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Homeostatic regulation of the proteasome via an Rpn4-dependent feedback circuit

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Abstract

The 26S proteasome is a complex protease consisting of at least 32 different subunits. Early studies showed that Rpn4 (also named Son1 and Ufd5) is a transcriptional activator of the *Saccharomyces cerevisiae* proteasome genes, and that Rpn4 is rapidly degraded by the 26S proteasome. These observations suggested that in vivo proteasome abundance may be regulated by an Rpn4-dependent feedback circuit. Here, we present direct evidence to support the Rpn4-proteasome feedback model. We show that proteasome expression is increased when proteasome activity is impaired, and that this increase is Rpn4-dependent. Moreover, we demonstrate that expression of a stable form of Rpn4 leads to elevation of proteasome expression. Our data also reveal that the Rpn4-proteasome feedback circuit is critical for cell growth when proteasome activity is compromised, and plays an important role in response to DNA damage. This study provides important insights into the mechanism underlying proteasome homeostasis.

Keywords: Proteasome; Ubiquitin; Rpn4; Proteolysis; Feedback regulation; DNA damage; Gene transcription

The ubiquitin–proteasome system is the primary intracellular machinery responsible for elimination of abnormal proteins and selective destruction of regulatory proteins involved in many cellular processes [1–3]. Most substrates attached with a multi-ubiquitin chain are degraded by the 26S proteasome, a ~2500 kDa self-compartmentalized multisubunit protease ([4] and refs. therein). The 26S proteasome consists of a barrel-shaped proteolytic core (the 20S core) with 4 stacked rings of seven subunits each in an $\alpha_7\beta_7\beta_7\alpha_7$ configuration. The 20S core possesses three types of catalytic activities: trypsin-like, chymotrypsin-like, and peptidylglutamyl peptide hydrolyzing activities, which are provided by 3 of the 7 distinct β subunits. The proteolytic active sites are located inside the chamber of the 20S core that is capped at one or both ends by the 19S regulatory particle (also known as cap or PA700) or the 11S particle [4,5]. The 19S regulatory particle is composed of at least 17 different subunits that are formed into two sub-complexes: the base and the lid [6–8]. The base is in contact with the 20S core and possesses 6 ATPase subunits (Rpt1 to Rpt6) and 2 non-ATPase subunits (Rpn1 and Rpn2). The lid including 8 non-ATPase subunits (Rpn3, Rpn5 to Rpn9, Rpn11, and Rpn12) is linked to the base partly via Rpn10, another non-ATPase subunit. The 19S regulatory particle mediates the binding and unfolding of ubiquitylated substrates before their translocation into the cavity of the 20S core for degradation. Recent reports have demonstrated that the 19S regulatory particle, specifically the Rpn11 subunit, also possesses a deubiquitylation activity, which is required for substrate degradation [9,10].

Biochemical analysis showed that the proteasome subunits are stoichiometrically present in vivo, suggesting a pattern of concerted regulation of the proteasome genes [6–8,11]. We and others recently discovered that

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the Rpn4 protein (also named Son1 and Ufd5) is a transcriptional activator required for normal expression of the *Saccharomyces cerevisiae* proteasome genes [12,13]. Interestingly, Rpn4 is extremely short-lived ($t_{1/2} \approx 2$ min) and degraded by the proteasome [12]. These results suggested a feedback circuit in which Rpn4 upregulates the proteasome genes and is degraded by the assembled active proteasome.

In the present study, we provide direct evidence to support the Rpn4-proteasome feedback model. We also demonstrate that the RPN4-proteasome feedback circuit is critical for cell growth and viability when proteasome activity is compromised. Interestingly, the Rpn4-proteasome feedback circuit also plays an important role in response to DNA damage.

