The proteasome biogenesis regulator Rpn4 cooperates with the unfolded protein response to promote resistance to endoplasmic reticulum stress

Rolf M. Schmidt and Sebastian Schuck*

Center for Molecular Biology of Heidelberg University (ZMBH), DKFZ-ZMBH Alliance and CellNetworks Cluster of Excellence, 69120 Heidelberg, Germany

* correspondence: Sebastian Schuck (s.schuck@zmbh.uni-heidelberg.de)
ABSTRACT

Misfolded proteins in the endoplasmic reticulum (ER) activate the unfolded protein response (UPR), which enhances protein folding to restore homeostasis. Additional pathways respond to ER stress, but how they help counteract protein misfolding is incompletely understood. Here, we develop a titratable system for the induction of ER stress in yeast to enable a genetic screen for factors that augment stress resistance independently of the UPR. We identify the proteasome biogenesis regulator Rpn4 and show that it cooperates with the UPR. Rpn4 abundance increases during ER stress, first by a post-transcriptional, then by a transcriptional mechanism. Induction of RPN4 transcription is triggered by cytosolic mislocalization of secretory proteins, is mediated by multiple signaling pathways and accelerates clearance of misfolded proteins from the cytosol. Thus, Rpn4 and the UPR are complementary elements of a modular cross-compartment response to ER stress.
INTRODUCTION

Cells continuously produce a large variety of proteins. To fulfill their functions, these proteins need to be properly folded, post-translationally modified, assembled into complexes and delivered to their final subcellular destinations. If errors occur in these maturation steps, compartment-specific quality control machineries clear the resulting misfolded or mislocalized proteins through refolding or degradation. These machineries are regulated by specialized adaptive responses, which adjust the folding and degradation capacity of particular organelles to ensure efficient elimination of aberrant proteins. If unresolved, protein misfolding in one compartment can disrupt overall cell homeostasis and threaten survival.

Protein maturation in the endoplasmic reticulum (ER), particularly in budding yeast, has served as a powerful paradigm to elaborate these general principles (Patil and Walter, 2001; Barlowe and Miller, 2013; Berner et al., 2018). Newly synthesized polypeptides containing ER sorting information, for example N-terminal signal sequences, are recognized by targeting factors such as signal recognition particle (SRP) and inserted into the ER (Aviram and Schuldiner, 2017). The process of protein insertion into the ER, termed translocation, is mediated by channel-forming translocon complexes (Rapoport, 2007). Translocation additionally requires ER-lumenal chaperones such as the highly abundant Kar2, which therefore have dual roles in protein import and folding (Gething, 1999; Young et al., 2001). Other components of the ER-resident folding machinery include oxidoreductases and glycosyltransferases (Braakman and Hebert, 2013). Proteins that have attained their native conformation are sorted into ER-to-Golgi transport carriers. Proteins that fail to fold properly become subject to ER-associated degradation (ERAD). Key steps in ERAD are protein retrotranslocation into the cytosol with the help of the AAA ATPase Cdc48, followed by degradation by the proteasome (Berner et al., 2018). If these mechanisms are insufficient, misfolded proteins accumulate. This condition constitutes ER stress and activates an adaptive response called the unfolded protein response (UPR) (Walter and Ron, 2011). The UPR is triggered when misfolded proteins are sensed by the ER transmembrane protein Ire1. Activated Ire1 initiates
non-conventional cytosolic splicing of the HAC1 mRNA, enabling production of the transcription factor Hac1. In turn, Hac1 induces numerous genes involved in ER function (Travers et al., 2000). The resulting increase in ER protein folding capacity resolves ER stress, closing a homeostatic feedback loop. The physiological significance of the UPR is demonstrated by yeast mutants lacking Ire1 or Hac1. When challenged by ER stress, these mutants exhibit a variety of defects in translocation, glycosylation, ERAD and ER-to-Golgi transport, and rapidly lose viability (Cox et al., 1993; Spear and Ng, 2003).

A number of UPR-independent pathways respond to, and help mitigate, ER stress. These pathways include MAP kinase signaling through Slt2/Mpk1 and Hog1, the Hsf1-dependent heat shock response and protein kinase A (PKA) signaling (Bonilla and Cunningham, 2003; Chen et al., 2005; Liu and Chang, 2008; Bicknell et al., 2010; Hou et al., 2014; Pincus et al., 2014). However, exactly how they counteract ER stress has been difficult to determine. For instance, ER stress is alleviated by augmented ER-to-Golgi transport and enhanced elimination of reactive oxygen species downstream of the heat shock response and also by reduced protein synthesis downstream of PKA signaling (Liu and Chang, 2008; Hou et al., 2014; Pincus et al., 2014). Yet, these mechanisms only partially explain the beneficial effects of the signaling pathways controlling them. Finally, the UPR can, by unknown means, be amplified by Ire1-independent induction of HAC1 transcription (Leber et al., 2004). Therefore, it remains to be fully defined which pathways cooperate with the UPR and how they contribute to ER stress resistance.

Here, we identify the proteasome biogenesis regulator Rpn4 as an important UPR-independent factor that promotes resistance to ER stress in yeast. We show that protein misfolding induces Rpn4 activity by transcriptional and post-transcriptional mechanisms, and provide evidence that Rpn4 complements the UPR by enhancing protein quality control in the cytosol.
RESULTS

A titratable system for the induction of ER stress

To identify pathways cooperating with the UPR, we searched for genes that can augment resistance to ER stress in UPR-deficient cells. Mutants lacking Ire1 or Hac1 grow normally under optimal conditions but cannot proliferate under even mild ER stress (Cox et al., 1993; Spear and Ng, 2003; Schuck et al., 2009). We hypothesized that UPR mutants can be protected against ER stress by overexpression of genes that complement the UPR. If so, such genes should be identifiable through a screen based on cell growth phenotypes.

To implement this idea, we established a titratable system for the induction of ER stress. We used CPY*, a folding-defective variant of the soluble vacuolar carboxypeptidase Y (Finger et al., 1993). We chose an HA-tagged mutant variant of CPY* that lacks all of its four N-glycosylation sites and is here referred to as non-glycosylatable (ng) CPY*. After translocation into the ER, this variant is unable to fold properly and is neither efficiently cleared by ERAD nor exported to the Golgi complex (Knop et al., 1996; Spear and Ng, 2005; Kostova and Wolf, 2005). As a result, ngCPY* accumulates in the ER lumen and burdens the ER protein folding machinery. We placed ngCPY* under the control of the GAL promoter and the artificial transcription factor Gal4-ER-Msn2 (GEM). This system allows regulation of gene expression with the exogenous steroid β-estradiol (Pincus et al., 2014). Estradiol-driven expression of ngCPY* caused dose-dependent activation of the UPR as measured with a HAC1 splicing reporter (Figure 1A; Pincus et al., 2010). HAC1 splicing obtained with ≥100 nM estradiol was similar to that elicited by 0.5 µg/ml of the general ER stressor tunicamycin (Figure S1A). For all estradiol concentrations tested, HAC1 splicing declined at later time points, suggesting that cells adapted to the stress and inactivated the UPR. Estradiol-induced expression of glycosylatable CPY*, which is degraded through ERAD and can be exported from the ER, yielded much weaker and more transient UPR activation (Figure S1B). Cycloheximide chase experiments confirmed that ngCPY* was substantially more resistant to degradation.
than glycosylatable CPY*, as reported (Figure 1B; Knop et al., 1996). Furthermore, ngCPY* tagged with superfolder GFP (sfGFP) was largely retained in the ER (Figure 1C). In contrast, CPY*(N479Q)-sfGFP, which lacks the glycosylation site required for efficient ERAD but contains the three glycosylation sites needed for ER export, escaped to the vacuole (Figure S1C; Kawaguchi et al., 2010).

Estradiol did not affect growth of wild-type or Δhac1 cells expressing only the artificial transcription factor GEM (Figure 1D). Furthermore, estradiol-induced expression of ngCPY* in wild-type cells caused only modest growth defects on solid media, even at the highest estradiol concentration tested. In Δhac1 cells, however, induction of ngCPY* expression with increasing concentrations of estradiol strongly retarded and eventually prevented cell proliferation. Growth assays in liquid media yielded similar results (Figures 1E and 1F). To determine whether the lack of proliferation of Δhac1 cells reflected a growth arrest or cell death, we assayed cell viability after expression of ngCPY*. No loss of viability of Δhac1 cells occurred, even with estradiol concentrations that completely blocked proliferation (Figures S1D). Hence, expression of ngCPY* merely caused a growth arrest. In summary, this estradiol-controlled system can be titrated to induce defined levels of ER stress and can be used to prevent growth of UPR-deficient cells.

