Minireview

Biting the hand that feeds: Rpn4-dependent feedback regulation of proteasome function

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Abstract

The 26S proteasome of eukaryotic cells mediates ubiquitin-dependent as well as ubiquitin-independent degradation of proteins in many regulatory processes as well as in protein quality control. The proteasome itself is a dynamic complex with varying compositions and interaction partners. Studies in *Saccharomyces cerevisiae* have revealed that expression of proteasome subunit genes is coordinately controlled by the Rpn4 transcriptional activator. The cellular level of Rpn4 itself is subject to a complex regulation, which, aside of a transcriptional control of its gene, intriguingly involves ubiquitin-dependent as well as ubiquitin-independent control of its stability by the proteasome. A novel study by Ju et al. [D. Ju, H. Yu, X. Wang, Y. Xie, Ubiquitin-mediated degradation of Rpn4 is controlled by a phosphorylation-dependent ubiquitylation signal, Biochim. Biophys. Acta (in press), doi:10.1016/j.bbamcr.2007.04.012] now revealed another level of complexity by showing that phosphorylation of a specific serine residue in Rpn4 is required for its efficient targeting by the Ubr2 ubiquitin ligase.
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The 26S proteasome serves an essential function in the ubiquitin/proteasome system (UPS) [1]. It mediates degradation of proteins tagged with poly-ubiquitin chains, which is linked to its role in controlling ubiquitin homeostasis [2]. The UPS is involved in the control of many short-lived proteins including critical regulators of the cell division cycle and of apoptosis. Because of this function, the proteasome has recently emerged as a promising drug target in the treatment of cancer [3,4]. As discussed in detail below, proteasome inhibitors that are used in such approaches, often in combination with other drugs, elicit a feedback regulatory response resulting in induction of proteasome gene expression [5]. Revealing the molecular details of this response is therefore of critical importance to understanding the effects of drugs that target the proteasome. In higher eukaryotes, the proteasome serves an important function also in the immune system. Peptides resulting from proteasomal breakdown of proteins derived from intracellular pathogens are presented to cytotoxic T cells on the surface of a cell as a signal of its infection [6,7]. Aside of these proteolytic functions,

subcomplexes of the 26S proteasome appear to have additional non-proteolytic functions in transcription, DNA-repair and chromatin remodeling (reviewed in [8]). These functions in DNA repair are probably a reason why many DNA repair genes and proteasome genes are controlled by the same transcriptional activator, Rpn4 (see below). The complexity of functions of the proteasome and its subcomplexes explains why there is a variety of elaborated mechanisms for their regulation. One such mechanism regulates enrichment of nuclear proteasomes. Studies in *Schizosaccharomyces pombe* led to the conclusion that the Cut8 protein tethers the proteasome to the nuclear envelope. For this to occur efficiently, Cut8 needs to be ubiquitylated by a Ubr1–Rad6 complex [9]. Proper targeting of the proteasome to the nucleus by this process appears to be required for efficient DNA damage resistance.

1. Structures and functions of proteasomal complexes

The 26S proteasome is a ∼2.5 MDa complex composed of a 20S core particle (CP) and two 19S regulatory particles (RP). The 20S proteasome (or CP) is a multicatalytic protease that, in its principle structure, is conserved from archaea to humans

