Copyright © 2010 by the Genetics Society of America
DOI: 10.1534/genetics.109.111419

Ssz1 Restores Endoplasmic Reticulum-Associated Protein Degradation in Cells Expressing Defective Cdc48–Ufd1–Npl4 Complex by Upregulating Cdc48

Eran Bosis,[1] Dor Salomon,[1] Orit Ohayon,[1] Gilad Sivan, Shoshana Bar-Nun[2] and Efrat Rabinovich

Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Manuscript received October 28, 2009
Accepted for publication December 22, 2009

ABSTRACT

The endoplasmic reticulum (ER)-associated protein degradation (ERAD) pathway eliminates aberrant proteins from the ER. The key role of Cdc48p–Ufd1p–Npl4p is indicated by impaired ERAD in *Saccharomyces cerevisiae* with mutations in any of this complex's genes. We identified *SSZ1* in genetic screens for *cdc48-10* suppressors and show that it upregulates Cdc48p via the pleiotropic drug resistance (PDR) network. A p*SSZ1* plasmid restored impaired ERAD-M of 6myc-Hmg2 in *cdc48-10*, *ufd1-2*, and *npl4-1*, while *SSZ1* deletion had no effect. Ssz1p activates Pdr1p, the PDR master regulator. Indeed, plasmids of *PDR1* or its target gene *RPN4* increased cdc48-10p levels and restored ERAD-M in *cdc48-10*. Rpn4p regulates transcription of proteasome subunits and *CDC48*, thus *RPN4* deletion abolished ERAD. However, the diminished proteasome level in Δ*rpn4* was sufficient for degrading a cytosolic substrate, whereas the impaired ERAD-M was the result of diminished Cdc48p and was restored by expression of p*CDC48*. The corrected ERAD-M in the hypomorphic strains of the Cdc48 partners *ufd1-2* and *npl4-1* by the p*CDC48* plasmid, and in *cdc48-10* cells by the p*cdc48-10* plasmid, combined with the finding that neither p*SSZ1* nor p*cdc48-10* restored ERAD-L of CPY*-HA, support our conclusion that Ssz1p suppressing effects is brought about by upregulating Cdc48p.

SECRETORY and membrane proteins are synthesized, folded, and assembled in the endoplasmic reticulum (ER) and are transported along the secretory pathway to their final destinations. Essential quality control mechanisms ensure that misfolded or damaged proteins are retained in the ER and eliminated by the ER-associated protein degradation (ERAD) pathway. Such proteins are dislocated back to the cytosol, where they are tagged and degraded by the ubiquitin–proteasome system (Bonifacino and Weissman 1998; Bar-Nun 2005).

The cytosolic Cdc48p–Ufd1p–Npl4p complex is one of the key ERAD players, and mutations in any of this complex's genes result in stabilization of ERAD-M and ERAD-L substrates, as shown for 6myc-Hmg2 and CPY*, respectively (Bays *et al.* 2001; Ye *et al.* 2001; Braun *et al.* 2002; Jarosch *et al.* 2002; Rabinovich *et al.* 2002). The yeast Cdc48p and its mammalian homolog p97, members of the AAA-ATPases family, participate in many cellular processes, including cell cycle regulation, homotypic membrane fusion, and proteasomal degradation (Ghislain *et al.* 1996; Patel and Latterich 1998; Vale 2000). Cdc48p/p97 provides the driving force for pulling ERAD substrates out of the ER (Ye *et al.* 2001; Elkabetz *et al.* 2004). This function concurs with the ability of AAA-ATPases to translate ATP hydrolysis into mechanical forces (Rouiller *et al.* 2002) and with the underlying activity of AAA-ATPases to unfold and disassemble protein complexes (Lupas and Martin 2002).

In our search for novel ERAD factors, we screened for suppressors that, when overexpressed, restored the impaired ERAD-M of 6myc-Hmg2 in the *Saccharomyces cerevisiae cdc48-10* conditional mutant. In our screen, we identified Ssz1p, a cytosolic member of the Hsp70 family. Ssz1p is tightly associated with the J-protein zuotin (Zuo1p) and the stable Zuo1p:Ssz1p complex (also known as the ribosome-associated complex, RAC) binds to the ribosome via Zuo1p, and, together with Ssbs, facilitates folding of nascent polypeptides as they exit the ribosome (Gautschi *et al.* 2001; Hundley *et al.* 2002; Gautschi *et al.* 2002; Shaner and Morano 2007).

Here we show that the restored ERAD-M in *cdc48-10* is brought about by the RAC-independent participation of Ssz1p in the pleiotropic drug resistance (PDR) network. PDR regulates the expression of many genes in response to various cytotoxic compounds, including cycloheximide, canavanine, and cadmium. Ssz1p is a posttranslational activator of the transcription factor Pdr1p (Hallstrom *et al.* 1998; Eisenman and Craig 2004), a PDR master regulator (Balzi *et al.* 1987; DeRisi *et al.*

---

[1] These authors contributed equally to this work.

[2] *Corresponding author:* Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. E-mail: shoshbn@tauex.tau.ac.il

TABLE 1

Yeast strain list

Strain	Genotype	Source
CDC48 (KFY100)	*MAT***a** *his4-619 leu2-3,112 ura3-52*	Rabinovich *et al.* (2002)
cdc48-10 (KFY194)	*MAT***a** *lys2-801 leu2-3,112 ura3-52 cdc48-10*^{ts}	Rabinovich *et al.* (2002)
SBN100 (*CDC48*)	*MAT***a** *KFY100 trp1::LEU2*	This study
SBN194 (*cdc48-10*)	*MAT***a** *KFY194 trp1::LEU2*	This study
DF5a	*MAT***a** *his3-Δ200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52*	Finley *et al.* (1987)
ufd1-2	*MAT***a** *his3-Δ200 leu2-3,2-112 lys2-801 trp1-1(am) ura3-52ufd1-2*	Finley *et al.* (1987)
NPL4 (RHY400)	*MAT***a** *ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::*P_{TDH3}*-6MYC-HMG2*	Bays *et al.* (2001)
npl4-1 (RHY554)	*MAT***a** *ade2-101 lys2-801 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 met2 ura3-52::*P_{TDH3}*-6MYC-HMG2 hrd4-1*	Bays *et al.* (2001)
Δ*hrd1* (RHY965)	*MAT***a** *his3Δ200 lys2-801 ade2-101 met2hmg1::LYS2 hmg2::HIS3 trp1::hisG leu2Δura3-52::6MYC HMG2 hrd1Δ::URA3*	Hampton *et al.* 1996)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Giaever *et al.* (2002)
Δ*rpn4*	*MAT***a** *BY4741 rpn4::KanR*	Giaever *et al.* (2002)
Δ*pdr1*	*MAT***a** *BY4741 pdr1::KanR*	Giaever *et al.* (2002)
Δ*ssz1*	*MAT***a** *BY4741 ssz1::KanR*	Giaever *et al.* (2002)