**A screen for genes promoting ER stress resistance in UPR mutants**

We exploited the ngCPY*+-induced growth arrest in UPR mutants to conduct a multicopy suppression screen. Cells lacking HAC1 and containing the titratable ER stress system were transformed with a genomic library in a high-copy vector. Transformants were first allowed to form colonies on estradiol-free solid medium and then replicated onto media containing a range of estradiol concentrations sufficient to block growth of the parental strain. Plasmids were retrieved from transformants able to grow on estradiol-containing plates and their inserts were sequenced. The obtained candidate genes were individually subcloned into the same high-copy vector and tested for their ability to suppress the toxicity of ngCPY* in Δhac1 cells.
As expected, the strongest suppressor was \textit{HAC1}, which complemented the \textit{HAC1} deletion in the parental strain (Table 1). The next strongest suppressors as judged by growth assays on solid and in liquid media were \textit{RPN4}, \textit{PDR1} and \textit{SSZ1} (Figure 2). Rpn4 is a transcription factor that localizes to the cytosol and the nucleus (Tkach et al., 2012). Its primary function is the induction of genes encoding proteasome subunits (Dohmen et al., 2007). Pdr1 is another transcription factor and controls genes involved in multidrug resistance (Prasad and Goffeau, 2012). Ssz1 is an atypical Hsp70 chaperone that is part of the ribosome-associated complex (Conz et al., 2007). \textit{SSZ1} overexpression activates Pdr1, and Pdr1 induces \textit{RPN4} transcription (Hallstrom et al., 1998; Owsianik et al., 2002). Therefore, Ssz1 and Pdr1 may promote resistance to ER stress by raising the levels of Rpn4. Alternatively, \textit{SSZ1} and \textit{PDR1} overexpression could relieve ER stress in our system by enhancing the expression of Pdr1 target genes other than \textit{RPN4}. Candidates include genes for multidrug transporters such as \textit{PDR5}, \textit{PDR10} and \textit{PDR15}, which may export estradiol from cells and thereby lower the levels of ngCPY*. Additional, but weaker suppressors included the transcription factor Yap1, which controls the oxidative stress response and activates \textit{RPN4} transcription (Owsianik et al., 2002), the Yap1 paralog Cad1, the F-box protein Saf1, the protein Mum2 involved in meiotic DNA replication and sporulation, and the essential nucleolar protein Nop56.

The identification of Rpn4 suggests that enhanced proteasome biogenesis promotes ER stress resistance. However, the entirety of genes controlled by Rpn4, here referred to as the Rpn4 regulon, encompasses many genes beyond those encoding proteasome subunits (Mannhaupt et al., 1999; Jelinsky et al., 2000). Rpn4 may therefore counteract ER stress by more than one mechanism. Indeed, Yap1 not only activates \textit{RPN4} but is itself activated by Rpn4 (Mannhaupt et al., 1999) and may aid stress resistance by preventing oxidative damage. Furthermore, we directly tested the Rpn4 target gene \textit{CDC48} (Bosis et al., 2010), even though it was not found in the screen. Overexpression of \textit{CDC48} also restored growth of \textit{Δhac1} cells expressing ngCPY*, although weakly compared to overexpression of \textit{RPN4} (Figures 2B - E). Overall, the outcome of the screen suggests that Rpn4 activity becomes limiting for cell proliferation during ER stress, at least in UPR mutants.
Rpn4 and the UPR cooperate to counteract ER stress

RPN4 is not controlled by Hac1 and hence not a UPR target gene (Travers et al., 2000; Pincus et al., 2014). This lack of a direct link suggests that Rpn4 acts in parallel to, rather than downstream of, the UPR (Ng et al., 2000). To better understand the interplay between Rpn4 and the UPR, we manipulated Rpn4 levels in wild-type and Δhac1 cells and challenged these cells with ngCPY* or tunicamycin (Figure 3A and 3B). Overexpression of RPN4 in wild-type cells increased resistance to both stressors. This observation indicates that Rpn4 activity becomes limiting for cell proliferation during ER stress also when the UPR is intact. Conversely, deletion of RPN4 caused a general growth defect and sensitized cells to ngCPY* and tunicamycin, consistent with previous reports (Wang et al., 2008; Hou et al., 2014; Rousseau and Bertolotti, 2016). As expected, Δhac1 cells were highly vulnerable to either ER stressor, and RPN4 overexpression provided a measure of protection. Δhac1 Δrpn4 cells grew very poorly already in the absence of ngCPY* or tunicamycin, and not at all in their presence. The synthetic sickness phenotype of Δhac1 Δrpn4 cells agrees with earlier reports and shows that Rpn4 and the UPR are functionally linked (Ng et al., 2000; Hou et al., 2014).

The slow growth of Δhac1 Δrpn4 compared with Δrpn4 cells even under optimal conditions implies that the UPR is constitutively active and physiologically important in Rpn4-deficient cells. Indeed, HAC1 splicing was elevated in untreated Δrpn4 cells, consistent with high throughput data (Figure 3C; Jonikas et al., 2009). The activation of the UPR in the absence of Rpn4 likely arises from inefficient ERAD and a subsequent buildup of misfolded proteins in the ER (Ng et al., 2000; Bosis et al., 2010). Accordingly, constitutive UPR signaling in Δrpn4 cells was similar to that in cells lacking the ubiquitin ligase Hrd1, which is essential for ERAD. Furthermore, quantitative real-time PCR showed that mRNA levels of the UPR target genes KAR2 and SIL1 were increased in untreated Δrpn4 cells (Figures 3D and 3E). Hence, cells compensate the lack of Rpn4 by activating the UPR.
To test whether the inverse is true and cells compensate the lack of a functionally sufficient UPR by increasing Rpn4 activity, we monitored Rpn4 abundance. Rpn4 is a short-lived protein that is rapidly turned over by the proteasome (Xie and Varshavsky, 2001). When proteasome capacity becomes limiting, Rpn4 accumulates and promotes proteasome biogenesis until its degradation is restored. This negative feedback loop homeostatically adjusts proteasome activity (Dohmen et al., 2007). The levels of chromosomally tagged Rpn4-HA in wild-type and Δhac1 cells were similar at steady state (Figures 4A and 4B). Upon treatment with 2 µg/ml tunicamycin, they increased two-fold within 15 minutes. Subsequently, Rpn4 abundance continued to rise but did so more strongly in Δhac1 cells, reaching more than four-fold basal levels after 60 minutes. To determine whether this rise involved an upregulation of RPN4 transcription, we analyzed RPN4 mRNA by quantitative real-time PCR. In wild-type cells, tunicamycin treatment for up to 60 minutes induced the UPR target genes KAR2 and SIL1, but not RPN4 (Figures 4C - E). Therefore, under these conditions, the increase in Rpn4 abundance in wild-type cells occurs by a post-transcriptional mechanism, presumably by slowed Rpn4 degradation. In Δhac1 cells, induction of KAR2 and SIL1 was strongly diminished, but RPN4 mRNA levels increased. While we cannot rule out changes in mRNA stability, we assume that this rise reflects enhanced transcription. This response likely contributes to the more pronounced stress-induced increase in Rpn4 abundance in Δhac1 cells. Importantly, prolonged ER stress induced with 5 µg/ml tunicamycin elevated RPN4 mRNA levels also in wild-type cells (Figure 4F). Hence, during ER stress, cells augment the UPR by enhancing Rpn4 activity. These results show that activation of the UPR and the Rpn4 regulon are two cooperating elements of the cellular response to ER stress.

**Rpn4 is upregulated by and protects against mislocalized secretory proteins**

We next asked how ER stress upregulates Rpn4. The stronger increase in Rpn4 abundance and the more sensitive RPN4 transcriptional response in Δhac1 cells may reflect more severe ER stress in these mutants. To test this assumption, we used protein translocation as readout for ER function. When the capacity of ER chaperones is exhausted, they can no longer assist protein import. As a result,
translocation is compromised, including that of Kar2 itself (Vogel et al., 1990). Western blotting showed a single band for Kar2 in untreated wild-type and \( \Delta hac1 \) cells (Figure 5A). Treatment with 2 \( \mu g/ml \) tunicamycin for up to 60 minutes did not change Kar2 levels. However, a second Kar2 band of slightly higher molecular weight appeared in \( \Delta hac1 \) cells, starting at 30 minutes. Since Kar2 is not glycosylated, this upshift indicates retention of its cleavable signal sequence (ss) and suggests that the slower migrating form corresponds to untranslocated ss-Kar2 (Ng et al., 1996). Hence, under these conditions, \( \Delta hac1 \) cells show signs of overwhelmed chaperone capacity. Treatment with 5 \( \mu g/ml \) tunicamycin increased Kar2 abundance in wild-type cells and caused the appearance of ss-Kar2 in both strains (Figures 5B). Therefore, strong ER stress impairs translocation also in wild-type cells. These results confirm that ER stress is initially buffered by the UPR in wild-type cells but rapidly disrupts ER function in \( \Delta hac1 \) cells.

