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Proteasome inhibition enhances resistance to DNA damage via upregulation of Rpn4-dependent DNA repair genes

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ABSTRACT

The 26S proteasome is an ATP-dependent multi-subunit protease complex and the major regulator of intracellular protein turnover and quality control. However, its role in the DNA damage response is controversial. We addressed this question in yeast by disrupting the transcriptional regulation of the *PRE1* proteasomal gene. The mutant strain has decreased proteasome activity and is hyper-resistant to various DNA-damaging agents. We found that Rpn4-target genes *MAG1*, *RAD23*, and *RAD52* are overexpressed in this strain due to Rpn4 stabilisation. These genes represent three different pathways of base excision, nucleotide excision and double strand break repair by homologous recombination (DSB-HR). Consistently, the proteasome mutant displays increased DSB-HR activity. Our data imply that the proteasome may have a negative role in DNA damage response.

1. Introduction

The 26S proteasome is an ATP-dependent, self-compartmentalised protease complex and consists of a 20S core particle that is capped at one or both ends by the 19S regulatory particle [1,2]. Proteasome abundance is regulated by a negative feedback mechanism. In yeast, the Rpn4 transcription factor and extremely short-lived proteasome substrate controls expression of proteasomal genes through interaction with its binding site, called PACE (Proteasome-Associated Control Element) [3–6]. Rpn4p can regulate numerous other genes, including those that function in the DNA damage response. Consistently, *RPN4* deletion sensitises yeast to various stressed conditions, including DNA damage [6–11].

The ubiquitin–proteasome system (UPS) also participates in multiple DNA repair pathways and thus plays an important role in the DNA damage response [12]. However, the role of the proteasome proteolytic activity in the DNA damage response is unclear. One dataset suggests that protein degradation is necessary for resistance to DNA damage [13]. A second dataset implies that the 19S but not 20S proteasome complex is involved in DNA repair and provides resistance to DNA damage [14]. Finally, a third dataset argues for negative roles for both proteasome subcomplexes in the DNA damage response [15–17]. However, none of these studies attempted to connect the phenotype of the proteasome mutants with the underlying molecular mechanism or vice versa.

Our aim was to clarify the role of the proteasome proteolytic activity and Rpn4 in cell resistance to DNA damage and to elucidate a potential underlying mechanism.

2. Materials and methods

2.1. Yeast strains

rpn4-Δ and *rad52*-Δ are BY4742 derivatives that were purchased from Euroscarf (Germany). YPL is a BY4742 derivative bearing *pre1–8* mutation that is PACE substitution to Pst I site in the *PRE1* promoter. WCG4a 11/22 is a WCG4a derivative carrying *pre1–1* and *pre2–2* temperature sensitive alleles of *PRE1* and *PRE2* proteasomal genes [18] (Table S1).

2.2. Western blotting

Cells were lysed by vortexing with glass beads (Sigma) in a 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl (pH 8.0),

Abbreviations: AZE, L-azetidine-2-carboxylic acid; MMS, methyl-4-methanesulfonate; 4NQO, 4-nitroquinoline 1-oxide; DSB-HR, double strand break repair by homologous recombination

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1 mM EDTA buffer. Cell lysates were clarified by centrifugation at 16000×*g* for 5 min. Supernatants were mixed with protein loading buffer and heated at 100 °C for 10 min. After separation in 7% PAAG, proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with non-fat milk and incubated with primary mouse anti-ubiquitin antibodies (1:3000, Ebioscience, San Diego, USA) and then with secondary anti-mouse antibodies (1:10000, Jackson Immunoresearch Laboratories, West Grove, USA). The membrane was then incubated with ECL reagents (Amersham Biosciences) and developed using Kodak film. α-Actin was used as a loading control and detected using primary mouse monoclonal antibodies (1:4000, Sigma) and secondary anti-mouse antibodies conjugated to HRP (1:10000, Jackson Immuno Research laboratories).

Real-Time PCR was performed as previously described in [9]. The primers are listed in Table S2.

