Regulatory mechanisms controlling biogenesis of ubiquitin and the proteasome

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Received 7 April 2004; accepted 23 April 2004

Available online 8 May 2004

Edited by Horst Feldmann

Abstract Analysis of several *Saccharomyces cerevisiae ump* mutants with defects in ubiquitin (Ub)-mediated proteolysis yielded insights into the regulation of the polyubiquitin gene *UBI4* and of proteasome genes. High-molecular weight Ub–protein conjugates accumulated in *ump* mutants with impaired proteasome function with a concomitant decrease in the amount of free Ub. In these mutants, transcriptional induction of *UBI4* was depending in part on the transcription factor Rpn4. Deletion of *UBI4* partially suppressed the growth defects of *ump1* mutants, indicating that accumulation of polyubiquitylated proteins is deleterious to cell growth. Transcription of proteasome subunit genes was induced in *ump* mutants affecting the proteasome, as well as under conditions that mediate DNA damage or the formation of abnormal proteins. This induction required the transcriptional activator Rpn4. Elevated Rpn4 levels in proteasome-deficient mutants or as a response to abnormal proteins were due to increased metabolic stability. Up-regulation of proteasome genes in response to DNA damage, in contrast, is shown to operate via induction of *RPN4* transcription.

Keywords: Ump1; Rpn4; Ubi4; Doa4; DNA repair

1. Introduction

Selective ubiquitin (Ub)-mediated proteolysis (UMP) is the dominating mechanism in the degradation of cytosolic and nuclear proteins in eukaryotic cells [1–3]. In this process, a substrate is tagged with a polyUb chain that mediates interaction with and degradation by the proteasome [4]. The functions of UMP range from a participation in protein quality control to the often timed and spatially controlled degradation of numerous regulatory proteins, including key cell cycle regulators and many transcription factors [1]. UMP is essential for viability of eukaryotic cells and it is becoming increasingly clear that malfunctions within the system are involved in the pathogenesis of severe human diseases [3].

To study the machinery involved in Ub-mediated proteolysis, we selected *ump* mutants with defects in this process. Two of the mutations directly affected proteasome subunits, one led to the loss of a proteasome maturation factor, the fourth one affected a deubiquitylating enzyme. The analysis of these mutants yielded insights into cellular buffer systems that compensate for changes in Ub levels or available proteasome capacity.

2. Materials and methods

2.1. Isolation of ump mutants

A selection-based screen that yielded *ump* mutants and the cloning of *UMP1* encoding a proteasome maturation factor has been described previously [5]. *ump2-1* is an allele of *PUP1* with a mutation leading to an exchange from Ala to Glu at position 130. The proteolytic defects of the *ump3-1* mutant were complemented by yeast genomic library plasmids containing the *UBI4* gene or the *DOA4* gene. *ump4-1* is an allele of *PRE4* containing a single point mutation leading to an exchange from Ser to Tyr at position 224.

2.2. Construction of yeast strains and plasmids

Strains expressing C-terminally 2xha (influenza virus hemagglutinin) tagged versions of the proteasome subunit β5/Pre2 instead of its untagged counterpart were constructed as described previously [5]. Strains expressing a C-terminally 3xha tagged variant of Rpn4 were constructed by a PCR-based method that used plasmid pFA-6a-3xha-HIS3-MX6 as a template [6]. The *ubi4-Δ::LEU2* strain has been described [9]. Replacement of *RPN4* with the *HIS3* marker was performed by an analogous strategy using plasmid pFA-6a-HIS3-MX6 as a template [6].

2.3. Determination of protein stability after expression shut-off (cycloheximide chase)

Yeast cells were grown at 30 °C either in YPD or SD liquid media to an OD_{600} of 0.8–1.2. Cycloheximide was added to each culture to a final concentration of 100 μg/ml. At the time points indicated in the figures, cell samples were withdrawn and extracts prepared by glass bead lysis in a buffer containing 50 mM Na–HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and "Complete Protease-Inhibitor cocktail" (Roche). Equal amounts of proteins were analyzed by SDS–PAGE and Western blotting.

2.4. RNA isolation and Northern-blot analysis

Yeast cells were grown at 30 °C in liquid YPD or SD medium to an OD_{600} of 0.8–1.2. Total RNA was prepared using the RNeasy kit (Qiagen). Northern-blot analysis of 10 μg RNA samples was carried out as described previously [7]. Radioactive signals were quantified on a Bio-Imaging Analyzer (Fujifilm BAS 1500).