The above results reveal a conspicuous correlation between impaired translocation and increased \( RPN4 \) mRNA levels. Both phenomena occur in \( \Delta hac1 \) cells exposed to 2 \( \mu g/ml \) tunicamycin, whereas 5 \( \mu g/ml \) are required in wild-type cells. Impaired translocation and increased \( RPN4 \) mRNA levels could be unrelated consequences of ER stress. Alternatively, their correlation could reflect a causal relationship, with translocation defects activating \( RPN4 \) transcription. To distinguish between these possibilities, we employed \( \text{sec65}-1 \) cells, which express a temperature-sensitive variant of the SRP subunit Sec65 (Stirling et al., 1992). Disruption of SRP function rapidly inhibits translocation and causes accumulation of secretory proteins in the cytosol, where they are unable to fold properly. As expected, Kar2 translocation was intact in \( \text{sec65}-1 \) cells at the permissive temperature of 25°C but impaired upon a shift to 30°C or above (Figure 5C; Ng et al., 1996). \( RPN4 \) mRNA levels did not change when wild-type or \( \text{sec65}-1 \) cells were shifted from 25°C to 28°C (Figure S2). However, shifts to temperatures of 30°C or above increased \( RPN4 \) mRNA levels specifically in \( \text{sec65}-1 \) cells (Figure 5D and S2). Importantly, \( HAC1 \) splicing was not activated under these conditions (Figure 5E). Therefore, disrupted translocation induces the \( RPN4 \) gene even in the absence of ER stress. This finding indicates that stress-induced translocation defects activate \( RPN4 \) transcription.
To examine the physiological significance of RPN4 expression in cells suffering from translocation defects, we analyzed growth of sec65-1 cells at different temperatures. Cells grew normally at up to 28°C but showed almost no growth at 30°C or above (Figure 6A). These observations are consistent with the described tight temperature sensitivity of the sec65-1 allele (Stirling et al., 1992). RPN4 overexpression restored some growth at 30 and 32°C, showing that the levels of Rpn4 were physiologically important under these conditions. Next, we tested whether elevated Rpn4 abundance promotes the degradation of misfolded cytosolic proteins. Cycloheximide chase experiments showed that RPN4 overexpression accelerated the degradation of Δss-ngCPY*-HA, which mislocalizes to the cytosol due to deletion of its signal sequence (Figures 6B and 6C). The same was true for Luciferase(DM)-mCherry, another misfolded cytosolic model protein (Figure 6D). Collectively, these experiments indicate that the Rpn4 regulon is activated by and protects against cytosolic mislocalization of secretory proteins.

**Multiple signaling pathways mediate RPN4 induction by ER stress**

Finally, we investigated through which signaling pathways ER stress induces the RPN4 gene. The RPN4 promoter contains well-characterized binding sites for Pdr1/3, Yap1 and Hsf1, called the Pdr1/3 response element (PDRE), Yap1 response element (YRE) and heat shock element (HSE), respectively (Owsianik 2002; Hahn et al., 2006). To test the relevance of these binding sites, we generated RPN4 reporters in which different RPN4 promoter variants controlled expression of the fast-maturing fluorescent protein mNeonGreen and measured mNeonGreen levels by flow cytometry. Steady-state activity of the RPN4 reporter was essentially unchanged by mutation of the two PDREs or the YRE but was reduced by 40% upon mutation of the HSE (Figure 7A). Tunicamycin treatment activated the RPN4 reporter, which was unaffected by mutation of any of the above promoter elements (Figure 7B). Hence, Hsf1 regulates basal RPN4 activity, but Pdr1/3, Yap1 and Hsf1 appear to be individually dispensable for RPN4 induction by ER stress.
Prolonged tunicamycin treatment inhibits PKA, resulting in derepression of the general stress response transcription factors Msn2 and Msn4 (Görner et al., 1998; Pincus et al., 2014). We therefore asked whether Msn2/4 downstream of PKA can activate the \textit{RPN4} gene. We used a strain in which the PKA isoforms Tpk1/2/3 had been modified such that their enlarged ATP binding pockets allowed specific inhibition of these kinases with the bulky ATP analog 1NM-PP1 (Hao and O’Shea, 2011). 1NM-PP1 treatment of cells harboring the analog-sensitive \textit{tpk1/2/3-as} alleles induced both the \textit{RPN4} reporter and the endogenous \textit{RPN4} gene (Figures 7C and 7D). As expected, deletion of \textit{MSN2/4} strongly reduced activation of the \textit{RPN4} reporter upon PKA inhibition (Figure 7C). Activation of the \textit{RPN4} reporter by tunicamycin was blunted in \textit{Δmsn2/4} cells but not blocked (Figure 7E), suggesting that prolonged ER stress activates partially redundant mechanisms to induce \textit{RPN4}. Accordingly, combined deletion of \textit{MSN2/4} and mutation of the YRE or the HSE additively reduced activation of the \textit{RPN4} reporter. Mutation of both the YRE and the HSE almost completely abolished reporter activation in a \textit{Δmsn2/4} background. These results show that multiple signaling pathways contribute to induction of \textit{RPN4} transcription during ER stress.
DISCUSSION

We have shown that Rpn4 and the UPR cooperate to counteract ER stress. Based on our findings, we propose the following model (Figure 7F). Protein misfolding in the ER burdens the proteasome by increased flux through the ERAD pathway, activates the UPR and eventually impairs protein translocation into the ER. As a result, secretory proteins mislocalize to the cytosol, where they are unable to fold properly and further strain proteasome capacity. Inefficient proteasomal degradation leads to an increase in Rpn4 abundance and activation of the Rpn4 regulon, which enhances proteasome biogenesis to clear misfolded ER and cytosolic proteins. Furthermore, persistent cytosolic protein misfolding induces RPN4 transcription through the transcription factors Msn2/4, Yap1 and Hsf1. The resulting rise in Rpn4 protein levels reinforces activation of the Rpn4 regulon.

Rpn4 abundance is controlled by rapid proteasomal turnover (Xie and Varshavsky, 2001) and by RPN4 gene activity. ER stress initially increases Rpn4 protein levels. Persistent ER stress, however, additionally raises RPN4 mRNA levels. These observations suggest a biphasic Rpn4 response. First, when misfolded proteins occupy the proteasome, Rpn4 is spared from degradation and activates its target genes, but RPN4 transcription remains unchanged. This scenario likely applies to early phases of ER stress and cases of mild stress. For instance, expression of the misfolded ER membrane protein Ste6* activates the Rpn4 regulon but not RPN4 transcription (Metzger and Michaelis, 2009). If slowed Rpn4 degradation is insufficient to resolve the stress, RPN4 transcription is upregulated in a second phase of the response, which provides another boost in Rpn4 target gene induction. Msn2/4, Yap1 and Hsf1 contribute to the stress-induced upregulation of RPN4, indicating that parallel pathways communicate protein misfolding to the RPN4 promoter. This situation is distinct from RPN4 induction after glucose starvation or heat shock, which requires Hsf1 but not Msn2/4 (Hahn et al., 2004a). Interestingly, the RPN4 promoter does not contain a canonical stress response element as binding site for Msn2/4 (Martinez-Pastor et al., 1996). Therefore, it is not clear whether Msn2/4 bind to the RPN4 promoter directly, although high throughput data hint at this
possibility (Harbison et al., 2004; Huebert et al., 2012). Furthermore, it remains to be determined exactly how cytosolic misfolding is sensed by Msn2/4, Hsf1 and Yap1. While the detection of misfolded cytosolic proteins by Hsf1 has been studied extensively, it is unknown how protein kinase A, which controls Msn2/4 and possibly also Hsf1 (Vergheese et al., 2012), may monitor protein folding. Finally, it is unclear whether Yap1 is activated by misfolded proteins. Alternatively, its contribution to RPN4 activation could reflect the fact that YAP1 is an Rpn4 target gene (Mannhaupt et al., 1999) and can reinforce RPN4 transcription as part of a positive feedback loop.

Rpn4 controls genes involved in many processes, including proteasome biogenesis, protein ubiquitination and DNA repair (Mannhaupt et al., 1999; Jelinski et al., 2000). Hence, the Rpn4 regulon could increase ER stress resistance through a combination of functional outputs. However, it has been shown that the sensitivity of Δrpn4 cells to various stresses, including ER stress, results from their inability to upregulate proteasome genes (Wang et al., 2008). Enhanced proteasome biogenesis is therefore likely to be critical for the promotion of ER stress resistance by Rpn4. Accordingly, overexpression of the Rpn4 target genes YAP1 or CDC48 increases ER stress resistance of UPR mutants much more weakly than RPN4 overexpression. Therefore, the relevant functions of Rpn4 must extend beyond inducing Yap1-driven oxidative stress tolerance or Cdc48-dependent ERAD, and our data support the notion that enhancing degradation of cytosolically mislocalized secretory proteins is a major factor. Nevertheless, the relative contributions of different components of the Rpn4 regulon to ER stress resistance remain to be delineated.

An effective UPR requires sufficient proteasome capacity to remove misfolded proteins through ERAD. The UPR controls genes encoding ERAD components but does not regulate proteasome biogenesis (Travers et al., 2000). Conversely, the Rpn4 regulon comprises most genes for proteasome subunits but only a small number of genes involved in ER protein folding (Mannhaupt et al., 1999). Hence, the transcriptional programs activated by the UPR and Rpn4 are largely distinct. They are, however, functionally complementary and represent two cooperating modules. This functional complementarity explains the strong negative genetic interaction
between HAC1 and RPN4. Remarkably, ER stress activates a second signaling pathway that promotes proteasome biogenesis. The Slt2/Mpk1 MAP kinase, which augments ER stress resistance, controls chaperones responsible for 19S regulatory particle assembly and increases the abundance of complete 26S proteasomes during stress (Bonilla and Cunningham, 2003; Chen et al., 2005; Rousseau and Bertolotti, 2016). Taken together, it is evident that proteasome biogenesis is an important UPR-independent process that enhances resistance to ER stress.