2.3. Measurement of the proteasome activity

Yeast cells were lysed by vortexing with glass beads (Sigma) in buffer A (50 mM Tris–HCl, pH 8.0, 5 mM Mg, 0.5 mM EDTA, 0.01% SDS, 10% glycerol). Lysates were clarified by centrifugation. Total protein concentration was determined spectrophotometrically according to the formula $C = (OD_{224} - OD_{233})/0.0496$. Fifty micrograms of total protein in buffer A was incubated with 20 μM of suc-LLVY-AMC for 1 h at 30 °C. Fluorescent signal was measured after addition of 1% SDS. In parallel, lysates activity were measured after a 30 min incubation with 20 μM lactacystin, a specific proteasome inhibitor. A drop in lysate activity after addition of lactacystin represented the specific activity of the proteasome.

2.4. Gap repair assay

A method previously described in [19] was used with minor modifications. Briefly, yeast cell cultures were grown to $OD_{600} \sim 0.4$ (instead of 1.0) and transformed with 0.5 μg XhoI/EcoRI cut pRPT5 by method described in [20]. To quantify the gap repair efficiency, the control cells were transformed with 0.5 μg of undigested pRPT5. The transformation mixture was spread onto selective media lacking uracil and incubated at 30 °C for 3 days. The efficiency of gap repair was calculated as the ratio of the number of colonies visible after transformation with cut plasmid to the number of colonies obtained with uncut plasmid.

2.5. Liquid β-galactosidase assay

The lacZ reporter gene was expressed under control of *RPT5* promoter or as a fusion with Rpn4 gene under control of *RPN4* promoter. LacZ activity was measured as previously described [21]. Briefly, cells were pelleted from the log-phase culture and were lysed in Z-buffer by vortexing with glass beads (Sigma). Cell lysates were clarified by centrifugation, mixed with ONPG at a final concentration of 2 mg/ml and incubated at 37 °C until a yellowish colour appeared. The reaction was stopped by the addition of Na_2CO_3. Total protein concentration and *O*-nitrophenoline concentration were measured spectrophotometrically using NanoDrop ND-1000.

2.6. Determination of protein half-life using a β-galactosidase assay

Overnight cell cultures were diluted to $OD_{600} = 0.25$ and grown in a synthetic medium containing 0.17% YNB without ammonium sulphate, 0.1% proline, amino acids, 2% glucose and 0.003% SDS for an additional 4 h at 30 °C. A concentration of 0.4 μg/ml polygodial was added to the cultures and incubated for 5 min to permeabilise the yeast cell membranes. Then, 600 μg/ml cycloheximide was added to yeast cells to halt protein synthesis and initiate the chase. The samples were withdrawn at the indicated time points and harvested by centrifugation. β-Galactosidase activity was measured as previously described [21].

2.7. DamID

Activity of Dam methylase fused to Rpn4 was measured in a model system that we had previously developed [22].

3. Results

3.1. Mutation of Rpn4 binding site deregulates PRE1

Using homologous recombination, we created stable YPL mutant strain. In this strain PACE was substituted with a PstI restriction site in the promoter of the *PRE1* gene encoding for an essential subunit of the 20S proteasome (Fig. 1A and Supplementary material).

To verify that the PACE mutation interferes with Rpn4 binding to the *PRE1* promoter, we used the highly sensitive DamID system [22]. We were unable to use conventional X-ChIP assay due to very low physiological level of Rpn4. DamID system detects Rpn4 binding by measuring the activity of Dam methylase that is fused to a transcription factor. The signal from Dam-Rpn4 chimeric factor is normalised to activity of the mutant protein

