

Feedback regulation of proteasome gene expression and its implications in cancer therapy

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Abstract Proteasomal protein degradation is one of the major regulatory mechanisms in the cell. Aberrant proteasome activity is directly related to the pathogenesis of many human diseases including cancers. How proteasome homeostasis is controlled is a fundamental question toward our understanding of proteasome dysregulation in cancer cells. The recent discovery of the Rpn4-proteasome negative feedback circuit provides mechanistic insight into the regulation of proteasome gene expression. This finding also has important implications in cancer therapy that uses small molecule inhibitors to target the proteasome.

Keywords Proteasome · Ubiquitin · Cancer therapy · Rpn4 · Gene expression · Feedback regulation

1 The 26S proteasome

The 26S proteasome is the major cellular protease responsible for degradation of regulatory and abnormal proteins [1–4]. It is involved in many cellular processes including but not limited to cell cycle control, transcription, DNA repair, apoptosis, quality control, and antigen presentation. The 26S proteasome is a 2.5-MDa protein complex consisting of a 20S core particle (CP or 20S proteasome) and one or two 19S regulatory particles (RP, also known as 19S proteasome or PA700). The RP is attached to one or both ends of the CP. Crystal structures of the CP from archaeal, actinobacterial species, and several eukaryotes have been solved [5–8]. The

CP is a barrel-shaped structure of a stack of four seven-subunit rings in an $\alpha_7\beta_7\beta_7\alpha_7$ configuration (Fig. 1). Both exterior rings contain one set of seven different α subunits; and both interior rings contain one set of seven different β subunits. The CP performs three types of catalytic activities inside its chamber: chymotrypsin-like, trypsin-like, and caspase-like activities, which are provided by β_5 , β_2 , and β_1 subunits. In immune responsive cells, the constitutively expressed β_1 , β_2 , and β_5 subunits are replaced by three induced β subunits (β_{1i} , β_{2i} , and β_{5i}) to form the immunoproteasome that has higher chymotrypsin-like and trypsin-like activities known to be favorable for antigen processing [9–11]. β_{5i} is replaced by another proteolytic active subunit β_{5t} in cortical thymic epithelial cells, forming the so-called thymoproteasome [12].

Protein substrates enter the CP chamber through a pore or gate at the center of the α subunit ring. The gate is closed in a free CP by interactions among the N-termini of the α subunits, blocking substrate entry into the proteolytic chamber [13]. In the 26S proteasome, the RP acts as an activator to facilitate the opening of the gate by interacting with the α subunits [13–15]. The RP complex can be divided into two subcomplexes called the base and the lid (Fig. 1). The base is in contact with the CP, consisting of six AAA(+)-type ATPases (Rpt1–6) and three non-ATPase subunits (Rpn1, Rpn2, and Rpn13). The primary functions of the base include substrate recruiting, unfolding, and translocation. The lid is formed by at least nine non-ATPase subunits (Rpn3, Rpn5–9, Rpn11, Rpn12, and Rpn15/Sem1). The Rpn11 subunit possesses a deubiquitylating activity, which is required for degradation of polyubiquitylated substrates. The connection between the lid and the base is stabilized by the Rpn10 subunit. Recent studies have shown that a number of ubiquitylating and deubiquitylating enzymes are associated with the 26S proteasome [16–19],

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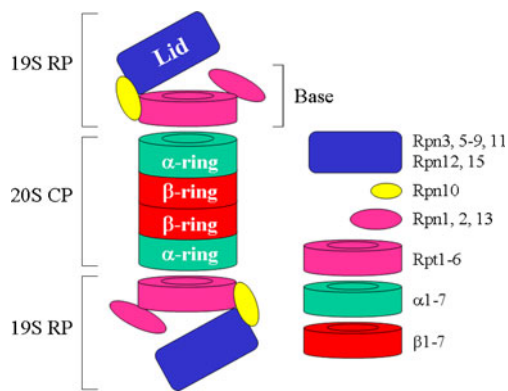


Fig. 1 Composition of the 26S proteasome. The 26S proteasome consists of the 20S proteasome (CP) and the 19S regulatory particle (RP). The CP is formed by four stacked rings: two outside α -rings and two inner β -rings. Each ring has seven different subunits. The RP is divided into two subcomplexes: the lid and the base. The subunits forming the lid and the base are shown. Rpn10 stabilizes the connection between the lid and the base

suggesting that the 26S proteasome is not only a “garbage disposal” but also plays an active role in ubiquitin chain remodeling. Clearly, the structure and activity of the proteasome system are highly regulated.

