# Structural Insights into Proteasome Activation by the 19S Regulatory Particle

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ABSTRACT: Since its discovery in the late 1970s, the ubiquitin–proteasome system (UPS) has become recognized as the major pathway for regulated cellular proteolysis. Processes such as cell cycle control, pathogen resistance, and protein quality control rely on selective protein degradation at the proteasome for homeostatic function. Perhaps as a consequence of the importance of this pathway, and the genesis of severe diseases upon its dysregulation, protein degradation by the UPS is highly controlled from the level of substrate recognition to proteolysis. Technological advances over the past decade have created an explosion of structural and mechanistic information that has underscored the complexity of the proteasome and its upstream regulatory factors. Significant insights have come from the study of the 19S proteasome regulatory particle (RP) responsible for recognition and processing of ubiquitinated substrates destined for proteolysis. Established as a highly dynamic proteasome activator, the RP has a large number of both permanent and transient components with specialized functional roles that are critical for proteasome function. In this review, we highlight recent mechanistic developments in the study of proteasome activation by the RP and how they provide context to our current understanding of the UPS.
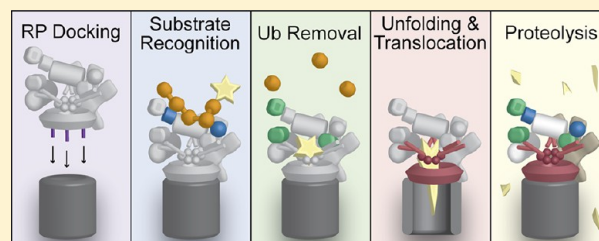
A major aspect of eukaryotic cell maintenance and viability is the rapid and selective turnover of misfolded, exogenous, or downregulated proteins. As a culmination of cell signaling events, these proteins are frequently targeted to the 26S proteasome, a 2.5 MDa protease responsible for catalyzing the decomposition of proteins into short peptides. Proteasome substrates are typically marked by covalent attachment of ubiquitin polymers, which are recognized by 26S machinery as a signal for destruction. Together, the ubiquitin–proteasome system [UPS (see Table 1 for abbreviations)] constitutes a major and highly conserved pathway for targeted protein degradation.[1−3] Improper function of the UPS can result in a variety of human pathologies, including autoimmunity and inflammation, neurodegeneration, and cancer;[4] thus, the proteasome is a promising therapeutic target. Bortezomib (Velcade) and carfilzomib (Kyprolis) are proteasome inhibitors used to treat certain hematological cancers, and other inhibitors are in clinical trials.[5,6]

A highly regulated enzymatic cascade coordinates the recognition of protein substrates, as reviewed in refs 7−9. These enzymes are responsible for both selection of proteasome targets and catalysis of the attachment of ubiquitin polypeptides. Ubiquitin moieties are typically conjugated through an isopeptide bond between their C-terminal glycine carboxylate and a free amine on another protein. Ubiquitination may occur either on a substrate or on another ubiquitin to form a polymeric chain. Ubiquitinating enzymes can function with strict chemical specificity for one of ubiquitin's seven lysines or its N-terminal methionine. Different linkage types confer unique chain topologies that are recognized by ubiquitin-binding proteins to select for substrates with a particular chain length or structure.[10−12] Some types, such as K63-linked chains, can signal for nondegradative events like cell trafficking, activation of NF-κB signaling, and DNA damage response.[13,14] Each of ubiquitin's seven lysines appears to target substrates for proteasomal degradation in yeast except K63,[15] whose extended chain conformation[16,17] binds factors that prevent proteasome association.[18] K48 and K11 constitute the largest relative cellular abundance of chains in yeast at ∼30% each, with K63 third at slightly more than half that amount.[15] A similar study in human embryonic kidney 293 cells revealed higher relative proportions of K48- and K63-linked chains of 52 and 38%, respectively.[19]

The basic component of the proteasome is the 20S core particle (CP),[20] a barrel-shaped protease composed of four heptameric rings of α and β subunits that stack in an $\alpha_7\beta_7\beta_7\alpha_7$ structure;[21] these subunits are related but unique in eukaryotes[22] (Figure 1A). Three interior chambers are formed at ring interfaces, including two antechambers at the $\alpha_7\beta_7$ ring interfaces and a catalytic chamber at the $\beta_7\beta_7$ ring interface (Figure 1B). The catalytic chamber contains the proteolytic activity of the proteasome,[23] whereas the antechamber maintains substrates in an unfolded state prior to their proteolysis.[24] Peptide hydrolysis is catalyzed by conserved threonines at the N-termini of the eukaryotic β1, β2, and β5