The mechanisms adjusting proteasome abundance in yeast and mammals share extensive similarities. Proteasome inhibition, which mimics proteasome overload, induces proteasome genes also in mammalian cells (Mitsiades et al., 2002; Meiners et al., 2003). This response is mediated by the transcription factor Nrf1 and the Slt2/Mpk1 homolog Erk5 (Radhakrishnan et al., 2010; Steffen et al., 2010; Rousseau and Bertolotti, 2016). Similar to Rpn4, Nrf1 is short-lived and activates many proteasome genes (Radhakrishnan et al., 2010; Steffen et al., 2010). Remarkably, Nrf1 turnover is mediated by ERAD, coupling proteasome biogenesis to the capacity of the ER quality control machinery (Steffen et al., 2010). Furthermore, understanding the links between the UPR and proteasome biogenesis is relevant for human disease. Multiple myeloma cells suffer from chronic ER stress and are highly sensitive to proteasome inhibition, implying that proteasome capacity is limiting for survival of these cells. This insight has led to major improvements in the treatment of plasma cell cancer through the use of proteasome inhibitors (Goldberg, 2012).

A simple perspective on adaptive responses is that protein misfolding in a particular subcellular compartment triggers a dedicated program that enhances quality control specifically in the troubled organelle. Our work supports a more holistic view that emphasizes two additional concepts: functional modularity and cross-compartment coordination. Regulated proteasome biogenesis is a functional module that serves as part of the cellular response to ER stress. Interestingly, proteasome biogenesis is also activated by impaired mitochondrial protein import (Wrobel et al., 2015; Wang and Chen, 2015). It may therefore be a functional module that is commonly employed when stress necessitates clearance of mislocalized proteins from the
cytosol. Autophagy and global attenuation of translation may be other such modules (Gasch et al., 2000). Furthermore, adaptive responses are not restricted to the compartment where stress initially arises. For example, as shown here, ER stress activates the UPR but also the Rpn4 regulon to safeguard against protein misfolding in the cytosol. Second, certain types of mitochondrial stress trigger the mitochondrial unfolded protein response but also activate the cytosolic heat shock response and promote proteasome biogenesis (Ho et al., 2006; Wrobel et al., 2015; Kim et al., 2016). Third, cytosolic folding stress activates the Hsf1-dependent heat shock response, which promotes protein folding in the cytosol but also controls the expression of major chaperones that function elsewhere, including Kar2 in the ER and Ssc1 in mitochondria (Yamamoto et al., 2005; Hahn et al., 2004b). Fourth, the UPR regulates genes that function in the entire secretory pathway (Travers et al., 2000). Many additional links between different organelle quality control systems exist in yeast and higher eukaryotes (Higuchi-Sanabria et al., 2018). A driving force for the evolution of these links may have been the need to prevent the spread of protein folding problems throughout the cell. Overall, it emerges that adaptive responses, although triggered by stress in one compartment, engage a combination of functional modules for comprehensive cell protection across compartment boundaries.
METHODS

Plasmids

Plasmids are listed in Table S1. Sequence information is available upon request. Sequences of oligonucleotides for plasmid generation are given in Table S2. To generate plasmids for estradiol-inducible expression, \( P_{GAL1} - T_{CYC} \) was amplified from pRS416-\( P_{GAL1} \) (Mumberg et al., 1994) with primers up_SacI_GAL/CYC_KpnI_down and cloned into AgeI-linearized pRS306-\( P_{ADH1} \)-GEM or ApaI-linearized pNH605-\( P_{ADH1} \)-GEM (Pincus et al., 2014) by ligation with the NEBuilder HiFi DNA assembly master mix (New England Biolabs, Ipswitch, Massachusetts), yielding pRS306-\( P_{ADH1} \)-GEM-\( P_{GAL1} \) and pNH605-\( P_{ADH1} \)-GEM-\( P_{GAL1} \). Plasmids for expression of HA-tagged CPY* variants were subsequently generated by amplification of ngCPY*-HA or CPY*-HA from pRS315-\( P_{CPY} \)-ngCPY*-HA or pRS315-\( P_{GAL1} \)-CPY*-HA (Spear and Ng, 2003) with primers EDY*_F2/EDY*_R and ligation with SmaI-linearized pRS306-\( P_{ADH1} \)-GEM-\( P_{GAL1} \), yielding pRS306-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-ngCPY*-HA and pRS306-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-CPY*-HA. Plasmids for expression of sfGFP-tagged CPY* variants were generated as follows: FLAG-sfGFP was amplified from pRS305-\( P_{ADH1} \)-Rtn1-FLAG-sfGFP (Szoradi et al., 2018) with primers FLAG-sfGFP_F/FLAG-sfGFP_R and ligated with SmaI-linearized pNH605-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-FLAG-sfGFP. Next, ngCPY*-HA was amplified from pRS315-\( P_{CPY} \)-ngCPY*-HA with primers EDY*_F2/EDY*_tag_R and ligated into pNH605-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-FLAG-sfGFP linearized with primers FLAG for 1/FLAG-open_R, yielding pNH605-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-ngCPY*-HA-sfGFP. Similarly, CPY*(N479Q)-HA was amplified from pRS305-\( P_{CPY} \)-CPY*(N479Q)-HA with primers EDY*_F2/EDY*_tag_R and ligated into pNH605-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-FLAG-sfGFP linearized with primers FLAG for 1/FLAG-open_R, yielding pNH605-\( P_{ADH1} \)-GEM-\( P_{GAL1} \)-CPY*(N479Q)-HA-sfGFP. To subclone genes of interest into YEp13, their coding regions together with upstream and downstream sequences were amplified from yeast genomic DNA with primers providing homologous ends (Table S2). The upstream and downstream sequences encompassed the entire sequence between the coding region of interest and the next upstream and downstream coding region, or at least 400 bp of upstream and 200 bp of downstream sequence. The resulting PCR products were
recombined with BamHI-linearized YEp13 through gap repair cloning in yeast. To generate pRS306-P<sub>ADH1</sub>-GEM-P<sub>GAL1</sub>-Δss-ngCPY*-HA, pRS306-P<sub>ADH1</sub>-GEM-P<sub>GAL1</sub>-ngCPY*-HA was linearized by PCR with primers CPY deltaSS fw/CPY deltaSS rev and religated with the NEBuilder HiFi DNA assembly master mix, thereby eliminating the CPY signal sequence. To generate pNH605-P<sub>RPN4</sub>-mNeonGreen, mNeonGreen was amplified from pFA6a-mNeonGreen-kanMX4 with primers Xho-neon/neon-BamHI and cloned between the XhoI and BamHI sites of pNH605-P<sub>RPN4</sub>-YFP. Similarly, pNH605-P<sub>RPN4</sub>-(HSEm)-mNeonGreen and pNH605-P<sub>RPN4</sub>-(PDREm)-mNeonGreen were generated by replacing YFP in pNH605-P<sub>RPN4</sub>-(HSEm)-YFP and pNH605-P<sub>RPN4</sub>-(PDREm)-YFP with mNeonGreen. To generate pNH605-P<sub>RPN4</sub>-(YREm)-mNeonGreen and pNH605-P<sub>RPN4</sub>-(YREm,HSEm)-mNeonGreen, pNH605-P<sub>RPN4</sub>-mNeonGreen or pNH605-P<sub>RPN4</sub>-(HSEm)-mNeonGreen were linearized with primers NheI-YRE fw/NheI-YRE rev, digested with Nhel and religated, thereby mutating the YRE to an Nhel site.

**Yeast strain generation**

Strains used in this study are listed in Table S3. Unless indicated otherwise, strains were derived from W303 mating type a (strain SSY122). Gene tagging and deletion was done with PCR products with homologous ends (Longtine et al., 1998; Janke et al., 2004). For irreversible single-copy genomic integration of CPY* expression plasmids, the P<sub>ADH1</sub>-GEM-P<sub>GAL1</sub> expression cassette was amplified with primers knock-in URA3 fw/knock-in URA3 rev (Table S2) and integrated into the URA3 locus. Other integrative plasmids were linearized by restriction digest before transformation. Similarly, the expression cassettes of pNH605 plasmids were excised with PmeI to facilitate integration. For experiments with sec65-1 cells, strain SSY002 (W303 mating type alpha) rather than SSY122 was used as a wild-type control because it has the same mating type and also is an ade2 mutant.

**Growth conditions**

Strains were cultured at 30ºC in SCD medium consisting of 0.7% yeast nitrogen base, 2% glucose and amino acids, lacking leucine where appropriate to maintain plasmid selection. Sec65-1 cells were grown in the same medium at 25ºC. For
steady state analyses, cultures were grown to saturation, diluted and grown for at least 9 h so that they reached mid log phase (OD\textsubscript{600} = 0.5 – 1). For induction of CPY\textsuperscript{*} expression, exponentially growing cells were diluted to early log phase (OD\textsubscript{600} = 0.1 – 0.5) and treated with 50 nM \(\beta\)-estradiol (Sigma-Aldrich, St. Louis, Missouri) for 4 h, unless indicated otherwise. For cycloheximide chase experiments, cells in mid log phase were treated with 50 \(\mu\)g/ml cycloheximide (Sigma-Aldrich). For tunicamycin treatment, exponentially growing cells were diluted to early log phase and treated with 2 or 5 \(\mu\)g/ml tunicamycin (Merck, Darmstadt, Germany) as indicated. For 1NM-PP1 treatment, exponentially growing cells were diluted to early log phase and treated with 3 \(\mu\)M 1NM-PP1 (Merck). For temperature shift experiments, cells grown to mid log phase at 25\(^\circ\)C were diluted to OD\textsubscript{600} = 0.2 and incubated at the indicated temperatures for 90 min.