2000; Devaux *et al.* 2001; Mamnun *et al.* 2002; Dohmen *et al.* 2007; Gulshan and Moye-Rowley 2007). Among others, the upregulated genes are involved in efflux of cytotoxic compounds (ABC and MFS transporters), stress response and, lipid metabolism (DeRisi *et al.* 2000; Gulshan and Moye-Rowley 2007). In particular, Pdr1p controls expression of the proteasome by upregulating Rpn4p, a transcriptional activator that binds to the proteasome-associated control element (PACE). This nonamer sequence (GGTGGCAAA) is located in the promoters of many genes including all proteasome subunits (Mannhaupt *et al.* 1999; Jelinsky *et al.* 2000; Kapranov *et al.* 2001; Xie and Varshavsky 2001; Owsianik *et al.* 2002; Ju *et al.* 2004; Dohmen *et al.* 2007; Gulshan and Moye-Rowley 2007; Hanna and Finley 2007;). Interestingly, the *CDC48* gene also contains the Rpn4p-binding PACE (Mannhaupt *et al.* 1999; Kapranov *et al.* 2001). Indeed, *CDC48* mRNA levels decrease upon *RPN4* deletion (Karpov *et al.* 2008). Here we provide evidence that the p*SSZ1* plasmid restores the impaired ERAD-M in mutants of the Cdc48p–Ufd1p–Npl4p complex and attribute this effect to upregulation of Cdc48p via the Pdr1p-dependent activation of Rpn4p.

MATERIALS AND METHODS

Strains and plasmids: Yeast strains used in this study are listed in Table 1. SBN100 and SBN194 were generated by replacing the *TRP1* gene with *LEU2* in KFY100 (*CDC48*) and KFY194 (*cdc48-10*), respectively. Strains Δ*ssz1*, Δ*pdr1*, Δ*rpn4*, and their parental BY4741 were obtained from the deletion library of all nonessential genes (Giaever *et al.* 2002). Yeast YEp24-based 2μ genomic libraries (Carlson/Botstein library) were used in the genetic screen for *cdc48-10* suppressors. The protein 6myc-Hmg2 was expressed either from the genome as indicated (Table 1) or from plasmids pRH244 (Rabinovich *et al.* 2002) or pER244 (generated by replacing the *URA3* gene with *LEU2* in pRH244). CPY*-HA (*prc1-1* allele) was expressed from plasmid pBG15 (Elkabetz *et al.* 2004) and ΔssCPY*-GFP was expressed from plasmid POW0668 (Lipson *et al.* 2008). Ssz1p was overexpressed either as an untagged protein (pHE31; generously provided by E. Craig) or as an N-terminally HA-tagged version (HA-Ssz1; generously provided by J. Frydman). The hyperactive Pdr1-3p mutant (F815S; Carvajal *et al.* 1997), was expressed from plasmid pSK (generously provided by W. S. Moye-Rowley). Low-copy plasmids were used to express Rpn4p either as an untagged protein (p314CUP1RPN4) or as a C-terminally FLAG-tagged Rpn4 (p314CUP1RPN4-FLAG) (generously provided by Y. Xie). Cdc48p was expressed from a high-copy plasmid pKF700 (generously provided K. U. Fröhlich) or from plasmid pOO700. The latter was generated by replacing the *LEU2* gene with *URA3*: pKF700 and a *URA3*-containing pBluescriptIISK+ (Stratagene) digested with *Bst*XI were filled in by T4 polymerase, and then digested with *Kpn*I and the excised *URA3* was inserted into pKF700. The myc-tagged cdc48-10p was expressed from plasmid pDS194 generated by amplifying *cdc48-10* from KFY194 genomic DNA with primers 5′-CCC GGA TCC ATG GGT GAA GAA CAT AAA CC-3′ and 5′-CCC GGT ACC CG ACTATACAAATCATCATCTTCC-3′. The PCR product was digested with *Bam*HI and *Kpn*I and inserted into these sites in pAMT20. All construct were sequenced.

Genetic screens for *cdc48-10* suppressors: The *cdc48-10* strain was transformed with Yep24-based genomic libraries and grown for 3 days at 30° on SD plates lacking uracil. The resulting colonies were replica plated and incubated for an additional 3 days at 37°. Survivors were collected and transformed with plasmid pER244 expressing 6myc-Hmg2, and turnover of 6myc-Hmg2 was measured at 37°. The DNA from cells that exhibited restored ERAD was extracted and reintroduced into naive *cdc48-10* cells. Plasmid DNA from the secondary transformants that exhibited restored ERAD was recovered and sequenced using primers flanking the inserts.

Growth sensitivity to cadmium: Yeast cells, grown at 30° to 1.0 A_{600} in the appropriate selective media, were spotted as 10-fold serial dilutions on plates supplemented with increasing concentrations of $CdCl_2$ (Jungmann *et al.* 1993; Wang and Chang 2003).

Degradation rates of 6myc-Hmg2, CPY*-HA, and ΔssCPY*-GFP: Degradation at either 30° or 37° was followed by immunoblotting of cells (3 A_{600}) collected and lysed at indicated time points after addition of cycloheximide (150 μg/ml), as previously described for 6myc-Hmg2 (Rabinovich *et al.* 2002), CPY*-HA (Elkabetz *et al.* 2004), or ΔssCPY*-GFP (Lipson *et al.* 2008). Ten percent of total cellular proteins was resolved by SDS–PAGE and electroblotted onto nitrocellulose. Blots were probed with anti-myc (clone 9E10), anti-HA (clone 12CA5), or anti-CPY (clone 10A5-B5; New Biotechnology) mouse antibodies followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson). The HRP was visualized by enhanced chemiluminescence (ECL) and the images were quantified by densitometry (ImageMaster 1D).

