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The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death

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The 26S proteasome is a large, ~2.5 MDa, multi-catalytic ATP-dependent protease complex that serves as the degrading arm of the ubiquitin system, which is the major pathway for regulated degradation of cytosolic, nuclear and membrane proteins in all eukaryotic organisms.

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As proteasome-mediated degradation regulates the turnover of numerous cellular proteins involved in essentially all cellular processes, its own regulation plays key roles in preserving homeostasis. Proteasome activity is regulated at several levels, ranging from its abundance, i.e., the synthesis of its subunits; the rate of its assembly and disassembly; its regulation by post translational modifications (PTMs); regulation of the events related to its proteolytic activity, i.e., substrate recognition and binding, subsequent conformational changes of the proteasome, substrate deubiquitination, unfolding, and translocation into the catalytic chamber; proteasome subcellular localization and its recruitment to specific organelles, and finally, the destruction of the proteasome itself accomplished either via degradation of individual subunits or by removal of the proteasome as a whole.

All the above events are responsive to the changing cellular environment and different pathophysiological conditions. In this review, we discuss the current knowledge regarding the regulatory processes that underlie the basis for proper proteasome function and its adjustment to the changing requirements of the cell.

Note that nomenclature of proteasomal subunits and effectors differs between organisms. Referring to proteasome subunits, we adopt the yeast protein terms: α, β, Rpn and Rpt subunits. Effector ortholog names are introduced once, and are later referred to using one representative name.

The 26S proteasome structure and function

The 26S proteasome consists of two distinct sub-complexes, a 20S core particle (CP) and a 19S regulatory particle (RP, also termed PA700). The 20S CP is composed of four axially stacked heteroheptameric rings (two outer α- and two inner β-rings), and has a barrel-shaped structure [1] (Figure 1). The outer α-rings contain seven similar, yet distinct α-subunits (α1-α7), and by forming a pore, they function as a tightly regulated "gate" for the entrance of substrates, and for removal of degradation products from the complex. This "gate" which is made of the N-termini of a subset of α-subunits, blocks the unregulated entrance of substrates into the catalytic chamber. A crucial role in the organization and activation of the "gate" is attributed to the N-terminus of the α3-subunit, since its deletion results in a constitutively open pore [2]. The mechanism of the "gate" opening and proteasome activity are regulated by the docking of proteasome regulators (such as 19S RP, PA28, PA200, ECM29 and PI31) containing an HbYX motif (where Hb stands for a hydrophobic residue; Y for tyrosine; and X for any amino acid) onto seven binding pockets formed by α-α interfaces on the 19S-facing surface of the outer α-rings [3, 4]. In addition, the outer α-rings form extra interior compartments, the "antechambers", which are connected to the central chamber of the "barrel" from each side, and can keep a

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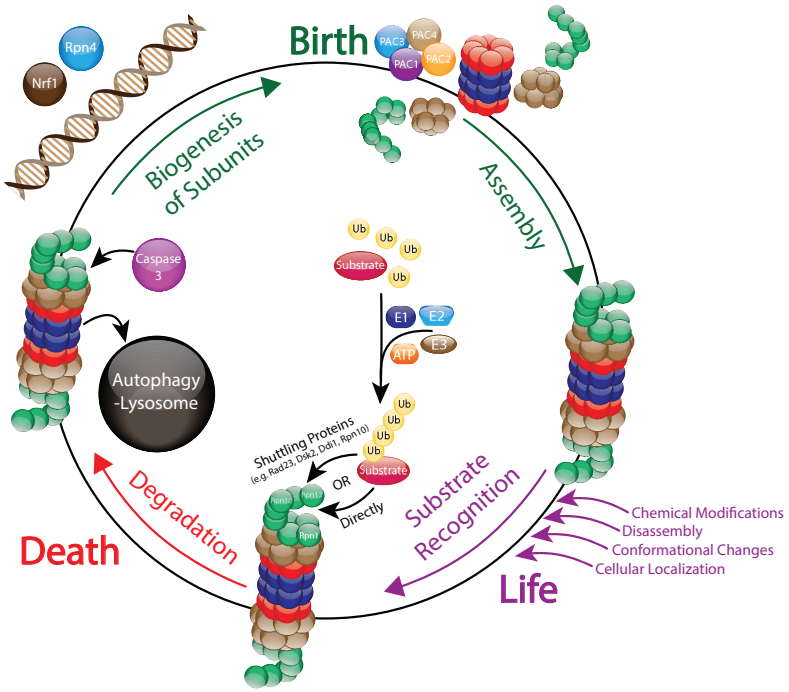

Figure 1 The life cycle of the proteasome. The "birth" of the proteasome is controlled by transcriptional regulation of its different subunits. The biogenesis is organized by transcription factors such as Nrf1 and Rpn4, which are sensitive to changing physiological conditions. The different subunits assemble in a coordinated manner to form the mature proteasome. The 26S proteasome recognizes ubiquitin conjugated substrates in a process mediated by intrinsic and extrinsic ubiquitin receptors. The recognition is regulated by different post-translational modifications, disassembly, conformational changes, and cellular localization that the proteasome undergoes. The "death" of the proteasome is at least partially mediated by the lysosome/vacuole/autophagy and cleavage by caspase(s). In the middle is the energy-dependent ubiquitin-substrate conjugate formation catalyzed by E1, E2, and E3.

certain amount of intact substrate or digested products [5, 6]. Also, several of the α-subunits have an important role in the subcellular localization of the proteasome by bearing a nuclear localization signal (NLS) [7-9].

Similarly, the inner β-rings consist of seven distinct β-subunits (β1-β7), which are flanked by the two outer α-rings. Three of the β-subunits, β1, β2 and β5, contain active sites with different proteolytic specificities: the peptidyl-glutamyl-hydrolyzing or caspase-like, the trypsin-like, and the chymotrypsin-like activity, respectively. The catalytic β-subunits are synthesized as precursors bearing N-terminal propeptides. The elimination of the propeptides during proteasomal maturation is required for exposure of the N-terminal catalytic threonine (Thr) residue. Hence, each mature eukaryotic proteasome has six proteolytic sites with three types of proteolytic activities [6, 10].