\textit{HAC1} splicing assay

To measure UPR activity, a \textit{HAC1} splicing reporter was used that translates Ire1 activity into the production of GFP (Pincus et al., 2010). To measure induction of the UPR, cells harboring this reporter were grown to mid log phase in 1 ml medium in 96 deep-well plates. Cells were diluted to early log phase, treated with estradiol or tunicamycin as described above, 100 \(\mu\)l aliquots were removed at each time point and GFP fluorescence after excitation with a 488 nm laser was measured with a FACS Canto flow cytometer (BD Biosciences, Franklin Lakes, New Jersey) equipped with a high-throughput sampler. In parallel, autofluorescence was determined with identically grown isogenic control strains not harboring the splicing reporter. Mean cellular GFP fluorescence was corrected for autofluorescence and normalized to the GFP fluorescence of untreated cells. To measure steady state UPR activity in different strains, cells expressing the \textit{HAC1} splicing reporter and cytosolic BFP under the control of the constitutive \textit{GPD} promoter were grown to mid log phase as above and GFP and BFP fluorescence were measured after excitation with 488 nm or 405 nm lasers. GFP fluorescence was corrected for autofluorescence and divided by BFP fluorescence to account for differences in protein translation capacity. Data were expressed relative to the GFP/BFP fluorescence ratio in wild-type cells.
Western blotting
Cell lysis and western blotting was done as described (Szoradi et al., 2018). In brief, cells were disrupted by bead beating, proteins were solubilized with SDS, protein determination was carried out with the BCA assay kit (Thermo Fisher Scientific Pierce, Waltham, Massachusetts), equal amounts of protein were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with primary and HRP-coupled secondary antibodies, developed with homemade ECL, and chemiluminescence was detected with an ImageQuant LAS 4000 imaging system (GE Healthcare, Chalfont St Giles, UK). Images were quantified with ImageJ and processed with Adobe Photoshop. Primary antibodies were rat anti-HA 3F10 (Roche, Basel, Switzerland), rabbit anti-Kar2 (Peter Walter, UCSF), rabbit anti-mCherry (Biovision, Milpitas, California) and mouse anti-Pgk1 22C5 (Abcam, Cambridge, UK).

Light microscopy
CPY* expression was induced with 25 nM estradiol for 4 h. Ten µM CMAC (Thermo Fisher Scientific, Waltham, Massachusetts) was added during the last 2 h of induction to stain the vacuole and cells were imaged live at room temperature. Images were acquired with a DMI8 inverted microscope (Leica, Wetzlar, Germany) equipped with a CSU-X1 spinning-disk confocal scanning unit (Yokogawa, Musashino, Japan), a ORCA-Flash 4.0 LT camera (Hamamatsu, Hamamatsu, Japan) and a HC PL APO 100x/1.4 NA CS2 oil objective lens (Leica). Background subtraction with a rolling ball algorithm was performed in ImageJ and images were processed in Adobe Photoshop.

Growth assays
Growth assays on agar plates and in liquid medium were done as described (Schuck et al., 2009; Szoradi et al., 2018). For growth assays on agar plates, dilution series with fivefold dilution steps were used. For quantification of growth in liquid medium, the cell density in arbitrary units was plotted against time and the area under the curve was calculated with the R package Growthcurver (Sprouffske and Wagner,
Data were normalized to the wild-type control and expressed as a growth index, which was set to 1 for wild-type cells.

**Viability assay**
Exponentially growing cells were diluted to $\text{OD}_{600} = 0.05$ and grown in the presence of different concentrations of $\beta$-estradiol for 24 h. Cultures were diluted to equal cell densities as judged by $\text{OD}_{600}$ measurements, equal numbers of cells were plated on solid YPD medium (1% yeast extract, 2% peptone, 2% glucose) and grown for 48 h. To determine cell viability, the number of colony-forming units from estradiol-treated samples was normalized to the number of colony-forming units from mock treated cells.

**Genetic screen**
Strain SSY1341 was transformed with a yeast genomic library in the YEp13 multicopy vector (Nasmyth and Tatchell, 1980; available from the American Type Culture Collection as ATCC 37323; kindly provided by Michael Knop, ZMBH). Transformants were plated onto SCD-Leu plates at approximately 200 colony-forming units per plate and grown at 30ºC. After 26 h, colonies were replicated onto SCD-Leu plates containing 50, 75 or 100 nM estradiol and grown for up to 36 h. Colonies that clearly grew better than the general background were restreaked onto SCD-Leu plates and replicated onto SCD-inositol to identify transformants that grew due to re-expression of $HAC1$. For confirmation, plasmids were retrieved from six transformants that grew in the absence of inositol and sequenced with primers YEp13 fw/YEp13 rev (Table S2). All contained $HAC1$. Transformants that failed to grow without inositol and hence lacked $HAC1$ were re-tested by growth assays on SCD-Leu plates containing 50 nM estradiol. Suppressing plasmids were retrieved from well-growing transformants and their inserts were sequenced. Inserts contained between one and six genes. To determine which genes were responsible for suppression, candidates were individually subcloned into YEp13 from genomic DNA from SSY122 and tested for growth on SCD-Leu plates containing 50 nM estradiol. Genes identified only once were discarded, with the exception of $PDR1$ and $YAP1$. 

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Quantitative real-time PCR

Isolation of mRNA, cDNA synthesis and quantitative real-time PCR were done as described (Szoradi et al., 2018). In brief, RNA from 5 ODs of cells was extracted with hot phenol, precipitated with ethanol and resuspended in 30 µl H2O. Synthesis of cDNA was done from 0.5 µg total RNA with the Protoscript II kit (New England Biolabs) using d(T)23 VN primers and PCRs were run on a LightCycler II 480 (Roche) using the SensiFAST SYBR No-ROX kit (Bioline, Luckenwalde, Germany). TAF10 mRNA served as internal standard to determine relative mRNA levels of KAR2, SIL1 or RPN4. Primer sequences are listed in Table S2. Information on PCR conditions is available upon request.

RPN4 reporter assay

Cells harboring an RPN4 reporter and expressing cytosolic BFP under the control of the constitutive GPD promoter were grown to mid log phase in 1 ml medium in 96 deep-well plates. To measure steady state RPN4 activity, 100 µl aliquots were removed and mNeonGreen and BFP fluorescence were measured after excitation with 488 nm or 405 nm lasers with a FACS Canto flow cytometer (BD Biosciences) equipped with a high-throughput sampler. In parallel, autofluorescence was determined with identically grown isogenic control strains not harboring an RPN4 reporter. Mean cellular mNeonGreen fluorescence was corrected for autofluorescence and divided by BFP fluorescence. Data were expressed relative to the mNeonGreen/BFP fluorescence ratio in cells harboring the wild-type RPN4 reporter. To measure induction of RPN4, cells were grown to mid log phase as above, diluted to early log phase and either left untreated or were treated with 5 µg/ml tunicamycin or 3 µM 1NM-PP1. At each time point, 100 µl aliquots were removed and mNeonGreen and BFP fluorescence were determined. For each time point, mNeonGreen/BFP fluorescence ratios were calculated as above and ratios in treated cells were normalized to those in corresponding untreated cells.
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FIGURE LEGENDS

Figure 1. A titratable system for the induction of ER stress. (A) Flow cytometric measurement of GFP levels in cells harboring the HAC1 splicing reporter and expressing ngCPY* under the control of the estradiol-inducible GAL promoter system. For each time point, data are normalized to untreated cells. Mean ± SEM, number of independent experiments (n) = 3. (B) Western blot of HA and Pgk1 from cells expressing CPY*-HA or ngCPY*-HA. Cells were treated with cycloheximide (CHX) for the times indicated. Pgk1 served as a loading control. (C) Images of cells expressing ngCPY*-sfGFP and the general ER marker Sec63-mCherry. Expression of ngCPY*-sfGFP was induced with 25 nM estradiol for 4 h and cells were stained with the vacuole dye CMAC. (D) Growth assay on solid media of wild-type (WT) and Δhac1 cells expressing the estradiol-inducible artificial transcription factor GEM and, where indicated, ngCPY* under the control of the GAL promoter. For each strain, series represent fivefold dilution steps. (E) Growth assay in liquid media of WT cells expressing ngCPY* under the control of the estradiol-inducible GAL promoter system. a.u., arbitrary units. (F) As in panel E, but with Δhac1 cells.

Table 1. Genes identified in the screen.

