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Molecular Architecture and Assembly of the Eukaryotic Proteasome

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Abstract

The eukaryotic ubiquitin-proteasome system is responsible for most cellular quality-control and regulatory protein degradation. Its substrates, which are usually modified by polymers of ubiquitin, are ultimately degraded by the 26S proteasome. This 2.6 MDa protein complex is separated into a barrel-shaped proteolytic 20S core particle (CP) of 28 subunits capped on one or both ends by a 19S regulatory particle (RP) comprising at least 19 subunits. The RP coordinates substrate recognition, removal of substrate polyubiquitin chains, and substrate unfolding and translocation into the CP for degradation. While many atomic structures of the CP have been determined, the RP has resisted high-resolution analysis. Recently, however, a combination of cryo-electron microscopy (cryo-EM), biochemical analysis, and crystal structure determination of several RP subunits has yielded a near-atomic resolution view of much of the complex. Major new insights into chaperone-assisted proteasome assembly have also recently been made. Here we review these novel findings.

Keywords

Proteasome; ubiquitin; assembly; ATPase; deubiquitylation; proteolysis

1. INTRODUCTION

The ubiquitin-proteasome system (UPS) is responsible for the majority of regulatory and quality-control protein degradation in eukaryotic cells. Nearly every cellular process is affected by the UPS (1, 2). The UPS utilizes ATP hydrolysis at several steps to mediate the selective destruction of its substrates. Degradation of a protein by the UPS is typically mediated by the energy-dependent covalent attachment of the small protein ubiquitin (Ub) to one or more lysines within the target protein via the concerted action of three enzymes, E1, E2, and E3 (Figure 1A). Additional Ubs can also be ligated to the initial Ub via one of its seven lysines (or its N-terminus), forming polyubiquitin (polyUb) chains. A chain of four or more Ubs is generally necessary and sufficient to create a targeting signal for delivery to the proteasome (3). The proteasome is responsible for recognizing the substrate, removing its polyUb tag, unfolding the substrate, and cleaving it into short peptides.

In this review, we focus on the structure and assembly of the proteasome, particularly the regulatory particle (RP), as there have been numerous breakthroughs in these areas over the past several years. For recent, more general reviews of the UPS, the reader is referred to Refs. 1, 2 and references therein.

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2. THE 26S PROTEASOME – ANATOMY OF A PROTEIN-DESTROYING MACHINE

The 26S proteasome is the largest and most complex member of an ancient superfamily of ATP-dependent chambered proteases found in all domains of life (4, 5). These proteases are characterized by an AAA+-family ATPase ring responsible for unfolding substrates and threading them through a narrow central pore into an interior proteolytic chamber, usually formed by an associated multimeric protein complex (Figure 1B). Sequestration of the proteolytic active sites within such a chamber prevents the unregulated destruction of folded cellular proteins. The ATPase ring converts the chemical energy of ATP hydrolysis into mechanical force for substrate unfolding, allowing a polypeptide end or interior loop to be translocated into the central chamber (Figure 1B). In the eukaryotic 26S proteasome (Figure 2A), the proteolytic chamber is referred to as the 20S core particle (CP), while the AAA+ ATPase ring is contained within a separable complex called the regulatory particle (RP). In addition to the ATPase ring, the RP contains many additional specialized subunits including ones that function as receptors for the polyUb tag, and others that cleave the tag from substrates prior to their degradation.

The 26S proteasome is highly conserved throughout the Eukarya where it is found in both the nucleus and cytoplasm. Simpler forms of the proteasome have been identified in archaea as well as in actinobacteria, although Ub has only been found in eukaryotes (6, 7). Several other protein complexes can bind the ends of the CP cylinder, but their precise contributions to the regulation of the CP remain less clear (Table 1). Because of space constraints, only RP-CP complexes, i.e., the 26S proteasome, will be discussed in this review.

2A. The 20S Core Particle

The twofold-symmetric CP comprises 28 related polypeptides encoded by 14 separate genes. Atomic structures of the CP from yeast and mammals have been solved (8–12), and their overall structures and subunit arrangements are virtually identical, despite millions of years of evolutionary divergence between these species. The CP consists of four axially stacked heteroheptameric rings (Figure 2B). The inner rings contain seven distinct α subunits (α -1–7), while the outer rings consist of seven different β subunits (β -1–7). The α , β , and β subunits contain the proteolytic active sites, and each site cleaves preferentially after particular amino acid residues (13). In mammals, four additional β subunits have been discovered: β i, β 2i, β 5i, and β t, where “i” and “t” stand for immuno- and thymo-, respectively (14, 15). These subunits are highly expressed in certain immune system tissues or are induced by particular stimuli, such as interferon- γ exposure, and they replace the canonical active site-bearing β subunits within the CP, altering CP proteolytic specificity. The β i, β 2i, β 5i-substituted CP, called the “immunoproteasome,” generates substrate cleavage patterns that enhance loading of peptides onto the class I major histocompatibility complex for immune presentation to killer T cells (16). “Thymoproteasomes,” which contain β i, β 2i, and β t, appear to increase the repertoire of “self” peptides for positive selection during T-cell development in the thymus (15).

In the crystal structures of the isolated CP, the entrances into the internal proteolytic chamber are usually occluded by the N-terminal tails of specific β subunits (8, 9). These tails form an ordered gate that must be opened for substrate entry. Thus, it is thought that free CP, which is detectable in cell extracts, has low activity by itself, at least against folded proteins (17). Atomic force microscopy analysis indicates that the gate exists in a dynamic equilibrium between open and closed states that is biased toward the latter (18). Many proteasomal activators, such as the RP, and proteins known to bind the β -ring surface, contain a conserved HbYX (hydrophobic, tyrosine, any amino acid) motif at their very C-

terminus (19–22). The HbYX motif inserts into a pocket formed at the interface of adjacent β subunits (Figure 2C), where the C-terminal carboxylate of the activator subunit forms a salt bridge with the β amine of a conserved lysine residue in the pocket (Figure 2D) (23, 24). These interactions help to maintain contact with the CP, and *in vitro* studies of CP activation by synthetic HbYX peptides suggest that their insertion into the β -ring pockets induces conformational changes in the β -ring that displaces (at least in part) the β -ring N-terminal tails from the pore of the CP, allowing substrate entry (25).

2B. The 19S Regulatory Particle (RP)

The RP is responsible for the binding, deubiquitylation, unfolding, and translocation of substrates into the CP, as well as opening the CP β -ring gate. As such, the RP functions as a highly regulated gatekeeper for the majority of proteasome substrates. It contains at least 19 subunits, each usually present in a single copy. The RP can be split under certain *in vitro* conditions into two subcomplexes, termed the lid and base (Figure 2A). The base consists of nine subunits: six RP Triphosphatases, Rpt1 – 6, and three RP Non-ATPases, Rpn1, 2, and 13. Rpt1 – 6 are paralogous AAA+ ATPases that form a heterohexameric ring (26). The ATPase ring directly contacts the surface of the CP β -ring, and exerts the ATP-dependent unfolding force upon substrates that is required for their translocation into the CP for degradation. Rpn1 and Rpn2 are the two largest subunits of the proteasome. The central portions of these subunits are composed of 11 β -helical proteasome/cyclosome (PC) repeats (27), thought to form scaffolds onto which substrates and other factors dock (Figure 3). Rpn13, as well as the Rpn10 protein, directly binds Ub, and both proteins function as polyUb-substrate receptors (28–32). In addition to Rpn10 and Rpn13, several extrinsic Ub receptors have been identified, including the UBA-UBL proteins Rad23, Dsk2, and Ddi1 (33–36). The Ub-associated (UBA) domains of these proteins bind the polyUb chains on substrates and shuttle them to the proteasome through interactions of their Ub-like (UBL) domains with the Rpn1 subunit (37, 38).

The lid consists of nine different Rpn subunits, Rpn3, 5–9, 11, 12, and Sem1 (Rpn15). The Rpn11 subunit is the only integral and essential deubiquitylating enzyme of the proteasome, (39, 40). The lid is structurally related to the COP9 signalosome (CSN) and the eIF3 translation initiation complexes (41, 42). While the composition of the CSN and eIF3 varies somewhat across eukarya (43, 44), the canonical form of each of these complexes contains six subunits with Proteasome/CSN/Initiation Complex (PCI) domains and two subunits with Mpr1/Pad1/N-terminal (MPN) domains. The composition of the lid appears invariant in eukaryotes, consisting of the canonical six PCI proteins (Rpn3, 5, 6, 7, 9, and 12) and two MPN subunits (Rpn8 and Rpn11), as well as Sem1, a small acidic protein with neither PCI nor MPN domains.

3. ARCHITECTURE OF THE 19S REGULATORY PARTICLE

An unprecedented view of RP structure and subunit architecture has recently emerged. Although the overall shape of the RP had been observed long ago by EM (45), the positions of its subunits and their high resolution structures were unknown. Over the past three years, a series of high-resolution cryo-EM-based reconstructions, along with biochemical experiments and crystal-structure determinations for several isolated subunits, have yielded a much clearer picture of RP architecture (Figure 3).

Surprisingly, the RP “lid” and “base” terminology (41) turns out to be somewhat misleading. The base includes subunits positioned further from the CP than any lid subunit (46–48). Rather than the lid capping the base, it instead straddles the long edge of the RP, contacting both the base and the CP β -ring (Figure 2A). Nevertheless, to avoid confusion, we will

continue to use the lid and base terms here. We describe the new proteasome structural data in the following sections.

3A. The RP Base

The Rpt ATPases—As was expected from structures of other AAA+ chambered proteases (5), the Rpt ATPase ring directly abuts the ends of the CP cylinder. Unexpectedly, however, the new EM models indicate that the pore of the ATPase ring does not align perfectly with the pore of the CP, being axially offset by as much as 30 Å and tilted on its vertical axis by approximately 5–10° (49, 50). The functional significance of this remains unknown. The six ATPases adopt a single arrangement in normal cells: Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5 (26). Although this arrangement was originally predicted based on sequence divergence among the proteasomal ATPases (51) and later by molecular modeling approaches (52), several alternative arrangements had been proposed based upon yeast two-hybrid (53), chemical crosslinking (54) and other (55) studies. Further, whether the ATPases normally adopt multiple arrangements had been unclear. The Rpt1-2-6-3-4-5 arrangement was unambiguously established only recently using engineered disulfide crosslinking (26); this analysis was guided by the structure of an archaeal homohexameric ATPase ring, proteasome-activating nucleotidase (PAN) (56, 57). The success of this approach implies a close evolutionary conservation of the core ATPase ring architecture.

The Rpts, PAN, and the actinobacterial ortholog ATPase forming Ring-shaped Complexes (ARC) share a similar domain organization (Figure 4A) (56, 57). Each contains an N-terminal region that includes a coiled-coil domain (CC), an oligonucleotide/oligosaccharide-binding (OB) domain, the AAA+ ATPase domain, and a C-terminal α-helical domain that sits on the outer surface of the ATPase ring. In AAA+ ATPases, the protein sequence N-terminal to the ATPase domain is typically specialized for its particular function, and as such, the combination of N-terminal CC and OB domains appears unique to the proteasomal ATPases. In PAN and ARC, there is a critical and highly conserved proline residue at the junction between the N-terminal CC region and the OB domain (Figure 4A) (56, 57). Proline is unusual in that the peptide bond it forms with the preceding residue can adopt either a *cis* or *trans* configuration. In the crystal structures of PAN N-domains, this peptide bond alternates between *cis* and *trans* conformations in adjacent subunits, allowing their N-terminal helices to form pairwise CCs. The ATPase subunits in the PAN crystal structure thus form a “trimer of dimers” (Figure 4B) (56, 57). This *cis-trans* pairing of ATPases appears to be conserved in the eukaryotic ATPase ring: Rpt2, Rpt3, and Rpt5 contain highly conserved prolines that align with the critical PAN proline (26, 56, 57) and take up alternating places within the ATPase ring (Figure 4C). In agreement, many isolated base subcomplexes, which are thought to be assembly intermediates, contain pairs of Rpt subunits, and in each case they are a predicted *cis-trans* pair.

