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Assembly, Structure and Function of the 26S proteasome

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Abstract

The 26S proteasome is a large multi-protein complex involved in the regulated degradation of ubiquitinated proteins in the cell. The 26S proteasome has been shown to control an increasing number of essential biochemical mechanisms of the cellular lifecycle including DNA synthesis, repair, transcription, translation and cell signal transduction. Concurrently, it is increasingly seen that malfunction of the ubiquitin proteasome system contributes to the pathogenesis of disease. The recent identification of four molecular chaperones, in addition to five previously identified chaperones, have provided mechanistic insight into how this cellular megastructure is assembled in the cell. These data, together with new insights into the structure and function of the proteasome, provide a much better understanding of this complex protease.

Introduction

Eukaryotic protein homeostasis, proteostasis (see Glossary), is central to cell development, ameliorates the rigors of cellular ageing and protects cells against disease. Deficiencies in proteostasis can lead to metabolic, oncogenic, neurodegenerative, and cardiovascular disorders¹. The 26S proteasome is at the heart of proteostatic mechanisms in the cell, because it is the major cellular protease. Therefore, the assembly, structure, and function of this regulated proteolytic machine is fundamental to the life process and when deranged contributes to disease.

The 26S proteasome (see text box 1) can be divided in two sub-complexes, the core particle (CP, 20S) and the regulatory particle (RP, 19S). The RP receives, assists in deubiquitination, and unfolds ubiquitinated protein substrates, that are subsequently translocated into an enclosed cavity formed by the CP. Here a variety of catalytic sites degrade the substrate into short peptides that are subsequently broken down to amino acids by peptidases and recycled by the cell. Recent developments in understanding proteasome assembly, structure and function will be highlighted in this review.

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Assembly of the proteasome

Assembly of the 26S proteasome is not a straightforward process; numerous proteins (at least sixty six²) have to assemble into a functional complex, whilst proteolytic active sites have to mature and be properly controlled during the assembly process. Additionally, three ring structures consisting of 6 or 7 unique, but homologous, subunits (ATPase subunit ring in RP and α -subunit ring and β -subunit ring in CP, see figure 1) need to form without errors. This is not a trivial supra-macromolecular task. Recent studies have shown that at least nine dedicated chaperones assist in the formation of the 26S proteasome (see table 1). These chaperones bind to proteasome sub-complexes and facilitate the formation of the 26S proteasomes, but (like other chaperones) do not form part of the final biological functional complex. Five of these proteins are dedicated to the assembly of the CP; these have been excellently reviewed recently and will only be briefly discussed here^{3–5}. The four remaining chaperones have been described only recently and will be discussed in more detail.

Core particle chaperones

Assembled CP has a cylindrical shape created by four seven subunit rings designated as follows: α_{1-7} , β_{1-7} , β_{1-7} , α_{1-7} (figure 1). The CP by itself is a functional entity that can associate with RP to form 26S, but also with other regulators, such as PA28/11S⁶. Consistent with this CP can assemble independently of the RP.

The assembly of the CP starts with the formation of a ring of α -subunits. However, the formation of this ring might be error prone. e.g. after *in vitro* translation, non-neighboring α -subunits have been shown to interact with each other⁷. Also, expression of some α -subunits in *E.coli* results in homoheptameric rings, that are stacked^{8, 9}. In yeast, deletion of the $\alpha 3$ subunit results in the incorporation of two $\alpha 4$ subunits¹⁰. Presumably, such promiscuity led to the evolution of chaperones to control ordered assembly of α -rings in the cell and to prevent aggregation of 'sticky' α -ring proteins. Indeed, deletion of the CP-chaperones from yeast results in lower levels of 20S proteasome, the accumulation of dead-end complexes, and the incorporation of a second $\alpha 4$ subunit instead of $\alpha 3$ ^{11–13, 14}.

Four CP-chaperones play a role in assembly of the α -ring; yeast Pba (proteasome biogenesis associated protein) 1–4 or their likely orthologs in human PAC (proteasome assembly chaperone)1–4. For simplicity we use only the human nomenclature here, but the assembly process seems largely conserved between yeast and humans. These proteins are only functional as heterodimers: PAC1-PAC2 and PAC3-PAC4^{11, 15}. The PAC3-PAC4 heterodimer is probably important early in assembly, when it attaches to $\alpha 5$ and assists in the recruitment and ordering of additional α -subunits^{14, 16}.

Upon completion of α -ring assembly, β -subunits are then incorporated. To allow incorporation of all β -subunits, the PAC3-PAC4 heterodimer needs to dissociate. Structural studies show that PAC3-PAC4 cannot bind to α -rings that contain $\beta 4$, because of steric hindrance between $\beta 4$ and PAC3¹⁶.

The PAC1-PAC2 heterodimer also assists in the formation of the α -ring, but unlike PAC3-PAC4 it remains associated with the complex upon incorporation of the β -subunits. PAC1-PAC2 can interact with $\alpha 5$ and $\alpha 7$ seems to bind the α -ring on the opposite side compared to PAC3-PAC4^{3, 13}.