3. Results and discussion

To study the genetics and physiology of selective UMP, we isolated and analyzed *ump* mutants with defects in this process.

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.078

Using a selection-based screen, four such mutants (*ump1* to *ump4*) were isolated. In contrast to wild-type cells, these mutants were unable to efficiently degrade substrates of the Ub-dependent N-end rule and UFD pathways [8,9] (data not shown). Both the *ump1* and the *ump3* mutants were severely hypersensitive to stresses such as heat and high concentrations of amino acid analogs or heavy metals, conditions known to elicit an increased generation of abnormal proteins, which are substrates of the Ub/proteasome pathway. The mutants *ump2* and *ump4* were less sensitive to such stresses (data not shown). One of these mutants (*ump1*) lacked a proteasome maturation factor as reported previously [5]. The *ump2* and *ump4* mutations, both of which affected the maturation (Fig. 2A) and activity of the proteasome, were mapped to the *PUP1* and *PRE4* genes encoding subunits β2 and β7 of this protease, respectively. The *ump3* mutation affected the deubiquitylating enzyme Doa4 [11]. The findings from the analyses of these mutants are the following.

3.1. Ump mutants and ubiquitin homeostasis

Analysis of cell extracts by anti-Ub immunoblotting revealed a striking accumulation of high-molecular weight Ub conjugates in *ump1*, *ump2*, and *ump4*, while at the same time levels of free Ub were reduced (Fig. 1A). Consistent with a defect in the degradation of such conjugates, in all of these mutants either the biogenesis or function of the proteasome is affected (see above). To determine whether the decreased availability of Ub in these mutants contributed to the observed