2 The discovery of the Rpn4-proteasome negative feedback circuit

The 26S proteasome is composed of 33 distinct subunits each encoded by a different gene. It was a difficult task to understand how the proteasome genes, in such a large number, are regulated. The central question was whether the proteasome genes are coordinately regulated by a master transcription factor or if they are activated by different transcription factors. Early biochemical studies have shown that the proteasome subunits are nearly stoichiometrically present in *Saccharomyces cerevisiae*, suggesting that the proteasome genes are coordinately regulated [20, 21]. Recent studies by Mannhaupt et al. and our own demonstrated that Rpn4 encoded by the *RPN4* gene (also named *SON1* and *UFD5*) functions as a transcription activator of the proteasome genes [22–24]. An Rpn4 binding site, a 9-bp motif known as proteasome-associated control element (PACE), is located in the promoters of the proteasome genes. Deletion of the PACE motif from one of the proteasome genes markedly reduces the level of assembled/active proteasome in the cell [25]. Interestingly, Rpn4 is an extremely short-lived protein ($t_{1/2} \leq 2$ min) and is degraded by the proteasome [23]. Moreover, stabilization of Rpn4 by inhibition of proteasome activity results in the upregulation of the proteasome genes [24, 26]. Together, these observations led to the formulation of a model in which proteasome homeostasis is regulated by

a negative feedback circuit (Fig. 2). On the one hand, Rpn4 induces the proteasome genes; on the other hand, Rpn4 is rapidly degraded by the assembled/active proteasome.

In addition to the proteasome genes, Rpn4 controls numerous non-proteasome genes involved in protein ubiquitylation, DNA repair, unfolded protein response, and other cellular processes [22, 27–40]. Interestingly, the *RPN4* gene itself is regulated by a wide range of signals. For instance, the *RPN4* promoter carries response elements for heat shock transcription factor (Hsf1), multidrug resistance-related transcription factors (Pdr1 and Pdr3), and Yap1, a transcription factor that plays an important role in response to oxidation, toxic metals, and DNA damaging agents [28–31]. It has become clear that environmental stressors activate these transcription factors to induce *RPN4* expression. These observations suggest that Rpn4 acts as a major mediator in a stress response network. The Rpn4-proteasome feedback circuit appears to be the core operator in this network. Not only does it regulate proteasome homeostasis but it also gauges the induction of other Rpn4 target genes by keeping the Rpn4 protein level in check. In fact, recent studies have demonstrated that inhibition of Rpn4 degradation severely reduces cell viability under stressed conditions [41, 42].

3 Feedback regulation of proteasome gene expression in higher eukaryotes

A question naturally arising from the discovery of the Rpn4-proteasome negative feedback circuit is whether the proteasome genes are regulated by an Rpn4-like transcription factor in higher eukaryotes. Bioinformatics analyses conducted by several groups revealed that Rpn4 sequence homologs and conserved PACE motifs exist only in *Hemiascomycetes*, especially among the *Saccharomyces* “sensu stricto” species [43, 44]. However, the proteasome genes in higher eukaryotes are also coordinately regulated by a feedback mechanism [45–53]. For example, knockdown of individual proteasome subunits by RNAi results in the upregulation of non-targeted subunits in *Drosophila* cells; and the feedback response is dependent on the 5'-untranslated regions of proteasome genes

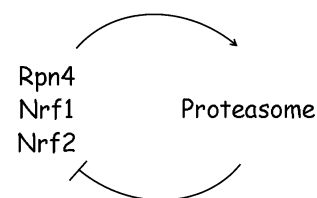


Fig. 2 Negative feedback regulation of proteasome homeostasis. The transcription factors Rpn4 (in yeast) and Nrf1 or Nrf2 (in mammalian cells) induce the expression of proteasome genes. The assembled/active proteasome in turn rapidly degrades the transcription factors

[47, 48]. In addition, suppression of the proteasome activity by proteasome inhibitors induces upregulation of proteasome genes in mammalian cells [45, 49, 51–53]. These experimental observations strongly suggest that there is a functional homolog of Rpn4 in higher eukaryotes.