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[1,10]. The active sites of this barrel-shaped ∼670 kDa protease reside in its inner chamber that is formed by two identical rings of so-called β subunits [11]. In vertebrates, the composition and peptide cleavage specificities of the CP change upon immune stress. In response to interferon γ, transcription of genes encoding three alternative active site subunits as well as of the proteasome assembly factor Ump1 is induced [7,12,13]. This leads to a rapid formation of so-called immunoproteasomes, which are important for the generation of certain antigenic peptides [14]. The outer rings of the 20S CP, which are composed of α subunits, form the entry port for proteins to be degraded inside the proteasome [15]. Unfolding and entry of proteolytic substrates into the 20S core particle is controlled by a gatekeeper subcomplex, the 19S RP (also known as PA700), which is composed of at least 19 subunits [16]. One of them, Rpn11 has deubiquitylating (DUB) activity [17–19]. The base of this subcomplex is formed by three non-ATPase subunits (Rpn1, Rpn2 and Rpn13) and a hexameric ring of ATPase subunits of the AAA type (Rpt1–Rpt6) [16,20]. The latter are thought to mediate unfolding of substrates and their threading through the entry port of the CP [16]. The visa a substrate has to present to the gatekeeper in order to gain access to the proteolytic chamber, in most cases, is a polyubiquitin chain. This tag is either recognized by the Rpn10 subunit of the 19S RP or is presented to this complex by ubiquitin shuttling factors of the Rad23 type [21–23]. There are, however, several substrates that are degraded by the proteasome without prior ubiquitylation. The best-studied example is ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis. Its targeting requires ODC antizyme and has been suggested to also involve ubiquitin binding sites in the proteasome, based upon the finding that binding of ODC to the proteasome *in vitro* was inhibited by the addition of ubiquitin chains [24]. Another protein that can be degraded by the proteasome via an apparently ubiquitin-independent pathway is Rpn4, a protein of central importance in the control of proteasome levels (see below).

Aside of the 19S RP, several other proteins or complexes have been described, which bind to α rings of proteasomal complexes. The ∼200 kDa PA200 protein or its *Saccharomyces cerevisiae* counterpart Blm10 were shown to stimulate the activity of 20S proteasomes [25,26]. Blm10, however, was also shown to bind to assembly intermediates of the 20S proteasome and to influence the rate of 20S particle formation [27,28]. In mammalian cells, another alternative proteasome activator (PA28) is synthesized as a response to interferon γ. Attachment of PA28 to the 20S proteasome appears to modify its activity in a way that the generation of certain antigenic peptides is fostered [29].

The 19S RP itself, apart of the above-mentioned functions, may be considered as an interaction platform. Proteins, for which binding to the 19S RP has been reported, include ubiquitin shuttling factors (Rad23, Dsk2), ubiquitin ligases (Ubr1, UFD4), deubiquitylation enzymes (Doa4, Ubp6), and Rpn4 [2,30–33]. Interaction of the 19S RP with shuttling factors and ubiquitin ligases is thought to escort substrates to the proteasome [23,31]. The Ubp6 protein has recently been shown to control substrate deubiquitylation during the process

of degradation thereby regulating ubiquitin turnover by the proteasome [2]. A novel study in *S. cerevisiae* revealed that regulation of the ubiquitin turnover function by the proteasome involves transcriptional control of the *UBP6* gene [34]. When cellular ubiquitin levels are low ("ubiquitin stress"), transcription of the UBP6 gene is induced. This leads to more proteasomes being charged with Ubp6, which in turn results in increased ubiquitin recycling. The intriguing dynamics of proteasome composition and interactions has been highlighted recently in two excellent reviews [16,35].