Materials and methods

Strains and plasmids. The *S. cerevisiae* strains used are listed in Table 1. YXY55 and YXY59 were derived from strains WCG4a and YHI29/1, respectively, by integration of plasmid RS306RPN1^F that contains a 3′ segment of the *RPN1* open reading frame (ORF) fused to sequence encoding the FLAG epitope. YXY122 and YXY130 were derived from strain JD52 by integration of plasmids RS306PRE2^F and RS306PRE6^F, respectively. RS306PRE2^F and RS306PRE6^F bear 3′ coding sequences of the *PRE2* and *PRE6* ORFs, respectively, fused to sequence encoding the FLAG tag. Upon site-specific integration of the plasmids into the yeast genome, the corresponding genes were disrupted; therefore, only tagged proteins were expressed in these strains. The plasmids that express C-terminally FLAG-tagged Rpn1 (Rpn1^F) and Pre6 (Pre6^F) from their native promoters were previously described [12]. The low-copy plasmid expressing C-terminally FLAG-tagged Rpn4 (Rpn4^F) from the P_{CUP1} promoter was also described before [12]. For overexpression of *RPN4*, the P_{CUP1}-*RPN4* cassette from the low-copy plasmid p314CUP1RPN4 [12] was subcloned into the high-copy vector RS423 [14]. The *RPN4* ORF along with ~500 base pair (bp) of promoter region was amplified by PCR and subcloned into low-copy vector RS314 [14], resulting in 314RPN4. The mutated *RPN4* allele encoding a stable version of Rpn4 (Rpn4$_{\Delta 1-10/R11}$), which lacks the N-terminal 10 amino acids and has the N-terminal 11 lysines mutated to arginines, was constructed by PCR-mediated site-directed mutagenesis [15]. Yeast mating, sporulation, and tetrad dissection were performed as described [16]. Colony formation efficiency was measured by plating assay. Specifically, ~300 cells of various strains were seeded on YPD plates and the resulting colonies were counted.

Immunoblotting, immunoprecipitation, and pulse-chase. *S. cerevisiae* cells were grown to A_{600} of 0.8–1.0, harvested and resuspended in lysis buffer (1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, and 50 mM Na–Hepes, pH 7.5) containing 1× protease inhibitor mix (Roche, Indianapolis, IN), and lysed by vortexing with glass beads. Equal amounts of extracts (based on Bradford assays) were separated by SDS–PAGE, followed by immunoblotting with monoclonal anti-FLAG antibody (Sigma, St. Louis, MO). The blots were stripped by incubation with 62.5 mM Tris–Cl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 75 °C for 30 min. Filters were re-probed with anti-yeast α-tubulin antibody (Serotec, Oxford, UK) to verify equal loading. 6%, 8%, and 12% gels were used for detection of Rpn1^F, Rpn4^F, and Pre6^F, respectively. Pulse-chase procedures were described before [12]. MMS (0.3%) treatment was carried out 30 min before labeling and remained in the course of chase. For immunoprecipitation of the 20S core, the lysis buffer contained 0.5% Triton X-100, 10% glycerol, 0.15 M NaCl, 5 mM $MgCl_2$, and 50 mM Na–Hepes (pH 7.5) with 1× protease inhibitor mix.

Northern analysis. *S. cerevisiae* cells were grown at 30 °C to A_{600} of ~1.0. RNA was isolated as described [17]. Northern hybridization was carried out using a NorthernMax system (Ambion, Austin, TX). The DNA probes amplified by PCR correspond to the segments of ORFs *PDA1* (bp 144–634), *RPN4* (bp 660–897), *PRE2* (bp 641–864), and *RPN1* (bp 2426–2979), respectively. DNA probes were labeled with [α-^{32}P]dATP using a Strip-EZ PCR labeling kit according to the