Table 1. Abbreviations

abbreviation	description
26S	19S regulatory particle with the 20S core particle
AAA ATPases	ATPases associated with diverse activities
Ar–ϕ	aromatic residue–hydrophobic residue
CC	coiled coil
CP	20S core particle
DUB	deubiquitinating enzyme
EM	electron microscopy
HbYX	hydrophobic–tyrosine–any amino acid
MPN	Mpr1 Pad1 N-terminal
NMR	nuclear magnetic resonance
OB	oligonucleotide/oligosaccharide binding
PAN	proteasome activating nucleotidase
PC	proteasome cyclosome
PCI	proteasome COP9 initiation factor 3
PDB	Protein Data Bank
Pru	pleckstrin-like receptor of ubiquitin
RP	19S regulatory particle
Rpn	regulatory particle Non-ATPase
Rpt	regulatory particle triphosphatase
UBL–UBA	ubiquitin-like–ubiquitin-associating
UIM	ubiquitin interacting motif
UPS	ubiquitin–proteasome system
VWA	von Willebrand factor type A

subunits with caspase, trypsin, and chymotrypsin-like specificity, respectively.[25] Bortezomib inhibits proteolysis through its ability to bind all three catalytic pockets, with the highest affinity for $\beta 5$[26,27] (Figure 1C). The CP is competent for cleaving unstructured proteins in a ubiquitin-independent pathway accounting for up to 20% of cellular targets,[28] but this process provides little means for substrate specificity and is ineffective for folded proteins, as the N-terminal region of the α ring subunits contains an axial gate that sterically occludes folded proteins from entering.[29] Upon CP activation by native proteins or chemical agents, the gating residues shift to a conformation that provides access to the CP interior.[30,31]

■ PROTEASOME ACTIVATION

Cells use a variety of proteins or protein complexes, known as activators, that dock into the CP, open its gate, and make it accessible for proteolysis.[32] Structural data have yielded insights into mechanisms of activator action on the CP,[33] although their substrate specificity, biological relation to each other, and the cellular triggers for their interaction with the CP remain under intense scrutiny.

Diversity of Activators. Endogenous CP activators include Blm10 (PA200),[34,35] 11S (PA28 or REG),[36,37] the RP (PA700),[38] and Cdc48 (p97).[39,40] Multiple activators share a common mechanism for CP binding in which a C-terminal HbYX motif docks into cavities formed at the α ring subunit interfaces and induces gate opening.[41,42] Blm10[43] contains a single C-terminal HbYX, whereas Cdc48[39] and the RP[41] contain a ring of six AAA+ ATPases that use HbYX motifs to dock into the CP's α ring pockets. The RP ATPase ring is composed of unique regulatory particle triphosphatase (Rpt) proteins, and the strict HbYX motifs of Rpt2, Rpt3, and Rpt5 dock the RP into the CP in mature holoenzymes.[44,45] PI31 and the Pba1–Pba2 (PAC1–PAC2) heterodimer also use HbYX motifs to dock into the CP, although these interactions lead to its inhibition.[46,47] The Pba1–Pba2 heterodimer promotes CP maturation,[48] and in contrast to activators, binding of its HbYX motif to the CP leads to a gate configuration that is not sufficiently opened for proteolysis of even small peptides.[49] Whereas PI31 inhibits the CP,[46] it stimulates proteolysis by CP–RP complexes.[50]

Studies of archaea have revealed a similar role for the HbYX motif of RP functional homologue proteasome activating nucleotidase (PAN),[41,51] but neither its HbYX motif nor that of archaeal Cdc48 is required for CP binding and activation.[39,40] Archaeal Cdc48 uses a bipartite binding mode to bind CP that includes its HbYX and a conserved AAA loop that is expected to be proximal to CP α ring gating residues.[40] A similar motif exists at this location in PAN and the Rpts,[51] and in PAN, these loops appear to allow CP binding when its HbYX motif is deleted.[40]
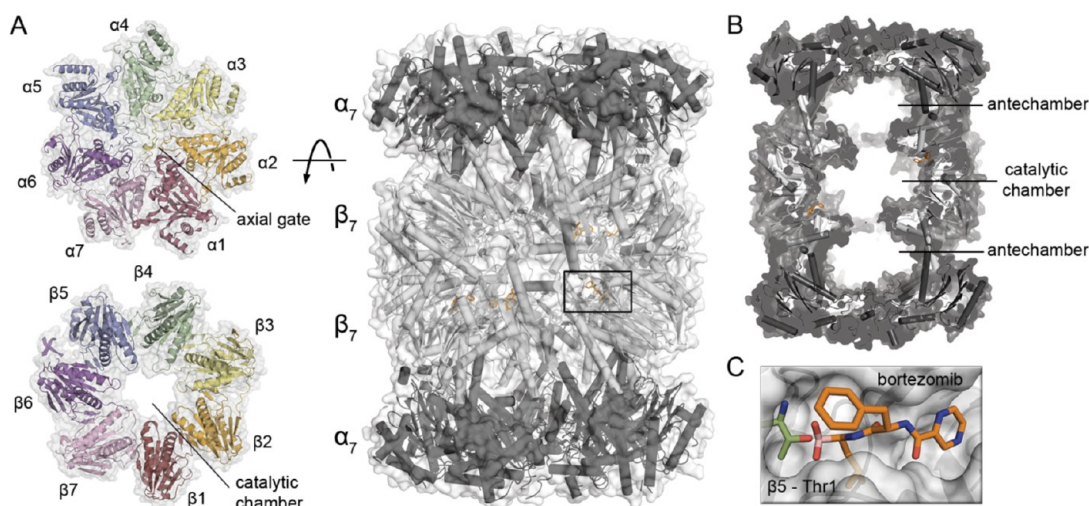