Rpn1 and Rpn2—Until recently, the structures and positions within the RP of Rpn1 and Rpn2 remained unclear and have been subject to extensive debate. The PC repeats of these proteins were anticipated to take on toroidal or horseshoe-like superfolds (58, 59) and had been hypothesized to be nestled inside the pore of the ATPase ring (or perhaps stacked on top) (58, 60). The strong structural similarity of the eukaryotic ATPase ring compared to PAN, however, argued against this possibility because the central pore of PAN is extremely narrow and unable to accommodate a large folded domain even at its widest point (26, 56, 57).

The crystal structure of yeast Rpn2 has recently been determined (27). It resembles an upside-down tobacco pipe (Figure 5A), with a central region of 11 PC repeats that make up the pipe’s barrel (green), bracketed by an extended N-terminal α-helical domain comprising

the stem (blue) and a small C-terminal globular domain that packs against the barrel (red). While the PC repeats of Rpn2 form a toroid as was predicted, their arrangement was quite unexpected – they adopt a tightly packed shape in which the pore formed by the PC repeats is plugged by two α -helices extending into the toroid (Figure 5B). This packing explains the highly hydrophobic character of these two central helices, as they are largely sequestered from solvent. Thus, the toroidal PC repeat domain of Rpn2 is very densely packed and is likely quite rigid. The molecular model of Rpn2 is readily docked into the RP EM density due to its large size and distinctive shape (Figure 3). Rpn2 is one of the most distally positioned subunits along the proteasomal long axis. Its N-terminal stem-like extension and part of the toroidal domain interacts with the N-termini of Rpt3 and Rpt6, while the center of the toroid and the C-terminal domain are positioned over the pore of the ATPase ring (48).

Comparison of purified Rpn2 and Rpn1 by EM indicates that these two proteins adopt very similar folds, consistent with their ~20% sequence identity (27, 58). In the 26S cryo-EM structures, Rpn1 also harbors a toroidal domain, presumably encoded by its PC repeats. Notably, all known docking sites of the extrinsic ubiquitin receptors lie within this toroid (37, 38). Thus, the Rpn1 (and perhaps Rpn2) toroids likely serve as loading platforms for incoming substrates or other proteins. In contrast to Rpn2, Rpn1 makes extensive contacts with the outside face of the ATPase ring in 26S holoenzyme EM structures (Figure 3) (46–48), and its density is somewhat variable, suggesting it may undergo movement. The close association of Rpn1 with the ATPase ring may help to control substrate docking by coupling conformational changes in Rpn1 with the binding and/or hydrolysis of ATP by the ATPases.

Rpn10, Rpn13, and the Extrinsic Shuttle Factors—Two general pathways exist for the delivery of ubiquitylated substrates to the proteasome: Ub recognition by the intrinsic receptors Rpn10 and Rpn13 or shuttling to the proteasome via the extrinsic receptors, which include Rad23, Dsk2, and Ddi1 (Figure 5C). While the cellular abundance of the extrinsic and intrinsic receptors are very similar, at least in yeast (61), the extrinsic receptors are generally substoichiometric on purified proteasomes while the occupancy of Rpn10 and Rpn13 on purified proteasomes is typically greater than 50% (62). As the extrinsic receptors have been suggested to bind the proteasome transiently, this low apparent occupancy could potentially reflect their steady-state binding levels *in vivo*. Rpn13 interacts with a short peptide segment in the C-terminal domain of Rpn2, positioning it far from the CP (Figure 3) (27, 63). Rpn13 adopts a pleckstrin-homology domain-like fold (Figure 3) named the pleckstrin-like receptor for Ub (PRU) (28). Rpn13 binds Ub chains with high affinity (~90 nM K_D for Lys48-linked diUb) via loops extending from the α -strands comprising the PRU domain (Figure 5D). Like Rpn13, Rpn10 directly binds Ub, but in this case via its α -helical Ub-interacting motif (UIM) (Figure 5D). Rpn10 contains one to three UIMs at its C-terminus depending on the species, but even Rpn10 orthologs with a single UIM prefer polyUb chains over monoUb (64, 65). Solution-structure studies indicate that the UIM region of Rpn10 is highly flexible (64), and this domain appears as a diffuse, poorly resolved density in EM structures of the 26S proteasome (62). In contrast, the N-terminal region of Rpn10 consists of a tightly folded von Willebrand factor A (vWA) domain that is readily resolved in these structures (Figure 3) (62, 66). Despite findings that loss of Rpn10 or disruption of the vWA domain destabilizes lid-base association within the RP (41, 53, 67), the vWA domain makes extensive contact only with subunits in the lid (46–48), suggesting it functions indirectly to promote or maintain RP stability (see below).

3B. The RP Lid

EM structures are now available for the RP lid as an isolated complex (46) and in the context of the 26S holoenzyme (46–48). The lid appears to act, in part, as a clamp to keep the RP and CP together (68). The lid looks similar to a horseshoe, with the arc formed by the

PCI domains of (from end to end) Rpn9, 5, 6, 7, 3, and 12 (Figure 6). The N-terminal helical extensions of each PCI subunit extend radially away from the horseshoe like fingers on a hand. Within the 26S proteasome, the Rpn5 and Rpn6 N-termini extend toward the CP and make extensive contact with the outer flanks of both the RP base and the CP (46, 68), suggesting the potential for coupling between the enzymatic activities of the lid and the rest of the proteasome. In the center of the horseshoe are the two MPN subunits, Rpn8 and Rpn11, which sit side-by-side to form a heterodimer positioned over the ATPase ring in the 26S holoenzyme (46, 69). Each MPN subunit makes extensive contact with several PCI subunits. Their N-termini appear to be flexible or disordered in the EM density of the isolated lid, but become more ordered in the 26S proteasome (46), suggesting their positioning is constrained upon their incorporation into the RP. Similarly, large movements in Rpn5 and Rpn3 likely occur as the lid is integrated into the RP (or 26S); these movements may be important for accommodation of base subunits upon RP assembly (46). While the exact position of Sem1 in the lid has not been determined, it has been found in a complex with Rpn3 and Rpn7 (67), indicating it directly binds one or both of these subunits.

4. STRUCTURAL INSIGHTS INTO SUBSTRATE SELECTION AND DEGRADATION

4A. Substrate Recognition and Positioning for Degradation

With a nearly complete three-dimensional model of the RP at subnanometer resolution, it now becomes apparent that the placement of subunits and proteasome-associated factors within the RP is likely organized in a way that facilitates movement of the substrate from the outer edges of the RP toward the CP for degradation. The subunits responsible for each successive step in substrate manipulation (i.e., recognition, deubiquitylation, unfolding, and translocation into the CP) are positioned sequentially closer to the CP pore. At the periphery of the RP are the Ub receptors Rpn10 and Rpn13. Both are located above the ATPase ring (“below” the ring is defined as the CP-binding surface) with an unobstructed potential substrate path toward the ATPase pore. Rpn13 is positioned above Rpt1 and Rpt2, while affinity labeling of the Rpn10 UIM domain indicates this region projects internally toward the Rpt4/Rpt5 CC (62). Given the placement of the Ub receptors, it is tempting to hypothesize that the CCs of specific pairs of Rpt subunits are responsible for binding substrates that dock at the proteasome via particular Ub receptors.

In contrast to Rpn10 and Rpn13, the positions and orientations of the shuttle factors upon binding the proteasome are unknown. Interpretation of how they dock onto the proteasome is complicated by their interaction with multiple subunits within the RP and the variability of Rpn1 density within the EM images (37, 64, 65, 70). While Rpn1 is considered the major binding site for Rad23, Dsk2, and Ddi1, the Ubl domains of the human orthologs of Rad23, Dsk2, or Ddi1 can be bound by the UIM and PRU domains of hsRpn10 and hsRpn13 (29, 65). The physiological significance of such shuttle factor Ubl-binding to Rpn10 and Rpn13 and whether these latter receptors cooperate with Rpn1 to process substrates bound to the shuttle factors remain to be explored. Deletions of the shuttle factor genes display synthetic genetic defects when combined with *RPN10* or *RPN13* deletions (29), arguing against a model in which the shuttle factors function exclusively by handing off substrates to the intrinsic receptors.

4B. Substrate Deubiquitylation

After a substrate binds the proteasome, its polyUb tag must be removed either prior to or potentially during substrate degradation. The proteasome harbors several deubiquitylating enzymes (DUBs): two non-integral DUBs Ubp6 (Usp14 in humans) and Uch37 (not in all species), and the Rpn11 subunit of the RP lid (Table I) (39, 40). Additionally, several

ubiquitin ligases, most notably Hul5, are found associated with proteasomes as well, strongly suggesting that the polyUb chains of substrates can undergo remodeling on the proteasome, perhaps as a substrate triage or quality control step (71). While the non-integral proteasomal DUBs trim polyUb chains from the distal end, Rpn11 instead removes whole chains at their point of attachment to substrate. This observation, and the severe growth phenotypes associated with attenuation of Rpn11 catalytic activity strongly suggests it is the primary DUB responsible for removal of polyUb chains immediately prior to or concomitant with the initiation of protein unfolding and degradation by the proteasome.

The catalytic metalloprotease domain of Rpn11 is related to the structurally determined MPN+/JAMM domain of AMSH, a functionally distinct DUB. Modeling of the atomic structure of the AMSH MPN+/JAMM domain into the EM density of Rpn11 in the 26S proteasome suggests that the Rpn11 active site is positioned 10–20 Å over the pore of the ATPase ring (46), and this positioning has recently been confirmed by near-atomic resolution EM (69). This proximity to the ATPase pore suggests a model in which substrates are unfolded until the polyUb chain-attachment site is pulled near the pore of the ATPase ring, bringing it into position for deubiquitylation by Rpn11. Conversely, it may be that positioning of the polyUb chain-attachment site and/or deubiquitylation triggers the initiation of unfolding and threading through the ATPase ring. The first model is supported by the finding that unfolding generally requires an unstructured or structurally destabilized region for initiation (72). Furthermore, in the case of substrates with polyUb chains attached at multiple sites, Rpn11 may need to act multiple times on the substrate; in this case, the threading action of the ATPases on the substrate could pull successive polyUb attachment sites into the Rpn11 active site during unfolding and translocation.

4C. Substrate Unfolding by the AAA+ ATPase Ring

Many inferences about the function and mechanism of the proteasomal ATPase ring have been drawn from the simpler homomeric ATPases from archaea and bacteria. Like the eukaryotic ATPases, PAN forms a hexameric ring that binds the archaeal CP in an ATP-dependent manner (73). Using PAN as a model, it has been deduced recently that the ATPase ring can bind a maximum of four nucleotides (ATP or ADP) at once, with the remaining two sites being empty due to negative allostery (74). Based on this finding and information about the relative rates of ATP-binding and hydrolysis, it appears that nucleotides tend to bind to the ATPase ring and be hydrolyzed in pairs, by subunits *para* to one another in the six-membered ring.

While PAN has been a powerful model for elucidating some of the basic features of the proteasomal ATPases, emerging structural, biochemical, and genetic data suggest that the eukaryotic ATPase ring diverges from PAN in several ways. Aside from the obvious diversification of eukaryotic Rpt sequences compared to PAN, a recent EM analysis of the 26S holoenzyme suggests that the ATPases actually take up a corkscrew-like arrangement similar to that seen in some ATP-dependent helicases, in which the pore residues form a downward-spiraling “staircase,” while the N-terminal OB-fold ring and the small C-terminal domains of the ATPases remain largely planar (46, 69). This observation raises the possibility that, instead of, or in addition to, the mechanical forces from the ATPases being exerted through small, local movements of pore loops as has been proposed for PAN (75), unfolding may be driven by movements of the entire large domains in which they proceed through the conformations observed in the staircase arrangement (46). Biochemical and phenotypic assays comparing mutations at paralogous positions in each eukaryotic ATPase subunit have unveiled Rpt-specific defects in substrate unfolding, CP gate-opening, and resistance to proteasome stresses (76, 77). It is unclear at the moment whether these apparently specialized roles of the ATPases reflects a mechanism allowing processing of substrates with more diverse sequences and structures than those unfolded by the archaeal

and bacterial ATPases, or if instead this functional diversification helps to couple unfolding and proteolysis to the activity of the proteasomal lid, which is not present in these simpler proteasomes.