Table 1
S. cerevisiae strains used in this study

Strains	Genotype	Source/references
JD52	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	[12]
EJY140	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 rpn4Δ::LEU2*	[27]
EJY141	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 rpn4Δ::LEU2*	[27]
EJY150	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 ufd4Δ::TRP1*	[27]
WCG4a	*MAT***a** *ura3 his3-11 15 leu2-3,112*	[19]
YHI29/1	*MATα pre1-1 ura3 his3-11,15 leu2-3,112*	[19]
CMY394	*MAT***a** *ura3-52 his3-Δ200 leu2Δ1 lys2-801 ade2-101 trp1Δ*	[20]
Y791	*MAT***a** *cim5-1 ura3-52 his3-Δ200 leu2Δ1*	[20]
JD126	*MAT***a** *UMP1-ha::YIplac128 PRE1-Flag-6His::YIplac211*	[32]
YXY55	*MAT***a** *ura3 his3-11, 15 leu2-3,112 RPN1-FLAG::URA3*	This study
YXY59	*MAT***a** *ura3 his3-11, 15 leu2-3 112 pre1-1 RPN1-FLAG::URA3*	This study
YXY122	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE2-FLAG::URA3*	This study
YXY130	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE6-FLAG::URA3*	This study
YXY186	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 pre1-1 rpn4Δ::LEU2*	Segregant of diploid produced by mating EJY140 with YHI29/1
YXY192	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE2-FLAG::URA3 rpn4Δ::LEU2*	Segregant of diploid produced by mating EJY140 with YXY122
YXY196	*MATα trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE6-FLAG::URA3 rpn4Δ::LEU2*	Segregant of diploid produced by mating EJY140 with YXY130
YXY206	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE1-Flag-6His::YIplac211*	Segregant of diploid produced by mating JD126 with EJY150
YXY210	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE1-Flag-6His::YIplac211 rpn4Δ::LEU2*	Segregant of diploid produced by mating YXY206 with EJY141

manufacturer's instructions (Ambion, Austin, TX). Hybridization patterns were detected by autoradiography and quantified by PhosphorImager (Molecular Dynamics). The *PDA1* transcript, whose level is almost constant under all conditions tested [18], served as internal control for RNA loading.

Results and discussion

Rpn4-dependent feedback control of proteasome expression

Our early work showed that Rpn4 is a transcriptional activator required for normal expression of the proteasome genes, that Rpn4 is extremely short-lived ($t_{1/2} \approx 2$ min), and that the degradation of Rpn4 is proteasome-dependent [12]. These results suggest that the in vivo proteasome abundance is maintained at a functionally appropriate level by a feedback circuit in which the Rpn4 protein level appears to be a key determinant. If this model is correct, a defect of the proteasome activity would be expected to lead to an increase in proteasome expression due to stabilization of Rpn4. We, therefore, decided to compare the proteasome levels between wild-type (wt) cells and proteasome mutants in which Rpn4 is stabilized.

Pulse-chase analysis showed that Rpn4 was stabilized in two *S. cerevisiae* proteasome mutants, *pre1-1* and *cim5-1* (Figs. 1A and B). *pre1-1* is a temperature-sensitive (*ts*) mutant with defective Pre1, a subunit of the 20S core [19]. The *cim5-1* mutant bears a *ts* mutation in Rpt1, an essential ATPase of the 19S particle [20]. A low-copy plasmid expressing C-terminally FLAG-tagged Pre6 ($Pre6^F$), a subunit of the 20S core, from its native promoter was transformed into these two proteasome mutants and wt cells. Immunoblotting with anti-FLAG monoclonal antibody was used to examine the steady-state levels of $Pre6^F$. As shown in Fig. 1D (top panel), the $Pre6^F$ level was significantly higher in the proteasome mutants than in the wt cells (compare lanes 2 and 1; 4 and 3). The blot was stripped and re-probed with antibody against yeast α-tubulin, verifying that comparable amounts of wt and mutant extracts were loaded (Fig. 1D, lower panel).