2. Rpn4 and the regulation of proteasome gene expression

Studies using cells of several species (yeast, *Drosophila* and humans) revealed that, apart of the many mechanisms that control proteasome function by regulation of interaction partners or facultative subunits, the essential genes encoding the subunits of house-keeping proteasomes are under a coordinated transcriptional control [36–38]. This conclusion was first derived from studies in the yeast *S. cerevisiae*. Feldman and colleagues noted the presence of a common sequence element (GGTGGCAAA) in the promoters of nearly all genes encoding proteasome subunits [36]. It was shown that these elements, termed PACE (*Proteasome Associated Control Elements*), are required for normal expression of proteasome genes. Rpn4, a 60 kDa protein containing a C2H2-type zinc finger motif and two acidic domains (Fig. 1B), was identified as the transcriptional activator that binds to PACE sequences [36]. Before this discovery, Rpn4, alias Ufd5, had already been linked to the UPS. *Rpn4/ufd5* mutations were found to inhibit the degradation of model substrates of the N-end rule and ubiquitin-fusion degradation (UFD) pathways [39]. These findings also provided a plausible explanation for an earlier observation that *rpn4* mutations (termed *son1* in this study) suppressed a conditional *sec63* allele [40]. An impairment of the UPS caused by the *rpn4/son1* mutation is likely to result in a stabilization of the thermolabile Sec63 protein [41]. An important study by Xie and Varshavsky (2001) revealed the principle of an Rpn4-mediated feedback regulation of proteasome gene expression by showing that Rpn4 is not only a transcriptional activator of proteasome genes but also a proteasome substrate [41]. This study also showed that Rpn4 binds to the Rpn2 subunit of the 19S RP. Experiments with truncated versions of Rpn4, however, indicated that this interaction is not required for proteolytic targeting of Rpn4. The observed interaction with the 19S RP, however, was consistent with results from a previous report that identified Son1/Rpn4 in preparations of the 26S proteasome [42]. The authors of this earlier study concluded that Son1/Rpn4 is a component of the 19S RP. Hence it was termed Rpn4 (for *Re*gulatory *p*article *n*on-ATPase subunit 4) [43]. Because a variety of studies failed to detect Rpn4 as a stoichiometric component of the proteasome, however, it appears to be more appropriate to classify Rpn4 as a proteasome-interacting protein [41].

Rpn4 is required for normal and induced expression not only of proteasome genes, but also of other genes of the UPS such as *UBA1, UBI4* and *CDC48*, as well a several hundred other genes,
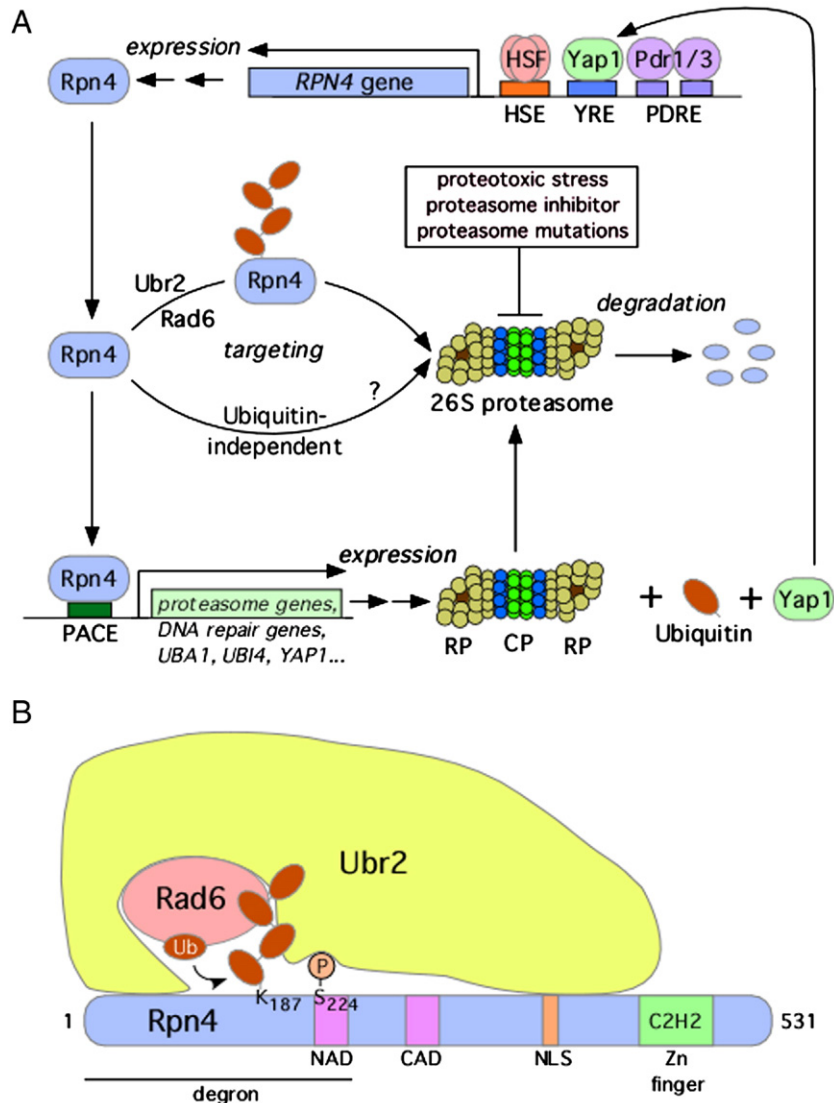
Fig. 1. Regulation of and by Rpn4. (A) Regulatory circuits involving Rpn4. The promoter of the Rpn4 gene contains elements, HSE, YRE and PDRE, which are recognized, respectively, by the transcriptional activators, HSF, Yap1, and Pdr1/3. Rpn4 itself stimulates expression of proteasome subunit genes as well as of genes involved in ubiquitylation, DNA repair and other stress responses. One of the latter genes encodes Yap1, a mediator of oxidative stress response. Rpn4 is a short-lived protein subject to Ubr2–Rad6-mediated ubiquitin-dependent degradation by the proteasome. Control of Rpn4 stability, in addition, involves a ubiquitin-independent targeting pathway that is not yet understood in its details. Inhibition of proteasome function leads to stabilization of Rpn4 and in turn to an up-regulation of its target genes. (B) Functional motifs of Rpn4 and its interaction with the Ubr2–Rad6 ubiquitylation complex. Rpn4 contains two acidic transactivation domains (NAD and CAD), a putative nuclear localization signal (NLS), and a zinc finger domain. Mapping studies have indicated that sequences at the N terminus and downstream of CAD are required for a stable binding of Rpn4 to Ubr2. For efficient ubiquitylation on lysine 187 (K187), however, binding of Ubr2 to phosphorylated serine 224 (S224) is required. Sequence extending from the N terminus to the end of NAD (1–229) contains two independently operating degrons sufficient to mediate either ubiquitin-dependent or -independent targeting to the proteasome (see main text for additional details).