Figure 2. Multicopy suppression of ngCPY* toxicity in Δhac1 cells. (A) Growth assay on solid media of wild-type (WT) and Δhac1 cells expressing ngCPY* under the control of the estradiol-inducible GAL promoter system and overexpressing RPN4, SSZ1 or PDR1 where indicated. (B) Growth assay in liquid medium of WT and Δhac1 cells expressing ngCPY* under the control of the estradiol-inducible GAL promoter system. Δhac1 cells additionally overexpressed the indicated genes. Cells were grown without estradiol. a.u., arbitrary units. (C) As in panel B, but in the presence of 50 nM estradiol. (D) Quantification of growth assays as shown in panel B. Data are normalized to WT cells. Mean ± SEM, n = 4. (E) Quantification of growth assays as shown in panel C. Data are normalized to WT cells. Mean ± SEM, n = 4.
**Figure 3.** Rpn4 and the UPR cooperate to counteract ER stress. (A) Growth assay on solid media of wild-type (WT), Δrpn4, Δhac1 and Δhac1 Δrpn4 cells, overexpressing RPN4 where indicated. (B) As in panel A, but on media containing different concentrations of tunicamycin (Tm). (C) Flow cytometric measurement of GFP levels relative to cytosolic BFP in WT, Δrpn4 and Δhrd1 cells harboring the HAC1 splicing reporter. Data are normalized to WT cells. Mean ± SEM, n = 3. (D) KAR2 mRNA levels in WT and Δrpn4 cells as measured by quantitative real-time PCR. Data are normalized to WT cells. Mean ± SEM, n = 3. (E) As in panel D, but for SIL1.

**Figure 4.** ER stress increases Rpn4 abundance and induces RPN4 transcription. (A) Western blot of HA and Pgk1 from wild-type (WT) and Δhac1 cells expressing Rpn4-HA and treated with 2 μg/ml tunicamycin (Tm) for the times indicated. (B) Quantification of Rpn4-HA protein levels relative to Pgk1 from western blots as shown in panel A. Data are normalized to WT cells at t = 0. Mean ± SEM, n = 5. (C) KAR2 mRNA levels in WT and Δhac1 cells treated with 2 μg/ml tunicamycin for the times indicated. Data are normalized to WT cells at t = 0. Mean ± SEM, n = 3. (D) As in panel C, but for SIL1. (E) As in panel C, but for RPN4. (F) As in panel C, but for RPN4 after treatment with 5 μg/ml tunicamycin for the times indicated.

**Figure 5.** RPN4 is upregulated by cytosolic mislocalization of secretory proteins. (A) Western blot of Kar2 and Pgk1 from WT and Δhac1 cells treated with 2 μg/ml tunicamycin for the times indicated. The asterisk indicates untranslocated ss-Kar2. (B) As in panel A, but with 5 μg/ml tunicamycin. (C) Western blot of Kar2 and Pgk1 from sec65-1 cells grown at 25°C and shifted to the indicated temperatures for 90 min. The asterisk indicates untranslocated ss-Kar2. (D) RPN4 mRNA levels in WT and sec65-1 cells grown at 25°C and shifted to the indicated temperatures for 90 min. Data are normalized to WT cells at 25°C. Mean ± SEM, n = 3. (E) Flow cytometric measurement of GFP levels in WT and sec65-1 cells harboring the HAC1 splicing reporter. Cells grown at 25°C were shifted to the indicated temperatures or treated with 2 μg/ml tunicamycin (Tm) for 90 min. For each strain, data are normalized to 25°C. Mean ± SEM, n = 3.
Figure 6. Rpn4 protects against cytosolic protein misfolding. (A) Growth assay on solid medium of sec65-1 cells grown at different temperatures and overexpressing RPN4 where indicated. (B) Western blot of HA and Pgk1 from cycloheximide-treated wild-type (WT) cells expressing Δss-ngCPY*-HA and additionally overexpressing RPN4 where indicated. Expression of Δss-ngCPY*-HA was induced with 100 nM estradiol for 4 h. The asterisk indicates a slower-migrating, post-translationally modified form of Δss-ngCPY*-HA. CHX, cycloheximide. (C) Quantification of Δss-ngCPY*-HA levels relative to Pgk1 from western blots as shown in panel B. For each strain, data are normalized to t = 0. Mean ± SEM, n = 3. (D) Luciferase(DM)-mCherry levels relative to Pgk1 and normalized to t = 0. Quantification is based on western blots of mCherry and Pgk1 from cycloheximide-treated cells expressing Luciferase(DM)-mCherry and additionally overexpressing RPN4 where indicated. Mean ± SEM, n = 3.

Figure 7. Multiple signaling pathways mediate RPN4 induction by ER stress. (A) Flow cytometric measurement of the activity of RPN4 reporter variants in untreated cells. Data are normalized to the reporter containing the native RPN4 promoter. The other reporters contain mutations in the two Pdr1/3 response elements (PDREm), the Yap1 response element (YREm) or the heat shock element (HSEm). Mean ± SEM, n = 4. (B) As in panel A, but after treatment with 5 µg/ml tunicamycin for the times indicated. Mean ± SEM, n = 3. (C) Flow cytometric measurement of the activity of the native RPN4 reporter in tpk1/2/3-as and tpk1/2/3 ∆msn2/4 cells treated with the ATP analog 1NM-PP1 for the times indicated. The analog-sensitive tpk1/2/3-as alleles enable specific inhibition of protein kinase A with 1NM-PP1. Mean ± SEM, n = 3. (D) RPN4 mRNA levels in wild-type (WT) and tpk1/2/3-as cells treated with 1NM-PP1 for the times indicated. Data are normalized to WT cells at t = 0. Mean ± SEM, n = 3. (E) Flow cytometric measurement of the activity of RPN4 reporter variants in WT and ∆msn2/4 cells treated with 5 µg/ml tunicamycin. Data are normalized to WT cells containing the reporter with the native RPN4 promoter. Mean ± SEM, n = 3. (F) Model for the cooperation of Rpn4 and the UPR. ER protein misfolding causes increased flux through the ERAD pathway, which strains proteasome capacity and
inhibits efficient protein degradation (1). In addition, the UPR is activated (2). Severe ER stress leads to translocation defects, causing mislocalization of secretory proteins to the cytosol, where they cannot fold properly (3). These cytosolic misfolded proteins further burden the proteasome (4). As a result, Rpn4 is stabilized, the Rpn4 regulon is activated and proteasome biogenesis is enhanced. If cytosolic misfolded proteins persist, the \textit{RPN4} gene is induced (5), providing a second mechanism to increase Rpn4 abundance and augment proteasome biogenesis.

\textbf{Figure S1.} (A) Flow cytometric measurement of GFP levels in cells harboring the \textit{HAC1} splicing reporter and expressing ngCPY* under the control of the estradiol-inducible \textit{GAL} promoter system. For each time point, data are normalized to untreated cells. Mean ± SEM, n = 3. (B) As in panel A, but with cells expressing glycosylatable CPY*. (C) Images of cells expressing CPY*(N479Q)-sfGFP and the general ER marker Sec63-mCherry. Expression of CPY*(N479Q)-sfGFP was induced with 25 nM estradiol for 4 h and cells were stained with the vacuole dye CMAC. (D) Viability of \textit{Δhac1} cells after induction of ngCPY* expression with the indicated estradiol concentrations for 24 h. Viability of untreated cells was set to 100%.

\textbf{Figure S2.} \textit{RPN4} mRNA levels in wild-type (WT) and \textit{sec65-1} cells grown at 25°C and shifted to the indicated temperatures for 90 min. Data are normalized to WT cells at 25°C.
Table S1. Plasmids used in this study. GEM = Gal4DBD-EstR-Msn2TAD.

| Plasmid                          | Alias       | Source                        |
|----------------------------------|-------------|-------------------------------|
| pRS416-P<sub>Gal1</sub>         | pSS031      | Mumberg et al., 1994          |
| pRS306-P<sub>ADH1</sub>-GEM      | pDEP001     | Pincus et al., 2014           |
| pNH605-P<sub>ADH1</sub>-GEM      | pDEP151     | David Pincus                  |
| pRS306-P<sub>ADH1</sub>-GEM-P<sub>Gal1</sub> | pSS476     | this study                    |
| pNH605-P<sub>ADH1</sub>-GEM-P<sub>Gal1</sub> | pSS474     | this study                    |
| pRS315-P<sub>Cpy</sub>-ngCPY*-HA | pPW2181     | Peter Walter                  |
| pRS315-P<sub>Gal1</sub>-CPY*-HA  | pES67       | Spear and Ng, 2003            |
| pRS306-P<sub>ADH1</sub>-GEM-P<sub>Gal1</sub>-ngCPY*-HA | pSS577  | this study                    |
| pRS306-P<sub>ADH1</sub>-GEM-P<sub>Gal1</sub>-CPY*-HA | pSS953  | this study                    |
| pRS305-P<sub>ADH1</sub>-Rtn1-FLAG-sfGFP | pSS421  | Szoradi et al., 2018          |
| pNH605-P<sub>ADH1</sub>-GEM-<sub>P</sub>Gal1-FLAG-sfGFP | pSS525  | this study                    |
| pNH605-P<sub>ADH1</sub>-GEM-ngCPY*-HA-sfGFP | pSS616  | this study                    |
| pRS305-P<sub>Cpy</sub>-CPY*(N479Q)-HA | pSS104  | Peter Walter                  |
| pNH605-P<sub>ADH1</sub>-GEM-P<sub>Gal1</sub>-CPY*(N479Q)-HA-sfGFP | pSS562  | this study                    |
| YEp13                             | pSS471      | Michael Knop                  |
| YEp13-RPN4                        | pSS811      | this study                    |
| YEp13-PDR1                        | pSS813      | this study                    |
| YEp13-SSZ1                        | pSS812      | this study                    |
| YEp13-MUM2                        | pSS822      | this study                    |
| YEp13-YAP1                        | pSS814      | this study                    |
| YEp13-CAD1                        | pSS815      | this study                    |
| YEp13-SAF1                        | pSS744      | this study                    |
| YEp13-NOP56                       | pSS818      | this study                    |
| YEp13-CDC48                       | pSS816      | this study                    |
| pRS306-P<sub>ADH1</sub>-GEM-P<sub>Gal1</sub>-△ss-ngCPY*-HA | pSS678  | this study                    |
| pRS306N-P<sub>TEF1</sub>-NES-Luciferase(DM)-mCherry | pSS637  | Szoradi et al., 2018          |
| pRS303K-P<sub>GPD</sub>-TagBFP   | pMaM227     | Michael Knop                  |
| pRS303H-P<sub>GPD</sub>-TagBFP   | pMaM245     | Michael Knop                  |
| pNH605-P<sub>Rpn4</sub>-YFP      | pSS975      | Felix Boos                    |
| pNH605-P<sub>Rpn4</sub>-(PDRem)-YFP | pSS977  | Felix Boos                    |
| pNH605-P<sub>Rpn4</sub>-(HSem)-YFP | pSS976  | Felix Boos                    |
| pFA6a-mNeonGreen-kanMX4           | pMAM375     | Michael Knop                  |
| pNH605-P<sub>Rpn4</sub>-mNeonGreen | pSS981  | this study                    |
| pNH605-P<sub>Rpn4</sub>-(PDRem)-mNeonGreen | pSS983  | this study                    |
| pNH605-P<sub>Rpn4</sub>-(YRem)-mNeonGreen | pSS987  | this study                    |
| pNH605-P<sub>Rpn4</sub>-(HSem)-mNeonGreen | pSS982  | this study                    |
| pNH605-P<sub>Rpn4</sub>-(YRem,HESem)-mNeonGreen | pSS984  | this study                    |
Table S2. Oligonucleotides used in this study.