We also integrated a sequence encoding the FLAG epitope at the 3′ end of the *RPN1* ORF expressed from its native chromosomal location in the *pre1-1* mutant and a congenic wt strain. We found that the level of $Rpn1^F$, a subunit of the 19S particle, was significantly higher in the *pre1-1* mutant than in the congenic wt strain (Fig. 1E, compare lanes 3 and 1). The higher steady-state levels of Pre6 and Rpn1 were not due to their stabilization in the proteasome mutants because these proteasome subunits are long-lived even in wt cells (data not shown). In agreement with the increase in protein levels, Northern analysis showed that the mRNA levels of *RPN1* and *PRE6* were higher (~2-fold) in the *pre1-1* and *cim5-1* mutants than in the wt cells (Fig. 1C). Thus, the proteasome expression is up-regulated in the proteasome mutants.

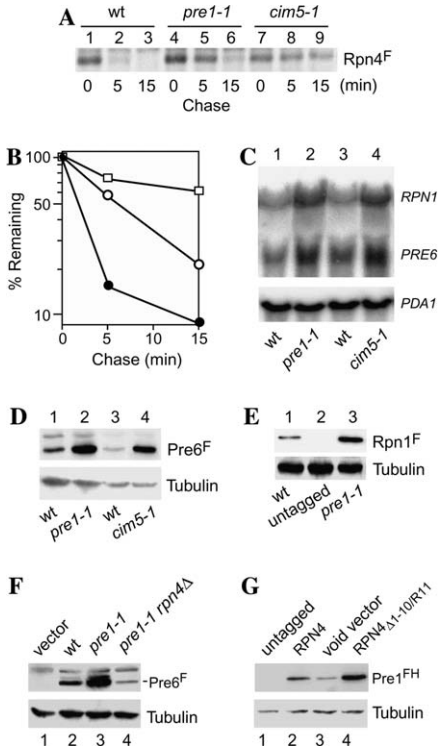

Fig. 1. The proteasome expression is regulated by an Rpn4-dependent feedback circuit. (A) Pulse-chase analysis of C-terminally FLAG-tagged Rpn4 ($Rpn4^F$) expressed from the P_{CUP1} promoter on a low-copy vector in wt strain (lanes 1–3), *pre1-1* (lanes 4–6), and *cim5-1* (lanes 7–9) mutants. The cells were labeled and chased at 30 °C. (B) Quantitation of the data from (A) by PhosphorImager to show the decay curves of Rpn4 in wt (●), *pre1-1* (○), and *cim5-1* (□). (C) Northern analysis of *PRE6* and *RPN1* transcripts in *pre1-1*, *cim5-1*, and their wt counterparts. Approximately equal amounts of different RNA samples were loaded, as verified by the *PDA1* transcript level. (D) Western analysis of C-terminally FLAG-tagged Pre6 ($Pre6^F$) in *pre1-1* (lane 2), *cim5-1* (lane 4), and congenic wt strains (lanes 1 and 3). Comparable amounts of wt and mutant extracts were used, as verified by stripping and re-probing the blot with antibody against yeast α-tubulin (lower panel). (E) Western analysis of C-terminally FLAG-tagged Rpn1 ($Rpn1^F$) expressed from its native chromosomal locus in wt strain (lane 1) and *pre1-1* mutant (lane 3). An untagged wt strain served as negative control (lane 2). (F) Increase of proteasome expression in *pre1-1* requires Rpn4. Western analysis of $Pre6^F$ expressed from its native promoter on a low-copy vector in wt (lane 2), *pre1-1* (lane 3), and the *pre1-1 rpn4Δ* double mutant (lane 4). A wt strain transformed with an empty vector was used as negative control (lane 1). (G) Stabilization of Rpn4 leads to an increase in proteasome expression. An *rpn4Δ* strain (YXY210) that expressed C-terminally FLAG-His_6 tagged Pre1 ($Pre1^{FH}$) from its chromosomal locus was transformed with a void vector (lane 3), or plasmids expressing either wildtype Rpn4 (lane 2) or a stable version of Rpn4 ($Rpn4_{\Delta 1-10/R11}$) (lane 4) from the *RPN4* native promoter in a low-copy vector. $Rpn4_{\Delta 1-10/R11}$ lacks the first 10 amino acids and has the N-terminal 11 lysines mutated to arginines [15]. Immunoblotting with anti-FLAG antibody was used to compare the steady-state levels of $Pre1^{FH}$ in these transformants. Cell extract from an untagged strain was used as negative control (lane 1).