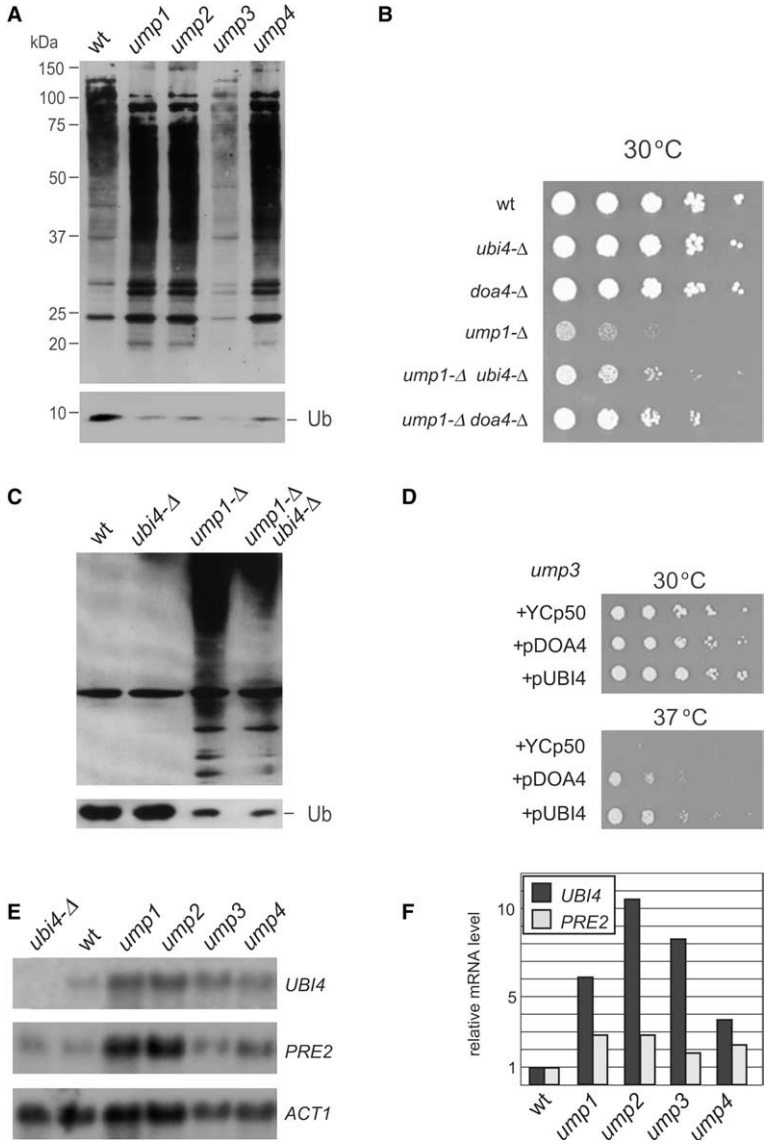

Fig. 1. Ub homeostasis in wild-type and *ump* mutant strains. A: Levels of Ub conjugates and of free Ub in extracts of wild-type and *ump* mutant cells were analyzed by anti-Ub Western blotting. To detect free Ub, the lower part of the gel was blotted for 10 min. Transfer of proteins from the upper part of the gel was carried out for 1 h. B: Deletion of *UBI4* or *DOA4* partially suppressed the growth defects of *ump1*-Δ C: Shown is the same analysis as in (A) but with a congenic set of wt, *ubi4*-Δ *ump1*-Δ and *ubi4*-Δ *ump1*-Δ strains. D: The *UBI4* gene is a suppressor of the *ump3-1* mutation. Shown is the complementation of the temperature sensitivity of *ump3* by plasmid-encoded *DOA4* (pDOA4) or *UBI4* (pUBI4). Plasmid YCp50 served as a vector control. E: Increased *UBI4* and *PRE2* transcript levels in *ump* mutants detected by Northern blotting. F: Quantitation of the results shown in (E). Signals for *UBI4* and *PRE2* were normalized to those of *ACT1*. mRNA values in *ump* mutants were calculated relative to those in wt, which were set to 1.

phenotypic defects, we asked whether a deletion of *UBI4* [10], which encodes the poly-Ub precursor of Ub, exacerbated or alleviated the phenotypes of the *ump1*-Δ mutant [5]. Surprisingly, *ubi4*-Δ partially suppressed the growth defects of the *ump1*-Δ strain, while in contrast increasing the synthesis of Ub by ectopic expression exacerbated these defects (Fig. 1B and data not shown). An anti-Ub Western blot revealed a less dramatic accumulation of Ub-protein conjugates in the *ump1*-Δ *ubi4*-Δ double mutant in comparison to the *ump1*-Δ single mutant (Fig. 1C). Taken together, these data indicated that an accumulation of Ub-protein conjugates underlies or at least contributes to the growth defects of the *ump1*-Δ mutant. Consistent with this assumption, we also obtained a partial suppression of the *ump1*-Δ growth defects when we introduced the *doa4*-Δ mutation (Fig. 1B), which interferes with Ub recycling and therefore reduces the availability of Ub for conjugation (see below). Comparable results were obtained for the *ump2* mutation, which was also partially suppressed both by *ubi4*-Δ and *doa4*-Δ (data not shown). A possible explanation for the observed detrimental effects of polyubiquitylated proteins accumulating in proteasome-deficient mutants is that they may sequester chaperones, which are required for protein folding and refolding. Another possibility is that accumulating conjugates may exert their growth-inhibitory effect by blocking the already impaired proteasome.

In contrast to the other *ump* mutants, we detected reduced levels of both free and conjugated Ub in the *ump3* mutant (Fig. 1A). Cloning of genes that complemented the defects of the *ump3* mutation resulted in the isolation of the *UBI4* and *DOA4* genes (Fig. 1D). Genetic analysis revealed that *UMP3* is identical to *DOA4*, while *UBI4* appears to be a dose-dependent suppressor of *ump3*/*doa4*. The double mutant *doa4*-Δ *ubi4*-Δ, however, did not display a severe reduction of exponential growth rates when compared to the *doa4*-Δ mutant but was extremely starvation sensitive (data not shown). These results showed that expression of the other Ub encoding genes *UBI1*-*UBI3* is insufficient to maintain a level of free Ub high enough to allow *ump3* mutant cells to survive at 37 °C or starvation conditions. It was reported that the half-life of Ub is significantly reduced in *doa4*-Δ cells because a significant fraction of conjugated Ub is degraded by the proteasome as well as by vacuolar proteases along with its substrates [11]. Consistent with this, our data showed that cells lacking the Ub-recycling function of Doa4 were unable to maintain normal levels of Ub although they showed increased expression of *UBI4* compared to wild-type cells (Fig. 1E and F). The latter data demonstrated that *UBI4* expression is subject to a feedback control that appears to mediate a response to reductions in the level of free Ub. An induction of *UBI4* was also detected in proteasome-deficient *ump* mutants. In these mutants, the up-regulation of *UBI4* expression depended in part on the transcription factor and proteasome substrate Rpn4 (see next section). Induction of *UBI4* is apparently detrimental to mutants impaired in proteasome function as it contributes to the accumulation of polyubiquitylated proteins (see above).