Additional "specializing" β-subunits have been identified in mammalian cells under specific conditions/organs: β1i, 2i, β5i and β5t, where "i" stands for immunoproteasome and "t" for thymoproteasome.

The thymoproteasome was found only in cortical epithelial cells of the thymus and is thought to play a vital role in the positive selection of $CD8^+$ T-cells. The configuration of the active site of the thymoproteasome is β1i-β2i-β5t and their chymotrypsin-like activity is lower in comparison with "standard" and immunoproteasomes [11, 12].

Immuno-β-subunits are commonly expressed in a broad variety of immune system-specific tissues like the spleen, thymus, lung, liver, kidney, colon, small intestine and antigen-presenting cells (APCs). Their expression can also be induced in non-immune tissues (or cells) by specific (e.g., IFN-γ, TNF-α, LPS) and less specific (e.g., aging and environmental stress factors) stimuli [11]. The proteolytic activity of the immunoproteasome has an altered specificity toward cleavage after basic and hydrophobic residues that are thought to increase the affinity of the substrate fragments to MHC class I molecules [13]. Analysis of proteasomes from different

tissues shows that a single 26S holoenzyme can be made of both "constitutive" and "immunological" β-subunits, thus generating an intermediate (or hybrid) proteasome subpopulation [11].

The "canonical" proteasome cap, the 19S RP, is a multifunctional complex which regulates proteasome function by identification, binding, deubiquitination, unfolding and translocation of substrates to the proteolytic chamber of the CP. The RP is further divided into two additional subcomplexes, the "base" and "lid". The base consists of six regulatory particle AAA ATPase subunits (Rpt1-Rpt6), organized into a ring, as well as four regulatory particle non-ATPase subunits (Rpn1, Rpn2, Rpn10 and Rpn13 (Adrm1)) [12]. Rpn1, Rpn10 and Rpn13 serve as ubiquitin receptors, recognizing substrates targeted to the proteasome [14-16]. The lid consists of nine different Rpn subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15 (Dss1/Sem1)), which form a horseshoe-shaped structure. A main function of the lid is deubiquitination of incoming substrates. This activity is carried out by the deubiquitinating enzymes (DUBs) Rpn11, Uch37 and Ubp6/Usp14 [17-21]. Interestingly, high-energy nucleotides are required in order to hold the 19S and 20S sub-complexes together and for opening the "gate" to the catalytic chamber by coordinating the timed separation and proper movement of the α-ring N-termini [22, 23]. Certain important functions of the 19S RP are energy-dependent; among them is "preparation" of the substrates and their translocation into the CP for degradation [1, 24]. The various functions of the 19S RP are described below, and are reviewed in details in a more biochemically based review [25].

Assembly of the 26S proteasome

The assembly of the proteasome is a highly complex, multi-step process, accompanied by proteasome-dedicated chaperones and maturation factors. The positioning of each individual subunit in the final structure of the mature proteasome is highly defined.

Assembly of the eukaryotic 20S proteasome is initiated by the formation of an α-ring, which is controlled and directed by two main heterodimeric chaperone complexes: proteasome-assembling chaperone 1 (PAC1)•PAC2 and PAC3•PAC4. One model for α-ring formation suggests that it is initiated by the interaction of the α5 and α7 subunits with the PAC1•PAC2 complex. The complex then mediates the incorporation of the rest of the α subunits, and prevents spontaneous dimerization of either α subunits or complete α-rings [26]. The PAC1•PAC2 complex stays bound to the outer side of the α-ring until the complete assembly of the 20S proteasome, and is thought to protect it from premature docking of activators [27]. The PAC3•PAC4 complex is also involved in early steps of the α-ring formation by acting along with PAC1•PAC2, preventing the incorrect incorporation of α-subunits during α-ring formation [28]. The PAC3•PAC4 complex is bound to the inner side of the α-ring. The complete formation of the heptameric α-ring initiates the assembly of the half-proteasome, consisting of one α- and one β-ring. In mammals, the α-ring serves as an assembly platform for the β-ring, the formation of which starts with β2, followed by β3, β4, β5, β6 and β1 subunits. The formation of the β-ring is initiated by incorporation of UMP1, another chaperone, into the α-ring prior or concomitant with β2. The incorporation of the β3 subunit triggers the release of the PAC3•PAC4 complex. One α-ring with incorporated β2, β3, and β4 subunits, forms an intermediate structure, termed the 13S complex [29]. UMP1 regulates the correct incorporation order of the other β subunits. In addition, UMP1 localizes the immature proteasome to the endoplasmic reticulum (ER), the main assembly site of the proteasome in mammalian cells [30]. Most of the β-subunits, excluding β3 and β4, are synthesized as precursors with N-terminal propeptides. The propeptides of β2 and β5 are essential for recruitment and incorporation of β3 and β6, respectively. The β5 propeptide is also necessary for specific interaction with UMP1 [31]. The propeptides of β1, β2 and β5 prevent the premature activity of their N-terminal catalytic Thr residue. Furthermore, the C-terminal tails of β-subunits have an important role in proteasome biogenesis by providing a specific interaction within or between β-rings. The β-ring formation is terminated by the incorporation of β7, hence forming a half 20S proteasome, called the 15S complex ($\alpha_{1\text{-}7}\beta_{1\text{-}7}$-UMP1-PAC1•PAC2). The incorporation of β7 induces the dimerization of two half-mers by insertion of its C-terminal tail into a groove between β1 and β2 in the opposite β-ring. Upon dimerization, the propeptides of the β-subunits undergo autocatalytic cleavage, exposing their catalytic Thr residue [24, 32]. Finally, the PAC1•PAC2 complex and UMP1 are degraded by the mature 20S proteasome [24, 26, 31].