Figure 1. Structure of the 20S core particle. (A) Structure of the *Saccharomyces cerevisiae* CP (α rings colored dark gray and β rings light gray) bound to bortezomib (orange; PDB entry 2F16), as well as a view of the α and β rings that highlight the axial gate and catalytic chamber. The unique subunits of the eukaryotic CP are indicated. (B) Cross section of the CP illustrating the catalytic chamber and both antechambers. (C) Close-up view of the catalytic pocket of the $\beta 5$ subunit showing its N-terminal threonine bound to bortezomib.

The RP is an ~19-subunit complex of stably and transiently associated proteins that caps the CP to form the 26S proteasome used by the UPS. It includes ubiquitin receptors to recognize substrates, deubiquitinating enzymes to remove ubiquitin, and its six Rpt proteins to facilitate substrate unfolding and translocation into the CP. The Rpts contain an N-terminal coiled coil (CC) that engages in extensive Rpt–Rpt contacts, an oligomer-binding (OB) domain, an ATPase domain, and a C-terminal domain (CTD) (Figure 2).
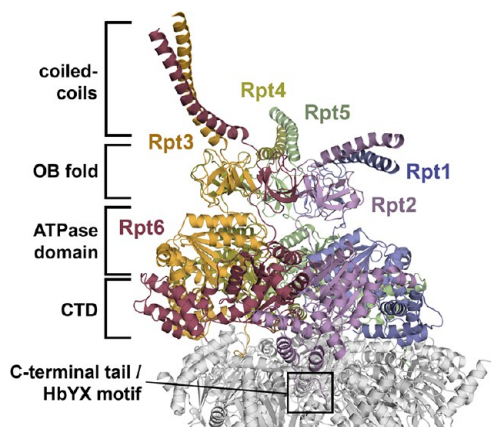

Figure 2. Arrangement of the Rpt ATPases. Model of the Rpt ATPases bound to the CP (light gray), determined by homology modeling from structures of PAN. Each subunit contains a coiled coil (CC), an OB fold, ATPase, and a C-terminal domain (CTD). Rpt2, Rpt3, and Rpt5 have a C-terminal HbYX motif docked into the CP. The ATPases were fit in a 1–2–6–3–4–5 arrangement into a cryo-EM reconstruction (PDB entry 4B4T) and form an asymmetric lock washer-like ring that is offset from the CP's axial pore.

Currently, no high-resolution structure is available for a full Rpt molecule. Cryoelectron microscopy (EM) data combined with cross-linking and interaction studies, as well as experimentally determined or modeled structures of RP components, have allowed full RP models to be determined to ~7–9 Å resolution.[44,45,52−55] In these models, the Rpts adopt an Rpt1–Rpt2–Rpt6–Rpt3–Rpt4–Rpt5[56] arrangement that forms an asymmetrical lock washer-like structure relative to the CP with Rpt2 at the lowest end and Rpt3 at the highest end; Rpt6 is at their interface in an intermediate configuration.[45]

PAN similarly forms an AAA+ hexameric ring structure, although it is a homohexamer, and a crystal structure of an 11S fusion protein containing the PAN HbYX motif bound to CP illustrates CP–HbYX motif contacts[57] that are critical for PAN activation[42] and are expected to be conserved in Rpt2, Rpt3, and Rpt5. Although not docked into the CP α ring in mature proteasomes, Rpt6 contains an HbYX motif variant that binds with high specificity to the α2α3 CP pocket during RP assembly, suggesting that the Rpt proteins are reconfigured during proteasome maturation.[58] Their reorganization may be facilitated by their own dynamic behavior, as EM variability maps demonstrate a high degree of heterogeneity in the Rpt region of the mature proteasome,[52] and ATP binding and hydrolysis elicit conformational changes in the ring.[55] Binding of ATPγS to subunits within PAN similarly induces conformational changes in the unbound subunits that reduce their ATPγS affinity.[59]

Crystallographic structures of the ATP-independent Blm10[43] and 11S activators[42,60,61] complexed with CP illustrate their mechanism of gate opening. The 11S activator binds the CP as a heptamer and uses its C-termini to dock into the same CP pockets used by HbYX motifs.[61] The 11S activator does not contain the HbYX motif and relies on the binding of activator loops adjacent to this pocket for gate opening.[62] This interaction initiates a conformational change in critical N-terminal gating residues Y8, D9, P17, and Y26 and opens the pore.[63] The 11S activator appears to perform ATP-independent proteolysis of short or intrinsically unfolded peptides to produce immune response factors such as MHC class I peptides.[64,65]

Yeast Blm10 caps the CP α rings as a single 246 kDa protein, surrounding the entrance pore with its C-terminal HbYX motif docked into the α5α6 pocket.[43] Its binding causes the gating residues to become disordered, partially opening the pore[43] and thus providing a mechanism for its proposed role in facilitating the degradation of unfolded proteins.[66] Blm10 has been implicated in DNA repair,[34] mitochondrial maintenance,[43] and CP assembly and maturation[67] and can be found in mixed Blm10–CP–RP proteasomes.[34]