The fifth CP-chaperone Ump1/POMP, which has no role in α -ring formation, seems to function as a quality control agent. The Ump1 protein prevents stable dimerization of two half CPs until all seven β -subunits are assembled on the α -ring (creating the half CP). The N-terminal pro-peptides of the β -subunits are crucial for dimerization of the half CPs. The formation of CP by

two half CPs is accompanied by the maturation of the β -subunits and the degradation of Ump1 and PAC 1-PAC³. In summary, the formation of CP is assisted by five chaperones.

Interestingly, 20S proteasome structures formed by only one or two α and β subunits, such as in prokaryotes, do not require chaperones for their formation³. Thus, the challenges of proper positioning of the seven homologous α -subunits and β -subunits in the eukaryotic CP structure seems to invoke the requirement for chaperones.

Regulatory particle chaperones

A third ring structure present in 26S proteasomes is an AAA-ATPase ring formed by the proteins Rpt1 to Rpt6 (see figure 1). These six proteins together with Rpn1, Rpn2, Rpn10 and Rpn13 form the so-called base complex. The base subunits Rpn10 and Rpn13 function as ubiquitin receptors and are the only non-essential base subunit in yeast². They are, therefore, expected to associate peripherally to the base. Rpn1 and 2 are homologous proteins, both forming a toroid structure¹⁷. They function as a scaffold as they bind several of the proteasome subunits as well as some proteasome associated proteins². All other RP subunits are contained in the lid complex (Figure 1). The function of this complex remains elusive, but includes de-ubiquitination of substrates by the Rpn11 subunit. The CP together with base and lid complexes can be combined *in vitro* to form proteasomes¹⁸; thus it is intuitive to think that 26S assembly proceeds through these sub-complexes. However, mammalian proteasomes have been successfully reconstituted from a different set of stable subcomplexes¹⁹. So, it remains to be seen how *de novo* formation of 26S proteasomes actually occurs *in vivo*.

Recently, a series of papers has revealed important roles for RP chaperones in proteasome assembly^{20–25}. These papers suggest that the lid complex and CP can form independently, as both readily accumulate in yeast mutant strains used in these and other studies^{14, 26}. However, base complex formation is compromised in the chaperone mutants.

At least four chaperones are involved in the RP assembly: Nas2/p27, Nas6/gankyrin/p28, Rpn14/PAAF1 and Hsm3/S5b (Figure 2a). While all these proteins have been reported previously to associate with proteasome complexes^{27–33} their absence from mature 26S proteasomes in yeast and their chaperone function had not been appreciated until these recent papers shed light on their mechanisms of action. For PAAF1 and, especially, gankyrin other proteasome functions (including a role as proteasome inhibitor and intermediate in proteasome binding respectively) have been described^{33–35}. The chaperones are evolutionary unrelated and structurally very different (see Figure 2A). Although they don't have recognizable enzyme activity, all four proteins contain domains known to be involved in protein-protein interactions^{36, 37}. This suggests that these chaperones mainly act by binding to specific partners. Indeed, three chaperones bind to the small C-domain present in the Rpt proteins (Figure 2B): Nas6-Rpt3, Rpn14-Rpt6, Hsm3-Rpt1, while Nas2 binds either the C-domain or the C-terminus of Rpt5^{20, 23, 25, 32}. Thus the chaperones might be important in the formation of the AAA-ATPase ring containing the Rpt1–6 subunits.

Most AAA-ATPases form hexameric rings (Figure 2b) consisting of six identical ATPases. They appear to require no assistance in ring formation. The AAA-ATPase ring present in eukaryotic proteasomes, however, is formed by six different, but related, ATPases. This might suggest that the chaperones have evolved in order to regulate the formation of the hetero-hexameric ring. Other AAA-ATPase assemblies containing six different ATPases include dynein, midasin, and the MCM helicase complex. Dynein and midasin have all six ATPase domains in one polypeptide, providing physical restriction to the order of the ATPases³⁸. The six MCM helicase proteins can assemble into a ring *in vitro*³⁹ without the assistance of chaperones. However, a subset of subunits, e.g. MCM 4 and 6 or MCM 4, 6, and 7, can also form rings *in vitro*. This could indicate that there is a need for factors capable of directing ring

assembly, although no chaperones have yet been identified. In summary, there is no precedent for the need of chaperones to assemble an AAA-ATPase hetero hexameric ring.

How the proteasomal chaperones assist in the ATPase ring formation is not fully understood and several potential mechanisms are discussed below. To assist directly in the formation of a complex each chaperone could either form a scaffold that binds multiple proteins or, alternatively, chaperone binding could induce a conformational change in a protein partner to create a new binding site: neither of these properties have been shown so far for these chaperones. Chaperones could also help assembly by stabilizing their binding partner(s), but RNAi studies in human tissue culture cell lines have shown that only depletion of Nas2 results in reduced levels of its binding partner²⁴. Finally, chaperones could shield otherwise exposed surfaces. This property is important to avoid undesirable interactions (e.g. to avoid aggregation) or premature binding of other binding partners. The prevention of premature interactions has been observed (similar to Ump1 in CP assembly). The absence of human Nas2 causes a premature association of the Rpt4–5 complex with other base components²⁴. Furthermore, the chaperones probably prevent premature binding of the Rpt subunits to the CP (Figure 3). Modelling studies suggest that binding of Nas6 to Rpt3 creates steric hindrance, so that the Rpt3 C-terminal tail cannot dock into its binding pocket on the CP21. Biochemical data support this and show a similar role for Rpn14 and Hsm321, 25. In a separate study it was found that human complexes containing p27 (Nas2) and S5b (Hsm3) did not bind CP. However, the role of the chaperones in this observation remains unexplored¹⁹. These processes are not mutually exclusive and each chaperone might employ a different set mechanisms.