3.2. Impaired proteasome function results in a transcriptional feedback regulation of proteasome gene expression mediated by the transcription factor Rpn4

During the analysis of *ump2* and *ump4* mutants, both of which are defective in proteasome maturation and function (see above), we observed an increase in the steady state levels of proteasome subunits (Fig. 2A). An analysis of proteasome gene mRNAs revealed that this regulation occurred at the level of transcription (Fig. 2A). Recently, it was reported that Rpn4 acts as a transcriptional activator of proteasome gene expression by binding to so-called PACE (Proteasome associated control element) sequences that are found in the promoters of proteasome subunit genes as well as genes of the Ub system such as *UBA1* and *UBI4* [12]. It was subsequently shown that Rpn4 itself is a substrate of the proteasome, suggesting that it is a key component in feedback regulation of the proteasome [13]. To test whether Rpn4 is indeed underlying the increased expression of proteasome genes detected in some of our *ump* mutants, we deleted the *RPN4* gene in wild-type and *ump* mutant strains. The most drastic effect of *rpn4*-Δ was observed in combination with *ump1*-Δ. Tetrad analysis revealed that these two mutations are synthetically lethal (Fig. 2B). The *ump2 rpn4*-Δ and *ump4 rpn4*-Δ double mutants showed strong synthetic growth defects already at 30 °C. In addition, they were highly sensitive to elevated temperature and conditions resulting in increased amounts of abnormal proteins (Fig. 2C). Our studies also revealed that Rpn4-mediated up-regulation of proteasome levels will often compensate primary defects in the proteasome, many of which might thus have escaped detection in phenotype-based assays. A deletion of the last 19 residues of the β7/Pre4 subunit, for example, caused a decrease in proteasome assembly and post-acidic activity, but did not lead to detectable growth defects [14]. However, when combined with a deletion of *RPN4*, this mutation resulted in a severe reduction in growth rates and viability (Fig. 2D).

In order to determine the importance of Rpn4 to the generation of basal levels of proteasomes under non-stressed conditions, we compared the proteasomal chymotryptic activity of congenic wild-type and *rpn4*-Δ strains. The *rpn4*-Δ mutation resulted in a reduction of this activity by ~50% (Fig. 2E), indicating that Rpn4 contributes significantly to the synthesis of basal levels of proteasomes. This reduction, however, does not appear to be detrimental for growth under non-stressed conditions (Fig. 2C). To determine whether the observed phenotypic effects of *rpn4*-Δ in *ump* mutant backgrounds could indeed be correlated to changes in the transcription rates of proteasome genes, we analyzed and quantified their mRNA levels by Northern-blot analysis. The results shown in Fig. 3 clearly established that *ump2* and *ump4* mutants are unable to up-regulate proteasome gene expression if they are lacking Rpn4. In addition, these double mutants showed a significantly reduced induction of *UBI4* transcription when compared to the *ump2* and *ump4* single mutants (Fig. 3). Rpn4 control of Ub expression is consistent with a previous report in which a PACE sequence was identified in the promoter of the *UBI4* gene [12].

The model of Rpn4-mediated feedback regulation of proteasome gene expression implicated a stabilization of Rpn4 as a result of impaired proteasome function. It was reported earlier by others that Rpn4 is stabilized in proteasome mutants and that degradation of Rpn4 seems not to involve ubiquitylation [13]. To confirm the relevance of the above-mentioned model to the observations made in our *ump* mutants, we therefore analyzed the level of epitope-tagged Rpn4 in these strains. Indeed, we found that steady state levels of Rpn4 were increased in *ump1*, *ump2* and *ump4*, but not in *ump3* strains (Fig. 4A). As described above, the *ump3* mutation leads to a reduction of the level of free Ub and thereby presumably does