Composition of the RP. The RP can be biochemically divided into lid and base subassemblies.[68] The base comprises the six Rpt proteins, regulatory particle non-ATPase (Rpn) proteins Rpn1 and Rpn2 (S2 and S1 in humans), and ubiquitin receptors Rpn10 (S5a)[69] and Rpn13.[70,71] The lid contains Rpn8, Rpn15 (DSS1 or Sem1), deubiquitinating enzyme (DUB) Rpn11 (Poh1),[72,73] and PCI domain proteins Rpn3, -5, -6, -7, and -9 with poorly understood functions and Rpn12, which has been implicated in the late-stage assembly of the RP[74,75] (Figure 3). The base and lid are laterally adjacent relative to the CP pore in EM-based models with the PCI C-terminal regions in the formation of a hexameric shield-like structure lying opposite from the translocation pore[44] (Figure
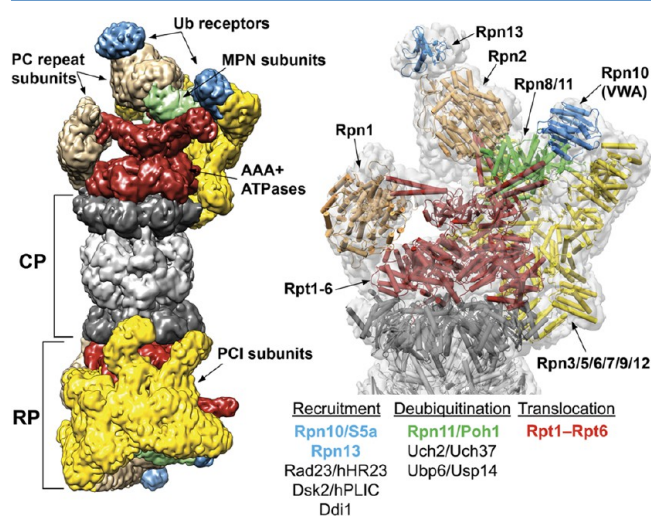

Figure 3. Electron microscopy models of the RP. Structure of the *S. cerevisiae* 26S proteasome by cryoEM (EMDB entry 2165), illustrating the CP (gray) bound to two RP caps. Each RP is composed of the Rpt ATPases (red), ubiquitin receptors Rpn10 and Rpn13 (blue), MPN domain proteins Rpn8 and Rpn11 (green), PC proteins Rpn1 and Rpn2 (beige), and PCI domain proteins (yellow). The subunits that perform ubiquitin recruitment, deubiquitination, and substrate translocation are indicated below and, for those present in the model, are colored accordingly.

3). PCI lid subunits contact ATPases (Rpn5−7) and the CP (Rpn5 and -6) in addition to Rpn11 and Rpn2;[44,76] thus, the base and lid form extensive contacts.

Rpn1 and Rpn2 have molecular masses of ∼100−110 kDa and are structurally similar;[77] the crystal structure of Rpn2 forms a toroidal conformation of two concentric α-helical PC repeats,[78] which is consistent with the fold produced by EM analysis of Rpn1.[78] Their positioning in the RP has been controversial.[77,79] EM models place Rpn1 along the perimeter of the Rpt ring and in contact with the Rpt1−Rpt2 dimer,[44,53] while Rpn2 extends from the N-terminal region of Rpt3 and -6, across Rpn8 and -11, to the periphery of the lid.[44,45,53] The electron density from Rpn1 in EM reconstructions is weak, suggesting that its location may be dynamic.[44] Rpn1 binds ubiquitin receptor UBL−UBA proteins Dsk2 and Rad23[80−82] as well as the DUB Ubp6,[83] and Rpn2 uniquely binds ubiquitin receptor Rpn13.[84−86]

■ SUBSTRATE RECRUITMENT

The RP has a diverse arsenal of ubiquitin receptors for substrate recognition. These include Rpn10 and Rpn13, which have dedicated binding sites in the RP, and UBL−UBA proteins, which appear to shuttle ubiquitinated cargo to the proteasome through multiple overlapping docking sites.

Ubiquitin Receptors in the RP. Rpn10 (S5a) was the first discovered RP ubiquitin receptor[69] and contains an N-terminal von Willebrand factor type A (VWA) domain that docks it into the RP and one or two ubiquitin interacting motifs (UIMs), depending on species. Human Rpn10's, two UIMs are composed of single helices connected by flexible regions that allow them to adapt to bind diverse ubiquitin chains[87,88] (Figure 4). This multivalency affords hRpn10 an 8-fold greater affinity for K48-linked diubiquitin than for monoubiquitin.[88] A second receptor, Rpn13, contains an N-terminal pleckstrin-like receptor for ubiquitin (Pru) domain that interacts with ubiquitin through a surface formed by three loops[70,71] (Figure 4). Rpn13 gains additional affinity upon binding to ubiquitin moieties engaged in a G76−K48 isopeptide bond, giving it a 3-fold greater affinity for K48-linked diubiquitin than for monoubiquitin.[71] Most ubiquitin-binding elements recognize a common surface that includes a hydrophobic pocket formed by L8, I44, and V70.[13] Rpn13's Pru and Rpn10's UIMs also bind to this surface;[70,87] however, Rpn13 engages in additional hydrogen bonds with ubiquitin's H68.[70]