Base assembly

The assembly of the base seems to occur by initial pairing of Rpt proteins (Figure 3). The Rpt1 and Rpt2 ATPases together with Rpn1 and Hsm3 have been repeatedly observed in cell lysates. The stability of this complex depends on Hsm3^{21–25}. Rpt5 might associate with this complex as well²¹. This entity, dubbed BP1, is a true assembly intermediate and not a dead-end product as it disappears in a chase experiment²¹. Most Rpt1-bound Hsm3 seems to associate with BP1 although some Hsm3 is found associated with base and RP^{23, 25}. A second pair formed by Rpt4 and Rpt5 with the chaperone Nas2 has also been characterized in detail^{22, 23}. Nas2 is important for the presence of this complex but dissociates upon further assembly. Rpt3 and Rpt6, the third pair of Rpt proteins, have been found in a complex with Nas6 and Rpn14, although these proteins have been more readily detected when associated with the base and/or RP^{23–25}. Each pair interacts with at least three other subcomplexes in assembled proteasomes as indicated by the green arrows in Figure 3. In what order these interactions are established is not well understood.

Some models suggest that these complexes, together with Rpn2 and Rpn13, form the base^{22–24}. In this process Hsm3 and Nas2 might dissociate. Upon association with the lid, RP is formed. When the RP associates with CP the Rpn14 and Nas6 proteins leave the RP, although Rpn14 dissociation might also occur at an earlier step. These models, however, do not address all observations. Mutants defective in CP assembly have shown defects in RP assembly¹⁴. Furthermore, deletion of the C-terminal amino acid of Rpt 4 and Rpt6 causes dramatic assembly defects²¹. The tails of the Rpt proteins dock into pockets in the CP^{40–42} and deleting the C-terminal amino acid disturbs this CP-Rpt interaction. Thus, there is a clear role for the CP in RP assembly (Figure 3). To ensure proper ordering of the Rpt proteins scaffolding proteins might play a role in assembly, however, such a role has not yet been shown for the chaperones. Rpn1 and Rpn2 are potential scaffolds^{21, 25}. Rpn1 can bind to Rpt1 and Rpt2, thus it might act as a scaffold for the BP1. Rpn2, Rpt6 and Rpt3 interact^{14, 43, 44} but these proteins do not appear to form a precursor complex together^{23, 24}. The CP itself might form another scaffold^{21, 25} with the pockets of the CP presumably having Rpt tail specificity^{40, 41} and thus directing Rpt

proteins to distinct positions in the ring. With CP as a scaffold, the chaperones could regulate the incorporation of the different precursors. However, if the precursor complexes assemble on the CP, free base and free RP observed in cell lysates must have dissociated from the CP and rebound to some of the chaperones. Two other recent papers report insights into the assembly process without using the chaperones as starting points^{19, 45}. Hendil, et al. used pulse chase in HeLa cells to nicely show a rapid formation of an Rpt3-Rpt6 pair⁴⁵. Furthermore, they argue for the formation of a precursor complex consisting of CP plus Rpn2, Rpn10, Rpn11, Rpn13 and Txnl1 (the last protein has recently been identified as associating with the proteasome) based on pulse chase and immunoprecipitation experiments. This proposed precursor complex has, however, not been biochemically identified. Their suggestion that pairs of Rpt proteins would subsequently join this assembly fits nicely with an important role for CP as a scaffold in RP assembly and a function for chaperones in regulating CP-Rpt interaction.

The second paper by Thompson, et al. identified three RP sub-complexes from red blood cells¹⁹. Consistent with the other studies, each sub-complex contains one Rpt pair. Ps-1 contains Rpt3 and Rpt6 together PAAF-1 and almost all RP subunits, except for Rpt1, 2, 4 and 5. Ps-2 is similar to BP-1, containing Rpn1, Rpt1, Rpt2 and S5b. Ps-3 is formed by Rpt4, Rpt5 and Nas2. *In vitro* these complexes can be reconstituted efficiently to form RP. While the role of these sub-complexes in assembly *in vivo* remains to be determined, it is very interesting that, apart from gankyrin, the RP chaperones are present in the different complexes. The role of the chaperones in reconstitution unfortunately has not been studied, but this study shows that CP is not required for this *in vitro* reconstitution.

In summary, it seems plausible that more than one pathway can lead to assembled 26S proteasomes. In demanding conditions, such as the high temperature used in one of the studies²³, a single preferred 26S proteasome assembly pathway might be used. Although the proteasome assembly pathways appear largely conserved from yeast to man, there are probably subtle differences in the process between different organisms. Clearly, more detailed mechanistic and structural studies are needed to completely understand the assembly process.

In addition to CP and chaperones the role of other molecular players in assembly need to be characterized. Hsp90 stimulates lid complex and 26S maintenance and assembly, but it is unclear how⁴⁶. Nob1 has a controversial role associating CP with RP in the nucleus^{47, 48}. Ecm29 could tether CP and RP as it stabilizes their association even in the absence of ATP^{18, 49}. The proteasome activator Blm10/PA200 has been reported to promote CP assembly and activation⁵⁰. There is no reason to assume this list is complete and there are likely to be more chaperones or modulators of the assembly process.