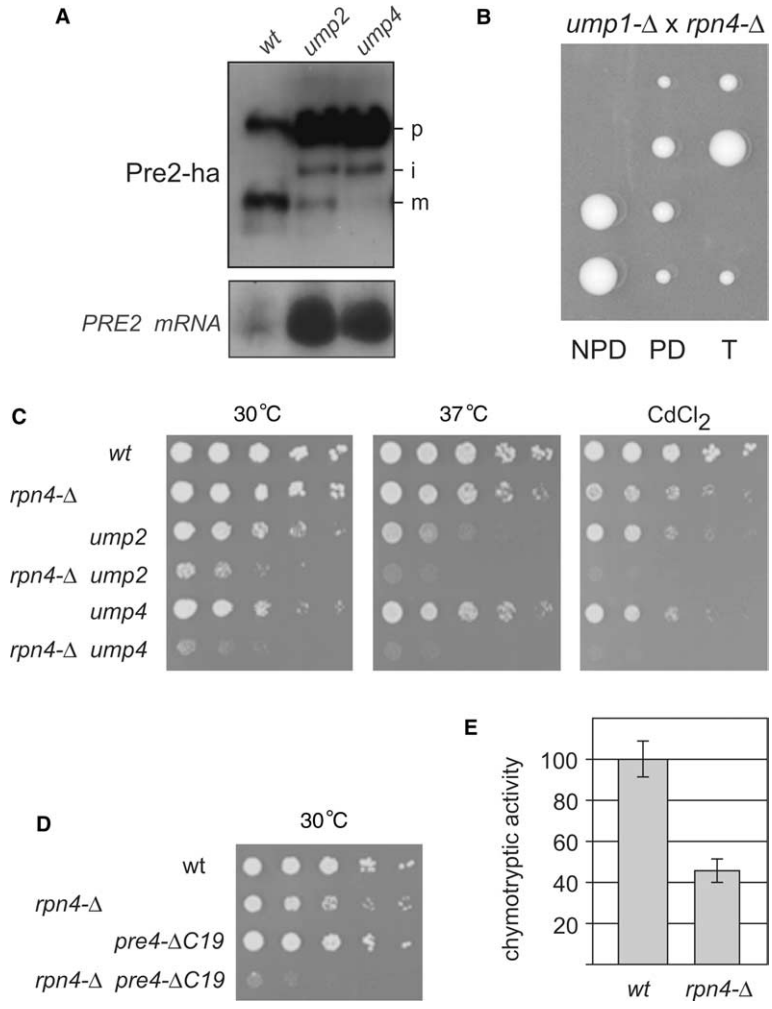

Fig. 2. Up-regulation of proteasome levels in *ump* mutants requires Rpn4. A: Elevated levels of β5/Pre2 were detected in crude extracts from *ump2* and *ump4* strains expressing Pre2-ha by anti-ha Western blotting (upper panel). p, Proβ5 precursor; i, incompletely processed β5; m, mature β5. The lower panel shows the detection of increased *PRE2* transcript levels in the same mutants by Northern blotting. B: *rpn4*-Δ and *ump1*-Δ are synthetically lethal. Tetrads of strain ML3 (*ump1*-Δ::*HIS3* × *rpn4*-Δ::*LEU2*) were dissected on YPD, and viable spore clones were analyzed for histidine and leucine prototrophy. No viable His+ Leu+ spore clones were obtained (not shown). NPD, non-parental ditype; PD, parental ditype; T, tetratype. C: Deletion of *RPN4* in the *ump2* and *ump4* mutants leads to strong synthetic growth defects and hypersensitivity to stresses. Serial dilutions of cells were spotted onto plates containing YPD medium with or without 30 μM CdCl2 and incubated for 2 days. D: Up-regulation of proteasome levels by Rpn4 masks the effect of the *pre4*-Δ*C19* mutation. Serial dilutions of cells were spotted onto YPD plates and incubated for 2 days. E: *RPN4* is required to maintain proteasome levels under normal growth conditions. Shown are the proteasomal chymotryptic activities in crude extracts. Values are the means of four independent measurements. S.D. are indicated.

not affect proteasome-mediated but Ub-independent protein degradation. The strong induction of *UBI4* transcription observed in *ump3* (Fig. 1F) therefore appears not to depend on increased levels of Rpn4, indicating that the polyUb gene is under multiple controls including the one mediated by Rpn4 [15,16].