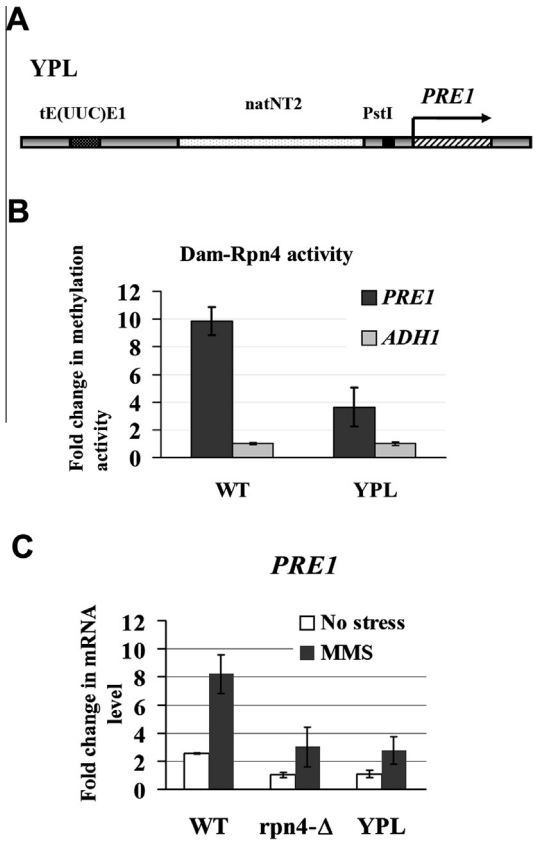

Fig. 1. PACE mutation decreases *PRE1* expression. (A) Scheme of *PRE1* mutated locus of the YPL PACE-less strain. (B) DamID shows that the PACE mutation inhibits Rpn4 recruitment to the *PRE1* promoter. Activity of the Dam-Rpn4 chimeric factor was normalised to the signal of Dam-Rpn4(C-A), in which Rpn4 is unable to bind DNA. Normalised Dam-Rpn4 activity on the *ADH1* promoter was used as a negative control and was arbitrarily set to 1. The standard deviation (S.D.) from the mean of three independent experiments is shown. (C) The expression of *PRE1* is decreased from PACE-less promoter. *ACT1* was used as a reference gene. The S.D. from the mean of three independent experiments is shown.

Dam-Rpn4(C-A). This mutant protein lacks of specific DNA-binding activity due to Cys to Ala mutations in the DNA-binding domain of Rpn4. Thus, normalisation to Dam-Rpn4(C-A) activity, allows us to detect specific binding of Dam-Rpn4. Low DNA methylation levels in YPL mutant (Fig. 1B) suggests that PACE mutation impairs Rpn4 recruitment to *PRE1*. Binding sites or protein–protein interactions in close proximity might account for the residual Dam-Rpn4 activity. As expected, RT-PCR shows that normal and stress-induced expression of PACE-less *PRE1* decreased to the level of the *rpn4*-Δ strain (Fig. 1C). Collectively, these data suggest that Rpn4 is unable to bind PACE-less promoter of *PRE1* and, therefore, fails to activate *PRE1* expression.

Upregulation of *PRE1* under MMS stress in *rpn4*-Δ and PACE-less strain (Fig. 1C) might be provided by another transcription factor(s).

3.2. Deregulation of PRE1 decreases proteasome activity and resistance to proteotoxic agents

Deregulation of *PRE1* should decrease proteasome activity. Accordingly, YPL strain shows the same hydrolysis rate of suc-LLYV-AMC, the fluorogenic artificial proteasome substrate, as in the *rpn4*-Δ (Fig. 2A) and accumulates polyubiquitinated proteins under heat shock conditions (Fig. 2B, lane 3). Consistently, YPL and *rpn4*-Δ strains are sensitive to heat shock and toxic analogues of amino acids (AZE and L-canavanine) that cause accumulation of misfolded proteins (Fig. 2C). Thus, *PRE1* deregulation impaires proteasome proteolytic activity.

3.3. Proteasome inhibition causes hyper-resistance to DNA damaging agents

The plating assay shows that proteasome mutant is resistant to MMS and hyper-resistant to 4NQO, whereas *rpn4*-Δ is unable to thrive under these conditions (Fig. 3A). The survival assay also shows that *rpn4*-Δ has the lowest survival rate upon DNA damage stress, and the YPL strain has the highest one (Fig. 3B and C). The introduction of a low copy plasmid encoding non-mutated *PRE1* gene decreases the resistance in mutant strain to the level of the wild-type strain (Fig. 3D). These data suggest that *PRE1* deregulation leads to hyper-resistance to DNA damage.

To verify the DNA stress resistant phenotype of proteasome-deficient strain, we used WCG4a 11/22 mutant strain bearing *pre1–1*, *pre2–2* mutations in the coding regions of *PRE1* and *PRE2* genes [18]. Fig. 3E shows that WCG4a 11/22 is more resistant to DNA damage than its isogenic wild-type strain.

We cannot ignore possible irreversible effects of genomic mutations that affect stress resistance. Therefore, we tested stress resistance of the wild-type strain grown in the presence of MG-132. MG-132 is a peptide aldehyde and reversible proteasome inhibitor [23]. We permeabilised yeast cells to MG-132 using polygodial at an innocuous concentration of 0.4 μg/ml [24]. MG-132 treatment does enhance cell resistance to DNA damage (Fig. 3F). Yeast permeabilised to MG132 by other methods also display increased resistance to 4NQO in the presence of MG132 (Fig. S1). Thus, different methods of proteasomal inhibition lead to increased resistance to DNA damage caused by MMS and 4NQO.