In contrast to Rpn10, Rpn13 uses its ubiquitin-binding domain to dock into the RP and does so through Rpn2.[85,89] With the exception of *S. cerevisiae*, Rpn13 contains a nine-helix C-terminal domain that recruits DUB enzyme Uch37 to the proteasome[85,89,90] and activates it.[89,90] Rpn13's two structural domains are connected by a flexible, low-complexity linker but interact when free of the RP, resulting in a reduced affinity for ubiquitin.[91] The interdomain interaction is along a surface similar to that at which Rpn2 binds, and docking to Rpn2 dissociates Rpn13's interdomain interaction and activates it for ubiquitin binding.[91]

The functional roles of the two receptors are not fully redundant, despite them both binding ubiquitinated substrates. Knockout of Rpn10 or its UIMs is embryonic lethal in mice,[92] and Rpn13 mice knockouts demonstrate growth defects and reduced litter size;[93] thus, loss of one receptor cannot be fully rescued by the other. One possible explanation is the existence of receptor-specific substrates, which have been reported for both Rpn13[94,95] and Rpn10.[96] In addition, Rpn10 was found to
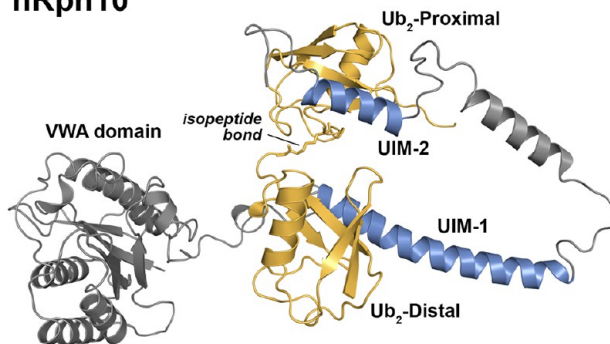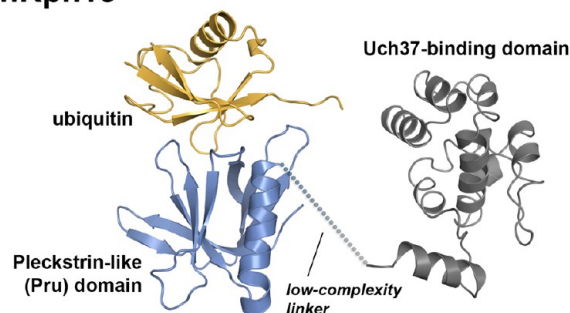

Figure 4. Structure of hRpn10 and hRpn13 bound to ubiquitin. Structure of human proteasome ubiquitin receptors hRpn10 (S5a) and hRpn13. hRpn10's UIMs (blue) are bound to ubiquitin moieties (orange) of K48-linked diubiquitin (PDB entry 2DKE). Its VWA domain was modeled separately (based on PDB entry 2X5N) to create full-length human Rpn10. The hRpn13 Pru domain (blue) is bound to monoubiquitin (orange, PDB entry 2Z59). A low-complexity linker adjoins hRpn13's Pru domain with its C-terminal Uch37-binding domain (PDB entry 2KQZ).

bind the DUB Rpn11 and proposed to be important for proteasome integrity.[97]

Rpn10 and Rpn13 may not simply work as independent ubiquitin-sensing components but rather coordinately bind ubiquitin chains on the same substrate. Both receptors bind simultaneously to K48-linked diubiquitin,[88] and in this ternary complex, Rpn13 binds to the ubiquitin with its K48 involved in an isopeptide bond (proximal); hRpn10's two UIMs interact dynamically with the other moiety (distal). In longer chains, it is likely that both UIMs interact cooperatively with different ubiquitins. EM models place the Rpn10 VWA domain above the translocation pore and adjacent to Rpn11, whereas Rpn13's Pru domain lies ∼90 Å away in a flaglike position at the distal end of the RP[44,45] (Figure 3). Ubiquitin chains that span this distance may allow dual binding of Rpn13 and Rpn10. It is not yet clear whether substrate binding alters the configuration of the ubiquitin receptors and DUBs to facilitate their coordinated activities.

