blue). This model illustrates how a ubiquitin chain could extend from Rpn10 to Rpn13 and along Rpn1. In addition to the two distinct ubiquitin-chain pathways displayed here, Rpn10's UIMs or Rpn13's Pru could bind to different ubiquitin chains of a substrate or alternatively, the ubiquitin chain could bind to just one receptor. The distribution of ubiquitins to the various ubiquitin-binding sites is expected to depend on the number of attached ubiquitin chains, as well as their length and linkage type. PDB **6WJD**, **6UYI**, **2N3V**, **1D3Z**, and EMDB **21691** were used to generate this figure. This figure was generated by using UCSF Chimera [182], PyMOL (PyMOL Molecular Graphics System, <http://www.pymol.org>), Adobe Illustrator (Adobe), and Adobe Photoshop (Adobe).





**Fig. 4.**

ATPase ring gymnastics. (A) Structural motifs of the proteasome ATPase ring. Left: A top view of the EM density for the Rpt1-Rpt6 hexamer in the s4 state (EMDB: **9045**), with the central channel of ATPase ring labeled. Middle: ATPase ring displayed as a ribbon diagram (PDB: **6EF3**) rotated 90° relative to the view on the left and highlighting the N-terminal coiled coils, N-ring, ATPase motor ring, and HbYX motifs. Right: ribbon diagram structure of Rpt5 and Rpt6 (PDB: **6EF3**) to illustrate the OB fold, Pore-1 loop (red), and nucleotide (oxygen, red; nitrogen, blue; phosphorus, orange). (B) Cutaway representations of the 26S proteasome in s1 (apo, EMDB: **3534**) and s4 (engaged, EMDB: **3537**) conformations, showing the different position of Rpn11 and change in alignment of the N-ring, ATPase motor ring, and CP. The central channel through the N-ring and ATPase motor ring is indicated by a dashed orange line. (C) Conformational switching of the human 26S proteasome between a ground state ( $S_A$ , PDB: **5VFS**) and substrate processing state ( $S_D$ , PDB: **5VFP**), with the CP aligned. During the transition from  $S_A$  to  $S_D$ , hRpn10's VWA domain rotates by ~30° towards the hRpt4/hRpt5 coiled coil. The color scheme in panels (B) and (C) follows that in Fig. 1B and Fig. 3D. This figure was generated by using UCSF Chimera [182], UCSF ChimeraX [183], Adobe Illustrator (Adobe), and Adobe Photoshop (Adobe).