Levels of Cdc48p and Rpt1p: Equal amounts of cells (1.5 A_{600}) were lysed, protein levels were estimated, and equal amounts of cellular proteins were resolved by SDS–PAGE and electroblotted onto nitrocellulose. Blots were probed by the following primary antibodies: mouse anti-myc (clone 9E10) to follow the levels of myc-tagged cdc48-10p; rabbit anti-Cdc48 (generously provided by K. U. Fröhlich); chicken anti-Rpt1 (generously provided by M. Glickman). A mouse anti-actin antibody (clone C4, MP Biomedicals) was used to monitor gel loading. The HRP conjugated to the secondary antibodies goat anti-mouse IgG (Jackson), goat-anti-rabbit IgG (Sigma), or rabbit anti-chicken (Chemicon) was visualized by ECL and quantified by densitometry.

RESULTS

A genetic screen for suppressors of *cdc48-10* defects in ERAD: The *CDC48* gene was originally identified as being involved in the cell-division cycle (Moir *et al.* 1982; Frohlich *et al.* 1991). Since mutations in *CDC48* that lead to cell-division arrest were also found to hamper ERAD (Rabinovich *et al.* 2002), we sought to identify novel ERAD factors by screening for suppressors that, when overexpressed, allow growth of the temperature-sensitive *cdc48-10* mutant under nonpermissive conditions. We transformed *cdc48-10* cells with a YEp24-based 2μ yeast genomic library, and following 3 days incubation at the permissive temperature (30°), transformants were replica plated and further incubated for 3 days at the restrictive temperature (37°). Out of ∼27,000 initial transformants, 17 colonies survived the restrictive temperature and were tested for degradation of the ERAD-M substrate 6myc-Hmg2. As previously reported (Rabinovich *et al.* 2002), *cdc48-10* cells failed to degrade 6myc-Hmg2 at 37° (Figure 1, A and B). Four out of the 17 surviving colonies exhibited restored ERAD-M at 37° (data not shown), suggesting that the temperature sensitivity of growth was not necessarily the outcome of impaired ERAD. The plasmids from these 4 colonies were isolated and individually reintroduced into naive *cdc48-10* cells, to confirm their capacity to restore ERAD. One of these secondary transformants, which contained the pDB2-1 plasmid, degraded 6myc-Hmg2 at 37° at rates similar to those measured in *CDC48* wild type (Figure 1, A and B).

The insert in pDB2-1 was identified by sequencing its boundaries and aligning the sequences against the yeast genomic database using the BLAST program (Altschul *et al.* 1997). pDB2-1 contained an insert of ∼5 kb of contiguous genomic DNA derived from chromosome VIII, spanning positions 222,686 to 227,948. Three full-length genes were found within this region (Figure 1C): *RPP1*, which encodes ribonuclease P protein 1 required for tRNA and 35S rRNA precursors processing (Stolc and Altman 1997); *PAN5*/YHR063C, a structural homolog of *Escherichia coli panE* 2-dehydropantoate 2-reductase that participates in the pantothenic acid pathway (White *et al.* 2001); and *SSZ1*, a cytosolic member of the Hsp70 family that associates with Zuo1p to form RAC, a stable complex that associates with ribosomes, and, together with Ssbs, is involved in facilitating folding of nascent polypeptides (Gautschi *et al.* 2001; Gautschi *et al.* 2002; Hundley *et al.* 2002; Shaner and Morano 2007). None of these genes has been previously reported to be associated with Cdc48p functions or with ERAD.

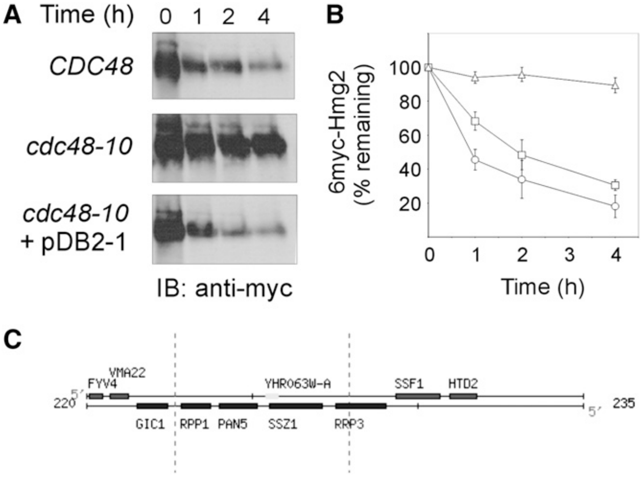

Figure 1.—Plasmid pDB2-1, identified in a genetic screen, restores ERAD-M in *cdc48-10* mutant. Turnover of 6myc-Hmg2 was measured in wild-type *CDC48* (KFY100; circles), *cdc48-10* (KFY194; triangles), and *cdc48-10* expressing plasmid pDB2-1 identified in the genetic screen (squares). The protein 6myc-Hmg2 was expressed from pER244. (A) Following a 90-min preincubation at 37°, cycloheximide (150 μg/ml) was added and cells further incubated at 37° were collected at the indicated time points. Cells (3 A_{600}) were lysed, total cellular proteins were resolved by SDS-PAGE, electroblotted and probed (IB) with a mouse anti-myc antibody followed by HRP-conjugated anti-mouse IgG, and HRP was visualized by ECL. (B) Blots represented by A were quantified by densitometry and 6myc-Hmg2 decay was plotted. The remaining 6myc-Hmg2 was calculated as the percentage of its level at the time of cycloheximide addition (100%). Values shown are means ± SEM of at least five independent experiments. (C) A genetic map of the insert in plasmid pDB2-1 from the Saccharomyces Genome Database (http://www.yeastgenome.org).