5. ASSEMBLY OF THE 26S PROTEASOME

The proteasomes of archaea and actinobacteria are structurally much simpler than those of eukaryotes (78). In most species, there is a single predicted type of β subunit, β subunit, and ATPase subunit. Thus, assembly of these compositionally simple proteasomes requires only that the correct number of subunits be placed in each ring, and that the rings stack properly. In contrast, eukaryotic proteasomes contain seven distinct copies of each β and each β subunit, and there are six different ATPases. Each subunit shows high similarity to its paralogs, yet it must typically occupy a single, defined site within the final structure. Thus, it is necessary to control the relative position of each paralogous subunit to form the appropriate subunit arrangement. Similarly, the task of finding the appropriate register between each ring and its neighboring rings is more complicated than in the simpler prokaryotic proteasomes, as the rotational symmetry within each ring is broken.

In many cases, a specific order of subunit incorporations into assembly intermediates may be important to prevent competition for similar binding sites between subunits or structural occlusion of binding sites during assembly. These alternative or incomplete arrangements could stall proteasome biogenesis or lead to dead-end assembly products. This may be especially true in the eukaryotic RP, which contains 13 non-ATPase subunits not present in simpler proteasomes. Finally, the high level of coordination of multiple enzymes within the proteasome requires that the subunits harboring enzymatic activity be restrained until the proteasome is fully assembled to prevent the decoupling of deubiquitylation, substrate unfolding, and proteolysis.

Eukaryotic cells have evolved several strategies to meet these demands. These generally fall into three categories: intrinsic regulatory elements such as certain CP β subunit appendages; extrinsic assembly chaperones that control the assembly of specific proteasomal subcomplexes; and hierarchical assembly mechanisms that act to regulate the order in which specific subunits or subcomplexes of the proteasome associate (78, 79). We present an overview of 20S CP (Figure 7) and 19S RP (Figure 8, Figure 9) assembly and highlight examples of how these strategies are utilized in proteasome biogenesis.

5A. 20S Core Particle Assembly

Assembly of the eukaryotic CP initiates with the formation of the heptameric β ring (Figure 7). The exact order of β subunit incorporation is unknown, and the possibility of assembling β rings with aberrant subunit arrangements (e.g., homoheptameric rings) is well known (80–82). β subunits, several of which are synthesized as precursors with N-terminal propeptides, then add to the β ring in an ordered fashion, creating a half-proteasome (83–86). Upon dimerization of two half-proteasomes to form the preholoproteasome (PHP), the active site-bearing β subunits undergo autocatalytic cleavage of their propeptides, generating the mature catalytic sites and completing CP assembly (87).

CP assembly is facilitated by at least three dedicated chaperone factors. Two heterodimeric chaperone complexes participate in the assembly of the β ring: Proteasome Biogenesis-Associated (Pba) 1/2 and Pba3/4 (PAC1/2 and PAC3/4 in mammals) (22, 88–92). Pba1/2 is a heterodimer that can bind isolated β subunits (89) and is found associated with proteasomal precursors but not the mature CP, suggesting that it is released upon maturation of the catalytic active sites (22). Pba1 and Pba2 both contain HbYX motifs, and bind to the β ring similarly to proteasomal activators (22, 93). Mutation of the critical lysine residue in

the pockets between α - β and β - β' appears to reduce binding of Pba1/2 to proteasomes, suggesting Pba1/2 binds CP precursors by HbYX tail insertion into these pockets (94). However, Pba1/2 does not enhance proteolytic activity (89). The interaction of Pba1/2 with the outer face of the α -ring suggests it may prevent premature docking of activators to the assembling CP. In mammalian cells, knockdown of PAC1/2 causes accumulation of large particles containing α -subunits but no β -subunits (89). This observation led to the proposal that Pba1/2 also function to prevents the formation of α -ring dimers that might stall proper proteasome biogenesis.

Pba3 and Pba4 also form a heterodimer, and it binds to β in vitro (88, 95). A crystal structure of Pba3/4 with β revealed that instead of binding to the outer α -ring surface, Pba3/4 instead binds to the inner surface recognized by the β -subunits (88). Modeling suggested that Pba3/4 would clash sterically with the incoming α -subunit, indicating that Pba3/4 is likely released prior to or concomitant with α -incorporation (Figure 7).

Whereas *PBA3/4* deletions enhanced the defects of numerous CP mutants, they did not exacerbate defects associated with loss of the β -subunit (95). In *a3* yeast, a second copy of α fills the slot normally occupied by β (96). Deletion of *PBA4* resulted in a large fraction of CPs bearing the alternative α - α configuration. Thus, Pba3/4 functions, at least in part, to ensure that β is inserted into its appropriate place in the α -ring. Intriguingly, cells with increased amounts of α - α proteasomes display a growth advantage under certain stresses, suggesting a potential physiological role for these alternative proteasomes.

Upon completion of the α -ring, β -subunits assemble onto the inner surface of the α -ring. Initiation of β -ring assembly is accompanied by the binding of Ump1 to the assembly intermediate (97). Ump1 may help prevent the premature dimerization of precursors containing incomplete sets of β -subunits (91, 98, 99). Completion of the β -ring overcomes this Ump1-dependent checkpoint (91, 99) and is followed closely by dimerization of half-proteasomes and degradation of Ump1 upon maturation of the CP catalytic sites (98). Systematic knockdown of β -subunits in mammalian cells suggested that β -subunit addition begins with β , and proceeds in a defined order, although the timing of β -addition was ambiguous (97). Ump1 was already associated with the α -ring in β -knockdown cells where the only β -subunit present on the α -ring is β , suggesting Ump1 binds with or before β . The ordered association of β -subunits is driven, at least in part, by the propeptides and peptide tails of certain β -subunits. Both the β -propeptide and C-terminal tail (which wraps around β) are required for efficient incorporation of the β -subunit in mammals (and probably also yeast) (97, 100). Similarly, the β -propeptide helps drive incorporation of β , and the C-terminal tail of β is required for efficient β -incorporation. The β -tail in conjunction with the β -propeptide also promotes the dimerization of fully assembled half-proteasomes to form the PHP.

5B. Assembly of the 19S Regulatory Particle

Like CP assembly, the RP also requires the assistance of dedicated chaperones for its efficient and accurate assembly in vivo. The lid and base subcomplexes of the RP appear to assemble independently and then associate with one another (and Rpn10) to complete the RP (67, 101). The recent RP EM structures show a dense mesh of protein-protein interactions between lid and base subunits. Therefore, it may be advantageous to form these subcomplexes prior to their association in order to limit assembly of RPs with aberrant compositions. This mode of assembly may also help restrict RP DUB and ATPase activities until assembly is completed, thereby ensuring the coupling of substrate deubiquitylation, unfolding, and degradation.

Base assembly—While a single arrangement of the proteasomal ATPases is observed in vivo (26), the Rpt subunits do not seem to encode within their primary structure all of the information required for their appropriate arrangement. For example, heterologously expressed Rpt4 or coexpressed Rpt1 and Rpt2 subunits have a propensity to form non-native enzymatically active structures (102). To help guide the proper formation of the base, eukaryotes utilize at least four dedicated **RP Assembly Chaperones (RACs)**: Rpn14, Nas6, Nas2, and Hsm3 (PAAF1, gankyrin/p28, p27, and S5b in mammals) (55, 103–107) (Table 1). Each RAC binds a distinct subset of base subunits (Figure 8A), but is not present in the 26S holoenzyme, at least in yeast, consistent with roles as assembly chaperones. Deletion of each RAC either alone or in combination impairs base formation but does not affect assembly of the CP or lid. Thus, the known RACs act specifically at the base assembly stage, although a role in lid-base and/or initial RP-CP joining cannot be excluded.

Our current understanding of base assembly derives primarily from studies of relatively stable base subcomplexes isolated from yeast mutants or mammalian cells subjected to siRNA-mediated knockdowns. Similar to the trimer of dimers proposed for the archaeal PAN complex, one of the earliest steps in base assembly seems to be the formation of Rpt *cis-trans* dimers: Rpt3-Rpt6, Rpt5-Rpt4, and Rpt2-Rpt1 (Figure 4C), and their association with their specific RACs (and for Rpt1-2, association with Rpn1) (78). It is unclear whether ATPase dimerization precedes Rpt binding to RACs or non-ATPase subunits, although the RACs can bind their cognate ATPase (or a fragment thereof) in the absence of the paired ATPase subunit (106, 108). Although ATP binding may not be required for formation of Rpt *cis-trans* pairs, it does appear to be important for association of these pairs with other ATPase pairs (109).

Following formation of the Rpn14-Rpt6-Rpt3-Nas6, Rpt4-Rpt5-Nas2, and Hsm3-Rpt1-Rpt2-Rpn1 complexes (referred to as the Rpn14/Nas6, Nas2, and Hsm3 modules, respectively), they associate with one another, along with Rpn2 and Rpn13, to form the base (Figure 8A). In yeast, the Rpn14/Nas6 and Nas2 modules appear to associate first, and upon incorporation of the Hsm3 module (or before), Nas2 is released from the complex (26). In mammalian cells, a complex between the Rpn14/Nas6 and Hsm3 modules has been observed that is specifically lacking Rpt4 and Rpt5 (55), possibly indicating that the order in which these modules come together to form the base may vary between species. Alternatively, the modules may assemble in multiple orders. Rpn14, Nas6, and Hsm3 each can be found bound to the fully assembled RP but not with the 26S proteasome, suggesting that they are normally released prior to or during RP-CP binding.

Structure and Function of the RACs—Each RAC contains distinct protein-protein interaction domains: Rpn14 contains seven WD40 repeats; Nas6 seven ankyrin repeats; Nas2 a PDZ domain, and Hsm3 eleven HEAT repeats (108, 110–113). These domains mediate interaction of each RAC with its cognate ATPase: Rpn14-Rpt6, Nas6-Rpt3, Nas2-Rpt5, and Hsm3-Rpt1. Crystal structures of Nas6 and Hsm3 bound to fragments of their cognate ATPases have been determined (Figure 8B and C) (111–113); the repeat domain in each recognizes the respective Rpt C-terminal domain (CTD). Rpn14 forms a propeller as expected (Figure 8D) (110). While Rpn14 has not been crystallized with its binding partner, yeast two-hybrid and protein pulldown assays indicate it interacts with the Rpt6 CTD. In contrast, recognition of Rpt5 by Nas2 depends on the Rpt5 HbYX motif; deletion of these three C-terminal residues is sufficient to abolish Nas2 binding (108).

Despite this structural information, the molecular mechanisms by which the RACs control base assembly are still poorly understood. In some models, docking of Nas6 and Hsm3 onto the ATPase ring would clash sterically with the 20S CP (106), perhaps explaining why these RACs are absent in the full 26S proteasome. Rpn14, Nas6, and Hsm3 appear positioned

either to mediate association of their cognate ATPases with their dimerization partners or perhaps to control the arrangement of the ATPase-heterodimer pairs in the ATPase ring. Hsm3 uses distinct elements to make contacts with both Rpt1 and Rpt2, and the integrity of both of these elements is required for efficient base assembly *in vivo* (112). Thus, Hsm3 appears to function, at least in part, by promoting the specific pairing of Rpt1 and Rpt2. In contrast, Rpt4 and Rpt5 appear to be quite stable together in the absence of their chaperone, Nas2 (105). The specific binding of Nas2 to the C-terminal tail of Rpt5 instead suggests a role in preventing the premature docking of this subunit, either alone or in complex with other base subunits, onto the CP (108).

The CP as a template for RP base assembly?—In addition to the RACs, the CP may also function as a base assembly chaperone by providing a template or scaffold for RP base formation. This hypothesis was first proposed because base subparticles accumulated when subunits or assembly chaperones of the yeast CP were mutated (95). In human cells, a nascent RP subcomplex containing Rpn2, Rpn10, Rpn11, and Rpn13 (and the proteasome-associated protein Txn11), but apparently lacking the remaining RP subunits, coimmunoprecipitates with the mature CP under some conditions (114). However, none of these subunits directly contacts the CP in the 26S holoenzyme, suggesting either that large-scale rearrangement of the RP-CP interface may occur during assembly or that the observed complex may be a dissociation product of the RP.