many of which are related to stress responses and DNA damage repair [5,36,44]. Rpn4 is required for normal basal level of proteasome gene transcription [36]. In addition, Rpn4 mediates a severalfold induction of proteasome gene transcription in response to heat shock, oxidative stress, other forms of proteotoxic stress such as treatment with amino acid analogues, proteasome inhibition or malfunction, and DNA damage [5,41,44–47].

3. Regulating the regulator

As illustrated in Fig. 1A, Rpn4 levels are controlled by multiple mechanisms that either regulate transcription of the RPN4 gene or the metabolic stability of the Rpn4 protein. Transcriptional induction of the RPN4 gene in response to various stresses is controlled by multiple transcription factors. Pdr1 and Pdr3 mediate a pleiotropic drug resistance mainly by controlling the expression of drug efflux pumps such as Pdr5. Forming homo- or heterodimers, these transcriptional activators bind to Pdr1/3 response elements (PDRE) in promoters of target genes [48]. The RPN4 promoter harbors two PDREs that mediate Pdr1/3-dependent regulation [45]. Yap1 (yeast AP-1) binds to a response element (YRE) in the promoter of RPN4 to induce expression upon oxidative stress [45]. The YAP1 gene itself, interestingly, contains a PACE box in its promoter and is a

target of Rpn4 [5], linking these two transcriptional activators in a positive feedback loop. A heat shock element (HSE) in the *RPN4* promoter that is recognized by shock transcription factor (HSF) is required for induction of *RPN4* transcription upon heat shock or methyl methanesulfonate (MMS) treatment [47]. Apart of this direct mode of regulation, HSF in addition amplifies *RPN4* expression indirectly by inducing the expression of Pdr3 [47].

Regulation of Rpn4 levels also involves a posttranslational control. Rpn4 is an extremely short-lived protein with a half-life of ~2 min in unstressed cells. The rate of Rpn4 turnover depends on the intracellular level of functional and available proteasome [41]. Inhibition of proteasome activity or overloading of the UPS as a result of proteotoxic stress results in a stabilization of Rpn4, and in turn, an up-regulation of its target genes [46,49]. In other words, Rpn4-mediated induction of proteasome genes compensates for a reduction or higher demand of proteasome activity. Cells lacking Rpn4 have about half the proteasome activity of a wild-type [46], and are hypersensitive to proteasome inhibition or proteotoxic stress [44,46,49].