By contrast, the assembly of the 19S proteasome is still not a well understood process. The lid and base sub-complexes of the 19S are assembled independently, which precedes their association with one another through Rpn10. The base formation is assisted by a group of base-dedicated chaperones, arranging the six ATPase subunits in a defined order in a ring, and also inhibiting premature DUBs and ATPase catalytic activities [1]. The base-dedicated chaperones expressed in yeast are Hsm3, Nas2, Nas6 and Rpn14, and their mammalian homologs are S5b, p27, gankyrin/p28 and Rpn14/PAAF1

(proteasomal ATPase-associated factor 1), respectively. In addition, Adc17 is a stress-induced chaperone found in yeast, which was shown to facilitate 19S RP assembly in response to changing demands [33]. These chaperones bind through their C-terminal protein-protein interacting domain to specific ATPase subunits [1, 32]. Base assembly starts with the formation of three Rpt *cis-trans* heterodimers (Rpt1:Rpt2, Rpt4:Rpt5, Rpt3:Rpt6), associated with specific chaperones (Hsm3/S5b-Rpt1:Rpt2-Rpn1, Rpt4:Rpt5-Nas2/p27, Nas6/gankyrin/p28-Rpt3:Rpt6-Rpn14/PAAF1), which are involved in the pairing process. Adc17 was also shown to support Rpt3:Rpt6 pairing [33]. The exact order of assembly of the different pairs to the final base structure is unclear, yet the process is accomplished by subsequent joining of all pairs, as well as that of Rpn2 and Rpn13 subunits [34].

The lid assembly is a much less understood process. However, it has been suggested that it also occurs in several steps. The assembly starts with formation of two modules: a core module, consisting of Rpn5-6, Rpn8-9 and Rpn11, and a second module, consisting of Rpn3, Rpn7 and Rpn15/Sem1. The joining of the two modules is mediated by interaction between Rpn3 and Rpn5. The incorporation of Rpn12 completes the lid formation [1]. In addition, a recent study showed that incorporation of the Rpn12 subunit triggers a conformational change in the forming lid (mediated by a single helix of Rpn12), which results in its association of the base [35]. No lid-specific assembly chaperones have been discovered yet. However, it has been suggested that Hsp90 contributes to its assembly in yeast [36].

Proteasomal regulation

Transcriptional regulation of proteasome biogenesis

Current knowledge regarding the basal rate of proteasome subunit biosynthesis is limited. It is believed that all subunits of the "canonical" proteasome are found in cells only in the context of their respective complex, the 20S or 19S, with the exception of the 19S subunit Rpn10/S5a, which is also present in a free state [37], though not necessarily in all cell types [38]. Since proteasomal subunits seem to incorporate into their sub-complexes with unassembled subunits being removed [39], it is difficult to determine whether the stoichiometry observed in intact complexes faithfully represents the rate of individual subunit's synthesis. Nevertheless, recent studies demonstrated the concerted, yet not necessarily even, upregulation of proteasome subunits in response to stress [40-42], contributing to the growing body of evidence for common signaling pathways regulating proteasome gene expression.

Upon proteasome inhibition, a concerted *de novo* synthesis of all 26S proteasomal subunits with subsequent whole proteasome formation was observed in several organisms [40, 43-46]. In mammals, it has been shown that nuclear factor erythroid 2-related factor 1 (Nrf1) is a transcription factor (TF) essential for the activation of proteasomal gene expression in response to proteasome inhibition [40, 47, 48]. Nrf1 was reported to be ubiquitinated by more than one E3 ubiquitin ligase (HRD1, Fbw7/FBXW7, and β-TrCP), and is possibly degraded by the proteasome [42, 47, 48]. Normally, Nrf1 is an ER-bound protein. Its release and translocation to the nucleus to activate transcription requires its deglycosylation, ubiquitination, and partial proteasomal degradation (i.e., processing). Interestingly, the addition of a low concentration of a proteasome inhibitor results in processing and nuclear localization of Nrf1, upregulation of proteasomal subunits, as well as of other proteasome-related genes, while such response does not occur in the presence of a high concentration of the inhibitor. In the absence of an inhibitor, Nrf1 is rapidly degraded [42].

Nrf1, and its homolog Nrf2, are basic leucine zipper TFs, known to bind antioxidant response elements (AREs) in promoters of target genes. Studies showed that many of the proteasomal subunit genes harbor putative AREs in their promoters, so that Nrf1-mediated proteasome upregulation following inhibition of the proteasome may rely on Nrf1 binding to these AREs [47, 48]. Nrf2 is also a substrate of the ubiquitin-proteasome system (UPS). Under normal conditions, it is bound to Keap1 which serves as the substrate-recognizing component of an SCF E3 ubiquitin ligase along with Cul3-Rbx1. The ligase ubiquitinates Nrf2, targeting it for proteasomal degradation. One mechanism suggested to underlie, at least in part, Nrf2 upregulation under oxidative stress is its dissociation from Keap1 in the presence of high concentration of antioxidants, which results in its stabilization and subsequent translocation to the nucleus, where it induces proteasomal genes transcription [49-51].

In *C. elegans*, the compensatory proteasome upregulation in response to both proteasome inhibition and oxidative stress was demonstrated to depend on a single TF, SKN-1, which is an ortholog of Nrf1 and Nrf2 [45, 52]. Expectedly, SKN-1 is also a proteasomal substrate, targeted for degradation following ubiquitination by the CUL4/DDB1 E3 ubiquitin ligase [53].