Ssz1 restores impaired ERAD in *cdc48-10* but it is not required for ERAD: Of the proteins encoded by pDB2-1, we focused on Ssz1p, speculating that it was the most plausible candidate to be involved in ERAD because, similar to Cdc48p, Ssz1p is a cytosolic protein that contains an ATPase domain (Hallstrom *et al.* 1998). To directly test this hypothesis, a plasmid harboring only

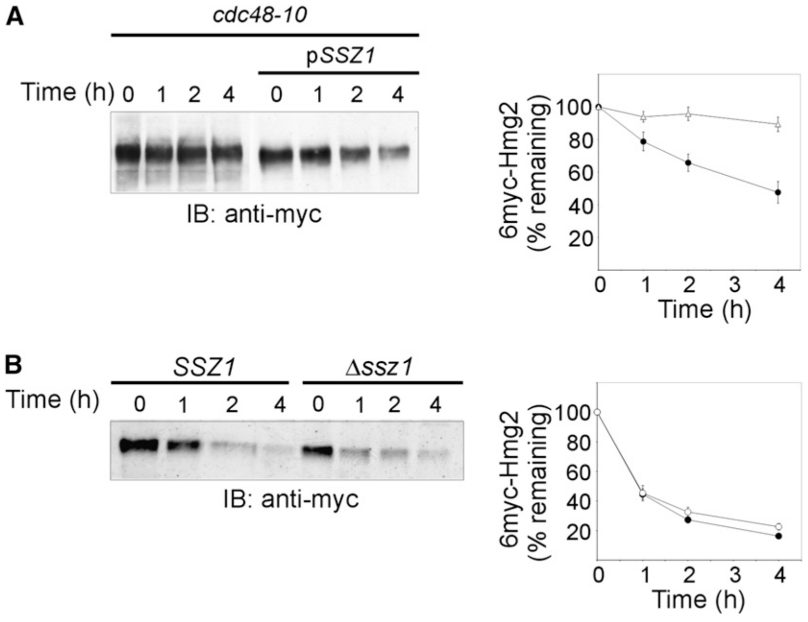

FIGURE 2.—p*SSZ1* plasmid restores ERAD-M in *cdc48-10* but Ssz1p is not required for ERAD-M. (A) Turnover of 6myc-Hmg2 was measured in *cdc48-10* (KFY194; open triangles) and *cdc48-10* expressing p*SSZ1* (closed circles). The degradation of 6myc-Hmg2 was followed at 37° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments. (B) Turnover of 6myc-Hmg2 was measured in wild-type BY4741 (closed circles) and Δ*ssz1* deletion strain derived from it (open circles). The degradation of 6myc-Hmg2 was followed at 30° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments.

the *SSZ1* gene was examined for its ability to restore growth and ERAD. The p*SSZ1* allowed growth of *cdc48-10* as well as *cdc48-3*, another temperature-sensitive *cdc48* allele, at the restrictive 37° (data not shown). Importantly, p*SSZ1* partially restored 6myc-Hmg2 degradation at 37° in *cdc48-10* cells (Figure 2A). We considered the possibility that the Hsp70 Ssz1p is a novel ERAD component. To examine whether it was essential for ERAD, 6myc-Hmg2 turnover was assessed in Δ*ssz1* cells. Clearly, deletion of *SSZ1* had no effect on the degradation of 6myc-Hmg2, which still turned over rapidly in Δ*ssz1* cells (Figure 2B).

Ssz1 restores impaired ERAD in *ufd1-2* and *npl4-1* but not in Δ*hrd1*: We next hypothesized that Ssz1p may act as a cytosolic chaperone, replacing the defective Cdc48p in its role in ERAD. This possibility was directly tested by introducing p*SSZ1* to strains harboring mutations in Ufd1p and Npl4p, the ERAD-dedicated Cdc48p partners. Evidently, p*SSZ1* partially restored the impaired 6myc-Hmg2 degradation in *npl4-1* (Figure 3A) and *ufd1-2* (Figure 3B) strains. This compensatory effect of p*SSZ1* was restricted to members of the Cdc48p–Ufd1p–Npl4p complex, as it did not correct the impaired 6myc-Hmg2 degradation in Δ*hrd1* cells (Figure 3C). We could have concluded that Ssz1p participated directly in an alternative pathway bypassing Cdc48p–Npl4p–Ufd1p altogether. However, Ssz1p was not an essential ERAD component (Figure 2B), and if it acted directly, Ssz1p should interact with ERAD substrates. Nonetheless, interactions of Ssz1p with 6myc-Hmg2 was never detected (data not shown), contrary to the physical interaction of Cdc48p with 6myc-Hmg2 (RABINOVICH *et al.* 2002). This may reflect a weak association of Ssz1p with ERAD substrates or suggests that Ssz1p plays another role in restoring ERAD when the Cdc48p–Ufd1p–Npl4p complex malfunctions.

The ability of Ssz1p to restore impaired ERAD could have been attributed to Cdc48p activation, facilitating proper folding of the temperature-sensitive cdc48-10p protein at the restrictive temperature. Such an activity should rely on interactions of Ssz1p with cdc4810p. Yet again, no such interaction was detected (data not shown). This, together with the unusual properties of Ssz1p as a chaperone, which functions within the Zuo1p:Ssz1p RAC complex as a J-protein that activates ATPases of other Hsp70s (GAUTSCHI *et al.* 2001, 2002; HUNDLEY *et al.* 2002; SHANER and MORANO 2007), suggested that Ssz1p plays an indirect role in restoring ERAD.

Pdr1 and Rpn4 restore impaired ERAD in *cdc48-10* but only Rpn4 is required for ERAD: An interesting function of Ssz1p that could account for its involvement in ERAD is its participation in the PDR network. Ssz1p is a post-translational activator of the transcription factor Pdr1p (HALLSTROM *et al.* 1998; EISENMAN and CRAIG 2004), a master regulator of PDR (BALZI *et al.* 1987; DERISI *et al.* 2000; DEVAUX *et al.* 2001; MAMNUN *et al.* 2002; DOHMEN *et al.* 2007; GULSHAN and MOYE-ROWLEY 2007). Importantly, in PDR activation Ssz1p neither functions as a chaperone nor as part of RAC, since Ssz1p and Zuo1p activate PDR only if their binding to the ribosome is abrogated (HUNDLEY *et al.* 2002; EISENMAN and CRAIG 2004). To determine whether p*SSZ1* restored ERAD-M in *cdc48-10* cells due to Ssz1p's role in PDR, we examined whether Pdr1p itself could restore ERAD. Indeed, when a plasmid expressing the Ssz1p-independent hyperactive *PDR1-3* mutant (CARVAJAL *et al.* 1997) was introduced into *cdc48-10* cells, 6myc-Hmg2 degradation at 37° was markedly accelerated (Figure 4A).