3.4. PACE-containing proteasomal genes are upregulated in the proteasome mutants due to Rpn4 stabilisation

We find that PACE-containing proteasomal genes are upregulated in YPL strain. The expression level of *RPT5* is increased in YPL (Fig. 4A). *RPT5* promoter-driven LacZ expression is more strongly induced in the mutant strain compared with the wild-type strain (Fig. 4B). We suggest that upregulation of PACE-containing genes is a consequence of Rpn4 stabilisation. To test this, we measured the Rpn4 protein level using our modification of LacZ-reporter assay [21]. The level of Rpn4 is six-fold higher in YPL than in the wild-type strain (Fig. 4C). The *RPN4* expression level remained unaffected in proteasome mutants under both normal and stressed conditions (Fig. 4D). These results imply that the level of Rpn4 is increased due to changes in posttranscriptional regulation. Accordingly, the cycloheximide chase with the Rpn4-LacZ reporter showed that Rpn4 half-life is, at least, two-fold higher in YPL mutant than in the wild-type strain (Fig. 4E). Note that Rpn4 half-life in wildtype strain is substantially higher than that one that was previously reported [5]. This could result from incomplete inhibition of protein synthesis and/or masking Rpn4 degradation signals in LacZ fusion.

We also tested the suggestion that Rpn4 stabilisation is sufficient to induce its target genes. We created stable Rpn4 variants (see Supplementary material). Rpn4* protein lacks *N*-degron and all six lysines involved in protein polyubiquitination [25]. Rpn4** protein lacks ubiquitin-dependent and ubiquitin-independent degradation signals [26]. Both Rpn4 variants expressed from low copy plasmid under control of the *RPN4* promoter increase mRNA level of *RPT5* in the wild-type strain under normal conditions (Fig. 4F), implying that Rpn4 stabilisation is sufficient to induce PACE-containing genes.

Rpn4 regulates not only proteasome but other components of the UPS including components of the ubiquitination system [27]. Therefore, we may expect that in *rpn4*-Δ strain all of the Rpn4-dependent UPS components are deregulated. On the contrary, in the YPL strain while the proteasome is deregulated, the ubiquitination system is upregulated by the stabilised Rpn4. Substantial imbalance between rates of protein ubiquitination and degradation may be the reason for increased accumulation of polyubiquitinated proteins in YPL compared with *rpn4*-Δ strain (Fig. 2B, lanes 2 and 3).

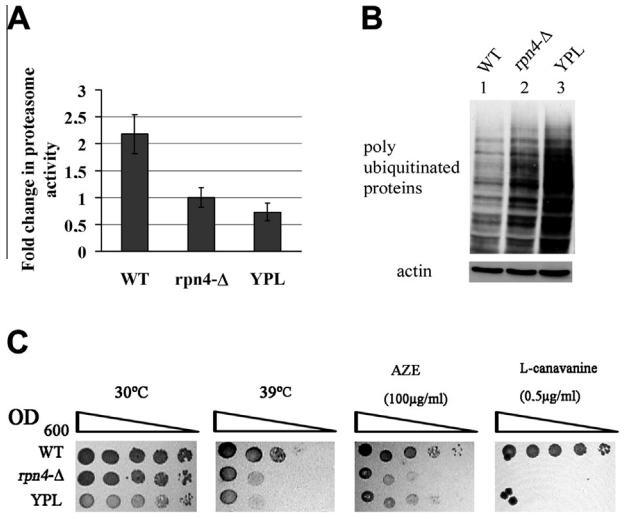

Fig. 2. YPL strain is defective in proteasome function. (A) Proteasome activity is decreased in crude lysates from the YPL strain. The S.D. from the mean of four independent experiments is shown. (B) *rpn4*-Δ and YPL strains accumulate polyubiquitinated proteins under heat shock. The strains were grown on YPD to OD_{600} = 1. Cell cultures were incubated at 39 °C for 2.5 h. Polyubiquitinated proteins were detected with anti-ubiquitin antibodies. Actin was used as a loading control. (C) YPL strain is sensitive to proteotoxic agents. Five-fold serial dilutions were prepared from overnight cultures starting from OD_{600} = 1.0. Subsequently, 4 μl of each dilution were spotted on appropriate media and incubated for 3–5 days. Growth at 39 °C was performed on YPD agar, whereas resistance to AZE (analogue for proline) or L-canavanine (analogue for arginine) was estimated on SM media lacking proline or arginine, respectively, at 30 °C.