Rpn4 is a short-lived protein that acts as a TF in *Saccharomyces cerevisiae*. Being a proteasomal substrate, ubiquitinated by the Ubr2 E3 ubiquitin ligase [54] or degraded in a ubiquitin-independent manner [55], Rpn4 levels are reciprocally correlated with proteasome activity. In face of compromised proteasomal function, Rpn4

level is elevated and its activity as a TF upregulated [56]. As it enters the nucleus, Rpn4 binds a unique sequence upstream to proteasomal genes, the proteasome-associated control element (PACE), thereby stimulating the expression of proteasomal genes [57]. As for transcriptional regulation of Rpn4 itself, it was found that many stress-induced TFs harbor recognition motifs upstream to the *RPN4* gene; among them are factors related to oxidative stress (*YAP1*), drug resistance (*PDR*s), and heat shock (*HSF1*). These findings suggest a possible role for proteasome upregulation under different stress conditions. Rpn4 was also found to bind upstream to the oxidative stress effector YAP1, further establishing the bilateral linkage between oxidative stress and proteasome level and function [58].

Besides common regulatory pathways affecting subunit abundance which respond to alterations in proteasome function or to general stress, an intriguing study suggested that overexpression of a single proteasome subunit, β5, may upregulate the level of other subunits, as well as proteasome assembly and activity. This results in improved cellular function as reflected by ameliorated response to oxidative stress and delayed senescence [59].

Another study showed that the upregulation of a single 19S subunit, Rpn6, also stimulates proteasomal activity in *C. elegans*, though not via elevation of other proteasomal subunits gene expression [60]. The same group also showed that human embryonic stem cells (hESCs) exhibit higher levels of proteasome activity, an elevation which disappears upon their differentiation in correlation with reduction in the level of Rpn6, with no change in the level of any other regulatory proteasomal subunit. Indeed, lowering Rpn6 level in hESCs resulted in reduced proteasomal activity. Induction of pluripotency (i.e., cell reprogramming into induced pluripotent stem cells, or iPSCs) led to increased proteasomal activity as well as to elevated Rpn6 level. It is suggested that Rpn6 stimulates proteasome activity by increasing the association between the 20S CP and the 19S RP. Accordingly, overexpression of Rpn6 resulted in increased proteasome assembly and activity, with a similar reciprocal effect observed following Rpn6 knockdown [61]. In both *C. elegans* and human, the regulation of proteasome assembly and activity through Rpn6 is mediated by the DAF-16/FOXO4 orthologous TFs [60, 61].

Proteasome activity in response to stress

A recent debate concerning the effect of mechanistic target of rapamycin (mTOR) on the proteasome is highlighting one of the signaling pathways regulating its activity. mTOR is a central regulator of cell growth and proliferation, affecting a broad array of activities, including protein synthesis and breakdown [62]. One study suggested that activation of mTOR and subsequent increase in protein synthesis, also results in Nrf1-mediated upregulation of proteasome biogenesis and activity [63]. On the other hand, a later study claimed that inhibition of mTOR, known to induce autophagy, results also in activation of proteasomal degradation, and that this upregulation is independent of *de novo* protein synthesis [64]. It is yet unclear whether these two apparently contradicting findings reflect differences in experimental approach and/or pathophysiological conditions of the cells [65, 66]. Nevertheless, it is clear that mTOR downstream effect on proteasome activity plays an important role in its regulation and more studies are required to define the different conditions and direction of the regulatory process.

Proteasome regulation by post-translational modifications

As a tightly regulated enzyme comprised of dozens of subunits, the proteasome is a target for many PTMs, having their number growing steadily.

In a recent meta-analysis, researchers generated a comprehensive map of proteasomal co- and post-translational modifications, detected via employment of proteomic analyses. More than 345 modifications, belonging to 11 different types, were shown to "decorate" the 26S proteasome in yeast [67]. It has also been shown that the same proteasomal modification site may serve as a target for more than one modification, suggesting a cross talk between different types of modification [68]. Such data demonstrate the complexity of studying proteasomal regulation by PTMs, while emphasizing the probable magnitude of the phenomenon.

Importantly, the mechanism by which the different modifications exert their effect on proteasomal function is largely unknown. For many of the modifications, even their effect on the proteasome and/or the site/subunit modified have not been deciphered [67]. Such are the cases, for example, for proteasome dephosphorylation by Ublcp1 (which regulates nuclear proteasome activity [69]), N-terminal acetylation of proteasomal subunits by N-acetyl transferases (NATs) in yeast (which mediates proteasome localization during aging [70] (and for which even the subunit(s) that is modified at its N-terminus has not been identified)), and ubiquitination of the proteasome (targeting it as a substrate for lysosomal degradation [71]).

Nevertheless, established cases of proteasomal PTMs and their role in proteasome regulation are already at hand. Table 1 lists different PTM types, of which both the target and the effect on proteasomal function are known. As some studies identified only the complex

Table 1 Proteasome PTMs with known target and effect

Modification type	Target subunit	Effect	Reference
Phosphorylation	α7	↓Substrate affinity, ↑26s stability	[72, 73]
Phosphorylation	19s	↑ATPase activity	[74]
Phosphorylation	Rpt6	↑Proteasome activity; ↑Proteasome assembly	[75, 76]
Phosphorylation	20s	↑Proteasome activity; ↓Proteasome activity	[77, 78]
Phosphorylation	Rpn2	↓Proteasome activity	[79]
Phosphorylation	α4	↓Proteasome activity	[80]
Phosphorylation	Rpn6	↑Proteasome activity	[81]
Ubiquitination	Rpn10	↓substrate binding	[82]
Ubiquitination	Rpn13	↓substrate binding	[83]
Acetylation	α6, β3, β6, β7	↑Proteasome activity	[84]
S-glutathiolation	α5	20S "gate" opening and ↑proteasome activity	[85]
N-myristoylation	Rpt2	Nuclear proteasome localization	[86]
O-GlcNAc	Rpt2	↓Proteasome activity	[87]
Poly-ADP ribosylation	20S	↑Nuclear proteasome activity and histone degradation	[88]

rather than the subunit that is modified, in these cases the table refers to that complex. This may explain an opposite effect (upregulation and downregulation) of a given modification occurring on different subunits.