To confirm that an increased half-life is underlying the higher steady state levels of Rpn4 observed in the *ump1* and *ump2* mutants, we followed its stability using expression shut-off experiments. The data presented in Fig. 4B and quantified as shown in Fig. 4E demonstrated that Rpn4 is degraded less efficiently in these mutants ($t_{1/2} \sim 16$ and 10 min in *ump1* and *ump2*, respectively) than in wild-type cells, in which its turnover is very rapid ($t_{1/2} \sim 2$ min). Note that much shorter chase time points were chosen for wild-type (wt) than for the mutants. The still relatively short half-life of Rpn4 in these mutants, which are severely impaired in the degradation of other proteasome substrates [5], may be due to its unique Ub-independent mode of degradation. Stress conditions leading to an accumulation of misfolded or damaged abnormal proteins may also require increased proteasomal activity. Therefore, we tested whether the addition of the arginine analog canavanine affects Rpn4 levels. Within minutes after addition of 1.5 μg/ml canavanine to an exponentially growing yeast culture, cellular levels of Rpn4 increased, whereas in untreated control cells the level of Rpn4 remained unchanged (Fig. 4C). Thirty minutes after the addition of canavanine, the level of Rpn4 started already to decrease again reaching the starting level after 60 min. Presumably, proteasomes had increased to a level that enabled the cells to cope with the situation and to adjust a new balance between Rpn4 degradation and the level of proteasomes. Expression shut-off experiments showed that canavanine treatment leads to increased stability of Rpn4 (Fig. 4D). Stabilization of Rpn4 was most likely due to an

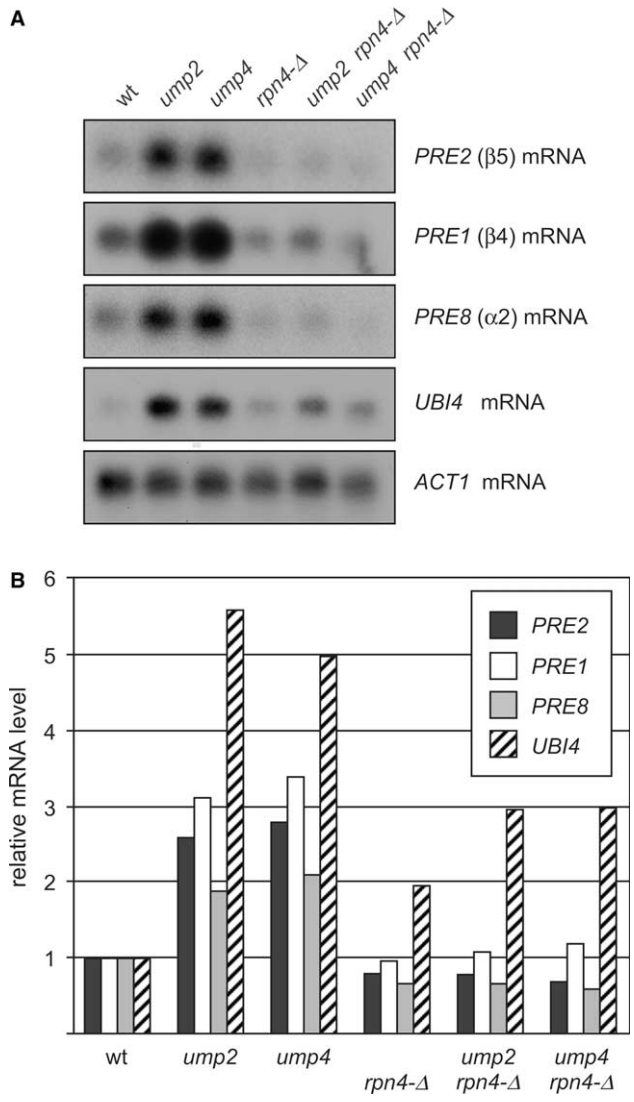

Fig. 3. Rpn4 mediates transcriptional up-regulation of proteasome gene expression in proteasome-deficient *ump* mutants. A: mRNA levels of proteasome subunit genes and *UBI4* detected by Northern blotting. B: Quantification of the results shown in (A). Signals were normalized to those of *ACT1*. mRNA values in mutant strains were calculated relative to those in wt, which were set to 1.