In yeast, deletion of the most C-terminal residue of specific ATPases greatly disrupts RP formation without significantly affecting their association with their cognate RACs (107, 108). As these mutations disrupt interaction of the RP with the CP, the CP was hypothesized to template base formation via insertion of the C-terminal tails of the assembling ATPases into specific surface pockets of the β -ring. Further, as purified CP was able to displace the RACs from their cognate ATPases, it was proposed that proper docking of ATPases onto the β -ring is the trigger for release of bound assembly chaperones. In contrast, others have assembled functional RP equally efficiently in the absence of the CP from RP intermediates purified from bovine erythrocytes, arguing against a stringent requirement for the CP in base assembly (115). While the data are clear that disruption of the CP or RP-CP interactions correlates with an accumulation of base assembly intermediates, whether this reflects a templating function for the CP in base assembly remains uncertain. Base intermediates might accumulate for other reasons. In yeast, proteasome gene expression is driven by the transcription factor Rpn4 (Nrf1 in humans), which is itself a substrate of the proteasome (116, 117). Thus, low proteasome activity will increase Rpn4 levels, which will in turn increase the cellular levels of proteasomes (and their assembly intermediates). While nearly all proteasome subunit genes contain Rpn4 binding sites in their promoters, the RAC genes do not. Thus, the chaperoning capacity of the RACs may become limiting when proteasome synthesis rates are very high, e.g., due to defective RP-CP association, leading to the accumulation of base assembly intermediates even though assembly *per se* is not compromised. Accordingly, deletion of *RPN4* in the context of specific ATPase C-terminal deletions reduced the levels of RP assembly intermediates without affecting the apparent levels of 26S proteasomes (107), although the levels of these intermediates were still higher than in wild-type cells. Lower effective RAC levels may also be engendered by tight binding to accumulating base or RP intermediates that do not allow their ready release. This could occur either if the RP-CP interface is compromised or if defects in CP formation lead to an excess of free RP. Hence, the CP-templating model for RP assembly, while attractive, will require additional experiments to address these ambiguities.

Lid assembly—In contrast to the base and CP, much less is known about lid assembly; moreover, no lid-specific assembly chaperones have been identified to date. Although the general molecular chaperone Hsp90 has been implicated in RP assembly in yeast (118), it is

not clear if it contributes directly or indirectly to RP biogenesis. Coexpression of the nine lid subunits with Hsp90 in *E. coli* is sufficient to yield a particle containing at least 8 of the 9 subunits (Sem1 was not detected) (46). The recombinant lid incorporates Zn²⁺ into the Rpn11 active site and, when incorporated into the 26S proteasome, displays DUB activity comparable to proteasomes containing yeast-derived lid, strongly suggesting that, other than potentially Hsp90, no eukaryote-specific chaperones are required.

The high-resolution EM structures of the lid and the mapping of the subunits within it are sufficient to allow the unambiguous identification of the winged-helix domains and adjacent α helices in each PCI subunit (27, 46, 47). Each PCI domain associates laterally with the PCI domain of its neighbor(s) to form a horseshoe-shaped lattice (Figure 6). While this lateral association could in principle initiate with any PCI subunit and finish in any order, only specific subcomplexes of the lid are observed in vivo (67, 119–121), pointing toward a potential hierarchical assembly mechanism in which subsequent subunit incorporations are triggered by the binding of the preceding subunits. In vitro binding studies, while still limited, support this idea (65) (see below). Assembly of specific subcomplexes may cause conformational changes that favor binding of subsequent subunits or may provide additional binding sites that enhance the affinity for other subunits. The MPN domain subunits Rpn8 and Rpn11 appear to contact several PCI subunits, and may help to stabilize specific interactions between PCI subunits or dictate the order in which the PCI subunits bind one another.

As with base assembly, our understanding of lid assembly derives primarily from the cataloging of the contents of lid subparticles that accumulate in yeast strains harboring mutations in specific lid subunits (67, 119–121). Lid assembly appears to begin via formation of two non-overlapping and complementary subcomplexes: Rpn5/6/8/9/11 (herein called Module 1 (122)) and Rpn3/7/Sem1 (called Lid Particle 3 (LP3)), (Figure 9A). The sequence(s) of subunit additions that form these two particles are unknown, although a Module 1-related complex lacking Rpn6 has also been observed in an *rpn6* mutant (121). In *rpn12* mutants, a lid subcomplex (LP2) containing all subunits of Module 1 and LP3 (but lacking Rpn12) accumulates (67, 119), and from this it was inferred that Module 1 and LP3 join one another to form LP2 (Figure 9A). Notably, mutation of any lid subunit (besides Rpn12) leads to the accumulation of free Rpn12, suggesting that Rpn12 addition to the lid is the final step in its assembly (67). Consistent with this, purified Rpn12 and LP2 associate with one another in vitro to form a particle indistinguishable from the lid. Notably, purified LP2 added to Rpn12-deficient yeast extracts fails to incorporate into 26S proteasomes even after extended incubation. In contrast, LP2 addition to LP2-deficient yeast extracts that have ample free Rpn12 causes rapid incorporation of both LP2 and Rpn12 into 26S proteasomes. These results support the idea of a hierarchical or ordered assembly mechanism in which LP2 will not associate with the base and continue RP assembly unless Rpn12 has first joined to complete lid formation.

Lid-base association and Rpn10 incorporation—The finding that LP2 is unable to incorporate into the RP until Rpn12 has joined suggests an important role for this subunit in governing lid-base association. A highly conserved C-terminal tail of Rpn12 is essential for efficient lid-base association in the absence of Rpn10, indicating that these two proteins have overlapping roles in securing the lid onto the base (67). Within the 26S proteasome, Rpn10 and Rpn12 are positioned on opposite sides of the Rpn2 structure (Figure 9B), and while Rpn12 makes extensive contact with Rpn2, Rpn10 does not (46, 47). Instead, Rpn10 interacts with the MPN domain of Rpn11, which in turn binds Rpn2. Thus, Rpn2 may act like a saddle horn onto which the lid attaches, in a manner aided by Rpn10-dependent stabilization of Rpn2-Rpn11 binding (Figure 9B). Of course, there are many points of interaction between the lid and base subcomplexes, but these two interactions may be the

first formed and may stabilize the lid until it can seat firmly onto the base. Further experiments will be necessary to clarify the contributions of distinct subdomains of lid and base subunits to this late stage of RP assembly.

5C. Assembly of alternative proteasome isoforms

As a detailed assembly map has developed over the past several years for the canonical 26S proteasome, an emerging question is how the assembly of the canonical proteasomes vs. the non-canonical immuno-, thymo-, and in the case of yeast (and perhaps humans), Ω - Ω proteasomes, is governed in cells. In cells expressing alternative proteasome subunits, such as the cortical thymus, all isoforms of the variant subunits (i.e., Ω , Ω i, Ω , Ω i, Ω , Ω i, Ω t) are readily detectable by immunoblot analysis of whole cell extracts, yet only certain configurations appear in mature CPs (15). For example, Ω t-containing proteasomes seem to include only the Ω i and Ω i subunits and not Ω and Ω . Although these subunits are expressed at higher levels than their canonical counterparts, this may not be sufficient to account for the specificity of proteasome formation observed. In support of this, immunoproteasome subunits are known to help recruit other immunoproteasome subunits into assembling CPs (123–126). This is to some extent explained by the different propeptides of these subunits (126), but other factors may also contribute. Isoform-specific assembly chaperones represent one intriguing but as yet untested possibility for such factors.

Besides the RP, the Ω -ring surface of CPs can bind at least three alternative regulators: PA200/Blm10, PA28, and REG Ω (only Blm10 is found in yeast) (reviewed in (127)). A common function of these regulators is to open the gate in the Ω -subunit ring; each regulator also forms a structure that contains an opening that could potentially provide a channel for passage of substrates through it to the CP (20, 21, 24, 128–130). In addition, the homo-hexameric AAA+-family ATPase Cdc48 (p97 in mammals) from archaea has recently been reported to bind and activate the archaeal CP (131, 132). As Cdc48 is highly conserved in eukaryotes, it may potentially function as a proteasomal activator in eukaryotes as well. In principle, assembling different regulators onto the ends of distinct CPs could generate a multitude of proteasomal species, although the range of such potential regulator-CP complexes in vivo and the control of their assembly remain unknown.

5D. Quality control of proteasome assembly

The evolutionary conservation of proteasome assembly chaperones across eukaryotes (and in some cases extending to archaea) suggests a considerable investment in controlling proteasome assembly. Invariably, however, assembly errors will occur – what becomes of these misassembled proteasomes? Recent work suggests that in addition to stringent cellular control over the assembly process, there may be post-assembly quality controls as well. The highly conserved, proteasome-associated protein Ecm29 is enriched on proteasomes in mutant yeast bearing certain proteasomal defects (94, 108, 133). Disruption of efficient proteasome assembly either by deletion of the CP assembly chaperone gene *UMP1* (133), mutation of the critical conserved lysine residues in the CP Ω -ring pockets (94), or deletion of the C-terminal residue of Rpt5 necessary for efficient docking into the CP surface (108) each resulted in enhanced Ecm29 association with RP-CP-related complexes. Deletion of *UMP1* resulted in the apparent absence of the Ω subunit in Ecm29-containing (?) RP-CP complexes and failure to properly process the Ω propeptide from its precursor form (133). As occupancy of CP active site(s) (by Ω -subunit propeptides or inhibitors) is thought to influence the conformation of the Ω -ring (22, 134), the aforementioned mutations would each be expected to affect the RP-CP interface. From EM reconstructions, Ecm29 appears to contact both the RP and CP (135), and it is not known to bind either in the absence of the other (94, 108, 133–135). Thus, it appears that Ecm29 may specifically recognize proteasomes with an aberrant RP-CP interface, at least in yeast.

Exactly why Ecm29 is associated with these defective proteasomes is still unclear. Ecm29 was reported to inhibit the catalytic activity of RP-CP complexes with which it was associated based on reduced liberation of AMC from a fluorogenic peptide substrate compared to *ecm29* proteasomes (108). However, it is possible that the proteasomes to which Ecm29 is bound are misassembled, rather than simply being less active. Similarly, addition of recombinant β subunit to Ecm29-bound proteasomes (which lacked β) resulted in its incorporation into the CP and release of Ecm29 (133). It remains unclear, however, whether Ecm29 simply sequesters defective proteasomes or is serving to facilitate their repair.

In mammalian cells, Ecm29 interacts with several proteins of the microtubule and molecular motor machinery, and decorates the surfaces of late endosomes presumably destined for the lysosome (136, 137). Normal proteasomes are slowly turned over by lysosomes in rat liver (138), and proteasomes are thought to associate with the vacuole in yeast (139). Thus, it is tempting to propose that Ecm29 may promote the recruitment and degradation of malformed proteasomes via the lysosome/vacuole. Ecm29 is not required for gross localization of proteasomes to the vacuole in yeast (139), but whether a defective subpopulation of proteasomes is degraded, repaired, or instead sequestered from general circulation in some way remains to be determined.

6. CONCLUDING REMARKS

The ever-growing list of human diseases in which protein homeostasis is disrupted is a testament to the importance of the UPS for normal cellular function and its potential as a therapeutic target. Notably, both inhibition and augmentation of protein degradation may have clinical value. Proteasome inhibition has been an accepted antineoplastic strategy since approval of bortezomib in 2003. In contrast, recent studies have indicated that enhancement of proteasome activity, typically by blocking proteasome-associated DUBs, may in principle be beneficial in protein misfolding disorders such as many forms of neurodegeneration, and may block replication of some viruses (140, 141). Similarly, unique types of proteasomes in lymphoid tissues provide novel targets for immune modulation via proteasome inhibition (10). Thus, modulation of proteasome biogenesis may also provide opportunities to regulate proteolysis in cells for therapeutic gain.