Substrate recognition and degradation

Ubiquitin receptors

Ubiquitinated substrates are recognized by ubiquitin receptors and targeted to the 26S proteasome for degradation. Ubiquitin receptors can be classified according to their association with the proteasome: the proteasome intrinsic receptors which are subunits of the 19S RP, and the extra-proteasomal proteins that bind ubiquitinated substrates as free entities and shuttle them to the 26S proteasome.

Proteasomal ubiquitin receptors

Three intrinsic proteasomal subunits have been shown to bind ubiquitinated substrates: Rpn13 [14], Rpn1 [15] and Rpn10 [16]. The 19S subunits Rpt5 [89] and Rpn15 [90] were also suggested as possible ubiquitin receptors, as they were shown to bind ubiquitin, but whether they actually recognize ubiquitinated substrates targeted to the proteasome is yet to be determined.

Rpn13 binds ubiquitinated substrates via its N-terminal pleckstrin-like receptor of ubiquitin (PRU) domain [91], while its C-terminal domain is known to bind and activate the DUB Uch37. Together, they function as a "proofreading" or "editing" machinery that enables the escape of poorly or inadvertently ubiquitinated substrates by removing their ubiquitin moieties [92, 93], or potentially by trimming the chain to a length that fits the proteasome better, directing the substrate for efficient degradation.

Besides its established role as a receptor for ubiquitinated substrates, Rpn1 has also been shown to mediate proteasomal interaction with several UPS components. Studies in yeast have shown that Rpn1 can serve as a binding site for the shuttle proteins radiation sensitivity abnormal 23 (Rad23), dual-specificity protein kinase 2 (Dsk2) [94, 95] and DNA damage-inducible 1 (Ddi1) [96]. Recent study characterized the binding sites of Rpn1 with different interactors, and found that its binding to both ubiquitin and Rad23 UBL domain occurs via the same binding site, T1, and that an adjacent binding site, T2, recognizes the ubiquitin-like (UBL) domain of the extra-proteasomal DUB Ubp6 [15].

Rpn10 is a unique ubiquitin receptor, as it functions both in its proteasome-bound form, as well as in its free state, as shown in *D. melanogaster* [97], *S. cerevisiae* [98], and *A. thaliana* [99]. Rpn10 binds polyubiquitin chains via its ubiquitin-interacting motif (UIM) [16, 97] and harbors a von Willebrand A (VWA) N-terminal domain, which facilitates its binding to the proteasome [100] and the subsequent degradation of several ubiquitinated substrates [16, 101]. Monoubiquitination of Rpn10 regulates its capacity to bind ubiquitinated substrates, as it promotes intramolecular interactions that hinder the UIM, reducing its affinity toward ubiquitinated substrates [82, 102, 103].

Non-proteasomal ubiquitin receptors and shuttle proteins

In addition to stoichiometric proteasomal ubiquitin

receptors, a group of non-proteasomal UBL-UBA (ubiquitin-associated) shuttle proteins also serves as ubiquitin receptors. Three of these receptors, Rad23, Dsk2 and Ddi1, have been characterized in detail in yeast. These UBL-UBA domain-containing proteins bind ubiquitinated substrates via their C-terminal UBA domain [104, 105], and associate with the proteasome via their N-terminal UBL domain [95, 106, 107]. This UBL-mediated interaction is carried out through binding to Rpn1 [95], Rpn13 [14] or Rpn10 [108, 109].

Although Dsk2 serves as a shuttle of ubiquitinated substrates to the proteasome, it has been surprisingly shown that its overexpression impairs proteolysis and exerts a cytotoxic effect [110-112]. This effect was shown to be attenuated by the binding of Rpn10 UIM (in its proteasome-unbound form) to Dsk2 UBL [112]. This association is believed to be regulated through Rpn10 monoubiquitination [113], resembling the mechanism that regulates UIM binding with ubiquitinated substrates [82, 102, 103].

Ubiquilins, the mammalian orthologs of the yeast Dsk2, are a family of four ubiquitin-like proteins that function as shuttle proteins targeting ubiquitinated proteins to the proteasome. This family is well conserved in frog, rat and human. Structurally, they harbor an N-terminal UBL domain by which they bind proteasomal Rpn10, and a C-terminal UBA domain which binds polyubiquitinated proteins [114, 115]. Ubiquilins have been shown to mediate the removal of damaged proteins following oxidative stress [116]. Abnormalities in ubiquilins, such as a compromised ability to bind Rpn10, were linked to elevated cellular levels of ubiquitinated proteins, leading to aggregate formation, which in turn was implicated in the pathogenesis of several neurodegenerative disorders (e.g., amyotrophic lateral sclerosis (ALS), Huntington's and Alzheimer's diseases [117-119]).

Rad23 contains two UBA domains: a centrally located UBA1 and a C-terminal UBA2 that bind mono- and polyubiquitinated substrates with different affinities. UBA1 binds K63-linked polyubiquitin chains with a slightly higher affinity than K48-linked chains, whereas the UBA2 domain preferentially binds K48-polyubiquitin chains [120]. Rad23 UBL domain binds Rpn1 [15, 95], positioning it at the center of the 19S base, close to the CP's entrance [121]. It has been suggested that the human orthologs of Rad23 (hHR23A and hHR23B) interact with Rpn10 [122]. As for the two yeast proteins, while it appears that they also interact with Rpn10 [15], a previous report suggests otherwise [122]. Rad23 interaction with the proteasome is inhibited by phosphorylation of its UBL domain, thereby affecting its activity [123]. Rad23 UBL domain was also found to bind other proteins. For example, its binding to the ubiquitin chain elongation factor Ufd2 facilitates proteasomal degradation of ubiquitin-fusion degradation (UFD) substrates [124], whereas its binding to the peptidyl tRNA hydrolase Pth2 antagonizes ubiquitin-dependent proteolysis, possibly by preventing association of Rad23 with the proteasome [122]. Rad23's role in degradation is controversial, with a number of studies suggesting that it acts as an inhibitor [125, 126], while others suggesting it promotes degradation by acting as a shuttle factor targeting proteins to the proteasome [15, 101, 106]. However, these studies clearly demonstrate that ubiquitinated proteins bound to Rad23 are protected from the modification of their ubiquitin chains, i.e., elongation and deubiquitination. This stabilization effect is hypothesized to mediate efficient substrate targeting to the proteasome by inhibiting unnecessary processing of the ubiquitin chain [127]. Rad23 also participates in ER-associated degradation (ERAD) by binding of its Rad4-binding domain to the deglycosylase Png1, forming a complex which mediates proteasomal degradation of a specific set of ER proteins [128].