To examine if the increase of proteasome abundance is Rpn4-dependent, we deleted *RPN4* from the *pre1-1* mutant and then examined the steady-state level of $Pre6^F$ expressed from its native promoter on a low-copy vector by immunoblotting. As expected, the steady-state level of $Pre6^F$ was higher in the *pre1-1* mutant than in the wt strain (Fig. 1F, compare lanes 3 and 2). Remarkably, the $Pre6^F$ abundance was lower in the *pre1-1 rpn4Δ* double mutant than in the wt strain (Fig. 1F, compare lanes 4 and 2). These results indicate that the increase of proteasome expression in *pre1-1* requires Rpn4.

Does stabilization of Rpn4 lead to increase of proteasome expression? To directly address this question, we generated an *rpn4Δ* strain that expressed C-terminally FLAG-His_6 tagged Pre1 ($Pre1^{FH}$) from its chromosomal locus. We transformed this strain with a void vector or plasmids expressing, respectively, wildtype Rpn4 and a stable version of Rpn4 ($Rpn4_{\Delta 1-10/R11}$) from the *RPN4* native promoter in a low-copy vector. $Rpn4_{\Delta 1-10/R11}$ lacks the first 10 amino acids and has the N-terminal 11 lysines mutated to arginines [15]. Immunoblotting with anti-FLAG antibody was used to compare the steady-state levels of $Pre1^{FH}$ in these transformants. As expected, the steady-state level of $Pre1^{FH}$ was higher in the presence of Rpn4 than in the absence of Rpn4 (Fig. 1G, lanes 2 and 3). Remarkably, the $Pre1^{FH}$ level was further increased in the cells expressing $Rpn4_{\Delta 1-10/R11}$ (Fig. 1G, compare lanes 4 and 2). Thus, a higher level of Rpn4 can enhance proteasome expression. These data (Fig. 1) establish that the proteasome expression is regulated by an Rpn4-dependent feedback circuit.

In line with our results, up-regulation of proteasome expression was also observed in *Drosophila* and mammalian cells treated with proteasome inhibitors or RNAi of proteasome subunits [21–24]. Although the molecular precision remains to be understood, it is likely that a yet-undiscovered functional homolog of yeast Rpn4 may play a similar role in regulating the proteasome expression in higher eukaryotes. The feedback regulation of proteasome homeostasis has important clinical relevance as proteasome inhibitors are being extensively tested for treating cancer and other diseases [25,26]. For instance, the efficacy of proteasome inhibitors may be compromised by feedback up-regulation of proteasome expression. It would be more efficient to target simultaneously the proteasome active site(s) and the transcriptional activator of the proteasome genes (i.e., Rpn4 homolog). We found that depletion of Rpn4 and decrease of the proteasome activity had a synthetic growth defect (see below).