While the recent advances in our understanding of proteasome structure will undoubtedly serve as a template for improved understanding of its function and assembly, detailed structural and biochemical information on the proteasome during its various catalytic steps will be necessary to more fully understand its mechanisms of action. Similarly, while knowledge of the architecture of subunits within the 26S holoenzyme provides a convenient endpoint for assessment of proteasome assembly, more detailed characterization of assembly intermediates is also needed. We remain far from understanding the basis for the impressive efficiency and specificity of proteasome assembly *in vivo*. Given the myriad factors involved in proteasomal assembly and proteolysis and the discovery of several uniquely configured proteasomes, the analysis of proteasome mechanism and assembly should continue to yield fundamental insights of both basic and clinical importance.

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Acronyms and Definitions

26S Proteasome	An ATP-dependent intracellular protease composed of a 20S proteolytic core particle (CP) and 19S regulatory particle (RP)
Ubiquitin (Ub)	A 76-residue protein that is covalently conjugated to substrates, often in the form of polymers (polyUb)
Proteasome/COP9 Signalosome/Initiator Complex (PCI) domain	A conserved domain with an N-terminal α -helical region followed by a winged-helix fold and found in six RP lid subunits
Mpr1/Pad1, N-terminal (MPN) domain	A conserved fold found in subunits of the RP lid and COP9 signalosome. The MPN+/JAMM variant has metalloprotease activity
ATPases Associated with various cellular Activities (AAA)+ ATPase	A family of ATPases forming oligomeric rings that unfold or remodel substrates
HbYX motif	A hydrophobic residue-tyrosine-any amino acid tripeptide at the C-terminus of proteins that bind the CP α -ring surface
Ubiquitin-associated (UBA) domain	A conserved domain found in many Ub-binding proteins that directly recognizes ubiquitin
Ubiquitin-like (UBL) domain	A protein domain that adopts a α -grasp fold similar to ubiquitin. This domain often mediates interaction with Ub-binding proteins
Proteasome/Cyclosome (PC) repeat	Alpha-helical repeats found in proteasome subunits Rpn1 and Rpn2 and the APC/cyclosome that fold into a toroid or superhelix
Proteasome Shuttle Factor	A polyUb-binding protein that also binds the proteasome RP and functions to deliver polyUb-modified substrates to the proteasome
Ubiquitin Interaction Motif (UIM)	A hydrophobic, α -helical motif that binds Ub and was originally identified in the proteasome Ub receptor Rpn10
Pleckstrin-like Receptor for Ubiquitin (PRU) domain	Domain found in the proteasome Ub receptor Rpn13. In many species, Rpn13 has a domain that binds the UCH37 DUB
Deubiquitylating enzyme (DUB)	A specialized protease that cleaves Ub from substrates such as other proteins and Ub precursors
Proteasome assembly chaperone	A dedicated assembly factor, not usually found in mature proteasomes, that assists in the biogenesis of the proteasome

Summary Points

1. Recent structural and biochemical analyses of the eukaryotic proteasome regulatory particle (RP) and its prokaryotic antecedents have provided a detailed view of RP subunit architecture.
2. The Rpt ATPase subunits, which are responsible for substrate unfolding for degradation, form a uniquely ordered heterohexameric ring that directly abuts the entrance to the CP.
3. The polyUb-substrate-binding sites of the RP are generally located most distally from the CP, while the subunits responsible for removing the polyUb tag and unfolding the substrate are poised over the entrances to the RP ATPase ring and CP ring, respectively.
4. Despite availability of atomic structures of several RP assembly chaperones bound to domains of their cognate Rpt subunits, the mechanistic basis of chaperone function in RP biogenesis remains poorly understood.
5. No dedicated chaperones have been found for RP lid assembly, and a hierarchical mechanism of subunit addition may largely account for high-fidelity lid assembly and lid-base joining.
6. Compositional plasticity in the 20S CP provides an additional layer of complexity to proteasomal assembly, and CP assembly chaperones may have key roles in mediating formation of these alternative forms.
7. Post-assembly proteasome quality controls may also be important in eukaryotes as suggested by recent work on the proteasome-associated Ecm29 protein in yeast.

Future Issues

1. How are the different enzymatic activities of the proteasome coordinated?
2. Why has the proteasome evolved six distinct ATPases while all other ATP-dependent proteases have homomeric rings? How does this heterogeneity contribute to substrate binding, unfolding, and translocation into the CP?
3. How do the multiple intrinsic and extrinsic ubiquitin receptors contribute to substrate recognition and processing?
4. What are the relative contributions of the CP and the RACs to RP base assembly?
5. How is association of the CP with its multiple regulator complexes governed in cells?
6. How is post-assembly proteasome quality control orchestrated and what are the pathways and mediators controlling it?
7. Where in the cell do proteasomal subparticles assemble and how are the localization dynamics of assembly and disassembly regulated?

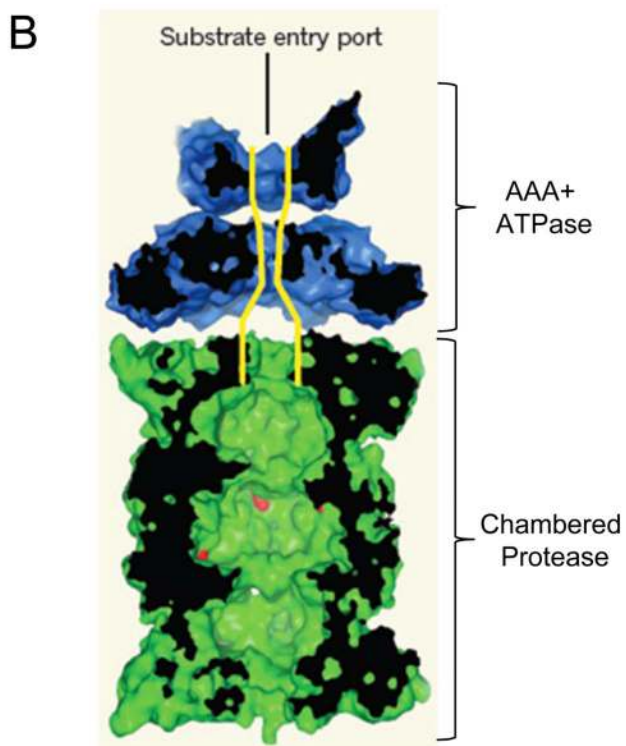
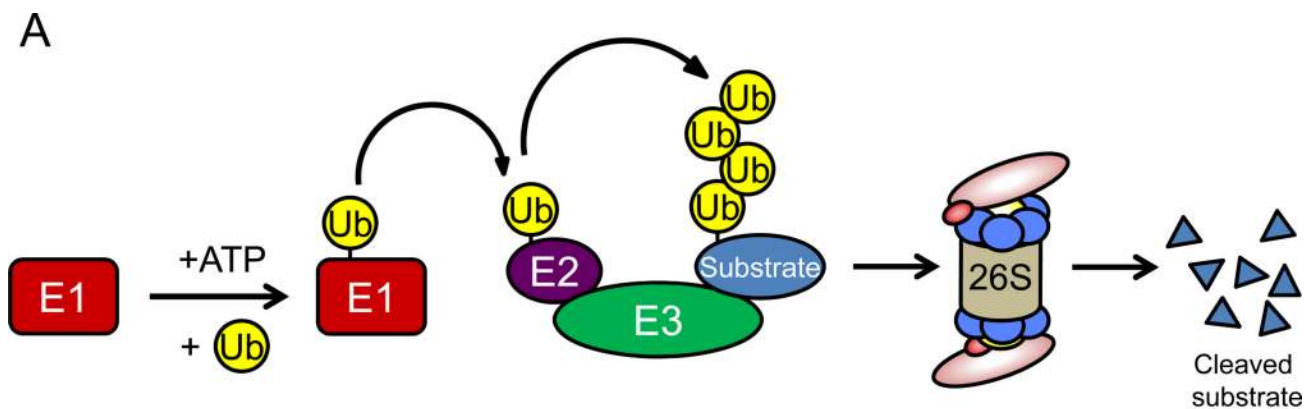


Figure 1. Schematics of the ubiquitin-proteasome system and a AAA+ chambered protease

A, Through the sequential actions of E1, E2, and E3 enzymes, a protein to be degraded is modified with a polyubiquitin chain, which serves as a targeting signal for the proteasome. *B*, A cutaway view of a AAA+ chambered protease, displaying the path of substrates through the ATPase ring and into the proteolytic chamber. The width of the passage into the protease chamber is delineated by yellow lines, and the proteolytic active sites are shown as red dots. In this protease, the catalytic chamber is bracketed by two antechambers. Adapted with permission from Nature Publishing Group, copyright 2012.

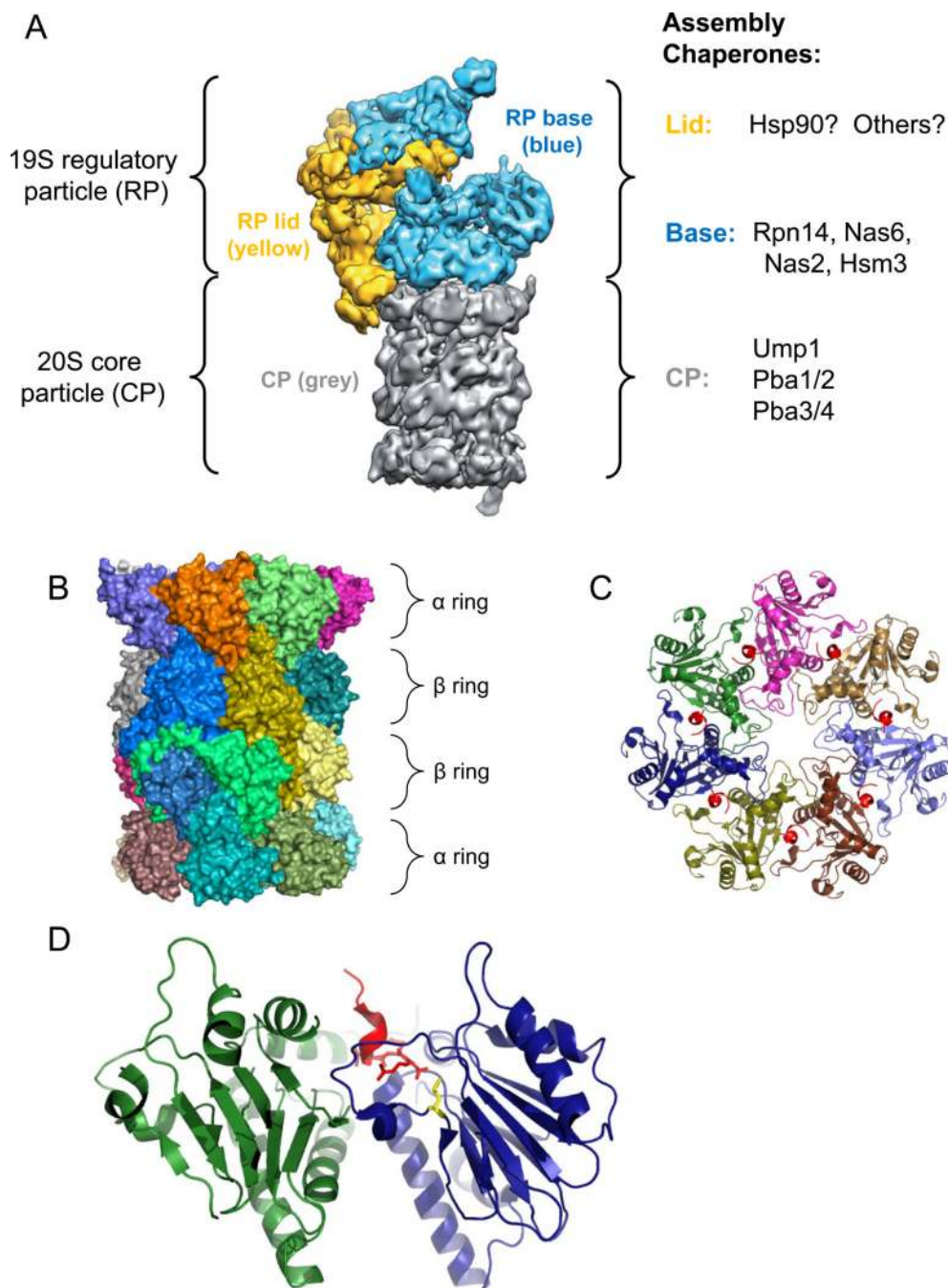


Figure 2. Structure of the 26S proteasome

A. The cryo-EM density of the 26S proteasome is shown. The 19S regulatory particle (RP) lid subcomplex is displayed in yellow, the RP base subcomplex in blue, and the 20S core particle (CP) in grey. Known and putative assembly chaperones for each subcomplex are displayed to the right. Adapted with permission from Nature Publishing Group (46), copyright 2011. *B.* Space-filling model of the 20S CP atomic structure from yeast (PDB ID = 1RYP). *C.* A view into the axial pore of the 20S CP from *T. acidophilum*. In this model (PDB ID = 3IPM), the archaeal ATPase HbYX motif (red ribbons) are inserted into the pockets formed at the interfaces of two adjacent β subunits, opening the CP gate. *D.* the HbYX motif interfaces with a critical lysine residue (shown in yellow) in the β pocket.