Even though UBL-UBA proteins interact with the proteasome, they are able to escape degradation. Several studies in yeast showed that the C-terminal UBA domain of both Dsk2 and Rad23 is responsible for their stability [129, 130], presumably by preventing initiation of their proteasomal degradation [130]. Introduction of long unstructured stretch (which serve as initiation sites for degradation) to Rad23 C-terminus, abolishes the protective effect of its UBA domain. However, when the C-terminal UBA domain of Rad23 (UBA2) was inserted downstream of the unstructured stretch, the protective effect was re-established. This effect was specific to UBA2, as insertion of the internal UBA domain (UBA1) downstream to the unstructured sequence retained no protective effect. Furthermore, introduction of UBA2 to the C-terminus of other substrates containing an unstructured region, diminished their degradation. Taken together, these findings demonstrate that UBA-mediated protection is dependent on its localization relative to the C-terminus of the harboring proteins [130].

In addition to the proteasomal intrinsic ubiquitin receptors and the non-proteasomal UBL-UBA shuttle proteins, there are also other proteins that have been implicated in shuttling of ubiquitinated substrates to the 26S proteasome. p97/VCP/Cdc48 is a highly conserved hexameric ATPase involved in numerous cellular functions, including DNA synthesis and repair, membrane fusion, disassembly of mitotic spindle, autophagic- and proteasome-mediated proteolysis, and ERAD [131]. Unlike other shuttle proteins which bind both to ubiquitin

and to the proteasome in an ATP-independent manner, p97 hydrolyses ATP and uses the resulting energy to structurally remodel or unfold its substrates, thus separating them from complexes, extracting them from cellular structures, or generating initial unstructured segments to facilitate degradation by the proteasome. The association of p97 with ubiquitinated substrates is mediated by several ubiquitin adaptors which recognize both p97 and the substrate [131, 132]. Several studies have demonstrated a rather more complex function of the adaptors, including binding of p97 to E3 ubiquitin ligases that ubiquitinate substrates, and an interaction with other ubiquitin-like modifiers [133]. One important type of p97 cofactors includes downstream processing proteins such as DUBs. They can either inhibit degradation of a given substrate by removing its ubiquitin moieties, or promote degradation by "editing" the substrates' ubiquitin chains to a length more suitable for proteasomal targeting [127, 132]. This suggests that p97 determines the fate of extracted proteins by playing a pivotal role in their ubiquitin-dependent degradation [134].

Sequestosome 1/p62 is a ubiquitin shuttling protein [135] that binds ubiquitinated substrates via its C-terminal UBA domain, associates with the RP subunits Rpt1 and Rpn10 via its N-terminal PB1 domain [136], thereby targeting proteins (e.g., tau) for proteasomal degradation [136, 137]. p62 also acts as a ubiquitin receptor in autophagy-mediated degradation, directly binding to LC3, a known mediator of autophagosome formation [138]. p62's role as a ubiquitin receptor in both proteasome- and autophagy-mediated degradation of ubiquitinated proteins is also supported by the finding that decreasing endogenous p62 levels results in the accumulation of ubiquitinated proteins [136].

Substrate deubiquitinaitng enzymes

During degradation, at least part of the ubiquitin chain moieties are rescued from degradation in a process mediated mostly by the deubiquitinating proteasome subunit, Rpn11 [17, 18]. Before deubiquitination of the substrate by Rpn11, two other DUBs can trim its ubiquitin chains: Uch37 and Ubp6/Usp14. Early removal of ubiquitin chains by these ATP-independent enzymes can antagonize substrate degradation, that in contrast to the activity of Rpn11 [139]. Uch37 is linked to Rpn2 via Rpn13 [93] and removes ubiquitin moieties from the distal end of the chain, releasing only monoubiquitin [19]. Shortening of ubiquitin chains affects the substrate's affinity for the proteasome, which may rescue poorly ubiquitinated proteins from degradation [140]. Ubp6 binds the proteasome via Rpn1 [20, 141] and was shown to cleave single ubiquitin moieties [142] as well as di- and tri-ubiquitins (and even longer oligomers) [139] form substrate-anchored chains. A recent study showed that Ubp6 can also remove ubiquitin chains *en bloc*, and that in both yeast and human cells it prefers substrates that are ubiquitinated at multiple sites [143]. Ubp6 was also shown to inhibit the degradation of 26S proteasome substrates in a non-catalytic manner [139]. One model, trying to explain Ubp6's non-catalytic effect, suggests that it is mediated via stabilization of the substrate-bound conformation of the proteasome and allosteric interference with the binding of the incoming substrate [139, 144].

After a polyubiquitin chain has been removed *en bloc* from the substrate by Rpn11 [18, 145], it must be further broken down to single recycling moieties. This function is mediated by IsoT/Ubp14/Usp5, which is a unique DUB that disassembles free polyubiquitin chains by hydrolyzing one ubiquitin at a time from the proximal end of the chain [146-148]. Optimal catalytic activity of this DUB was shown to require its zinc-finger ubiquitin binding domain (ZnF UBP), which recognizes the C-terminal Gly-Gly residues of an unanchored ubiquitin [149]. IsoT suppression was found to result in accumulation of free ubiquitin chains and stabilization of proteins, such as p53, a *bona fide* 26S proteasomal substrate [148, 150].