Recent studies have suggested that nuclear factor erythroid-derived 2-related factor 2 (Nrf2) may be the sought Rpn4 counterpart in mammalian cells [51, 54–57]. Nrf2 is a cap “n” collar-basic leucine zipper transcription factor that activates its target genes through a common *cis*-acting element named antioxidant response element (ARE) [58]. Previous studies have shown that Nrf2 is the most potent transcription factor in inducing the expression of antioxidant enzyme genes when the cell is under oxidative and electrophilic stress [59, 60]. Kensler and co-workers found that administration of antioxidant 3H-1,2-dithiole-3-thione, which is known to activate Nrf2, enhances the expression of proteasome genes in the liver of wild-type, but not Nrf2^{-/-} mice [54]. This observation suggests that the proteasome genes are induced by Nrf2 in stress response. The same research group further demonstrated that the Nrf2-dependent induction of proteasome subunit PSMB5 by 3-methylcholanthrene requires the AREs of the PSMB5 promoter in murine neuroblastoma Neuro2A cells [55]. Enhanced expression of proteasome genes by Nrf2 was also detected in human fibroblasts and colon cancer cells [56, 57]. Like Rpn4, Nrf2 is very short lived and degraded by the proteasome. The cellular Nrf2 level increases in response to stress because its interaction with Keap1 is disrupted. Keap1 is an adaptor protein required for Nrf2 ubiquitylation by the Cul3-Rbx1 ubiquitin ligase. These features make Nrf2 a perfect candidate for coordinating the feedback expression of the proteasome genes. Indeed, the work by Kraft et al. suggested that feedback induction of proteasome genes in response to proteasome inhibitor MG132 is mediated by Nrf2 in human skin fibroblast cells [51]. In addition to Nrf2, other cap n collar-basic leucine zipper transcription factors may also be involved in the feedback regulation of proteasome genes. A recent study has revealed that Nrf1 but not Nrf2 is required for the feedback induction of proteasome genes in response to proteasome inhibitors in mouse embryonic fibroblasts (MEFs) [53]. It is possible that the transcription factors required for the feedback proteasome gene expression are cell type dependent: The feedback regulation is conducted by Nrf1 in some cell types and by Nrf2 in others.

Unlike Rpn4, which controls both basal and inducible proteasome expression in yeast [24, 25], neither Nrf1 nor Nrf2 appears to play a major role in basal expression of proteasome genes in mammalian cells. Both Nrf1^{-/-} and Nrf2^{-/-} MEFs and the hepatic cells of Nrf2^{-/-} mice display similar endogenous levels of proteasome subunits as their wild-type counterparts [53, 54]. These observations suggest that there may be two distinct systems to regulate the expression of the proteasome genes in mammalian cells: one for the basal level

and the other for the feedback upregulation. Whereas recent studies have provided some clues to understand the feedback regulation mechanism, more work needs to be done to identify the pathway that controls the basal level expression of the proteasome genes in mammalian cells.

4 Implications of feedback regulation of proteasome gene expression in cancer therapy

The proteasome has emerged as a drug target for cancer therapy [61]. The illustration of the feedback regulation of proteasome gene expression has several important implications in cancer therapy that targets the proteasome. First, it provides a clue to understand the cause of proteasome overexpression often detected in cancers [62–66]. It is possible that the feedback induction, which normally occurs only when the proteasome activity is suppressed, may become constitutively active in cancer cells. In support of this hypothesis, Xu et al. demonstrated that the feedback expression of proteasome genes in response to proteasome inhibitors is less inducible in breast cancer cells than in noncancerous breast epithelial cells [49]. Second, the feedback mechanism may contribute to the emergence of bortezomib resistance in cancer therapy. Bortezomib is the only FDA-approved proteasome inhibitor in clinical use. Although this drug has shown promising results in the treatment of multiple myeloma and mantle cell lymphoma, it has limited efficacy in other types of cancers [61, 67]. The bortezomib resistance can be explained by multiple mechanisms. One possible mechanism is the feedback expression of proteasome genes. Bortezomib is a reversible inhibitor and is rapidly cleared from the patients' blood [61, 68, 69]. The transiently inhibited proteasome activity recovers after drug clearance and drug dissociation from the active site. The recovery is conceivably boosted by new proteasome synthesis induced by the feedback mechanism, thereby reducing the extent and duration of proteasome inhibition. Whereas the compromised efficacy of bortezomib may be strong enough to kill myeloma tumor cells, it may not be sufficient to be effective in other cancers, especially solid tumors. Third, the feedback pathway is a potential target for cancer therapy. An early study by Ju et al. has already raised the concern that the efficacy of proteasome inhibitors may be compromised by the feedback upregulation of proteasome genes [24]. Ju et al. have also suggested that simultaneous targeting of the proteasome active sites and the transcription factor of the proteasome genes would be an efficient regimen for the treatment of cancer. This idea originated from the observation that the depletion of Rpn4 displays a strong synthetic growth defect with impairment of proteasome activity [24]. In support of this prediction, a recent study demonstrated that knockdown

of Nrf1 enhances the cytotoxic effect of proteasome inhibitor YU101 in MDA-MB-231 breast cancer and U2OS osteosarcoma cell lines [53]. It will be of interest to examine whether this combined treatment is applicable to other types of cancer cells and whether it has a beneficial effect in clinical trials.