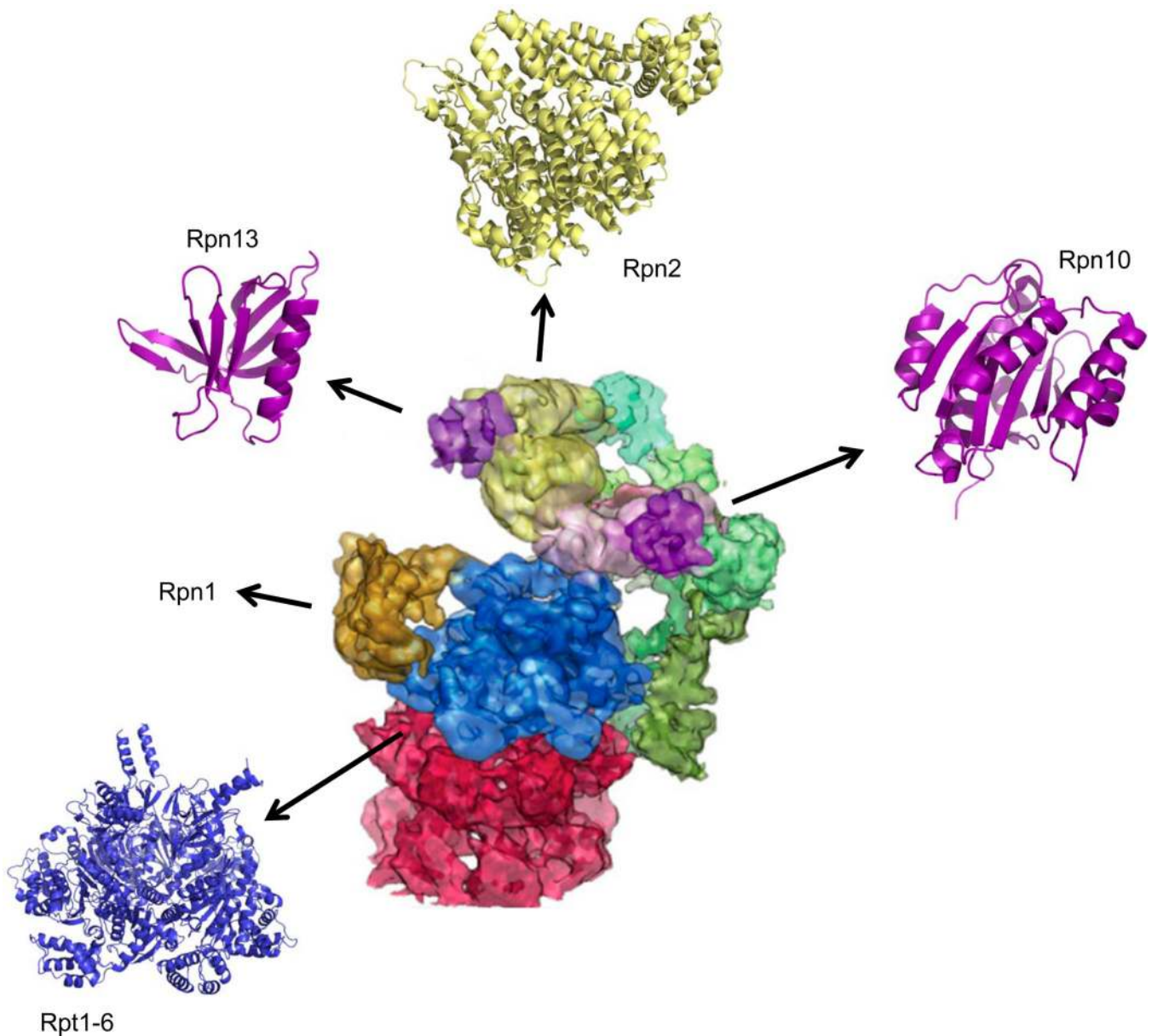


Figure 3. Organization of the RP, with atomic and pseudoatomic models of base subunits

A bird's eye view of the 26S proteasome EM density is shown. Where available, the atomic or pseudoatomic models of Rpt1-6 (modeled using PDB ID = 3H4M, 3H43), Rpn2 (PDB ID = 4ADY), Rpn10 von Willebrand domain (PDB ID = 2x5N), and Rpn13 PRU domain (PDB ID = 2R2Y) are shown and colored the same as their respective EM densities. Adapted with permission from *Proceedings of the National Academy of Science of the U.S.A* (47), copyright 2012.

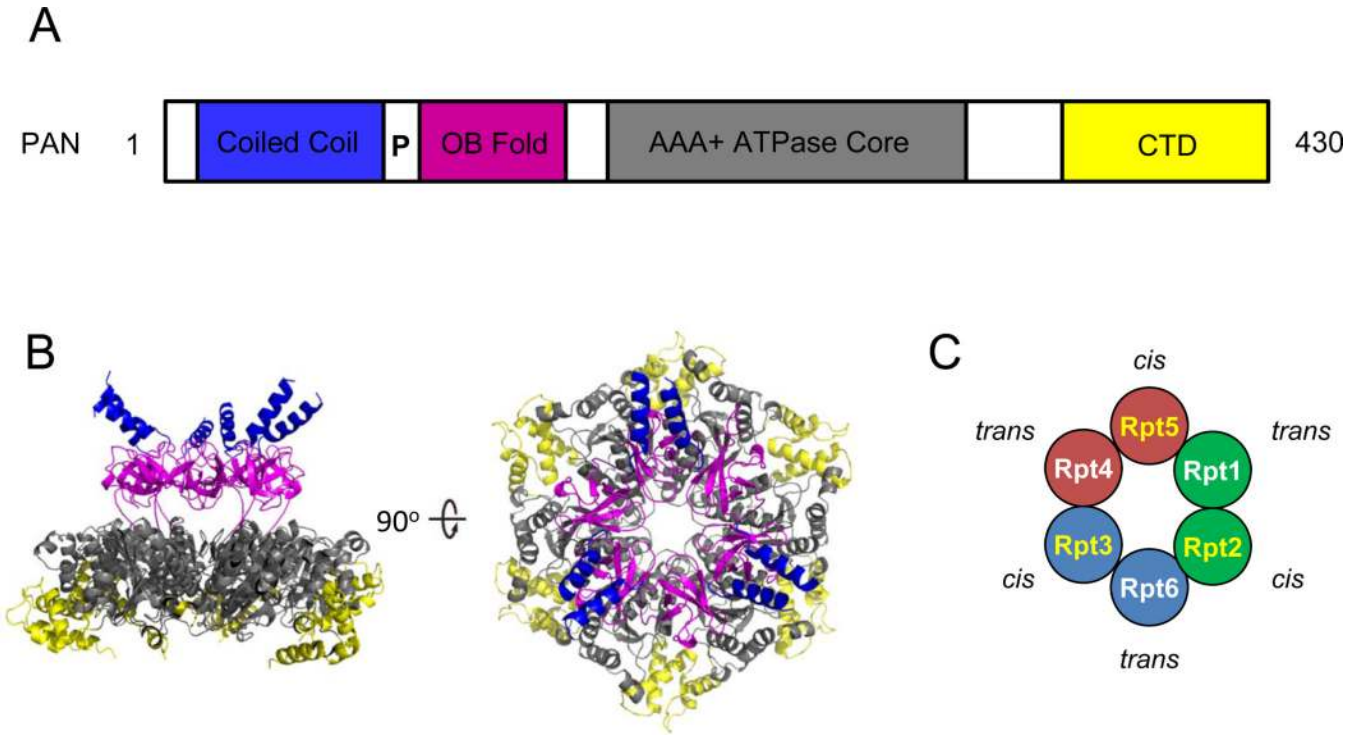


Figure 4. Proteasomal AAA+ ATPase structure and hexameric ring organization

A. The domain architecture of the *M. jannaschii* proteasome-activating nucleotidase (PAN) is shown. The eukaryotic ATPases Rpt1-6 share a similar domain organization as PAN. *B.* A pseudoatomic model of the proteasomal ATPase ring (modeled as in Figure 3) is shown. Domains are colored as in (*A*) *C.* Arrangement of the eukaryotic ATPases in the heterohexameric ring. The inferred proline *cis/trans* isomerism of each Rpt subunit is listed. Subunits forming pairs in the “trimer of dimers” model are similarly colored. See text for details.