One of the target genes of Pdr1p is *RPN4*, encoding a transcriptional activator of the proteasome (MANNHAUPT

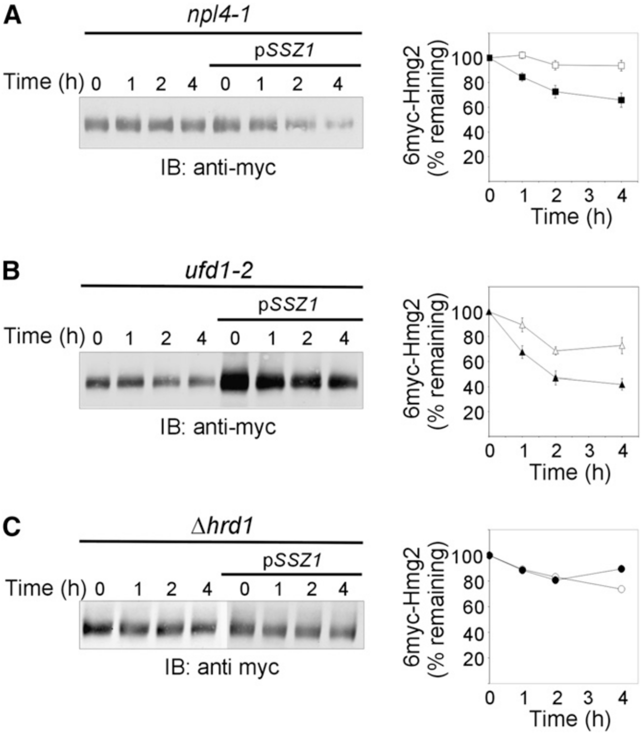

FIGURE 3.—p*SSZ1* plasmid restores ERAD-M in *npl4-1* and *ufd1-2* mutant strains but not in Δ*hrd1*. Turnover of 6myc-Hmg2 was measured in (A) *npl4-1* (open squares) and *npl4-1* expressing p*SSZ1* (closed squares), (B) *ufd1-2* (open triangles) and *ufd1-2* expressing p*SSZ1* (closed triangles), and (C) Δ*hrd1* (open circles) and Δ*hrd1* expressing p*SSZ1* (closed circles). The degradation of 6myc-Hmg2 was followed at 30° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments.

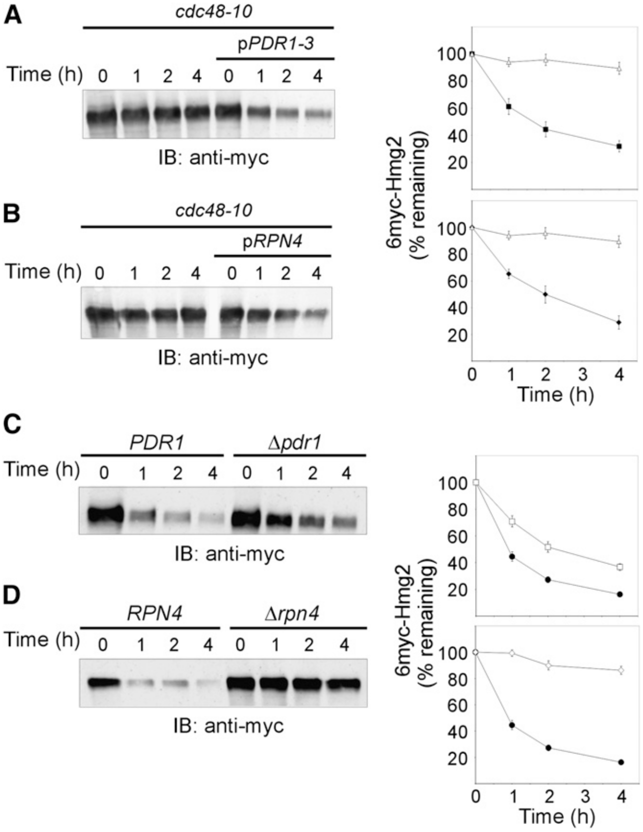

FIGURE 4.—Plasmids p*PDR1* and p*RPN4* restore ERAD-M in *cdc48-10* but only Rpn4p is required for ERAD-M. Turnover of 6myc-Hmg2 was measured in (A) *cdc48-10* (KFY194; open triangles) and *cdc48-10* expressing p*PDR3-1* (closed squares) and (B) *cdc48-10* (SBN194; open triangles) and *cdc48-10* expressing p*RPN4* (closed diamonds). The degradation of 6myc-Hmg2 was followed at 37° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments. Turnover of 6myc-Hmg2 was measured in wild-type BY4741 (closed circles) and the deletion strain derived from it: (C) Δ*pdr1* (open squares) and (D) Δ*rpn4* (open diamonds). The degradation of 6myc-Hmg2 was followed at 30° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments.

et al. 1999; KAPRANOV *et al.* 2001; XIE and VARSHAVSKY 2001; OWSIANIK *et al.* 2002; JU *et al.* 2004; DOHMEN *et al.* 2007; GULSHAN and MOYE-ROWLEY 2007; HANNA and FINLEY 2007). Therefore, we surmised that the link of Ssz1p to ERAD was mediated by Rpn4p. This possibility was confirmed by introducing the p*RPN4* plasmid into *cdc48-10* cells and showing that the impaired ERAD-M of 6myc-Hmg2 at 37° was restored (Figure 4B). Taken together, these results suggest that the suppressing effect of Ssz1p in *cdc48-10* can be mediated by Pdr1p and Rpn4p.