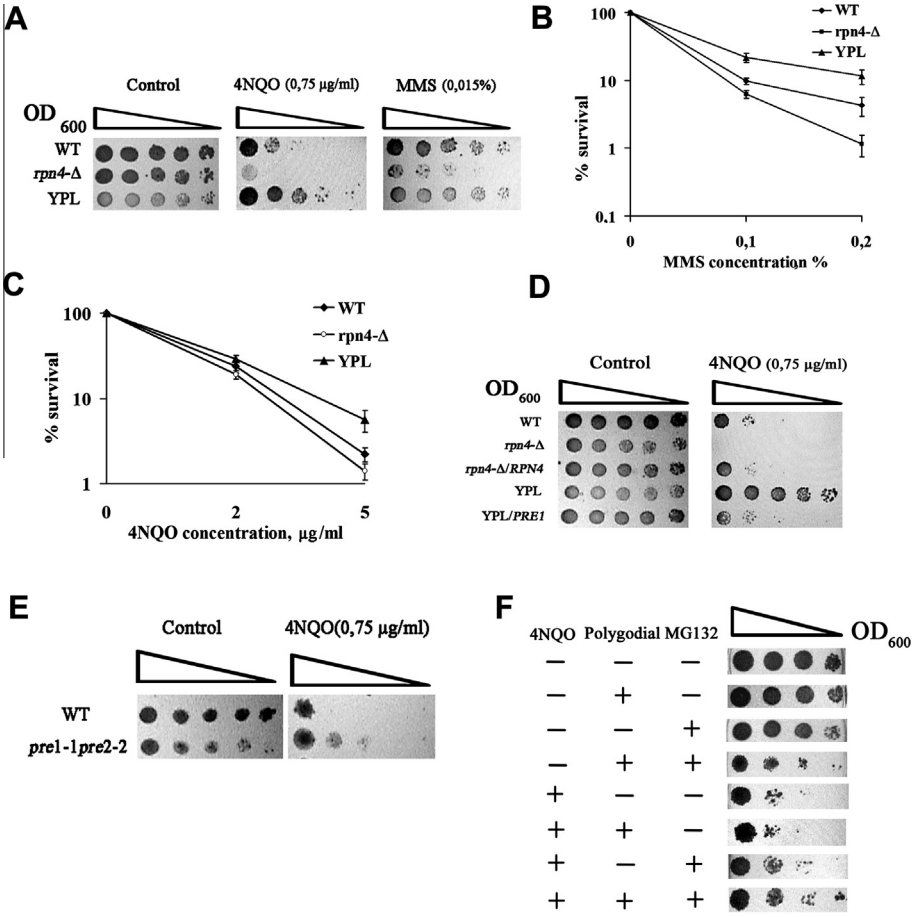

Fig. 3. YPL strain is resistant to DNA-damaging agents. (A) Adaptation to MMS or 4NQO on YPD. Five-fold serial dilutions of cultures were spotted on YPD and incubated at 30 °C for 3 days. The control plate was incubated with no stressing agents. (B) YPL strain shows enhanced survival rate upon 4NQO treatment. The survival test was performed according to Le Tallec et al. [42], with minor modifications. Early-exponential-phase cells grown on YPD were exposed to 4NQO at indicated concentrations for 2 h. Cell aliquots with $OD_{600} \sim 1–10 \times 10^{-5}$ were spread onto YPD plates. After incubation for 3 days at 30 °C, viable colonies were counted. Error bars are S.D. from the mean of three independent experiments. (C) YPL has increased survival rate upon MMS treatment. The test was performed as previously described for 4NQO treatment except that the cell cultures were treated with MMS for 45 min. Error bars are S.D. from the mean of three independent experiments. (D) Adaptation to 4NQO on SM media. Five-fold serial dilutions of yeast transformant cultures were spotted on SM agar and incubated at 30 °C for 3 days. (E) Mutations in coding regions of *PRE* genes enhance resistance to 4NQO. Five-fold serial dilutions of overnight cultures were spotted on YPD and incubated for 4 days at 25 °C. (F) The proteasome inhibitor increases resistance to chronic exposure of 4NQO. Ten-fold serial dilutions of overnight culture BY4742 were spotted on SM plates prepared with the indicated chemical combinations. Polygodial was used to permeabilise yeast cells. The plates were incubated at 30 °C for 3 days.