Cross-linking studies have also implicated proteasome subunits Rpt5,[98] Rpt1,[99] and Rpn1[99] in binding ubiquitin chains, and the recruitment of ubiquitin chains to the proteasome by Rpn13 or Rpn10 may allow additional interactions through avidity effects.[100] Rpt1 and -5 and Rpn1 would provide additional docking sites for the ubiquitin chain near the CP, according to EM structures (Figure 3). Additional ubiquitin receptors have been demonstrated to exist in the RP

as deletion of Rpn10's and Rpn13's ubiquitin binding capacity does not eliminate RP binding of ubiquitinated proteins.[71,91]

UBL−UBA Ubiquitin Receptors That Interact with the RP. UBL−UBA proteins appear to contribute an additional layer of regulation to degradation by the UPS. They contain a ubiquitin-like domain (UBL) named for its homology to ubiquitin, and ubiquitin-associating domains (UBA), which bind ubiquitin.[101,102] There are three UBL−UBA proteins in yeast, Rad23, Dsk2, and Ddi1, which have multiple orthologs in higher eukaryotes. UBL−UBA proteins have diverse ubiquitin chain specificities.[103] hHR23a's (human Rad23) C-terminal UBA domain prefers K48-linked chains[104] and inserts between neighboring ubiquitin moieties,[105] whereas the hPLIC1/ ubiquilin-1 (human Dsk2) UBA domain binds with significantly higher affinity to monoubiquitin than does hHR23 and does not exhibit a notable preference for K48- versus K63-linked ubiquitin chains.[106]

Rad23 and Dsk2 UBL domains bind Rpn10,[107,108] Rpn13,[71] and Rpn1,[82] and proteomics studies of human cells identify hPLIC1 and hHR23B associated with the RP.[109] Furthermore, hHR23B is reported to stimulate proteasome binding of K48-linked chains[18] and is essential for viability in more than 90% of mice.[110] Like Rpn13, hHR23a's ubiquitin-binding domains engage in interdomain interactions, in this case with its UBL domain.[111] This interaction may convey selectivity for higher-affinity ubiquitin interactions, as hHR23a's affinity for ubiquitin is increased when its UBL domain is deleted.[112] Depending on their protein levels, UBL−UBA proteins can facilitate or inhibit the degradation of ubiquitinated substrates,[113] suggesting a complex role with the UPS. hHR23 proteins have long flexible linker regions connecting their functional domains,[111] but their noncomplex amino acid composition protects these proteins from proteolysis by the proteasome.[114] UBL−UBA proteins share redundant functions[115−118] and can bind each other,[119] but they also have distinct roles, such as for Rad23 in DNA repair[120] and Dsk2 in neuropathology.[121]

■ SUBSTRATE PROCESSING

The axial channel of the CP is ~13 Å wide when open,[21] a space sufficiently narrow to prevent the passage of the majority of ubiquitinated substrates in their native form. Moreover, ubiquitin forms a very stable structure with a melting temperature in excess of 80 °C,[122] and turnover of stably folded proteins proceeds slowly because of the high energy requirement for their thermodynamic destabilization.[123] Thus, DUB removal and recycling of ubiquitin is coordinated with substrate unfolding and translocation into the CP for proteolysis.

Deubiquitination. The RP lid metalloenzyme Rpn11 (Poh1) is critical for degradation-coupled substrate deubiquitination,[72,73,124] and its activity in mature proteasomes requires ATP hydrolysis.[72,73] It is placed above the ATPases and near the translocation pore by EM[44,45,54] (Figure 3). Cross-linking studies of *Schizosaccharomyces pombe* colocalize the Rpn11 C-terminal domain with the N-terminal end of Rpt3,[52] potentially linking its activity to this ATPase. ATP hydrolysis by the Rpt proteins may initiate a "commitment step",[125] whereby an unstructured end of the substrate is bound tightly through conformational changes in the Rpts that position it in an orientation to allow deubiquitination.[52] Consistent with this model, Rpn11 preferentially hydrolyzes isopeptide linkages at the ubiquitin proximal to the substrate, thus cleaving the ubiquitin chain as a whole.[72]

Rpn11 and Rpn8 both contain N-terminal MPN domains, and these two proteins interact in the RP.[44,45] The MPN domain in Rpn11 coordinates a zinc ion and catalyzes deubiquitination,[72,73] whereas Rpn8 lacks a zinc-binding motif and is unlikely to perform DUB activity.[124] Overexpressed Rpn8 can partially rescue phenotypes of the Rpn11 C-terminal domain mutation,[126] suggesting that it may aid Rpn11 through a noncatalytic role. The exact function of Rpn8 and its link to Rpn11 is not known.

Although removal of ubiquitin is an important step in RP processing, it is antagonistic for degradation if it occurs prematurely. If the chain is shortened or eliminated prior to substrate commitment, the target protein may escape. The RP houses two additional DUBs responsible for shortening ubiquitin chains. Ubp6 (Usp14)[83,127] and Uch2 (Uch37)[128] cleave short polymers from ubiquitin chains, which may delay proteolysis.[129] Curiously, Ubp6 appears to maintain inhibitory effects on substrate turnover even in catalytically inactive mutants,[129] suggesting that it may assume noncatalytic roles in proteasome regulation. Studies have linked Ubp6 to RP assembly[130] and channel opening.[131]

Uch37 (also known as UCHL5) binds to the C-terminal domain of Rpn13, which activates it and brings it into the proximity of RP-bound substrates.[85,89,90] It is reported to selectively remove single ubiquitin moieties from the distal end of chains, and it therefore may be responsible for processing poorly ubiquitinated or unproductively bound substrates.[128] The fact that Uch37 binds and is activated by Rpn13 raises the potential for its selective activity on substrates docked via Rpn13.