The effect of p*PDR1-3* or p*RPN4* plasmids in restoring 6myc-Hmg2 degradation in *cdc48-10* cells could have resulted from their direct participation in ERAD. *RPN4* was already demonstrated to be essential for the proteasomal elimination of two model substrates of the N-end rule and the UFD pathways (JOHNSON *et al.* 1995) and the ERAD-L substrate CPY* (NG *et al.* 2000). However, Pdr1p, the upstream activator of Rpn4p, was never implicated in ERAD. Deletion of *PDR1* caused some effect on ERAD-M, as indicated by the twofold prolonged half-life of 6myc-Hmg2 in Δ*pdr1* cells (Figure 4C). Conversely, ERAD-M was completely inhibited in Δ*rpn4* cells and 6myc-Hmg2 turned into a practically stable protein (Figure 4D). These results suggest that the ability of Ssz1p to restore ERAD-M when the Cdc48p is defective is mediated by Rpn4p, most likely via Pdr1p.

Cdc48p is a major limiting ERAD component in Δ*rpn4*: The critical function of Rpn4p in proteasomal degradation in general and ERAD in particular could be a direct consequence of its established role in regulating the expression of the proteasome subunits (MANNHAUPT *et al.* 1999; KAPRANOV *et al.* 2001; XIE and VARSHAVSKY 2001; OWSIANIK *et al.* 2002; JU *et al.* 2004; DOHMEN *et al.* 2007; GULSHAN and MOYE-ROWLEY 2007; HANNA and FINLEY 2007; KARPOV *et al.* 2008). Hence, the impaired ERAD-M observed in Δ*rpn4* cells (Figure

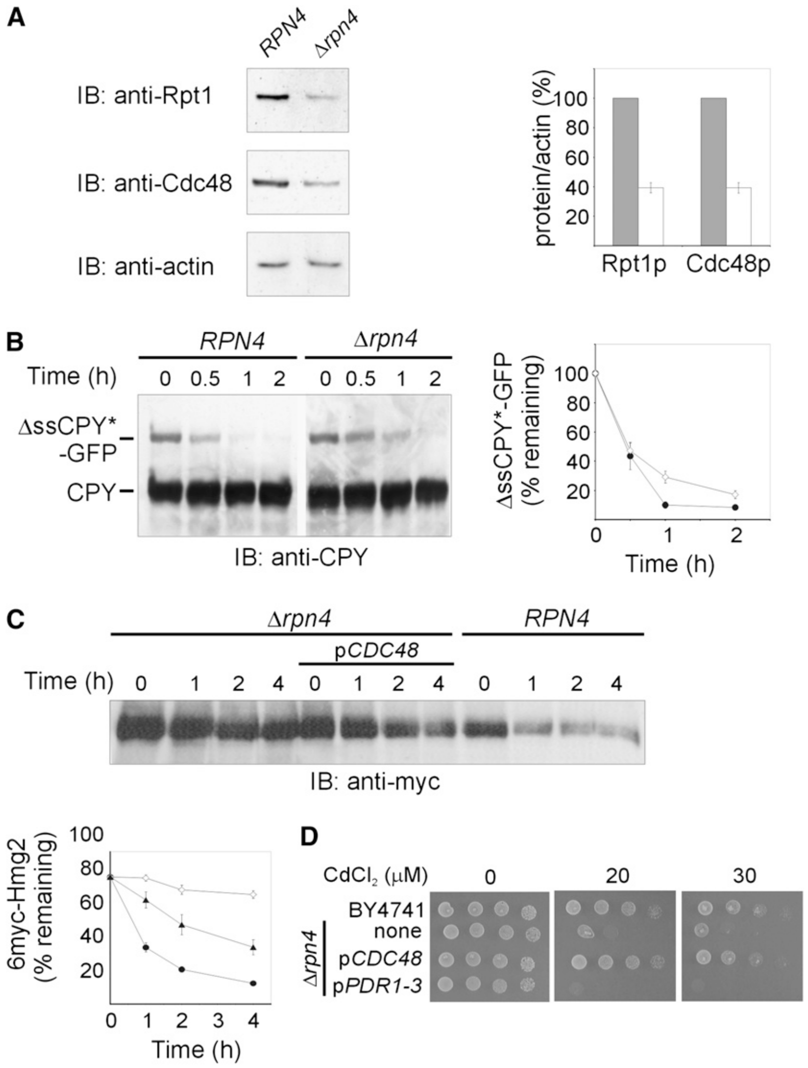

FIGURE 5.—Cdc48p is a major limiting ERAD component in Δ*rpn4*. (A) Equal amounts of wild-type *RPN4* (BY4741) and Δ*rpn4* cells were lysed, equal amounts of total cellular proteins were resolved by SDS–PAGE, electroblotted and probed (IB) with the indicated primary antibodies, followed by HRP-conjugated secondary antibodies, and HRP was visualized by ECL. Anti-actin antibody was used to monitor cellular protein loads. Blots were quantified by densitometry, and the bars represent the ratio of Cdc48p or Rpt1p to actin in wild-type (100%; gray bars) or Δ*rpn4* cells (white bars). Values shown are means ± SEM of four independent experiments. (B) Proteasomal degradation of the cytosolic substrate ΔssCPY*-GFP was measured in wild-type *RPN4* (BY4741; closed circles) and Δ*rpn4* (open diamonds) cells. Equal amounts of *RPN4* or Δ*rpn4* cells expressing ΔssCPY*-GFP were lysed, total cellular proteins were resolved by SDS–PAGE, electroblotted and probed (IB) with a mouse anti-CPY antibody followed by HRP-conjugated anti-mouse IgG, and HRP was visualized by ECL. Endogenous CPY indicates protein loads. Blots were quantified by densitometry and the decay of ΔssCPY*-GFP was plotted. The remaining ΔssCPY*-GFP was calculated as the percentage of its level at the time of cycloheximide addition (100%). Values shown are means ± SEM of at least three independent experiments. (C) Turnover of 6myc-Hmg2 was measured in wild-type *RPN4* (BY4741; closed circles), Δ*rpn4* (open diamonds) and Δ*rpn4* cells expressing p*CDC48* (pKF700; closed triangles). The degradation of 6myc-Hmg2 was followed at 30° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least three independent experiments. (D) The growth of 10-fold serial dilutions of wild-type *RPN4* (BY4741), Δ*rpn4* and Δ*rpn4* expressing either p*CDC48* or p*PDR1-3* plasmids was monitored on plates supplemented with the indicated concentrations of $CdCl_2$.