3.5. Stabilised Rpn4 upregulates DNA repair genes

Stabilised Rpn4 may also upregulate non-proteasomal stress responsive genes. We selected nine DNA repair genes *NTG1*, *APN1*, *POL31*, *RAD10*, *RAD23*, *RAD50*, *RAD51*, *RAD52*, and *MAG1*. Promoters of most of these genes have Rpn4 binding site(s) and Rpn4 binding to or Rpn4-dependent expression of these genes has been shown in whole genome studies [10,28–30]. According to our RT-PCR results, only three genes *RAD23*, *RAD52*, and *MAG1* are regulated in an Rpn4-dependent manner (Fig. 5A–C). These genes represent three different DNA repair pathways. *MAG1* encodes 3-methyladenine DNA glycosylase that initiates base excision repair by excision of methylated DNA bases [31]. *RAD23* encodes a protein that acts in nucleotide excision repair [32]. *RAD52* encodes a key player of DNA double-stranded break repair by homologous recombination [33]. These genes are upregulated 1.5–3-fold in YPL compared with the wild-type strain under normal conditions, as well as upon DNA damage stress (Fig. 5A–C). Our data corroborate the earlier observations that *RAD52* is overexpressed in proteasome mutants [34]. Other potential Rpn4 target genes do not differentially expressed in *rpn4*-Δ strain under normal condition and DNA damage stress (Fig. S2).

3.6. Activity of DNA double-strand break repair by homologous recombination (DSB-HR) is increased in proteasome mutants

We suggest that transcriptional activation of DNA repair genes contributes to induction of corresponding DNA repair pathways. We tested this suggestion in case of *RAD52* using a gap repair assay that utilises homologous recombination with the genomic locus to repair the gapped plasmid (Fig. 5D). *rad52*-Δ strain has the lowest observed DSB repair activity. The low DSB repair activity is also observed for the *rpn4*-Δ strain with abolished Rpn4-dependent regulation of *RAD52*. YPL strain shows the highest DSB repair activity (Fig. 5E).

4. Discussion

We find that disrupting the Rpn4-dependent regulation of *PRE1* proteasomal gene enhances resistance to such DNA damaging agents as MMS and 4NQO. We describe a novel mechanism underlying this phenotype. Proteasome inhibition stabilises Rpn4, which in turn, upregulates DNA repair genes. Overexpression of DNA repair genes seems to upregulate the corresponding DNA repair pathways. For *RAD52*, we observed induced DSB-HR. This