Different regulators of the proteasome

PI31, a proline-rich protein, was first described as an inhibitor of proteasomal activity, partially through inhibition of the binding between 19S (or PA28) regulatory particle to the 20S CP [151, 152]. It was later suggested as a modulator of the immunoproteasome with no effect on the constitutive proteasome [153]. A study in *D. melanogaster* has shown that PI31, in complex with the SCF E3 ubiquitin ligase Nutcracker, regulates proteasome function by exerting a positive effect on the 26S activity, and a negative effect on the activity of the free 20S CP [154]. While this may resolve the apparent contradicting findings regarding PI31 effect on proteasome function, another study found that regardless of its level, PI31 has no effect on either proteasome content or function, and that such an effect may be specific to certain physiologic conditions or proteasome pools [155].

Ecm29 is a large, 205 kDa, protein associated with the proteasome [20] and regulating its function via several mechanisms. It was shown to directly inhibit proteasome activity in yeast, partially via inhibition of the 19S ATPase activity [156, 157]. On the other hand, a positive effect on proteasome function was also described in yeast. It was found that Ecm29 supports proteasome assembly, as it stabilizes a 20S-19S intermediate in which 20S maturation is delayed due to temporary shortage of specific

β subunits [158]. Other studies showed that Ecm29 is recruited to the 19S RP in response to oxidative stress, and induces disassembly of the 26S proteasome [159, 160]. It was suggested that degradation of oxidized proteins is mediated mainly by the 20S rather than the 26S proteasome [161, 162]. According to this model, Ecm29-dependent disassembly of the 26S holoenzyme serves to increase the abundance of 20S, allowing cells to cope with the stress. In mammals, Ecm29 (encoded by the *KIAA0368* gene) promotes proteasome dissociation under oxidative stress [163], and associates with various molecular motors and endosomal components. This association may be involved in its ability to recruit 26S proteasomes to distinct cellular locations, such as the ER and the centrosome [164, 165].

Besides the suggested role for free 20S CP, it is also active as part of other complexes different from the 'canonical' 26S proteasome. Proteasome regulatory particles other than the 19S, such as PA28αβ, PA28γ and PA200, bind the 20S to form different proteasomal complexes, and may thereby facilitate the degradation of certain substrates under different physiological conditions, and/or of those that are degraded less efficiently by the "canonical" proteasome and/or of those that may not need prior ubiquitination for their degradation [166]. For example, PA28γ mediates the degradation of the steroid receptor coactivator-3 (SRC3) and the cell-cycle regulator p21 [167-169]. It was recently suggested that in several mammalian cell types, a considerable fraction of the 20S may reside in non-26S forms: either in its free form, or in complexes where it is capped by activators other than the 19S [170, 171].

Structural changes induced by substrate binding

In recent years, cryo-EM-based studies established the molecular architecture of the 26S proteasome [172-174]. Though these studies generated a near-atomic resolution structural models, they also implied dynamic heterogeneity rather than a single static proteasomal structure. Recent classification of a large data set provided researchers with the ability to dissect this structure, and to discern between three coexisting proteasomal conformational states, S1, S2 and S3 [175]. Prior to this advancement, it has been suggested that conformational changes are part of the mechanism by which the proteasome deubiquitinates, unfolds, translocates, and degrades substrates [176, 177]. The conformational state S1 is believed to represent the substrate-unbound proteasome and is strikingly abundant in some tissues [178]. Ubiquitinated substrates binding to S1, and the subsequent engagement of their initiation site within the ATPase pore, seems to induce conformational changes, resulting in S3 proteasomal conformation, corresponding with the substrate-bound form of the proteasome [175]. The S2 form probably corresponds to an intermediate/hybrid state between S1 and S3 [175], and in fact was not defined in a later structural model of endogenous proteasome in yeast, which describes only substrate-bound (M1) or -unbound (M2) states [179].

Importantly, following substrate binding, the 19S RP translocates the substrate into the catalytic chamber of the 20S CP, while unfolding it and removing its conjugated ubiquitin. This requires opening of the α-ring gate, which is dependent on binding of C-terminal peptides of Rpt 19S subunits to the 20S surface [3, 4, 180, 181].

Proteasomal degradation foci

Besides direct regulation by various mechanisms, proteasome function is also dependent on its recruitment to specific cellular sites, where selective proteolytic activity is required.

Many neurodegenerative diseases are characterized by inclusion bodies enriched with ubiquitinated proteins and proteasomal subunits [182-190], which raised the hypothesis that protein degradation is impaired in these disorders [191]. It was suggested that proteasomes are recruited to ubiquitinated aggregated proteins in order to degrade them, but for whatever reason are stalled; yet, this point is still debated [192-195].

In addition to its possible involvement in the degradation of aggregated proteins, the proteasome has additional roles in neuronal function. It has been shown that proteasome recruitment to synapses supports neuroplasticity, as it regulates the local turnover of both pre- and post-synaptic proteins [196-198]. Researchers showed that the synaptic protein GluN2B, an NMDA receptor subunit, is critical for NMDA receptor function in synaptic stress and plasticity, which is important for learning and memory formation. It has been shown that GluN2B mediates proteasome synaptic anchoring, thus enhancing its local activity [199].