The Rpn4-proteasome feedback circuit is critical for cell growth of proteasome mutants

Previous studies have shown that Rpn4 is required for proteolysis of several model substrates and that the *rpn4Δ* cells grow modestly slower than congenic wt cells [12,27,28]. Colony formation assay showed that the viability of *rpn4Δ* mutant is similar to those of wt cells, strain, suggesting that subnormal abundance of proteasome is largely sufficient for cell growth and viability under normal growth conditions. The compensatory increase of proteasome expression in the proteasome mutants (Fig. 1) implies that the Rpn4-dependent feedback circuit may be essential for proteasome mutants. We then decided to examine the growth of proteasome mutants in the absence of Rpn4. The *pre1-1* mutant grew slightly slower than a congenic wt strain at permissive temperature (30 °C) (Fig. 2A, compare panels c and a). The colony formation efficiency of *pre1-1* was found to be comparable to that of the congenic wt strain, indicating that the decrease of proteasome activity caused by *pre1-1* mutation can be tolerated. Remarkably, we found that deletion of *RPN4* in the *pre1-1* mutant significantly impaired the cell growth (Fig. 2A, compare panels d and b). The colony formation efficiency of the *pre1-1 rpn4Δ* double mutant was less than 75% of that of the *pre1-1* mutant in plating assays. Thus, depletion of Rpn4 and the *pre1-1* mutation exhibit a strong synthetic defect in cell growth and viability.

The synthetic growth defect of *rpn4Δ* and mutations of proteasome subunits was also observed in an *rpn4Δ* mutant that expresses a C-terminally FLAG-tagged

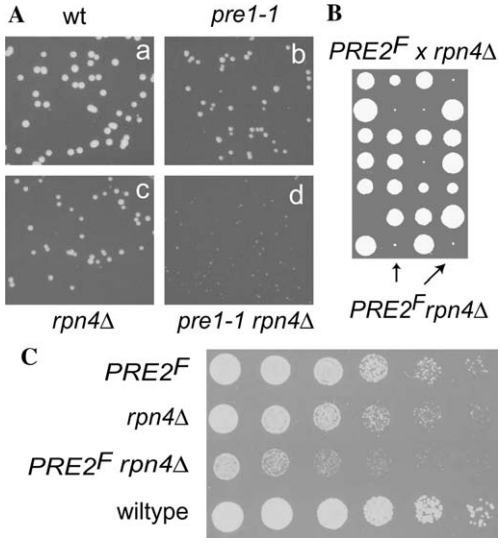

Fig. 2. Rpn4 is essential for cell growth and viability of proteasome mutants. (A) Deletion of *RPN4* and the *pre1-1* mutation exhibited a synthetic growth defect. Pictures were taken 36 h after plating of wt, *pre1-1*, *rpn4Δ*, and the *pre1-1 rpn4Δ* double mutant on YPD plates. (B) Tetrads of the diploid generated by mating *$PRE2^F$::URA3* (YXY122) and *rpn4Δ::LEU2* (EJY140) haploids were dissected on YPD plates. Viable spore clones were analyzed for uracil and leucine prototrophy. The slow growing colonies on YPD (indicated by arrows) were identified as Ura^+ Leu^+ spore clones that carry both *$PRE2^F$::URA3* and *rpn4Δ::LEU2* alleles. (C) Deletion of *RPN4* and addition of a FLAG epitope to the C-terminus of Pre2 led to a severe growth defect. Serial dilutions of cells as indicated were spotted on YPD plates and incubated for 2 days at 30 °C.