4D) could have resulted from limiting amounts of the proteasome. Indeed, proteasomal Rpt1p was diminished to ~40% of its wild-type levels in Δ*rpn4* cells (Figure 5A), consistent with decreased mRNA levels of two additional proteasome subunits, *RPT4* and *PRE1*, in Δ*rpn4* cells (KARPOV *et al.* 2008). However, *CDC48* is among the many genes that contain the Rpn4p-binding PACE sequence (MANNHAUPT *et al.* 1999; KAPRANOV *et al.* 2001) and Cdc48p was also diminished to ~40% of its wild-type level in Δ*rpn4* cells (Figure 5A), consistent with a 2.5-fold decrease in its mRNA (KARPOV *et al.* 2008). Hence, the impaired ERAD upon *RPN4* deletion could be the outcome of lower levels of Cdc48p. To distinguish whether the proteasome or Cdc48p was the major ERAD limiting factor in Δ*rpn4* cells, proteasomal activity was examined by the turnover of the cytosolic substrate ΔssCPY*-GFP (MEDICHERLA *et al.* 2004), whose degradation was independent of Cdc48p (LIPSON *et al.* 2008). Evidently, the 2.5-fold lower levels of the proteasome in Δ*rpn4* cells (Figure 5A and KARPOV *et al.* 2008) were not limiting for proteasomal proteolysis of ΔssCPY*-GFP, as its short half-life was hardly affected by *RPN4* deletion (Figure 5B).

The diminished level of Cdc48p in Δ*rpn4* cells (Figure 5A), along with the efficient proteasomal proteolysis of ΔssCPY*-GFP, which was independent of either Rpn4p (Figure 5B) or Cdc48p (LIPSON *et al.* 2008), suggested that Cdc48p might be a major ERAD-M limiting component in Δ*rpn4* cells. Indeed, expression of the p*CDC48* plasmid significantly accelerated ERAD-M in Δ*rpn4* cells (Figure 5C). However, the shortened half-life of 6myc-Hmg2 was still longer than that measured in the wild-type *RPN4* strain, indicating that Cdc48p may not be the only limiting Rpn4p target involved in ERAD. Impaired degradation of abnormal proteins by the ubiquitin–proteasome system is also reflected by sensitivity to cadmium (JUNGMANN *et al.* 1993). Cadmium reacts with thiol groups and can displace zinc, iron, or copper from certain metalloproteins (VIDO *et al.* 2001). The correlation between cadmium sensitivity and ER stress was inferred from activation of the unfolded protein response (URANO *et al.* 2002) and upregulation of ERAD-related proteins, such as Cdc48p, Kar2/BiP/GRP78, and 26S proteasome subunits, in response to cadmium (Vido *et al.* 2001). Increased cadmium sensitivity upon *SSZ1* deletion was previously reported but Ssz1p-mediated copper tolerance indicated that neither *PDR1* nor *PDR5* was involved (Kim *et al.* 2001). We found that Δ*rpn4* cells were highly sensitive to cadmium (Figure 5D). In accordance with the remarkable sensitivity of Δ*rpn4* cells to many xenobiotics, which exceeds the sensitivity of *PDR1* deletion mutants (Teixeira *et al.* 2008), we show that this increased sensitivity was not alleviated by the p*PDR1-3* plasmid (Figure 5D), although Pdr1p upregulates ABC transporters and other genes that participate in efflux of cytotoxic compounds (DeRisi *et al.* 2000; Gulshan and Moye-Rowley 2007). Conversely, the cadmium hypersensitivity of Δ*rpn4* cells was alleviated by the p*CDC48* plasmid (Figure 5D), correlating the cadmium sensitivity of Δ*rpn4* cells with impaired ERAD that results from limiting amounts of Cdc48p rather than insufficient proteasome. Interestingly, we found that the *cdc48-10* and the Δ*rpn4* are synthetically lethal mutations (data not shown), indicating that cells can tolerate defective Cdc48p or diminished levels of this essential AAA-ATPase but cannot survive when the amounts of the compromised Cdc48p are limiting.

ERAD-M but not ERAD-L is restored by excess cdc48-10p, or Cdc48p in *cdc48-10*, or the hypomorphic *ufd1-2* and *npl4-1* strains: Our results thus far demonstrate that Cdc48p level is regulated by Rpn4p and that Cdc48p is the major limiting ERAD-M factor in Δ*rpn4* cells. We also show that p*RPN4*, p*PDR1*, and p*SSZ1* similarly restore ERAD-M in *cdc48-10* cells. Combined, these findings could be explained by the ability of Ssz1p to increase the level of cdc48-10p via the post-translational activation of Pdr1p, which, in turn, activates *RPN4* transcription, hence upregulating *cdc48-10* expression. Indeed, we observed ~70% increase in the levels of the cdc48-10p protein in *cdc48-10* cells expressing p*SSZ1*, ~40% in cells expressing p*PDR1-3* and nearly 180% increase in cells expressing p*RPN4* (Figure 6A). These findings suggested that ERAD-M could be restored by an excess of the mutated cdc48-10p protein that compensated for its poor activity and somewhat reduced level in *cdc48-10* cells (Figure 6A). To directly test this possibility, we cloned the *cdc48-10* gene and introduced the p*cdc48-10* plasmid into *cdc48-10* cells. Clearly, the approximately twofold excess cdc48-10p (Figure 6A) partially restored the impaired ERAD-M of 6myc-Hmg2 at 37° (Figure 6B). Sequencing of p*cdc48-10* identified two missense mutations, P257L and T413R. These substitutions may hamper hexamerization, since P257 in the D1 Walker A motif is highly conserved and proximal to the ATP-binding K261. In mammalian p97, D1 and its ATP-binding lysine participate in hexamerization (Wang *et al.* 2003; DeLaBarre *et al.* 2006). Thus, hexamerization of cdc48-10p may nevertheless be driven by its excess upon p*cdc48-10* expression.