It is worthy of note that the downregulation of one of the proteasome genes by deletion of the Rpn4-binding site from the *PRE1* promoter dramatically reduces the assembled proteasome level and proteasome activity [25]. This finding is of clinical relevance because it provides a potentially important approach to reduce the proteasome activity in cancer cells. To date, proteasome inhibitors attacking the catalytic sites of the proteasome are the only tool to reduce the proteasome activity. However, many types of cancers are resistant to proteasome inhibitors by a variety of mechanisms. Knockdown of individual proteasome genes may present a promising alternative to proteasome inhibitors in cancer therapy. In fact, a recent study revealed that knockdown of proteasome subunits by RNA interference impairs cancer cell growth and exhibits a synthetic lethal phenotype when combined with proteasome inhibitors [70].

5 Combined therapy via targeting the pathways that display synthetic lethal phenotypes with the proteasome

Combined therapy is a feasible approach to increase the efficacy of proteasome inhibitors in the treatment of cancers. Unfortunately, current regimens of combined therapy are simply an application of a second known anti-cancer agent together with bortezomib, and have failed to highlight a significant synergistic effect [61, 67]. The key to the success of combined therapy is to target a specific pathway, which is essential for cell growth and survival when the proteasome activity is inhibited. Whereas it is difficult to define such pathways in mammalian cells, recent studies in *S. cerevisiae* have provided the needed information.

The *S. cerevisiae* proteasome genes are coordinately activated by Rpn4. Loss of Rpn4 results in decreased expression of proteasome genes and consequently lower proteasome activity, which mimics the effect of proteasome inhibitors. Genome-wide analyses have shown that the deletion of *RPN4* exhibits synthetic growth defects with mutations in numerous genes involved in a variety of pathways [71, 72]. Specifically, yeast cells lacking Rpn4 and one of these genes are either inviable or grow much slower than the mutants that carry a single mutation. Since most of these yeast genes have homologs in human cells, it is reasonable to speculate that simultaneous targeting of the proteasome and one of the human homologs may display a synergistic effect against cancer cells. As a proof of concept,

Ju et al. explored the relevance of the synthetic growth defect caused by a double deletion of *ERG24* and *RPN4* to combined therapy [73]. *ERG24* encodes the C-14 sterol reductase, which is required for ergosterol biosynthesis in yeast [74]. There are two Erg24 homologs in human cells with C-14 sterol reductase activity, including 3 β -hydroxysterol C-14 reductase and lamin B receptor [75, 76]. To test if Egr24 could be a potential target for combined therapy with proteasome inhibitors, Ju et al. took advantage of two recently identified Erg24 inhibitors, dyclonine and alverine citrate [77]. Dyclonine and alverine citrate are the major components of two over-the-counter medicines. Dyclonine is an oral anesthetic found in throat lozenges, whereas alverine citrate is a commonly used smooth muscle relaxant for the treatment of irritable bowel syndrome. Ju et al. found that dyclonine and alverine citrate substantially enhanced the cytotoxic effect of the proteasome inhibitor MG132 in breast cancer cells [73]. The combined treatment with MG132 and dyclonine or alverine citrate markedly induced apoptosis. This study presents a powerful yeast genetic approach to identification of potential targets for combined therapy with proteasome inhibitors.

6 Concluding remarks

The discovery of feedback regulation of proteasome gene expression provides insight into how proteasome homeostasis is regulated. This finding also raises a concern in using proteasome inhibitors as a single regimen for the treatment of cancers, that is, feedback upregulation of proteasome genes may compromise the efficacy of proteasome inhibitors. This problem can be overcome by combined therapy that targets another gene or pathway to produce synthetic lethal phenotypes with inhibition of the proteasome. It is worthy of note that the proteasome activity can be regulated at different levels. In addition to gene expression, proteasome assembly is an important process to control the cellular proteasome activity. Moreover, post-translational modifications of proteasome subunits such as oxidation, phosphorylation, glycosylation, acetylation, and myristoylation have been reported to alter the proteasome activity [78–81]. Further investigation of the biological significance and mechanisms of these modifications will provide more choices for proteasome-targeting cancer therapy.

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