Unfolding and Translocation. Interaction of the substrate with the Rpt proteins is thought to be initiated via intrinsically unfolded regions[132] and occur through a stepwise process that couples ATP hydrolysis with unfolding. PAN[133] and the bacterial AAA ATPases HslU,[134] ClpA,[135,136] and ClpX[137,138] contain Ar−φ loops in their ATPase domain that undergo a conformational switch in response to ATP hydrolysis, thus coupling chemical energy to mechanical force. A comparison of 26S EM structures with hydrolyzable versus nonhydrolyzable ATP demonstrates conformational mobility of the Rpts,[55] consistent with this model (Figure 5). Mutation of the Ar−φ sequence in yeast RP ATPases results in accumulation of ubiquitinated protein conjugates,[133] and as in the bacterial ATPases, these loops are flexible.[52] ATP binding and hydrolysis have been proposed to proceed stepwise or "wobble" around the Rpt ring, based on a study that found ATP to bind optimally to two PAN subunits at a time and a maximum of four PAN subunits.[59] Mutations in these loops for single Rpt proteins provide a range of phenotypes that vary depending on the particular Rpt affected,[139] suggesting their functional asymmetry. The requirement for the C-terminal HbYX motif also differs according to the Rpt protein with Rpt2 and -5 HbYX motifs sufficient for gate opening[41,140] and the Rpt3 HbYX motif essential for RP assembly.[141]

Structures of the CTD fragments of PAN,[51,142] Rpt1,[143,144] Rpt3,[145] and Rpt6[146] each contain a similar four-helix bundle adjoined by nonstructured loop regions. Electron density was not observed for the C-terminal region containing their HbYX motifs, and the corresponding Rpt6 tail was directly observed to be randomly coiled and flexible by NMR.[146] Isolated yeast Rpt6 CTD exhibits helix−coil transitions that influence its interaction with assembly chaperone Rpn14, and conformational exchange was similarly observed in yeast Rpt4 and Rpt5
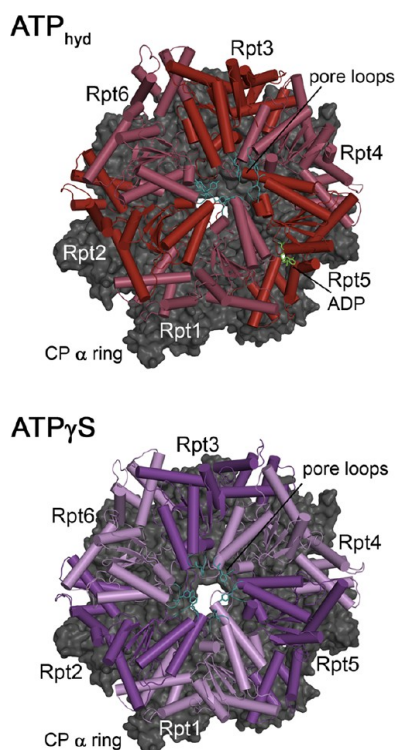
Figure 5. Model of the Rpt unfoldase pore. View of the Rpt ring illustrating the translocation pore relative to the CP (gray), with hydrolyzable ATP (red; adapted from PDB entry 4B4T) or nonhydrolyzable ATPγS (purple; adapted from PDB entry 4BGR) and the CC and OB regions omitted for the sake of clarity. The pore loops that contain the Ar−φ motif are highlighted (teal). A modeled ADP molecule (green) is shown bound to Rpt5 of the EM reconstruction generated with hydrolyzable ATP; its placement is based on that observed in PAN (PDB entry 3H4M).

CTDs.[146] Rpt6 may play an important role in nucleating formation of the RP base,[58,147] a process that its CTD dynamics may facilitate.[146] Upon attachment of the lid, the yeast Rpt−CP interface appears to be reconfigured such that a specific interaction of Rpt6 with the α2α3 pocket is broken to accommodate stable binding of Rpt2, -3, and -5 in mature proteasomes.[58]

An NMR study demonstrated the CP from *Thermoplasma acidophilum* exhibits a high degree of conformational variability, rapidly interconverting between multiple conformers that differ along contiguous regions that connect activator-binding sites to the catalytic site.[148] Binding of the 11S variant and mutation of residues that contact activators shift the relative population of these conformers and result in changes in substrate hydrolysis.[148] Similarly, the cleavage preference of the human CP varies depending on the 11S variant present;[149] humans have REGα, REGβ, and REGγ 11S activators. Thus, an allosteric pathway appears to span the ∼60 Å distance between the activator-binding and catalytic regions of the CP, and this allostery provides a mechanism by which activators influence product formation.