| Oligonucleotide       | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| up_Sacl_GAL           | CAAGCATTTAGTAATGACTATCAAAAgactgtcaggttacgtagag                         |
| CYC_KpnI_down         | ATAGCCTTATCGATGAGTCACCGGgtaccggccgaatttaag                              |
| EDY*.F2               | CTAGAACTAGTGATGCCATCCCCTTtagtagtagtaggtgtagttagttagttagtactctacc       |
| EDY*.R                | ATATCGAATTCCCTGGACCAACCCTTGGATGactgtcataaatctctaatctcaag               |
| FLAG-sfGFP_F          | CTAGAACTAGTGATGCCATCCCCTTtagtagtagtaggtgtagttagttagttagtactctacc       |
| FLAG-sfGFP_R          | ATATCGAATTCCCTGGACCAACCCTTGGATGactgtcataaatctctaatctcaag               |
| EDY*.tag_R            | GTCATCGTCGTCTTTGTTAGTCacccacaaagccaatagcc                             |
| FLAG for 1            | GACTACAGAACAGCAGATGAC                                                   |
| FLAG-open_R           | gggtatatagttctactctctactctactctactctactctactctactctactctactctactctactc |
| gap_RPN4-fw           | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_RPN4_rev          | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_PDR1-2-fw         | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_PDR1-2_rev        | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_SS21-fw           | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_SS21_rev          | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_MU2-fw            | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_MU2_rev           | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_YAP1-fw           | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_YAP1_rev          | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_CAD1-fw           | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_CAD1_rev          | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_SAF1-fw           | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_SAF1_rev          | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_NOP56-fw          | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_NOP56_rev         | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_CDC48-fw          | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_CDC48_rev         | TGGGCGCCTGATGCCCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_CPY_deltaSS-fw    | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| gap_CPY_deltaSS_rev   | CTATCGAATCAGCGATCGACCGACCGACCAACGTCTGGTAGttcataaatctctaatctcaag       |
| knock-in URA3-fw      | ATTTATGGTGAAGGATAAGTTTTGACCATCAAAGAAGGTTagcttgtctgtaagcggatg          |
| knock-in URA3_rev     | ATTTATGGTGAAGGATAAGTTTTGACCATCAAAGAAGGTTagcttgtctgtaagcggatg          |
| Yep13-fw              | CGTTGATGAATACACTGAAATGC                                                 |
| Yep13_rev             | AAGGAATGGTGATGAAATGC                                                  |
| pPCR_RPN4-fw          | AGTAGGAGACAGACACACATCC                                                  |
| pPCR_RPN4_rev         | GATTTGCTGTATGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGT
| Xho-neon              | TTTCAGCATTGCATGTAGCTTGCTCGATGAC                                       |
| neon-BamHI            | AAGGAATGGTGATGAAATGC                                                  |
| NheI-YRE fw           | aaaGCTAGCattgcacctacgtagtattac                                      |
| NheI-YRE rev          | aaaGCTAGCattgcacctacgtagtattac                                      |
Table S3. Yeast strains used in this study. For strains with extra-chromosomal plasmids only the genotype of the parental strains without plasmid is given. GEM = Gal4DBD-EstR-MsnTAD, SR = splicing reporter.

| Strain | Relevant genotype | Source |
|--------|-------------------|--------|
| SSY122 | ADE2 leu2-3,112 trp1-1 ura3-1 his3-11,15 MAT a | Szoradi et al., 2018 |
| SSY1338 | URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-ngCPY*-HA | this study |
| SSY2491 | URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-ngCPY*-HA LEU2::HAC1-SR | this study |
| SSY2489 | URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-CPY*-HA | this study |
| SSY2490 | URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-CPY*-HA LEU2::HAC1-SR | this study |
| SSY2364 | Sec63-mCherry::TRP1 LEU2::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-ngCPY*-HA-sfGFP | this study |
| SSY2363 | Sec63-mCherry::TRP1 LEU2::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-CPY*(N479Q)-HA-sfGFP | this study |
| SSY1337 | URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub> | this study |
| SSY1340 | hac1Δ::HIS3 URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>* | this study |
| SSY1341 | hac1Δ::HIS3 URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-ngCPY*-HA | this study |
| SSY1062 | hac1Δ::HIS3 | this study |
| SSY784 | rpn4Δ::HIS3 | this study |
| SSY2144 | rpn4Δ::HIS3 hac1Δ::hph | this study |
| SSY2357 | HIS3::P<sub>GPD</sub>-TagBFP-hph LEU2::HAC1-SR | this study |
| SSY2359 | rpn4Δ::HIS3 HIS3::P<sub>GPD</sub>-TagBFP-hph LEU2::HAC1-SR | this study |
| SSY2511 | hrd1Δ::HIS3 HIS3::P<sub>GPD</sub>-TagBFP-hph LEU2::HAC1-SR | this study |
| SSY1545 | RPN4-3HA::kan | this study |
| SSY2147 | hac1Δ::hph RPN4-3HA::kan | this study |
| SSY1500 | ade2-1 leu2-3,112 trp1-1 ura3-1 his3-11,15 MAT alpha | Peter Walter |
| SSY1507 | sec65-1, ura3-52, trp1-1, leu2-3,112, ade2, his3-11 MAT alpha | Stirling et al., 1992 |
| SSY2452 | ade2-1 LEU2::HAC1-SR | this study |
| SSY2453 | sec65-1 LEU2::HAC1-SR | this study |
| SSY2444 | URA3::P<sub>ADH1</sub>*-GEM-P<sub>GAL1</sub>*-Δss-ngCPY*-HA | this study |
| SSY1521 | HIS3::P<sub>GPD</sub>-TagBFP-hph ura3::P<sub>TEF1</sub>Luciferase(DM)-mCherry-nat | Szoradi et al., 2018 |
| SSY1482 | tpk1/2/3-as | David Pincus |
| SSY1484 | tpk1/2/3-as HIS3::P<sub>GPD</sub>-TagBFP-kan | this study |
| SSY2621 | tpk1/2/3-as HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>-mNeonGreen | this study |
| SSY2622 | tpk1/2/3-as HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(HSEm)-mNeonGreen | this study |
| SSY2629 | tpk1/2/3-as HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(PDREm)-mNeonGreen | this study |
| SSY2630 | tpk1/2/3-as HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(YREm)-mNeonGreen | this study |
| SSY1483 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 | this study |
| SSY1485 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 HIS3::P<sub>GPD</sub>-TagBFP-kan | this study |
| SSY2623 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub*-mNeonGreen | this study |
| SSY2624 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(HSEm)-mNeonGreen | this study |
| SSY2632 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(PDREm)-mNeonGreen | this study |
| SSY2633 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(YREm)-mNeonGreen | this study |
| SSY2634 | tpk1/2/3-as msn2Δ::nat msn4Δ::TRP1 HIS3::P<sub>GPD</sub>-TagBFP-kan LEU2::P<sub>RPN4</sub>*(YREm,HSEm)-mNeonGreen | this study |
Figure 1. A titratable system for the induction of ER stress. (A) Flow cytometric measurement of GFP levels in cells harboring the HAC1 splicing reporter and expressing ngCPY* under the control of the estradiol-inducible GAL promoter system. For each time point, data are normalized to untreated cells. Mean ± SEM, number of independent experiments (n) = 3. (B) Western blot of HA and Pgk1 from cells expressing CPY*-HA or ngCPY*-HA. Cells were treated with cycloheximide (CHX) for the times indicated. Pgk1 served as a loading control. (C) Images of cells expressing ngCPY*-sfGFP and the general ER marker Sec63-mCherry. Expression of ngCPY*-sfGFP was induced with 25 nM estradiol for 4 h and cells were stained with the vacuole dye CMAC. (D) Growth assay on solid media of wild-type (WT) and ∆hac1 cells expressing the estradiol-inducible artificial transcription factor GEM and, where indicated, ngCPY* under the control of the GAL promoter. For each strain, series represent fivefold dilution steps. (E) Growth assay in liquid media of WT cells expressing ngCPY* under the control of the estradiol-inducible GAL promoter system. a.u., arbitrary units. (F) As in panel E, but with ∆hac1 cells.
Table 1. Genes identified in the screen.