Proteolytic control of Rpn4 levels is mediated by two seemingly independent targeting mechanisms that recognize distinct degradation signals (or degrons). One degron resides within the 151 N-terminal residues or Rpn4 and can either be inactivated by addition of an N-terminal tag or by deletion of the first 10 residues [50]. Targeting of Rpn4 to the proteasome by the N-terminal degron remarkably is not affected by inhibition of cellular ubiquitylation or mutation of lysine residues in Rpn4 suggesting that this mechanism is ubiquitin-independent [41,50]. How this degron mediates degradation of Rpn4 by the proteasome is not known. The second degron comprises the N-terminal one (NAD) of two acidic domains and a ubiquitylatable lysine (K) residue, preferentially K187 [51]. Recognition of this degron and ubiquitin-mediated targeting to the proteasome is mediated by the Ubr2 ubiquitin ligase and the Rad6/Ubc2 ubiquitin-conjugating enzyme [52]. Inactivation of either pathway alone only leads to a modest reduction of Rpn4 turnover, whereas simultaneous inactivation of both results in a drastic stabilization [52]. Inhibition of the proteasome obviously inhibits both pathways and therefore results in a strong activation of a feedback control of proteasome gene expression (see above). Ubiquitin depletion might trigger a more modest Rpn4-dependent up-regulation of proteasome genes that together with increased expression of the poly-ubiquitin gene (*UBI4*) and of the *UBP6* gene may be sufficient to reestablish appropriate levels of ubiquitin [16,46]. The function of the ubiquitin-independent mechanism of Rpn4 degradation remains obscure. One possibility is that it is required to keep Rpn4 levels low under certain conditions that lead to a redirection and therefore limitation of ubiquitylation, but that should not induce increased proteasome levels. Another possibility might be that different subpopulations of Rpn4, e.g. in the cytosol or the nucleus, are preferentially targeted by one or the other pathway.

An interesting question regarding the mechanisms that target Rpn4 for degradation is whether they are regulated by

means other than proteasome inhibition. The new study by Ju et al. has revealed that phosphorylation on one of two serine (S) residues (in particular S220) in NAD of Rpn4 is required for efficient ubiquitylation by the Ubr2–Rad6 complex [53]. Interestingly, phosphorylation is not essential for binding of Ubr2 to Rpn4, as it occurs in the absence of NAD and apparently requires the presence of structural elements both of the N- and C-terminal parts of the protein. It could be shown, however, that when this high affinity interaction was eliminated, phosphorylation within NAD enhanced binding to Ubr2. These data indicated that not binding to Ubr2 *per se* but correct positioning of phosphorylated Rpn4 is important for efficient ubiquitylation. Since S214 and S220 are surrounded in NAD by acidic residues, it was tested whether Rpn4 is a substrate of caseine kinase 2 (CK2). *In vitro* experiments showed that CK2 can phosphorylate Rpn4 on these two serine residues [53]. These findings suggested that Ubr2-mediated degradation of Rpn4 may be regulated by phosphorylation.

Our current understanding of the complex regulation of Rpn4 is already elaborated but still incomplete. Several new questions have been raised by recent studies that led to the discovery of both ubiquitin-dependent and -independent mechanisms in the post-translational control of Rpn4. Among the questions raised by the findings of Ju et al. is whether phosphorylation of Rpn4 leading to its Ubr2-dependent ubiquitylation is a process that is regulated in response to changes in a cell's physiology. Another important question is what the significance and function of a second and ubiquitin-independent pathway of Rpn4 degradation is and whether regulation of this mechanism integrates other signals than the Ubr2-dependent pathway. Finally, to extend our knowledge from the yeast model system up to a cancer patient treated with proteasome inhibitor, it will be of critical importance to identify the transcription factors that control expression of proteasome genes in mammalian cells, and to study whether these regulators are regulated by mechanisms similar to those discovered for Rpn4 in yeast.

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