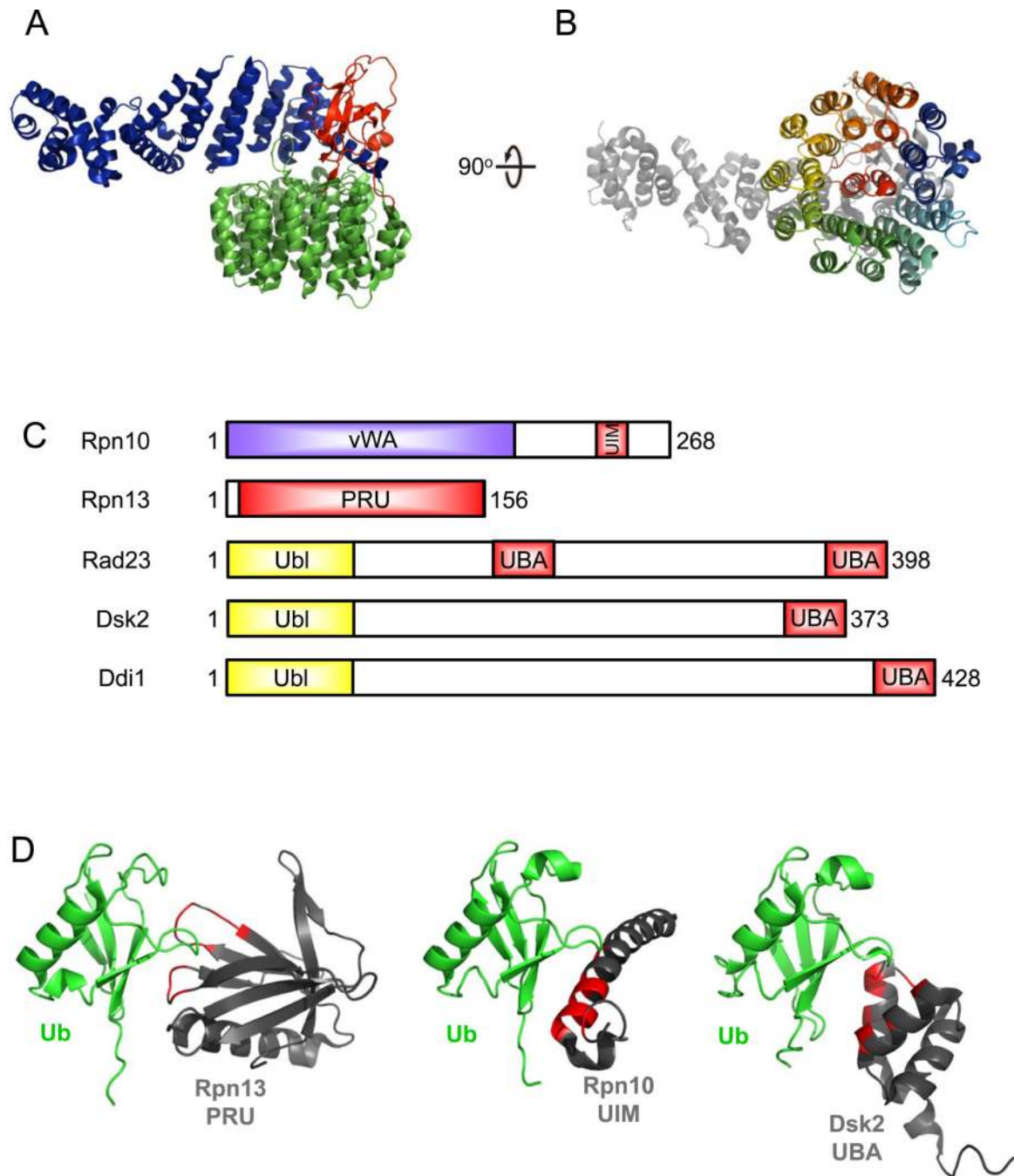


Figure 5. 19S base ubiquitin receptor and scaffold subunit architecture

A. Ribbon structure of Rpn2 from *S. cerevisiae* (PDB ID = 4ADY). The N-terminal helical domain is colored blue; the toroidal domain green; and the C-terminal domain red. *B.* An axial view of the Rpn2 toroid, illustrating the central α helices. PC repeats are colored blue (N-terminal) through orange (C-terminal), with the central helices in red. *C.* Domain organization of the intrinsic ubiquitin receptors Rpn10 and Rpn13, and the extrinsic receptors Rad23, Dsk2, and Ddi1. Domains and amino acid numbering is according to the *S. cerevisiae* gene products. vWA, von Willebrand factor A domain; UIM, ubiquitin-interacting motif; PRU, Pleckstrin-like receptor for ubiquitin domain; Ubl, ubiquitin-like domain; UBA, ubiquitin-associated domain. *D.* Distinct modes of interaction with Ub are

utilized by each ubiquitin receptor. Amino acids of each Ub-binding domain (shown in grey) that contact Ub (green) are highlighted in red. (PDB ID = 2Z59, 2KDE, 1WR1 for PRU, UIM, and UBA, respectively).

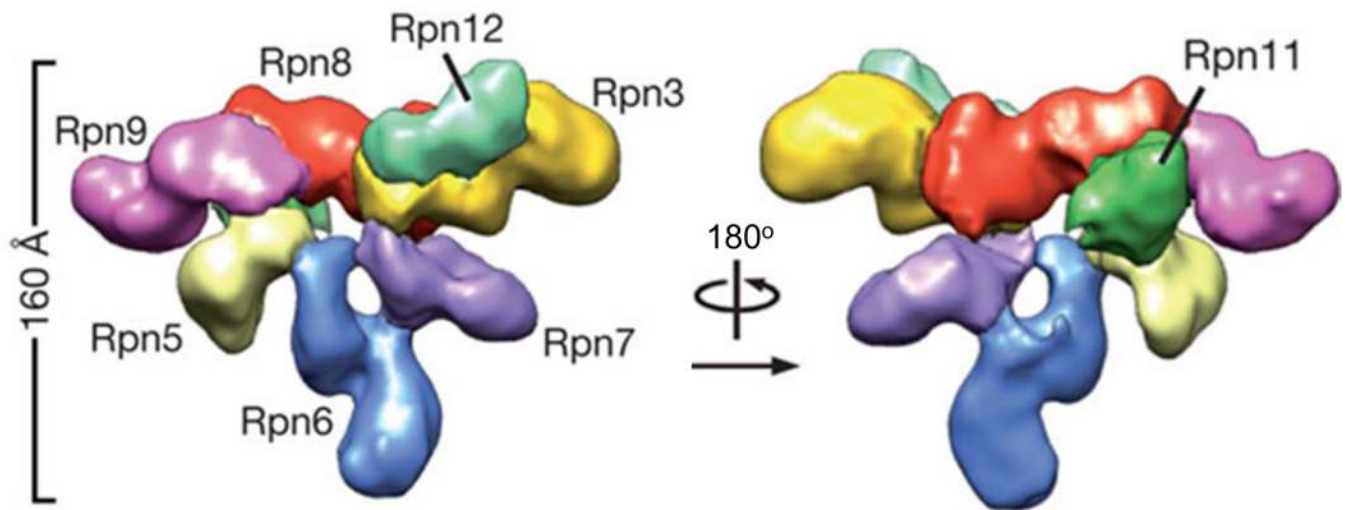


Figure 6. Molecular architecture of the proteasome lid

A. The EM structure of the purified lid complex and the densities contributed by each subunit are shown. *B.* Atomic structure of the *D. melanogaster* Rpn6 PCI domain PDB ID = 3TXN), illustrating the N-terminal helical region and the C-terminal winged helix domain. *C.* Atomic structure of the human Mov34 MPN domain (PDB ID = 2O95). Adapted with permission from Nature Publishing Group (46), copyright 2011.

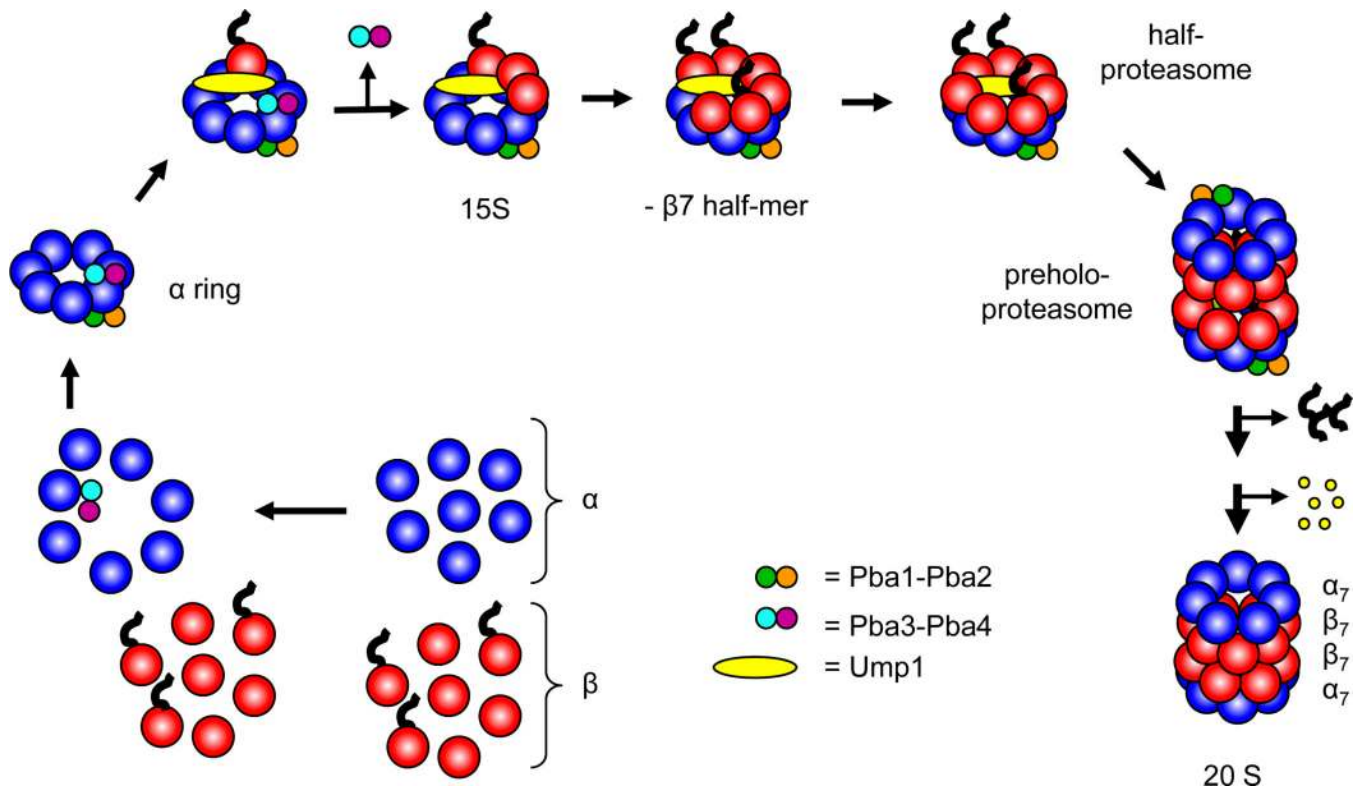


Figure 7. The 20S CP assembly pathway
See text for details.

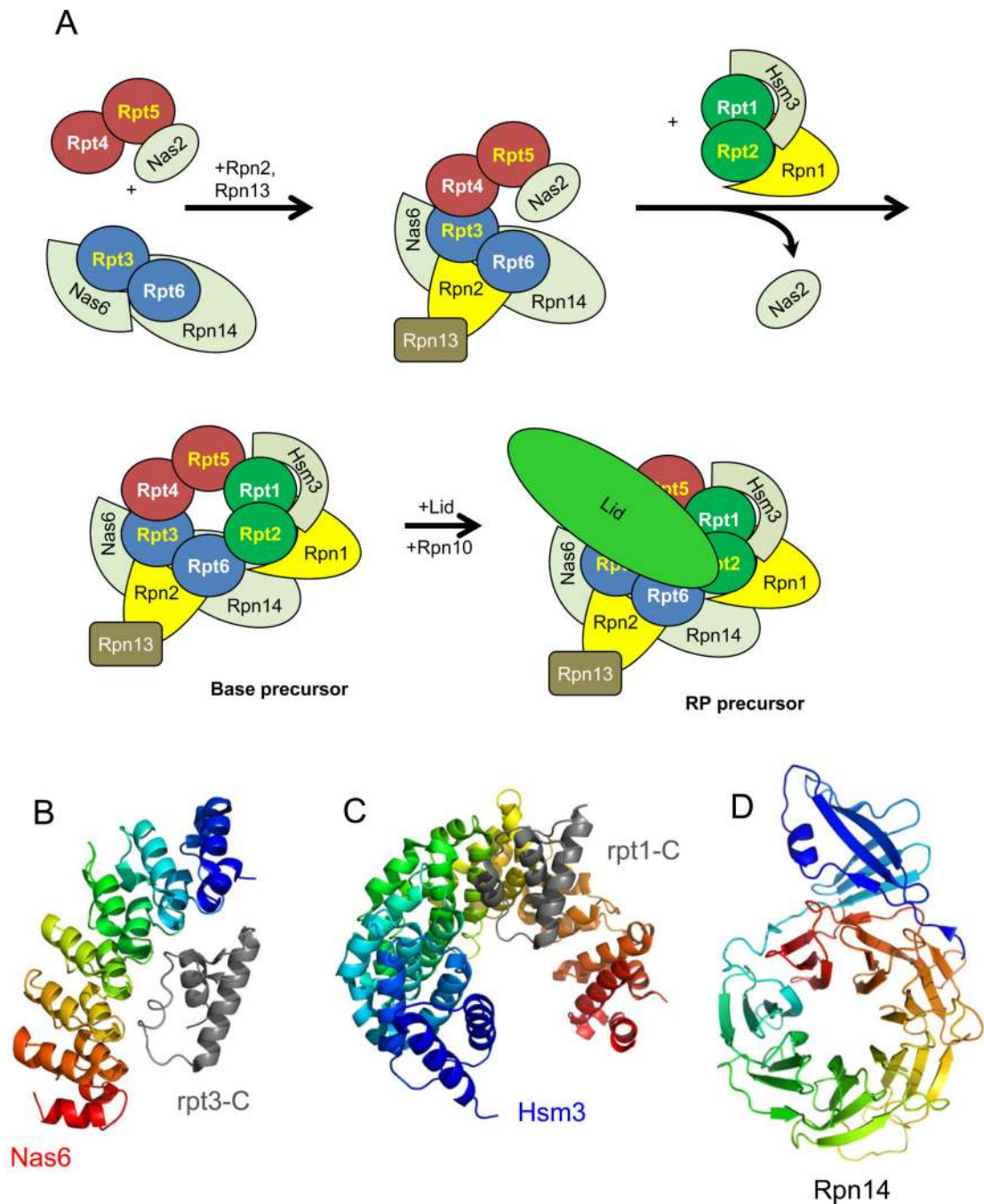


Figure 8. Assembly of the RP base is mediated by RP assembly chaperones

A, The RP base assembly pathway in yeast. See text for details. *B*, *C*. Interaction between (*B*) *S. cerevisiae* Nas6 and the C-domain of Rpt3 (PDB ID = 2DZN) and (*C*) *S. cerevisiae* Hsm3 and the Rpt1 C-domain (PDB ID = 3VLF). C-domains are similarly oriented. *D*. Atomic structure of *S. cerevisiae* Rpn14 (PDB = 3ACP).