| gene   | description                                                                 |
|--------|-----------------------------------------------------------------------------|
| HAC1   | transcription factor, regulates the unfolded protein response                |
| RPN4   | transcription factor, stimulates expression of proteasome genes             |
| PDR1   | transcription factor, regulates the pleiotropic drug response, induces RPN4 transcription |
| SSZ1   | Hsp70 chaperone, part of ribosome-associated complex, overexpression activates Pdr1 |
| MUM2   | protein essential for meiotic DNA replication and sporulation               |
| YAP1   | transcription factor, regulates response to oxidative stress, induces RPN4 transcription |
| CAD1   | transcription factor, involved in stress responses, paralog of YAP1         |
| SAF1   | F-box protein, subunit of SCF ubiquitin ligase complexes                     |
| NOP56  | essential nucleolar protein                                                  |
Figure 2. Multicopy suppression of ngCPY* toxicity in ∆hac1 cells. (A) Growth assay on solid media of wild-type (WT) and ∆hac1 cells expressing ngCPY* under the control of the estradiol-inducible GAL promoter system and overexpressing RPN4, SSZ1 or PDR1 where indicated. (B) Growth assay in liquid medium of WT and ∆hac1 cells expressing ngCPY* under the control of the estradiol-inducible GAL promoter system. ∆hac1 cells additionally overexpressed the indicated genes. Cells were grown without estradiol. a.u., arbitrary units. (C) As in panel B, but in the presence of 50 nM estradiol. (D) Quantification of growth assays as shown in panel B. Data are normalized to WT cells. Mean ± SEM, n = 4. (E) Quantification of growth assays as shown in panel C. Data are normalized to WT cells. Mean ± SEM, n = 4.
Figure 3. Rpn4 and the UPR cooperate to counteract ER stress. (A) Growth assay on solid media of wild-type (WT), \( \Delta rpn4 \), \( \Delta hac1 \) and \( \Delta hac1 \Delta rpn4 \) cells, overexpressing RPN4 where indicated. (B) As in panel A, but on media containing different concentrations of tunicamycin (Tm). (C) Flow cytometric measurement of GFP levels relative to cytosolic BFP in WT, \( \Delta rpn4 \) and \( \Delta hrd1 \) cells harboring the HAC1 splicing reporter. Data are normalized to WT cells. Mean ± SEM, n = 3. (D) KAR2 mRNA levels in WT and \( \Delta rpn4 \) cells as measured by quantitative real-time PCR. Data are normalized to WT cells. Mean ± SEM, n = 3. (E) As in panel D, but for SIL1.
Figure 4. ER stress increases Rpn4 abundance and induces RPN4 transcription. (A) Western blot of HA and Pglyk1 from wild-type (WT) and Δhac1 cells expressing Rpn4-HA and treated with 2 µg/ml tunicamycin (Tm) for the times indicated. (B) Quantification of Rpn4-HA protein levels relative to Pglyk1 from western blots as shown in panel A. Data are normalized to WT cells at t = 0. Mean ± SEM, n = 5. (C) KAR2 mRNA levels in WT and Δhac1 cells treated with 2 µg/ml tunicamycin for the times indicated. Data are normalized to WT cells at t = 0. Mean ± SEM, n = 3. (D) As in panel C, but for SIL1. (E) As in panel C, but for RPN4. (F) As in panel C, but for RPN4 after treatment with 5 µg/ml tunicamycin for the times indicated.
**Figure 5.** *RPN4* is upregulated by cytosolic mislocalization of secretory proteins. (A) Western blot of Kar2 and Pgk1 from WT and Δhac1 cells treated with 2 µg/ml tunicamycin for the times indicated. The asterisk indicates untranslocated ss-Kar2. (B) As in panel A, but with 5 µg/ml tunicamycin. (C) Western blot of Kar2 and Pgk1 from sec65-1 cells grown at 25°C and shifted to the indicated temperatures for 90 min. The asterisk indicates untranslocated ss-Kar2. (D) *RPN4* mRNA levels in WT and sec65-1 cells grown at 25°C and shifted to the indicated temperatures for 90 min. Data are normalized to WT cells at 25°C. Mean ± SEM, n = 3. (E) Flow cytometric measurement of GFP levels in WT and sec65-1 cells harboring the *HAC1* splicing reporter. Cells grown at 25°C were shifted to the indicated temperatures or treated with 2 µg/ml tunicamycin (Tm) for 90 min. For each strain, data are normalized to 25°C. Mean ± SEM, n = 3.
Figure 6. Rpn4 protects against cytosolic protein misfolding. (A) Growth assay on solid medium of sec65-1 cells grown at different temperatures and overexpressing RPN4 where indicated. (B) Western blot of HA and Pgk1 from cycloheximide-treated wild-type (WT) cells expressing Δss-ngCPY*-HA and additionally overexpressing RPN4 where indicated. Expression of Δss-ngCPY*-HA was induced with 100 nM estradiol for 4 h. The asterisk indicates a slower-migrating, post-translationally modified form of Δss-ngCPY*-HA. CHX, cycloheximide. (C) Quantification of Δss-ngCPY*-HA levels relative to Pgk1 from western blots as shown in panel B. For each strain, data are normalized to t = 0. Mean ± SEM, n = 3. (D) Luciferase(DM)-mCherry levels relative to Pgk1 and normalized to t = 0. Quantification is based on western blots of mCherry and Pgk1 from cycloheximide-treated cells expressing Luciferase(DM)-mCherry and additionally overexpressing RPN4 where indicated. Mean ± SEM, n = 3.
Figure 7. Multiple signaling pathways mediate RPN4 induction by ER stress. (A) Flow cytometric measurement of the activity of RPN4 reporter variants in untreated cells. Data are normalized to the reporter containing the native RPN4 promoter. The other reporters contain mutations in the two Pdr1/3 response elements (PDREm), the Yap1 response element (YREm) or the heat shock element (HSEm). Mean ± SEM, n = 4. (B) As in panel A, but after treatment with 5 µg/ml tunicamycin for the times indicated. Mean ± SEM, n = 3. (C) Flow cytometric measurement of the activity of the native RPN4 reporter in tpk1/2/3-as and tpk1/2/3 Δmsn2/4 cells treated with the ATP analog 1NM-PP1 for the times indicated. The analog-sensitive tpk1/2/3-as alleles enable specific inhibition of protein kinase A with 1NM-PP1. Mean ± SEM, n = 3. (D) RPN4 mRNA levels in wild-type (WT) and tpk1/2/3-as cells treated with 1NM-PP1 for the times indicated. Mean ± SEM, n = 3. (E) Flow cytometric measurement of the activity of RPN4 reporter variants in WT and ΔPVQ24 cells treated with 5 µg/ml tunicamycin. Data are normalized to WT cells containing the reporter with the native RPN4 promoter. Mean ± SEM, n = 3. (F) Model for the cooperation of Rpn4 and the UPR. ER protein misfolding causes increased flux through the ERAD pathway, which strains proteasome capacity and inhibits efficient protein degradation (1). In addition, the UPR is activated (2). Severe ER stress leads to translocation defects, causing mislocalization of secretory proteins to the cytosol, where they cannot fold properly (3). These cytosolic misfolded proteins further burden the proteasome (4). As a result, Rpn4 is stabilized, the Rpn4 regulon is activated and proteasome biogenesis is enhanced. If cytosolic misfolded proteins persist, the RPN4 gene is induced (5), providing a second mechanism to increase Rpn4 abundance and augment proteasome biogenesis.
Figure S1. (A) Flow cytometric measurement of GFP levels in cells harboring the *HAC1* splicing reporter and expressing ngCPY* under the control of the estradiol-inducible *GAL* promoter system. For each time point, data are normalized to untreated cells. Mean ± SEM, n = 3. (B) As in panel A, but with cells expressing glycosylatable CPY*. (C) Images of cells expressing CPY*(N479Q)-sfGFP and the general ER marker Sec63-mCherry. Expression of CPY*(N479Q)-sfGFP was induced with 25 nM estradiol for 4 h and cells were stained with the vacuole dye CMAC. (D) Viability of ∆hac1 cells after induction of ngCPY* expression with the indicated estradiol concentrations for 24 h. Viability of untreated cells was set to 100%.
Figure S2. RPN4 mRNA levels in wild-type (WT) and sec65-1 cells grown at 25°C and shifted to the indicated temperatures for 90 min. Data are normalized to WT cells at 25°C.