Proteasome in Action. Decades of concerted effort have yielded snapshots of the CP docked with various activators. These snapshots reveal shared mechanistic features and highlight an important role for protein dynamics at the proteasome. In addition to the act of activator binding, dynamics are propagated at the proteasome by transient

associations, ATP binding and hydrolysis, and DUB activity, as discussed in this review. Other factors may also drive proteolysis and motion, including post-translational modifications of proteasome subunits[150−152] and substrate interactions.[153,154] Recent studies have also identified the involvement of multiple chaperones in RP nucleation and base assembly that interact with the CTD of specific Rpt subunits, as reviewed in refs 155 and 156. Attachment of the lid complex may drive conformational changes that alter the Rpt−CP interaction profile and promote holoenzyme maturation.[58,74] The timing and regulation of base assembly and lid attachment have the potential to regulate substrate turnover in response to specific physiological conditions. This area holds promise for the development of therapeutics that specifically target the activation of proteasomes, the use of different activator types, or the formation of heterogeneous doubly capped CP, which may alter the degradation rate or substrate preference.

Recent advances in cryo-EM have provided structures of RP−CP holoenzymes at subnanometer resolution, allowing more accurate models of the topology of the RP as it recognizes and processes substrates (Figure 6). One of these studies found
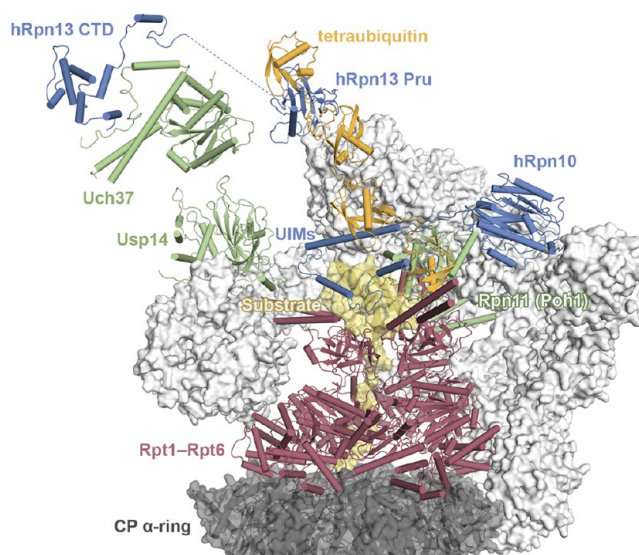

Figure 6. Modeling the RP as it processes the substrate. Model structure of the human RP (adapted from PDB entry 4B4T) as it recognizes and processes a ubiquitinated substrate. A K48-linked tetraubiquitin chain (orange) connected to a substrate (yellow) is bound simultaneously by ubiquitin receptors hRpn13 and hRpn10 (blue). The ATPase ring (red) is bound to a nonstructured end of the substrate, committing it to degradation. The ubiquitin−substrate isopeptide bond is placed adjacent to Rpn11 (Poh1, green) for hydrolysis as the substrate is unfolded and translocated into the CP (dark gray). hRpn13's C-terminal domain is bound to Uch37 (green; adapted from PDB entry 3IHR), which along with Ubp6 (Usp14, green, PDB entry 2AYN) deconjugates chains. The remaining RP subunits are displayed with a light gray surface.

differences between the two RP caps,[54] in agreement with its dynamic nature. The transient nature of proteasome interacting proteins, altered conformations upon ATP binding and hydrolysis, and functional subunit redundancies may produce many independently functioning "minor" states that represent a part of the dynamic portrait of the 26S proteasome in action.

The roles of certain RP components and their conformation may be related to properties of the substrate and ubiquitin

chain. As its UIMs are essential,[92] Rpn10-mediated substrate recognition may be critical for localizing the proximal ubiquitin near Rpn11, which in turn places the substrate at the mouth of the ATPase ring. It is not known exactly how the proteasome locks onto the substrate, although the Rpt OB folds are positioned at the most distal end of the substrate's path (Figures 2 and 6). These folds are defined by their ability to form versatile interactions,[157] and those of the Rpts are viable candidates for this. The presence of other subunits may be necessary only under certain conditions; for example, Rpn13 may be required for degrading certain substrates or orienting substrates with extended ubiquitin chains. K48-linked chains require a minimum of four ubiquitins to interact with both receptors in the current model (Figure 6), and it seems likely that chain length would affect the catalytic roles of Uch37 and Ubp6, although the specifics are unclear. It is not yet known what factors influence RP composition under various contexts.

Other questions are inspired by structural models of the RP−CP proteasome. The doubly capped RP$_2$−CP proteasome contains an intrinsic rotation between the two RP assemblies because of the subunit-level asymmetry of the eukaryotic CP (Figure 3). The 11S activator is a homoheptamer, and the homohexameric PAN binds to the symmetric archaeal CP; however, the unique Rpts interact with partial specificity for unique CP pockets.[58,158] It is not known what this RP orientation may mean for substrate proteolysis. Similarly, little of the functional role of the PCI hexameric shield is known (Figure 3), and it is difficult to envision its conservation if it is serving solely as a scaffold. Each proteasome structure determined is a snapshot of the process that occurs during proteolysis, and the ability to integrate structure, dynamics, and function may hold the key to fully understand its molecular mechanisms.

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Notes
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