Structure of the 26S proteasome

To understand proteasome assembly and function in general, structural information about the proteasome is essential. All high resolution structures of proteasome complexes have so far been of the CP or the CP associated with PA26 or Blm10, both proteasomal activators^{51–53}. How the RP is organized is still unclear. EM studies of CP with the RP subunits Rpn1 and Rpn2 show the Rpn1 subunit of the 19S regulator on an interface with the central surface of the 20S core with the Rpn2 subunit on top of Rpn1⁵⁴. The ATPases then have to surround this Rpn1/2 core structure. However, this proposed architecture of the base has been challenged based on detailed EM structures of complete 26S proteasomes^{44, 55}. These reports indicate that there is insufficient space within the ATPase ring to accommodate Rpn1/2. We also know that the C-termini of the Rpt proteins dock into pockets located on the CP, thus the Rpt proteins need to be in close proximity to the CP^{40–42}. As has been observed previously, and described as “wagging”⁵⁶, the recent EM structural papers also show a non-symmetrical positioning of the ATPase ring on the CP^{44, 55}. This suggests that each Rpt may interact differently with the

CP. Such asymmetry could be coordinated with the catalytic state of the ATPases. In summary, it is clear we still lack important understanding of how the six-member ATPase ring interacts with the seven-member α ring of the CP.

Additionally, a previously proposed order of the proteasomal ATPases of Rpts 2-1-3-5-4-6,^{43, 57} seems in conflict with some of the assembly data described above. It has also been challenged recently using structural information from PAN-AAA-ATPase structures (Figure 2b)^{44, 58, 59}. Extrapolating from this data, the ATPase ring should be regarded as a trimer of dimers in which the different subunits of each dimer need to have a different conformation of their coiled-coil domain. Based on this information the following order of ATPases has been suggested: Rpt1-2-6-3-4-5^{44, 55}. Although this order remains to be confirmed experimentally, it shows nice consistency with the Rpt pairs that form during assembly (Figure 3), the evolutionary relationship between the Rpt proteins⁶⁰, and many of the published interaction data^{44, 55}.

Function: how does the 26S proteasome perform its role?

Conformation changes in assembled proteasomes

The mechanism by which the 26S proteasome engages ubiquitinated proteins and feeds them into the 20S catalytic core is not understood.

It has been suggested that yeast 26S particles may dissociate during the catalytic cycle⁶¹, which might suggest a role of some of the chaperons in the catalytic cycle. However, it appears that (at least mammalian) 26S proteasomes can degrade polyubiquitinated proteins without dissociation⁶². Also for yeast proteasomes it has been shown that engagement of the proteolytic active sites actually stabilizes the 26S complex¹⁸. In either case, the six ATPases in the base of the regulatory particle may undergo conformational changes during ATP binding and hydrolysis⁶³. These conformational changes may be used for substrate unfolding and translocation².

The binding of polyubiquitinated substrates can cause small structural changes in the proteasome. Substrates targeted for degradation are generally modified with at least an ubiquitin chain-length of four ubiquitins. These chains bind to the 26S particle⁶⁴ and an unstructured region initiates the degradation of the protein⁶⁵ or sometimes of a binding partner⁶⁶. Polyubiquitinated model substrates bound to the proteasome enhance their own degradation by facilitating gate opening and allosterically activating the peptidase activities⁶⁷. Thus, assembled proteasomes are dynamic structures that undergo conformational changes during degradation as well as substrate binding.

The role of ubiquitin binding and chains

Substrates can be delivered to the proteasome *via* different paths as well as with different types of ubiquitin chains. At least two subunits of the regulatory particle, Rpn13/ADRM1 and Rpn10/S5a, bind polyubiquitinated proteins, but roles for Rpn1 and Rpt 5 have also been suggested^{2, 3}. In yeast, a large amount of Rpn10/S5a is not associated with the 26S proteasome which may imply that this protein has a function in substrate shuttling to the 26S particle⁶⁸. There are also many extra-proteasomal ubiquitin binding proteins which may shuttle polyubiquitinated proteins to the 26S proteasome for degradation, e.g. Rad23 and Dsk2. Many of these proteins contain ubiquitin-like (Ubl) domains, which may bind to the proteasomal ubiquitin receptors, and an ubiquitin binding domain (Uba), to bind to polyubiquitinated substrates.

Interestingly, when DSK2 is induced (over-expressed) Lys 48-linked ubiquitin conjugates accumulate with impaired proteolysis and cytotoxicity. These data suggest that the excess of