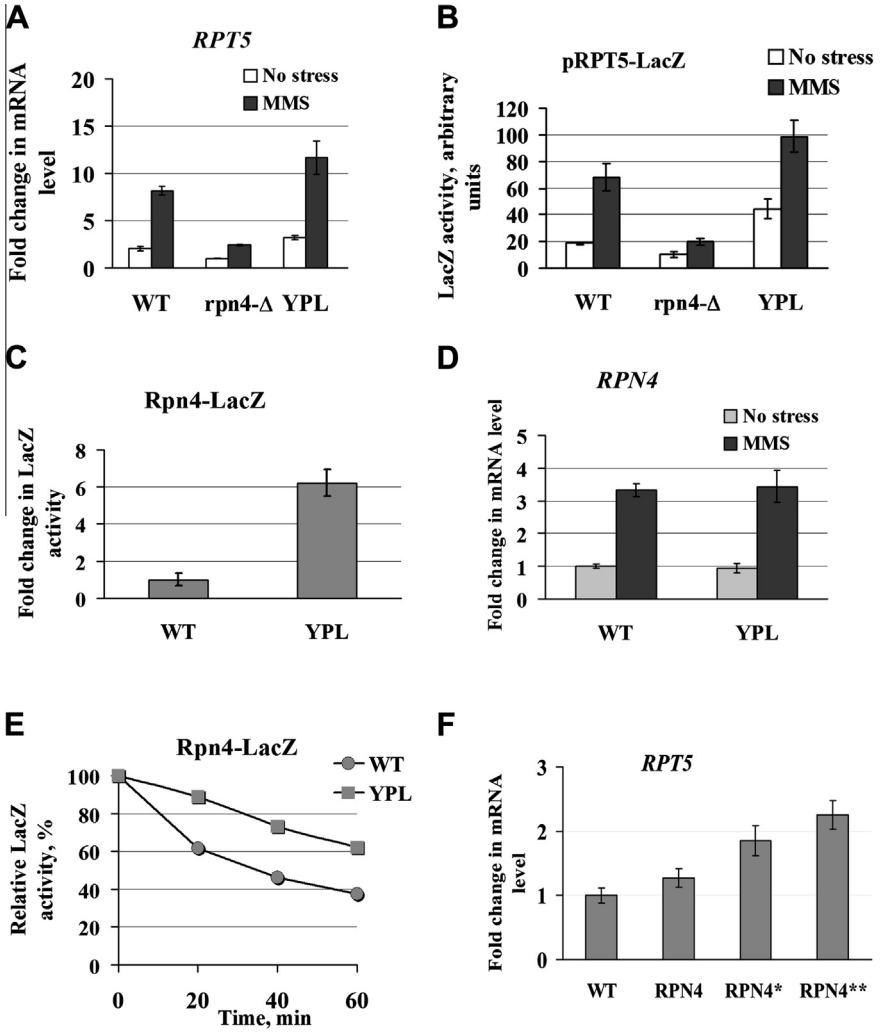

Fig. 4. PACE-containing proteasomal genes are overexpressed in *PRE1* mutant due to Rpn4 stabilisation. (A) *RPT5* is induced in the YPL strain under normal conditions or MMS stress. The S.D. from the mean of three independent experiments is shown. (B) The activity of the LacZ reporter is increased from the non-mutated *RPT5* promoter in the YPL strain under normal conditions or MMS stress. The S.D. from the mean of three independent experiments is shown. (C) Activity of the Rpn4-LacZ reporter is increased in YPL mutant. The S.D. from the mean of three independent experiments is shown. (D) *RPN4* expression level is the same in the YPL and wild-type strains under normal and stressed conditions. The S.D. from the mean of three independent experiments is shown. (E) The half-life of Rpn4 is increased in *PRE1* mutant. (F) *RPT5* expression is increased in the wild-type strain producing stabilised Rpn4 forms from low copy plasmid under control of *RPN4* promoter. The S.D. from the mean of three independent experiments is shown.

mechanism suggests a negative role for the 26S proteasome in DNA damage response via degradation of Rpn4.

The proteasome may play a negative role in DNA damage response via degradation of DNA repair proteins. A well-known example is Rad4, a component of the DNA damage sensor complex. Rad4 degrades rapidly under normal conditions and is stabilised in complex with Rad23 upon DNA damage [35]. Therefore, proteasome inhibition may lead to hyper-resistance to DNA damage by stabilisation of DNA repair proteins [15–17,36,37].

There are several arguments for the positive role of the proteasome in DNA damage response. Proteolysis may drive the multistep DNA repair process by eliminating proteins that work on preceding steps that may inhibit subsequent steps [12]. Proteolysis is also required to remove proteins cross-linked with DNA or stalled RNA-polymerase II [38,39]. Accordingly, the *rpn4*-Δ strain, defective in proteolysis, is highly sensitive to a broad range of DNA damaging agents [6–11].

However, there is no strong evidence that stalled RNA-polymerase, proteins cross-linked with DNA or stabilised components of the DNA repair pathways are sufficient to cause hypersensitivity of proteasome mutants to DNA damage. Moreover, deletion of the *RPN4* gene disrupts regulation of not only proteasomal genes but also a number of DNA repair genes. Therefore, we cannot positively conclude that the deregulation of what genes, proteasomal or non-proteasomal, causes *rpn4*-Δ strain sensitivity to DNA damage. To address this question, Wang et al. inhibited proteasome function by disrupting Rpn4-dependent regulation of the *PRE1* proteasomal gene [13]. They found that mutants were sensitive to UV irradiation and MMS. However, all experiments were performed on selective media because of the instability of genomic modification. We also find that YPL is sensitive to MMS and UV irradiation on selective media (Figs. S3A and S4C). It seems that SM media does not affect hyperresistance of YPL to other DNA damaging agents like camptothecin and zeocin (Fig. S4A). Currently, a mechanism(s) by which SM sensitises proteasome mutants to UV or MMS is unknown. In addition, we found that *UBI4* is highly induced in the YPL grown on the selective media (Fig. S3B). *UBI4* overexpression may serve as an indicator for a stress that required the enhanced activity of the ubiquitin–proteasome system [40]. Thus, our data show that synthetic selective media may act as a stress factor and affect the DNA damage resistance of, at least, some proteasome-deficient strains. Earlier observations suggest that stable proteasome