version of Pre2 ($Pre2^F$), a catalytic subunit of the 20S core. To construct an *rpn4Δ* mutant that expresses $Pre2^F$ from its chromosomal location, we crossed two haploids, *PRE2^F::URA3* and *rpn4Δ::LEU2*, both derived from the same genetic background except for opposite mating types (see Materials and methods and Table 1). Tetrads were dissected on YPD plates (Fig. 2B), and viable spore clones were analyzed for uracil and leucine prototrophy. Interestingly, the slow growing spore clones on YPD (Fig. 2B) turned out to be Ura^+ Leu^+ cells that carry both *PRE2^F::URA3* and *rpn4Δ::LEU2* alleles. Serial dilution assays also confirmed that the *PRE2^F rpn4Δ* double mutant grew much slower than both *PRE2^F* and *rpn4Δ* single mutants (Fig. 2C). Plating assays further revealed that the colony formation efficiency of the *PRE2^F rpn4Δ* double mutant was less than 60% of that of the *PRE2^F* mutant. Therefore, addition of a FLAG epitope to the C-terminal of Pre2, when combined with a deletion of *RPN4*, resulted in a severe reduction in cell growth and viability. Similarly, addition of a FLAG epitope to the C-terminus of Pre6 expressed from its chromosomal locus also severely impaired the growth and viability of *rpn4Δ* cells but not congenic *RPN4* wt cells (data not shown). The simplest explanation for these observations is that modification of Pre2 or Pre6 with the FLAG epitope may cause a subtle defect of the proteasome, which is not recognized in *RPN4* wt cells due to a compensatory increase in the proteasome abundance. However, the subtle proteasome defect is augmented in *rpn4Δ* cells, which have low abundance of proteasome and are unable to induce the proteasome genes in response to the proteasome defect. These observations (Figs. 2A–C) reveal the importance of the Rpn4-dependent feedback regulation of proteasome expression to the cells that have a compromised proteasome activity.

It is noteworthy that addition of a FLAG-His6 tag to the C-terminus of Pre1 did not impair the growth of *rpn4Δ* cells (data not shown), indicating that some proteasome subunits but not the others can perfectly tolerate the addition of a C-terminal epitope. Strains that express epitope-tagged proteasome subunits at native chromosomal loci are now widely used for affinity purification of proteasome if their doubling times are similar to that of untagged parental strains [6]. Our results, however, suggested that proteasome defects may not be reflected by phenotype-based assays such as cell growth and viability in the presence of Rpn4. Therefore, the proteasomes purified from these strains may not be fully active and suitable for in vitro studies. It is important to determine if modification of a proteasome subunit would cause a synthetic growth defect with *rpn4Δ* before an otherwise *RPN4* wt strain bearing the modified proteasome subunit is used for proteasome preparation. Our observations also imply that *rpn4Δ* cells may be utilized to sensitize in vivo assays that monitor the biological effects of proteasome inhibitors.

The Rpn4-proteasome feedback circuit in response to DNA damage

Recent reports showed that the proteasome is involved in DNA repair, and that some of the target genes of Rpn4 are required for DNA repair [29–31]. Consistent with these reports, we observed that several