Dsk2 might bind and sequester Lys-48-linked chains away from the proteasome. Alternatively, the binding of Dsk2 to the proteasome, e.g. *via* Rpn10/S5a, may interfere sterically with the binding of substrate-bound Ubl-containing shuttle proteins or Lys 48-linked chains of so modified substrates⁶⁹. It is not excluded either that Dsk2 might stimulate the degradation of substrates linked *via* non-canonical chains, i.e. other than Lys 48-based. Ubiquitin has seven lysine residues (and a free amino terminus) all of which can be involved in ubiquitin chain formation. Chain linkages can be recognised by many proteins containing ubiquitin binding domains⁷⁰ and by deubiquitinating enzymes⁷¹. Under the conditions used in the Dsk2 study the majority of conjugated ubiquitin in the cell lysate was linked *via* Lys 48 and Lys 63⁶⁹. Lys-63 linked chains are generally considered to be involved in non-proteolytic functions⁷². Interestingly, the 26S proteasome does bind Lys 63 as well as Lys 48 polyubiquitinated proteins⁷³. Also substrates modified with Lys 63-linked chains by the yeast ubiquitin protein ligase Rsp5 *in vitro*, are degraded by the 26S proteasome. Furthermore, the membrane anchored transcription factor precursor Mga2, an Rsp5 substrate, contains high levels of Lys63-linked chains *in vivo* that are sufficient for Mga2 processing by the 26S proteasome⁷³. Thus, Lys 63-linked chains are not necessarily excluded from proteasomal degradation.

In contrast to the earlier described paper⁶⁹, Xu et al found that unconventionally-linked ubiquitin chains are abundantly attached to proteins in yeast under their experimental conditions, with Lys 11 (28%) being almost as abundant as Lys 48 (29%)⁷⁴. Their data suggests that all the non-Lys 63-based linkages may target proteins for degradation by the 26S proteasome. Specific roles for Lys 11-linked chains have been reported, e.g. the anaphase promoting complex (APC/C) triggers substrate degradation by assembling Lys 11-linked ubiquitin chains⁷⁵. Another example, in yeast, is that Ubc6 attaches Lys 11-linked ubiquitin chains to proteins extracted from the endoplasmic reticulum (ER) for ER-associated degradation (ERAD)⁷⁴.

Recent studies corroborate these findings since UBX proteins that deliver ubiquitinated proteins in ERAD to the cdc48/VCP/p97 complex for transfer to the 26S proteasome associate more prominently with Lys 11 linkages than Lys 48 linkages⁷⁴. The p97 complex can assemble with all of the 13 known mammalian UBX-domain proteins. The UBX proteins that bind ubiquitin conjugates also interact with dozens of E3 ubiquitin ligases. The large number of ubiquitin ligases found associated with UBX proteins suggests that p97 plays a far broader role than previously anticipated in the global regulation of protein turnover⁷⁶. This presumably explains why mutations in p97 cause a progressive autosomal dominant disorder called inclusion body myopathy (IBM) associated with Paget disease of the bone (PDB) and frontotemporal dementia (FTD)⁷⁷.

Finally, there appear to be differences in the linkage-repertoire of ubiquitin chains in proteins in yeast and mammalian cells. Exemplifying this, when the yeast 26S proteasome is inhibited chemically or genetically there is no change in Lys 63 linkage levels⁷⁴. In contrast, in cultured mammalian cells accumulation of Lys 63-linked chains occurs, although at a slower rate than Lys 48 or Lys 11 chains, when proteasomes are inhibited by MG132⁷⁸. In Huntington's disease, Lys 63- and Lys 11-linked polyubiquitin chains also accumulate in human brain (with an early accumulation of Lys 48 chains) as well as in brains of the R6/2 mouse model of the disease⁷⁹. Lys 63 specific antibodies show the accumulation of Lys 63-linked ubiquitinated proteins in inclusions in Huntington's disease and in neurofibrillary tangles in Alzheimer's disease but rarely in Lewy bodies in Parkinson's disease. However, neuronal inclusions in brain, resembling "pale bodies", which are considered precursors of Lewy bodies, contain Lys 63-linked chains in a mouse model of neurodegeneration⁸⁰ when the 26S proteasome is genetically obliterated^{81, 82}. These contrasting findings may indicate that Lys 63 linked ubiquitin chains are utilised more frequently for proteasomal degradation in mammalian cells than in yeast⁷⁴.

Promiscuous 26S proteasomal subunits

To understand the full range of proteasomal functions it is necessary to discover all the proteins in the cell that interact with the 26S proteasome as well as those proteins that may exist freely or in other complexes in the cell besides interacting with the 26S proteasome (in addition to proteins that may bring ubiquitinated proteins to the proteasome for degradation, e.g. Rpn10 and RAD23). In yeast, quantitative analyses of tandem affinity-purified cross-linked protein complexes has shown that at least 471 proteins, including weak and transient interactors, associate with the proteasome⁸³. As might be expected, there are also an increasing number of proteins that are found in other complexes in the cell as well as in the 26S proteasome. Many of these proteins are known to have roles in human disease (Table 2).

One such example is the human DSS1 protein (deleted in split hand/split foot-1) known in yeast as suppressor of exocytosis mutations (SEM1). The Sem1 protein is found in at least three protein complexes in the cell: with the COP9/signalosome-associated protein Csn12 in an mRNA splicing complex, in a Thp1/Sac3 mRNA export/transcription complex, and in the proteasome^{84, 85}. Some protein subunits of these complexes are structurally related, e.g. the different subunits of the proteasomal lid and those of the COP9/signalosome are structurally highly homologous. In the proteasome Sem1/DSS1 binds to the Rpn3 subunit of the lid sub-complex and maintains the stability of the 26S proteasome^{86–89}.