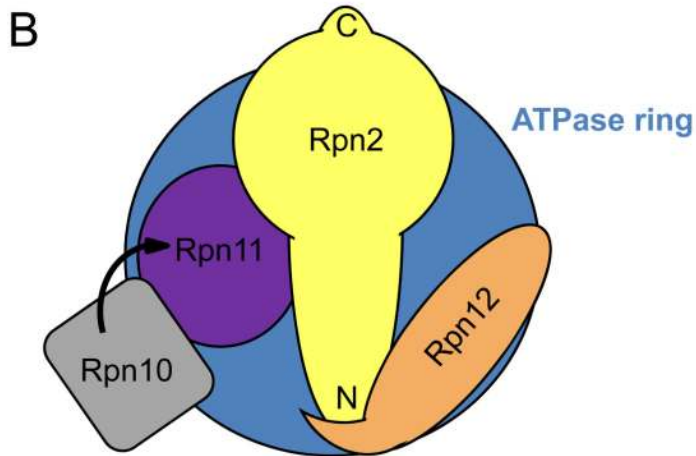
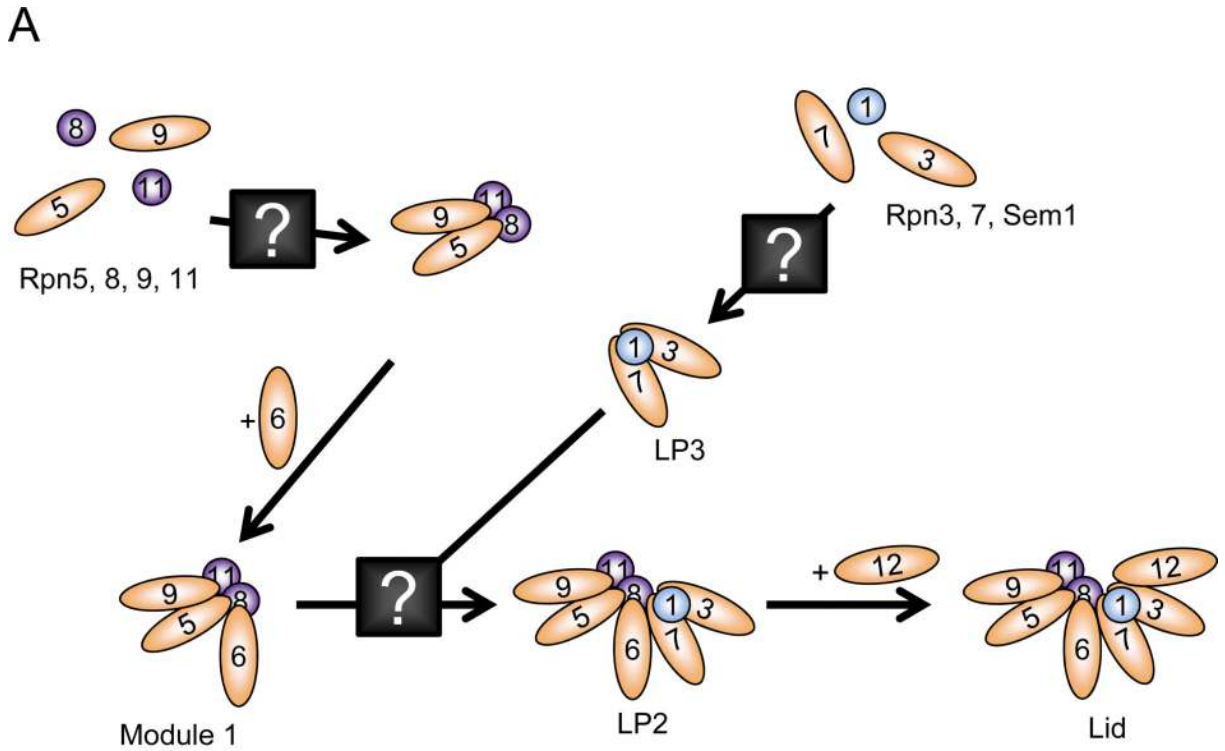


Figure 9. Assembly and attachment of the RP lid

A, The RP lid assembly pathway inferred from studies in yeast. MPN subunits are shown in purple, PCI domain subunits are shown in peach, and Sem1 is shown in light blue. See text for details. *B*, A model for the stabilization of lid-base interaction by Rpn10 and the Rpn12 C-terminal tail via interaction with Rpn2 during RP assembly. In this model, Rpn10 positions Rpn11 to interact with Rpn2 (curved arrow), while the C-terminal tail of Rpn12 directly contacts the Rpn2 N-terminal extension (straight arrow). The N- and C-termini of Rpn2 are marked.

Standardized Name	Yeast Gene Name(s)	Human Standardized	Other Mammalian Names	Function
20S CP Subunits				
Subparticle				
Alpha Ring				
α1	<i>SCL1</i>	PSMA6		
α2	<i>PRE8</i>	PSMA2		
α3	<i>PRE9</i>	PSMA4		
α4	<i>PRE6</i>	PSMA7		
α5	<i>DOA5; PUP2</i>	PSMA5		N-ail is major component of 20S gate
α6	<i>PRE5</i>	PSMA1		
α7	<i>PRE10</i>	PSMA3		
Beta Ring				
β1	<i>PRE3</i>	PSMB6		Postacidic protease activity
β2	<i>PUP1</i>	PSMB7		Trypsin-like protease activity
β3	<i>PUP3</i>	PSMB3		
β4	<i>PRE1</i>	PSMB2		
β5	<i>PRE2</i>	PSMB5		Chymotrypsin-like protease activity
β6	<i>PRE7</i>	PSMB1		
β7	<i>PRE4</i>	PSMB4		
20S-associated proteins				
Pba1	<i>PBA1; POC1</i>	PAC1		Alpha ring chaperone
Pba2	<i>PBA2; POC2; ADD66</i>	PAC2		Alpha ring chaperone
Pba3	<i>PBA3; POC3; DMP2; IRC25</i>	PAC3		Alpha ring chaperone
Pba4	<i>PBA4; POC4; DMP1</i>	PAC4		Alpha ring chaperone
Ump1	<i>UMPI; RNS2</i>	POMP; hUMP1		Beta ring chaperone
Bim10	<i>BLM10</i>	PA200		CP regulator
19S RP Subunits				
Subparticle				
Standardized Name				
Base				
Rpt1	<i>RPT1; CIM5; YTA3</i>	PSMC2	S7; p48	Unfoldase
Rpt2	<i>RPT2; YHS4; YTA5</i>	PSMC1	S4; p56	Unfoldase
				Other names <i>CIM5; S7; p48; PSMC2</i> S4; p56; PSMC1

20S CP Subunits Subparticle	Standardized Name Alpha Ring	Yeast Gene Name(s)	Human Standardized	Other Mammalian Names	Function
Rpt3	<i>RPT3</i> ; <i>YNT1</i> ; <i>YTA2</i>	PSMC4	S6; S6b; p47	AAA+ ATPase	Unfoldase S6; S6b; p47; PSMC4
Rpt4	<i>RPT4</i> ; <i>CRL13</i> ; <i>PCS1</i> ; <i>SUG2</i>	PSMC6	S10b; p42	AAA+ ATPase	Unfoldase S10b; p42; PSMC6
Rpt5	<i>RPT5</i> ; <i>YTA1</i>	PSMC3	S6; s6a; p50	AAA+ ATPase	Unfoldase S6; S6a; p50; PSMC3
Rpt6	<i>RPT6</i> ; <i>CIM3</i> ; <i>SUG1</i>	PSMC5	S8; p45	AAA+ ATPase	Unfoldase S8; p45; PSMC5
Rpn1	<i>RPN1</i> ; <i>HRD2</i> ; <i>NAS1</i>	PSMD2	S2; p112	Scaffold	Ub receptor docking S2; p112; PSMD2
Rpn2	<i>RPN2</i> ; <i>SEN3</i>	PSMD1	S1; p97	Scaffold	Rpn13 docking S1; p97; PSMD1
Rpn10	<i>RPN10</i> ; <i>MCB1</i> ; <i>SUN1</i>	PSMD4	S5a; p54	vWA domain; UIM domain	Ub receptor S5a; p54; PSMD4
Rpn13	<i>RPN13</i>	ADRM1		PRU domain	ADRM1 Ub receptor; Uch37 receptor; Ub receptor docking
Lid					
Rpn3	<i>RPN3</i> ; <i>SUN2</i>	PSMD3	S3; p58	PCI domain	Scaffold? S3; p58; PSMD3
Rpn5	<i>RPN5</i> ; <i>NAS5</i>	PSMD12	p55	PCI domain	Scaffold? p55; PSMD12
Rpn6	<i>RPN6</i> ; <i>NAS4</i>	PSMD11	S9; p44.5	PCI domain	Scaffold? S9; p44.5; PSMD11
Rpn7	<i>RPN7</i>	PSMD6	S10a; p44	PCI domain	Scaffold? S10a; p44; PSMD6
Rpn8	<i>RPN8</i>	PSMD7	S12; p40; MOY34	MPN domain	Scaffold? S12; p40; MOY34; PSMD7
Rpn9	<i>RPN9</i> ; <i>NAS7</i>	PSMD4	S11; p40.5	PCI domain	Scaffold? S11; p40.5; PSMD4
Rpn11	<i>RPN11</i> ; <i>MPRI</i>	PSMD14	S13; Poh1	MPN+ domain; DUB activity	Substrate deubiquitylation S13; PSMD14; Pad1; Poh1
Rpn12	<i>RPN12</i> ; <i>NIN1</i>	PSMD8	S14; p31	PCI domain	Scaffold? S14; p31; PSMD8
Sem1	<i>SEMI</i> ; <i>HOD1</i>	PSMD9	S15; p27L; DSSI	Largely unstructured	Scaffold? S15; p27L; Rpn15; Dss1; PSMD9
RP-associated proteins					
Dsk2	<i>DSK2</i>	PLIC-2		Ubl domain; UBA domain	Extrinsic Ub receptor
Rad23	<i>RAD23</i>	hHR23b		Ubl domain; UBA domain	Extrinsic Ub receptor
Ddi1	<i>DDI1</i> ; <i>VSM1</i>	Ddi1		Ubl domain; UBA domain	Extrinsic Ub receptor
Ubp6	<i>UBP6</i>	USP14		Ubl domain; DUB activity	pUb chain editing
Uch37	No ortholog	UCH37		UCH domain; DUB activity	pUb chain editing
Hul5	<i>HUL5</i>	KIAA10/E3a		HECT family E3 ligase activity	E3 ligase; substrate triage?

20S CP Subunits Subparticle	Standardized Name	Yeast Gene Name(s)	Human Standardized	Other Mammalian Names	Function
Ecm29	<i>ECM29</i>	<i>Ecm29</i>	<i>Ecm29</i>		HEAT repeats Proteasome stabilizer?
Sp5	<i>SPG5</i>	<i>SPG5</i>	No obvious ortholog		Unknown
Rac1	<i>RPN14</i>	<i>RPN14</i>	<i>PAAF1</i>		RP assembly chaperone
Rac2	<i>NAS6</i>	<i>NAS6</i>	<i>PSMD10</i>	p28; gankyrin	RP assembly chaperone
Rac3	<i>NAS2</i>	<i>NAS2</i>	<i>PSMD9</i>	p27; BRIDGE	RP assembly chaperone
Rac4	<i>HSM3</i>	<i>HSM3</i>	<i>S5b</i>		RP assembly chaperone