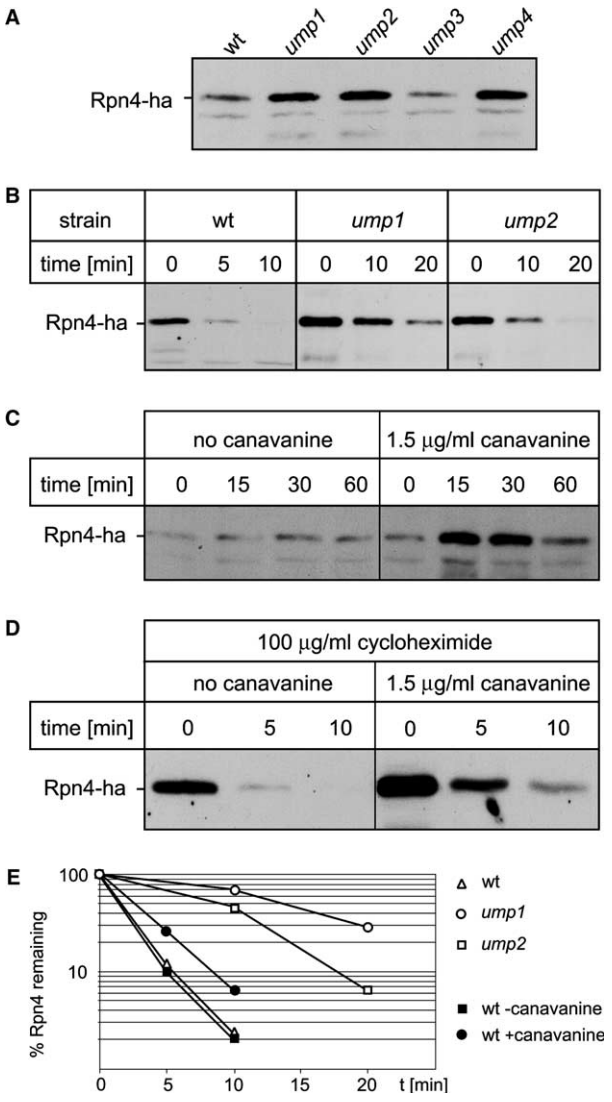

Fig. 4. Defects and overloading of the proteasome induce elevated cellular Rpn4 levels by affecting its metabolic stability. A: Rpn4-ha steady state levels in wild-type and *ump* mutant strains were detected by anti-ha Western blotting. B: Rpn4-ha stability was detected after shutting off expression using cycloheximide. C: Overloading of the proteasome with abnormal proteins leads to an increased level of Rpn4. Strain ML26-2B (Rpn4-ha) was grown to mid-logarithmic phase in SD medium lacking arginine. 1.5 μg/ml canavanine was added to one half of the culture and cells were withdrawn at the indicated time points. Levels of Rpn4-ha were detected by anti-ha Western blotting. D: Increased stability of Rpn4 in cells treated with canavanine. Cells were grown as in (C). Rpn4 stability was determined by expression shut-off analysis as in (B) except that 1.5 μg/ml canavanine was added 25 min prior to cycloheximide addition. E: Quantitation of Rpn4-ha signals shown in (B) (open symbols) and (D) (closed symbols).

overloading of the proteasome with high levels of abnormal proteins resulting from the incorporation of canavanine into newly synthesized polypeptides.

It was reported recently that aside of the genes mentioned Rpn4 controls, directly or indirectly, the expression of more than 700 genes, most of which are associated with responses to a variety of stresses [17]. A large group of these Rpn4-controlled genes is involved in DNA damage repair. This and earlier studies suggested a link of DNA damage repair and the functions of the proteasome [18–20]. Consistent with these studies, we observed that *ump* as well as *rpn4* mutants were hypersensitive to the DNA damage inducing drug MMS (Fig. 5A). This prompted us to ask whether and how the responses to DNA damage and the control of proteasome levels by Rpn4 are connected. Western-blot analysis showed that after addition of 0.1% MMS, the cells immediately responded by increasing the steady state level of Rpn4 protein. This is apparently due to a striking increase of *RPN4* mRNA (Fig. 5B). At the same time the stability of Rpn4 protein was unaffected (Fig. 5C). An increase in transcription of proteasome subunit genes after MMS treatment was detected as a consequence of the elevated levels of Rpn4 (Fig. 5B). Rpn4 itself has been reported to be positively regulated by the transcription factors Pdr1, Pdr3 and Yap1 that mediate multidrug and oxidative stress resistance [21]. Interestingly, *YAP1* itself was reported to contain a PACE sequence in its promoter