Chromatin immunoprecipitation in yeast shows that Sem1 is recruited along with 19S and 20S proteasomes and double-strand break (DSB) repair proteins to DSBs *in vivo* suggesting a direct role for proteasomes in DSB repair⁹⁰. Interestingly, DSS1 interacts with the breast cancer protein BRCA2 (breast cancer associated gene 2)^{91–92} and both DSS1 and BRCA2 participate in DSB repair^{92–93}. BRCA2 is part of a large BRCC (BRCA1- and BRCA2-containing complex) complex that has ubiquitin protein ligase activity⁹⁴. Thus, DSS1 may serve to anchor an ubiquitin ligase involved in DNA repair to the 26S proteasome. The very acidic region in Sem1/Dss1 might control access of ssDNA to BRCA2 during double strand break repair or more generally control access of nucleic acids to Sem1/DSS1 containing complexes^{84, 92}. Adding to the multiplicity of its role is the recent finding that Sem1 and the proteasome-associated deubiquitinating enzyme Ubp6 are involved in telomeric silencing by modulating histone ubiquitination and acetylation and therefore recruitment of silencing factors⁹⁵. On top of this, it appears that 26S proteasomes containing DSS1 bind and target p53 for ubiquitin-mediated degradation via the gankyrin-MDM2 pathway⁸⁸.

The oncoprotein gankyrin, which is over-expressed in hepatocellular carcinoma, also seems to have multiple distinct functions. It is one of the RP chaperones described earlier, but it also binds to the retinoblastoma protein (pRb), cyclin-dependent kinase 4 (CDK4), the p53-ubiquitylating enzyme MDM2 and the RelA subunit of NFκB^{32, 96–98}.

It is currently unclear how gankyrin, a chaperone involved in 19S regulator biogenesis, yet not present in 26S proteasomes, can connect 26S proteasomes to the stability and function of pRb, CDK4, MDM2 and RelA. However, when the C-terminus of Rpt3 does not engage in its complementary pocket in the CP, gankyrin/Nas6 remains bound to the 26S proteasome^{21, 25} providing a potential mechanism for gankyrin association with the 26S particle. Furthermore pRb, CDK4, MDM2 and RelA are not found in yeast, leaving open the possibility of pleiotypic functions of gankyrin and the 26S proteasome in higher eukaryotic cells and their relationship to cell physiology and disease. How these different functions of gankyrin are regulated and how they relate to the oncogenicity of gankyrin remain important questions to address.

A third protein with apparent functions in different complexes is the 26S proteasomal lid subunit Rpn11 (human POH1). This deubiquitylating enzyme has a function through its C-terminal residues in controlling mitochondrial structure and function independent of the N-

terminal MPN+/JAMM catalytic motif⁹⁹. A pool of non-proteasomal Rpn11 that associates with mitochondria may exert this function¹⁰⁰. Clearly, given the importance of mitochondrial dynamics in normal cell biology and disease processes, e.g. optic atrophy¹⁰¹, further studies to corroborate the non-proteasomal activities of Rpn11/POH1 and mitochondria are needed in yeast and in human cells.

20S proteasomes and protein degradation in the cell

Studies in reticulocyte lysates have indicated that added purified 20S proteasomes can degrade more than 20% of extracted proteins¹⁰². More specifically, 20S proteasomes may degrade oxidized or otherwise damaged proteins^{103, 104} as found in several diseases such as inflammation, ischemia, and neurodegeneration. Interestingly, in yeast an aberrant form of free 20S proteasomes has been observed in mutants of the 20S chaperone PAC3-PAC4. Mutants also show an increased resistance to high levels of cadmium¹⁴. Further studies will have to address if the aberrant 20S proteasome has a role in the acquired resistance and whether wild type cells can regulate the formation of this 20S form. Studying neurons in mouse brains, the conditional regional ablation of a proteasomal ATPase gene causes depletion of 26S but not 20S proteasomes and recapitulates the neuropathology and neurochemical deficits of Parkinson's disease and dementia with Lewy bodies. There might be many reasons why reduced 26S proteasome levels in these neurons leads to cell death because of the extensive different activities of 26S proteasomes in the cell. However, remaining 20S proteasomes are not sufficient for neuronal viability^{81, 105}. Besides important cellular roles for 26S proteasomes, free 20S proteasomes, and free RP has several reported roles within the cell, for example in transcriptional elongation¹⁰⁶. The RP-chaperones described earlier might play a role in regulating cellular free RP, however this aspect has only briefly been touched upon up to now²⁵. It will be interesting to see how functions of free RP are impacted in yeast strains with the strongly reduced RP levels in chaperone mutants. With the presence of cellular functions for free RP and 20S proteasomes, in addition to all the important roles of the 26S proteasome, it is clear that it will be essential to understand what regulates the levels of 20S, RP, and 26S in cells. These levels will depend on the assembly pathways (with important roles for the chaperones), but will also be affected by the equilibrium between 20S proteasomes and other activators such as BLM10/PA200 or PA28/REG/11S. These dynamics and interactions are still poorly understood.