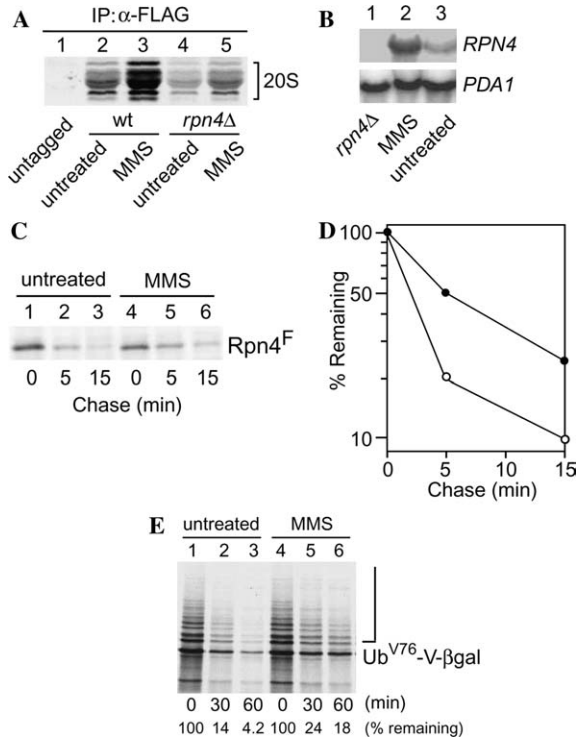

Fig. 3. The Rpn4-proteasome feedback circuit in response to DNA damage. (A) Immunoprecipitation of 20S core from wt (YXY206) and *rpn4Δ* (YXY210) strains expressing C-terminally FLAG-His6-tagged Pre1 ($Pre1^{FH}$) from the native chromosomal locus, either untreated (lanes 2 and 4) or treated (lanes 3 and 5) with 0.3% MMS. The untagged parental strain (JD52) was used as control (lane 1). $Pre1^{FH}$ and co-immunoprecipitated 20S core proteins by anti-FLAG antibody were separated by SDS–PAGE. The intensity of 20S core was quantified by PhosphorImager. (B) Northern analysis of *RPN4* expression in the presence (lane 2) and absence (lane 3) of MMS treatment. An *rpn4Δ* strain (lane 1) was used as negative control. The *PDA1* transcript whose level is not changed by MMS [31] served as an internal control for RNA loading. (C) Rpn4 was partially stabilized by MMS treatment. Cells expressing $Rpn4^F$ from the P_{CUP1} promoter in a low-copy vector were treated with 0.3% MMS for 30 min before labeling. The MMS-treated and untreated cells were labeled with [^{35}S]methionine/cysteine for 5 min and chased for 0, 5, and 15 min in the presence of cycloheximide and excessive cold methionine/cysteine. (D) Quantitation of the data from *C* by PhosphorImager to show the decay curves of Rpn4 with (●) and without (○) exposure to MMS. (E) The turnover rate of UFD substrates (Ub^{V76}-V-βgal) was decreased in response to MMS treatment. Pulse-chase analysis was performed as described in (C) with different chase times. Ubiquitylated species of Ub^{V76}-V-βgal were indicated by a half square bracket. PhosphorImager was used to quantify the amount of ^{35}S in the bands of Ub^{V76}-V-βgal (plus the first 5 of its ubiquitylated derivatives) at each time point, and plotted as a percentage of the amount at time zero.

proteasome mutants as well as *rpn4Δ* cells are sensitive to DNA damaging agents including MMS (data not shown). This prompted us to investigate the behavior of the Rpn4-proteasome feedback loop in response to DNA damage. We examined if the proteasome expression level would be altered in response to MMS, and if Rpn4 is required for the possible change. We found that the proteasome abundance in wt cells was increased ~3-fold after MMS treatment (Fig. 3A, compare lanes 3 and 2). The proteasome abundance was also higher but less significant (~1.6-fold) in the MMS-treated *rpn4Δ* cells than in the untreated counterparts (Fig. 3A, compare lanes 5 and 4). These results and previous DNA microarray analysis [31] indicate that proteasome expression is induced by MMS, and Rpn4 is required for a maximal induction.

We then asked if the Rpn4-proteasome feedback circuit is triggered in response to MMS. Specifically, we wished to test if the proteasome activity is impaired by MMS treatment, leading to stabilization of Rpn4 and a consequent increase in proteasome expression. To examine the effect of MMS on the Rpn4 stability, we measured the turnover rate of Rpn4 in MMS-treated cells by pulse chase analysis. As shown in Figs. 3C and D, the turnover of Rpn4 was slower in the MMS-treated cells ($t_{1/2}$ >5 min) than in the untreated cells ($t_{1/2}$ ≈2 min). Similarly, the turnover rate of the UFD pathway substrate (Ub^{V76}-V-βgal) was also decreased in response to MMS treatment (Fig. 3E). Therefore, the proteasome activity is indeed impaired by the MMS treatment, which subsequently triggers the Rpn4-proteasome feedback circuit.

Interestingly, the MMS treatment also induced the transcription of the *RPN4* gene (~2.5-fold) (Fig. 3B, compare lanes 2 and 3) in addition to partial stabilization of the Rpn4 protein. This two-layered MMS inducibility likely provides the cell with a swift response to the MMS challenge by increasing the proteasome expression to a sufficient level in a short period of time. Further studies on the mechanism underlying *RPN4* induction will shed new light on how cells respond to DNA damage.

Acknowledgments

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