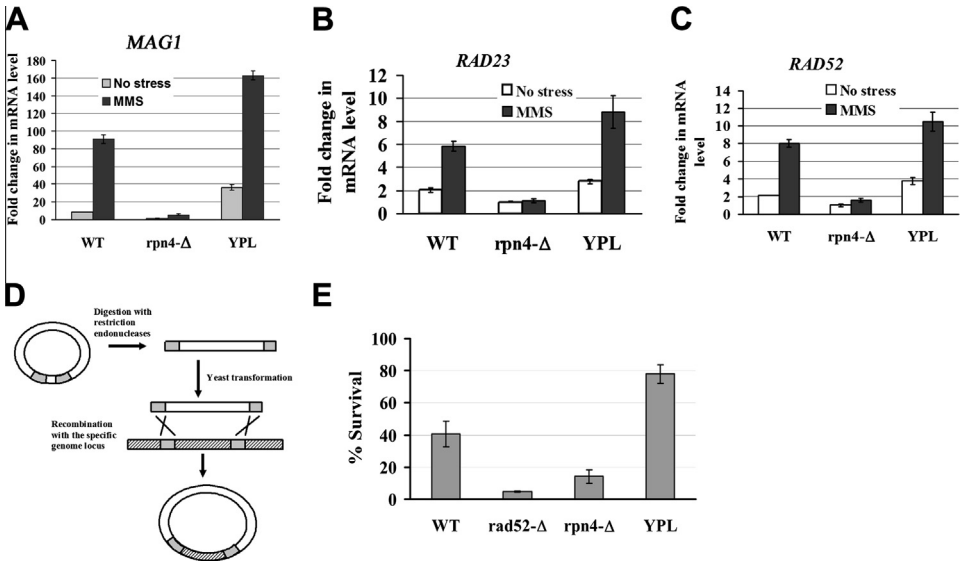

Fig. 5. Rpn4-dependent DNA repair genes are upregulated when proteasome function is impaired. The expression level of *MAG1* (A), *RAD23* (B), and *RAD52* (C) in the wild type and mutant strains was measured by real-time PCR under normal conditions or after treatment with 0.1% MMS during 30 min. *ACT1* was used as a reference gene. The S.D. from the mean of three independent experiments is shown. (D) The principle of the gap repair assay. A plasmid with fragments homologous to the genomic locus is gapped. After transformation, the linearised plasmid undergoes homologous recombination with the corresponding genomic locus. The major product of recombination, circularised plasmid, enables yeast transformants to grow on the selective media. The control cells are transformed with the same quantity of undigested plasmid. The ratio between the number of colonies obtained with the linearised plasmid to the number of colonies in the control reflects the efficiency of DSB-HR. (E) DSB-HR efficiency in proteasome mutants. Yeast cells were transformed with EcoRI/XhoI digested pRPT5. The control cells were transformed with undigested pRPT5. Yeast transformants were grown on complete selective media lacking uracil for 3 days. DSB-HR efficiency is estimated as the ratio between the number of colonies obtained with digested plasmid and the number of colonies obtained in the control. The S.D. from the mean of six independent experiments is shown.

mutants grown on rich YPD media often display hyper-resistance to DNA damage [15,17,41,42].

We used different methods to show that yeasts with compromised proteasome function display increased resistance to DNA damage. Disruption of negative feedback proteasome regulation stabilises Rpn4. We found that genes of three different DNA repair pathways are upregulated by stabilised Rpn4. Though, individual genes are slightly overexpressed under stressed conditions, we believe that the synergistic effect of Rpn4-dependent upregulation of a number of DNA repair genes provides a dramatic increase in overall cell resistance to DNA damage.

Recently, it has been demonstrated that the stabilisation of Pap1 in proteasome mutants of *Schizosaccharomyces pombe* increases resistance to oxidative stress [43]. We can generalise the idea that the proteasome may play a negative role in the stress response pathways that have transcription regulators as proteasome substrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.007.

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