Concluding remarks

In the last 21 years it has become clear that the cellular “multicatalytic proteinase”, conserved from archaeobacteria to eukaryotes¹⁰⁷, has central roles in all aspects of cell physiology. The assembly, structure and function of the protein degradative 26S proteasome is now seen to be as sophisticated as its cellular protein synthesising counterpart the ribosome. The discovery of chaperones involved in the biogenesis of both the CP and RP serves to illustrate the complexity of the assembly process but still leaves open questions concerning alternative routes to the building of the mega structure. There is still a need to determine the complete structure of the 26S proteasome, but given the natural dynamic fluctuation of the complete particle⁵⁶, this may prove difficult. In higher eukaryotic cells with multiple compartments the outstanding questions include the elucidation of the interactions of 26S proteasomes with cellular organelles and movement between compartments (Box 2). Nowhere can this complexity be better seen than in the nervous system where the movement of 26S proteasomes into dendritic spines¹⁰⁸ to facilitate synaptic plasticity and memory is controlled by the interaction of the particles with an abundant regulatory enzyme of the nervous system called calcium calmodulin dependent kinase II α that regulates memory deposition¹⁰⁹. The world of proteasomal interactors and dynamics deserves much more investigation.

Text Box 1 The ubiquitin proteasome system

The ubiquitin proteasome system is the major cellular system for the regulated degradation of proteins in the cell. Proteins are ubiquitinated as a signal for degradation by the 26S proteasome. Protein ubiquitination has many other roles in the cell including the regulation of chromatin structure, nucleic acid metabolism, autophagy, and receptor mediated endocytosis¹¹¹.

The ubiquitination of proteins is carried out by the sequential activities of specific enzymes: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin protein ligases (E3). There are also many deubiquitinating enzymes that remove ubiquitin from proteins. The E3 ligases are the ultimate arbiters of protein stability in the cell. There are similar numbers of E3 ligases in the cell (~600 hundred) as there are protein kinases. Protein ubiquitination/deubiquitination and protein phosphorylation/dephosphorylation are intimately linked to control all aspects of cell physiology, as are other functional group and functional protein post-translational protein modifications.

The Proteasome

The proteasome is the major cellular protease. The proteolytically active sites reside within the 20S core particle (CP), a cylindrically shaped structure formed by four stacked rings, ordered α_{1-7} , β_{1-7} , β_{1-7} , α_{1-7} . Subunits β_2 , β_5 and β_1 have proteolytic activity, displaying trypsin-like, chymotrypsin-like and caspase-like peptidase activity respectively. Importantly, these active sites are sequestered to the inside of the CP to protect the cell from non-specific degradation. The N-termini of the α subunits close the ends of the cylinder, often referred to as the gate. While some proteins can be degraded by the CP, most degradation depends on ATP. The only ATP hydrolyzing proteins in the proteasome are the six ATPases present in the regulatory particle (RP). The 19 subunits of the RP are divided between two sub-structures, the base and lid complexes. The lid contains the Rpn11 subunit that has deubiquitinating activity. The base consists of a ring of AAA-ATPases. The C-termini of these ATPases dock into pockets present between the α subunits of the CP. This results in a translocation of the α tails and an opening of the gate. An interesting architectural problem is that this six-membered ATPase ring sits on a seven-membered α -ring. Although sequence alignments suggest that one pocket remains empty, it's not clear how these rings interdigitate. The CP can associate with one or two RPs, but there are other activators that may also bind to the 20S CP particle. These include the Bln10/PA200 protein that is a large heat-repeat containing protein and the 11S/PA28 complex, itself a heteroheptamer, either of which may dock directly with the CP.

Box 2 Outstanding Questions

Outstanding questions in proteasomal cell biology are: (i) how many protein assemblies, including 19S, 20S and 26S proteasomal complexes, control key events in the cell like DNA synthesis, DNA repair and transcription?; (ii) how are the presence and dynamics of these different interdependent assemblies regulated?; and (iii) when are 26S proteasomes (or sub-complexes) involved in these processes in a non-proteolytic or proteolytic manner?

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Glossary Box

Proteostasis(protein homeostasis)	a process regulating proteins within the cell in order to maintain the health of both proteome and the organism itself and maintained by a highly inter-connected network comprising pathways that control protein synthesis, folding, trafficking, aggregation, disaggregation, and degradation.
Ubiquitin	a small covalent protein modifier that is used to modify (ubiquitinate) proteins in the cell by a process similar to that of protein phosphorylation.
Ubiquitin chain	an ubiquitin attached to a lysine within another ubiquitin creates an ubiquitin chain. Often chains are referred to based on the sequence number of the lysine in ubiquitin to which a subsequent ubiquitin is attached. Chains can be found with single and mixed linkages (e.g. K11 and K48 linkage together in a chain). Besides linear chains, also forked chains exist, in which one ubiquitin has two or more ubiquitins directly attached to different lysines within a single ubiquitin. Ubiquitin chains can be attached to proteins or found free in the cell ¹¹⁰ .
26S proteasome	a large protein complex that recognizes and degrades polyubiquitinated proteins.
20S proteasome	the catalytic core of the 26S proteasome consisting of a $\alpha_7\beta_7\beta_7\alpha_7$ cylindrical complex. Commonly referred to as core particle (CP).
19S regulator	a multiprotein complex that can be attached to one or both ends of the 20S CP for the recognition of ubiquitinated proteins, their deubiquitination and their subsequent degradation in the 20S core. Commonly referred to as regulatory particle (RP).
Polyubiquitination	modification of a protein by a chain of polyubiquitin anchored to a single lysine residue on a target protein.
19S regulator structure	this consists of a base complex and lid complex. The base contains a putative ring of six ATPases and two non-ATPases. The lid contains many proteins, including those with deubiquitinating activity, involved in the recognition of ubiquitinated proteins and their processing for eventual degradation.
ATPase	an enzyme that hydrolyses ATP for a biochemical process.
Proteasomal ATPases	proteasome subunits that are members of the AAA (ATPases associated with various cellular activities) superfamily of ATPases that control numerous processes in the cell.
Chaperone	a protein that assists in the (un)folding or (dis)assembly of other proteins, but that is not part of the final biological complex.