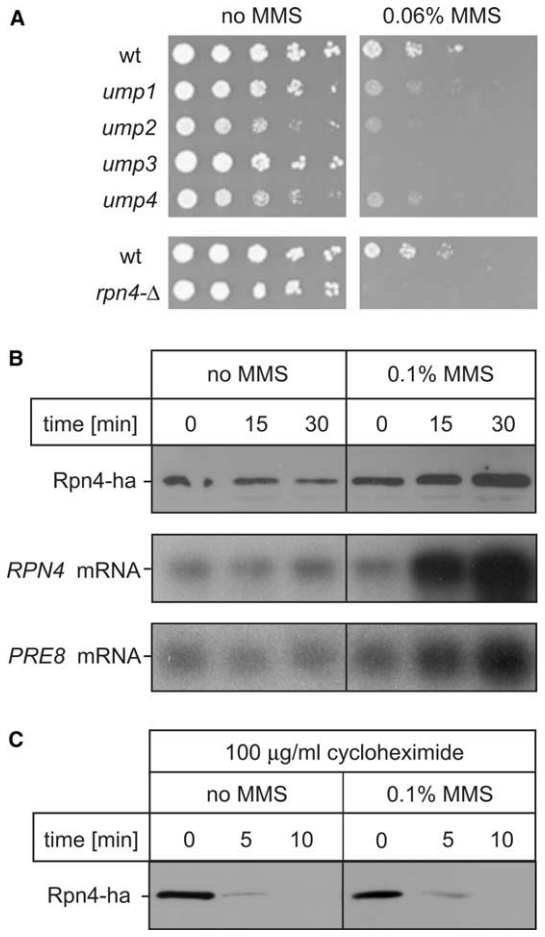

Fig. 5. Rpn4 mediates the up-regulation of the proteasome in response to DNA damage. A: *rpn4*-Δ and *ump* mutant strains are hypersensitive to the DNA damaging drug methyl methanesulfonate (MMS). Serial dilutions of cells were spotted onto plates containing yeast rich medium with or without MMS and incubated for 2 days at 30 °C. B: Induction of Rpn4 and the proteasome after MMS treatment. Strain ML26-2B (Rpn4-ha) was grown to mid-logarithmic phase in YPD. The culture was divided and 0.1% MMS added to one half. Cells were withdrawn at the indicated time points and levels of Rpn4-ha protein analyzed by anti-ha Western blotting. *RPN4* and *PRE8* (encoding proteasome subunit α2) mRNA levels were analyzed by Northern blotting. C: Rpn4-ha stability was assayed after shutting off expression with cycloheximide 10 min after addition of MMS.

[12] suggesting that, in a second level of regulation, the Rpn4 protein may positively regulate expression of its own gene indirectly via induction of Yap1. It remains to be seen whether the observed induction of Rpn4 transcription after MMS treatment involves these transcription factors. Taken together, the data presented here demonstrated that Rpn4 is a sensor of proteasome capacity that controls the levels of proteasomes and of the *UBI4* gene in response to defects and overloading of the proteasome. In addition, Rpn4 mediates up-regulation of proteasome synthesis in response to DNA damage as a consequence of transcriptional induction of *RPN4* itself. Up-regulation of proteasome gene expression has also been observed after treatment of both yeast or mammalian cells with proteasome inhibitors, indicating that feedback regulation of proteasomes occurs in both cell types [22,23]. For mammalian cells, this response is of medical relevance as proteasome inhibitors are being tested as drugs in the treatment of diseases such as cancer and stroke [22–24].

Acknowledgements: We are grateful to Erica Johnson (University of Philadelphia) and Mark Hochstrasser (Yale University, New Haven, CT), respectively, for the gifts of the *rpn4/ufd5* and *doa4* mutant strains, and to Elisabeth Andrews for assistance in cloning *UMP* genes. R.J.D. is indebted to Alexander Varshavsky (Caltech, Pasadena, CA) in whose laboratory this study was initiated. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Do 649) to R.J.D. and from Fundação para a Ciência e Tecnologia (POCTI/BME/32621/2000) to P.C.R.

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