Promyelocytic leukemia-nuclear bodies (PML-NBs) are spheres located in nuclei of many cell types. They are surrounded by the PML protein, which is also an oncogene involved in a chromosomal translocation that results in its fusion with retinoic acid receptor α (RARα), which is the underlying cause of promyelocytic leukemia. Normally, retinoic acid binds to RARα and abrogates its inhibitory effect on gene expression, thus leading to expression of proteins that mediate differentiation. The PML-RARα fusion retains RARα inhibitory effect, also in the presence of retinoic acid, thus inhibiting differ-

entiation. Notably, the PML-RARα fusion also disrupts PML-NBs, as it dimerizes with native PML, probably adding to its deleterious effect [200].

PML-NBs recruit several key regulators such as p53, DNA repair factors, and the ubiquitin-like protein SUMO. Therefore, they were suggested to take part in the regulation of several processes, such as DNA damage response, cell survival and senescence [201, 202]. They have also been shown to contain proteasome [203, 204], as well as its transcription regulator Nrf2. Following SUMOylation, Nrf2 is ubiquitinated by the RNF4 E3 ubiquitin ligase and degraded by the PML-NB-localized proteasome, which represents an example for mutual regulation of the proteasome and Nrf2 [49]. PML, SUMO and RNF4 were also suggested to cooperate in the proteasomal-mediated removal of misfolded proteins in the nucleus, which were also described to co-localize with PML-NBs. PML was shown to have a SUMO ligase activity, facilitating SUMO-dependent ubiquitination by RNF4 and subsequent degradation of misfolded proteins by the proteasome [205]. How proteasomes are recruited into PML-NBs, and whether this recruitment regulates additional cellular activities is yet to be determined.

Another suggested focus for both basal and stress-induced proteasome activity is the centrosome, a perinuclear organelle composed of a pair of centrioles that are surrounded by pericentriolar material, and serves as a microtubule organizing center. This organelle is enriched in proteasome, ubiquitin and other regulators, all of which are recruited to the centrosome from the cytosol in response to proteasome inhibition and increase in the level of misfolded proteins [206]. Rpn10 has been shown to regulate centrosomal proteasome activity in neurons, thereby facilitating dendrite elaboration in rodent brain [207].

Proteasomes were also shown to be tethered to ER membrane as part of their role in ERAD [208-210], as well as to the outer mitochondrial membrane. This later association enhances the degradation of mitochondrial substrates [211], and is upregulated in response to mitochondrial stress [212]. FK506-binding protein 38 (FKBP38) was suggested as a proteasome anchor to organellar membranes [213]. As is discussed above, Ecm29 is also involved in proteasome recruitment to different organelles, such as the ER and centrosome [164, 165].

The proteasome as a substrate for degradation

The proteasome is known to be a stable complex. Whereas much is known about its biogenesis, its degradation pathway(s) is still poorly understood.

Following induction of apoptosis in human cell lines, it was shown that the 19S proteasomal subunits Rpt5, Rpn2 and Rpn10 are cleaved by caspase-3. The cleavage of Rpn10 and Rpn2, which together connect the lid and base, results in impaired proteasome activity and accumulation of ubiquitinated proteins [214]. In *D. melanogaster* cells it was shown that caspase-3 activation results in the cleavage of α2, α4, and β4 subunits of the 20S, and the Rpt1 subunit of the 19S. Also, the PA28γ complex was identified as a caspase-3 substrate [215]. An additional degradation pathway of non-functional proteasomal subunits was recently identified in yeast. It was shown that free, unassembled subunits, are degraded by the UPS itself, and that ubiquitination of the subunits is essential for their degradation. The heat-shock protein Hsp42 was shown to mediate the degradation of the unassembled subunits by sorting them into cytoprotective compartments, such as insoluble protein deposits (IPOD), where they are degraded by the proteasome [39].

Lysosomal degradation of the entire 26S proteasome complex was also described. Using a rat model, the accumulation of the proteasome within lysosomes was identified following leupeptin treatment or nutrient starvation. Moreover, it was postulated that the delivery of the proteasome to lysosomes is mediated by autophagy [216]. Nevertheless, the mechanism behind this process has remained elusive. Recent study in yeast has identified the independent targeting of the 19S and 20S sub-complexes for vacuolar degradation through autophagy upon nitrogen starvation [217]. The vacuolar degradation of the 20S proteasome depended on the DUB Ubp3, while that of the 19S proteasome was not. Furthermore, the elimination process of the proteasome following nitrogen starvation involved dissociation of the 19S and 20S proteasomes and their nuclear export [217]. In addition, the vacuolar targeting of a chemically or genetically inactivated 26S proteasome by autophagy has been recently described in *A. thaliana*. In this plant, Rpn10 can act also as a selective autophagy adaptor that simultaneously binds both ATG8 (LC3 in mammals, an autophagosomal receptor) and the proteasome, the ubiquitination of which is stimulated following its inhibition. Unlike proteasome engulfment induced by its own inhibition, the mechanism that underlies the vacuolar targeting of the proteasome upon nitrogen starvation in *A. thaliana* is independent of Rpn10 [71]. It appears therefore that the lysosomal degradation of the 26S proteasome via autophagy pathway is an evolutionarily conserved process.

Future perspectives

Although the proteasome has been studied extensively, much has still remained unknown. While proteasomal

activators, inhibitors, and PTMs are discovered frequently, the mechanisms that underlie their function are still elusive. The same is true for several of the proteasomal subunits, including the long sought after ubiquitin-binding ones. Even proteasome assembly and trafficking between cellular compartments (e.g., cytosol and nucleus) are not fully understood.

One important missing piece of information is a high resolution and dynamic structure of the proteasome along with a native ubiquitinated substrate. Such structure will provide insights on the attachment of the ubiquitin chain, the position of the substrate and its initiation of unfolding, insertion and degradation.

As the UPS is involved in nearly all cellular processes, it will be interesting to identify the one(s) that regulates proteasome biosynthesis and activity, as one may expect that changing pathophysiological conditions may affect the proteasome as well, most probably via direct regulatory relationships. Importantly, even for conditions known to regulate/affect proteasome biogenesis, assembly or function, a detailed mediating mechanism is still missing.

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