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Figure 1.

Composition of the 26S proteasome. Proteasome is formed by two regulatory particles abutting cylindrically shaped core particle. Core particle is formed by two α -rings and two β -rings. Regulatory particle consists of a base complex and a lid complex. Rpn10 is at the interface between these complexes (shown in light blue). The lid (green) contains indicated subunits and its function is not well understood, with the exception of rpn11, which functions as a de-ubiquitinating enzyme. The base contains a ring formed by six AAA-ATPases, Rpt1–6 (purple ring), and the subunits Rpn1, Rpn2 and Rpn13 (shown as orange box; see text for more details). The proteasome contains three heteromeric ring structures, each present twice. The α and β -ring are formed by seven subunits; scissors indicate the active sites and the dots in the α ring show the binding pockets for the Rpt tails (see text). The pocket between α_7 and α_1 lacks a Lys typically found in the pocket and might not harbour an Rpt C-terminus⁴¹. The Rpt-ring is formed by six AAA-ATPases.

Nas6

7 ANK repeats

Hsm3

Armadillo-like repeats

Rpn14

7 WD40 repeats

Nas2

PDZ domain

Figure 2.

(A) Domain topology of Nas6, Rpn14, Hsm3 and Nas2. Below the topology is shown a 3D-structure of the domains to illustrate the structural difference. Only for Nas6 a structure been determined, for the others the structure of a similar domain from a different protein have been used (pdb coordinates used 1IXV for Nas6, 2H12 for WD40 repeat, 3GRL for Armadillo like repeats, 1G9O for PDZ domain). Hsm3 shows little conservation and detailed analysis of the domain can be found in Le Tallec et al.¹⁶. Surprisingly, while structurally unrelated, these chaperones bind to the same small C-domain in the AAA-ATPases. (B) The domain topology of the proteasomal AAA-ATPases. cc is coiled coil region, OB is the OB-domain, ATPase is the ATPase domain containing the walker A and walker B motifs and the C indicates the C-domain, typically found in AAA-ATPases behind the ATPase domain. The structure shown is a ring formed by the ATPase (Blue) and C-domains (red) of six proteasome-activating nucleotidase (PAN) AAA-ATPases from the archaea *Methanocaldococcus jannaschii*⁴⁴. The CP cartoon is shown to illustrate the expected interface of the CP and the ATPase ring. Cartoons and structures are not drawn to scale.

Figure 3.

Cartoon showing the components known to be involved in the RP-assembly. The pathways and order of events are still unclear. Therefore an interaction map is displayed instead. The known potential interactions between the different complexes are shown in green. The chaperones are known to interfere with the interactions indicated in red, suggesting these proteins have a quality control role in assembly. Nas2 has also been shown to stabilize Rpt4 and 5 (purple arrow). In the middle of the cartoon are the seven pockets of the CP surrounding the gate (which is shown in an open conformation, although generally this is closed without activators bound to the CP). The dark pocket indicates the only pocket without a conserved positive charge expected not to be able to host a tail. Rpt tails that dock in the pockets are shown as dark orange extensions from the Rpt proteins. Numbers 1 to 6 indicate the different Rpt proteins, H3 Hsm3, R14 rpn14, N6 Nas6 and N2 Nas2.

Table 1

Names of the chaperones

Saccharomyces cerevisiae		Homo Sapiens		
name	Systematic	alternatives	name	HGNC ¹ alternatives
Pba1	YLR199C	Poc1	PAC1	PSMG1 DSCR2, e21-LRP, LRPC21,
Pba2	YKL206C	Add66 , Poc2	PAC2	PSMG2 HCCA3, MDS003, MGC15092, CLAST3, HsT1707 TNFSF5IP1
Pba3	YLR021W	Poc3, Dmp2, Irc25	PAC3	PSMG3 C7orf48, MGC10911, MGC10911
Pba4	YPL144W	Dmp1, Poc4	PAC4	PSMG4 C6orf86
Ump1	YBR173C	Rns2	UMP1	POMP HSPC014
Hsm3	YBR272C		S5b	PSMD5 KIAA0072
Nas2	YIL007C		P27	PSMD9
Nas6	YGR232W		Gankyrin	PSMD10 P28
Rpn14	YGL004C		PAAF1	FLJ11848, Rpn14, WDR71

In Bold are the SGD standard names (<http://www.yeastgenome.org/>).
¹Names based on HUGO gene Nomenclature Committee name assignment (<http://www.genenames.org/>).

Table 2

26S proteasomal interactors and Human Disease

Name	Disease
DSS1/SEM1	Developmental abnormality (Deleted in split hand/split foot)
Gankyrin/Nas6p	overexpressed oncoprotein (liver and colon cancer)
P97/cdc48	Paget's disease of bone, dementia, inclusion body myositis

See text for functions of the proteins