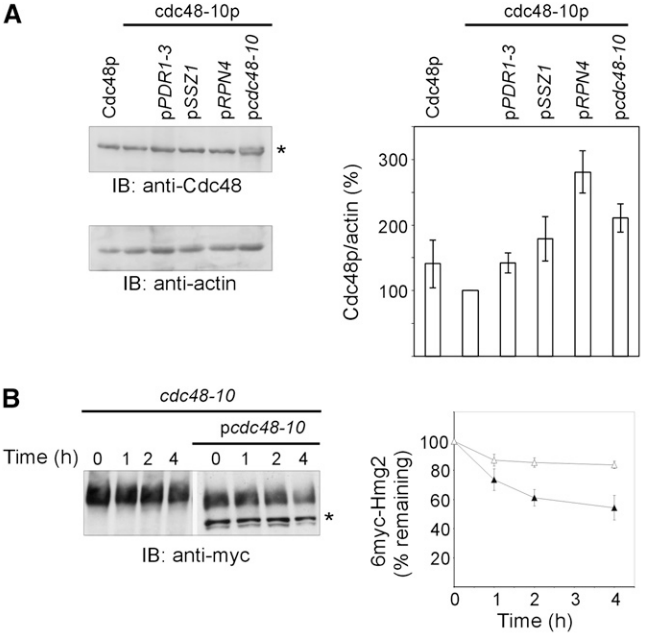

Figure 6.—Increased cdc48-10p protein levels restore ERAD-M in *cdc48-10* cells. (A) Equal amounts of total cellular proteins from *CDC48* wild-type and *cdc48-10* cells or *cdc48-10* cells expressing p*PDR1-3*, p*SSZ1*, p*RPN4*, or p*cdc48-10* plasmids were analyzed as describe in Figure 5A. Total amounts of wild-type Cdc48p or cdc48-10p proteins were probed with anti-Cdc48. Anti-actin was used to monitor protein load. Blots were quantified by densitometry, and the bars represent the ratio of Cdc48p or cdc48-10p to actin, with the ratio in naive *cdc48-10* set as 100%. Values shown are means of three independent experiments ± SEM (B) Turnover of 6myc-Hmg2 was measured in *cdc48-10* (SBN194; open symbols) and *cdc48-10* expressing excess *cdc48-10* from p*cdc48-10* (pDS194; closed symbols). The degradation of 6myc-Hmg2 was followed at 37° (triangles), as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments. The myc-tagged cdc48-10 expressed from the plasmid is marked by an asterisk.

The ERAD-dedicated partners of Cdc48p are Npl4p and Ufd1p. Therefore, the ability of p*SSZ1* to restore ERAD-M in the *npl4-1* or *ufd1-2* strains (Figure 3) could have similarly resulted from upregulating *npl4-1* and *ufd1-2* expression. Although no PACE was found in the *NPL4* or *UFD1* promoters, *UFD1* is considered among the Rpn4p-target genes (Beyer *et al.* 2006; Metzger and Michaelis 2009). Alternatively, the reduced activity of the entire Cdc48p–Npl4p–Ufd1p complex in the *npl4-1* or *ufd1-2* strains may have resulted from impaired interaction of the mutated hypomorphic component with Cdc48p. If this was the case, the activity of the Cdc48p–Npl4p–Ufd1p complex should be rescued by an excess of Cdc48p. Indeed, our results show that the impaired ERAD-M of 6myc-Hmg2 in *npl4-1* (Figure 7A) and *ufd1-2* (Figure 7B) was restored upon expression of the p*CDC48* plasmid. This suggests that excess Cdc48p

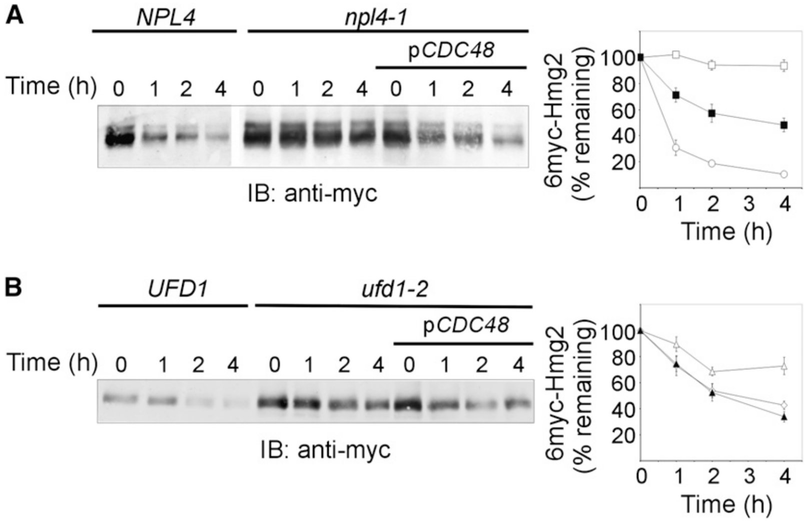

Figure 7.—*CDC48* plasmid restores ERAD in *npl4-1* and *ufd1-2* hypomorphic mutant strains. Turnover of 6myc-Hmg2 was measured in (A) wild-type *NPL4* (open circles), *npl4-1* (open squares), and *npl4-1* expressing p*CDC48* (pOO700; closed squares). (B) Wild-type *UFD1* (*DF5a*; open diamonds), *ufd1-2* (open triangles), and *ufd1-2* expressing p*CDC48* (pOO700; closed triangles). The degradation of 6myc-Hmg2 was followed at 30° as described in Figure 1A and plotted as described in Figure 1B. Values shown are means ± SEM of at least four independent experiments.

protein may compensate for its weakened interaction with the mutant npl4-1p and ufd1-2p proteins, thus providing a plausible explanation for the restoring effect of the p*SSZ1* plasmid by upregulating Cdc48p.

ERAD-L and ERAD-M are distinguished by their substrates' topology and although they share some components, other proteins are specific to ERAD-L and are not required for ERAD-M (Carvalho *et al.* 2006; Ismail and Ng 2006; Nakatsukasa *et al.* 2008; Nakatsukasa and Brodsky 2008). Our initial attempt to restore the impaired ERAD-L with p*SSZ1* by following degradation of CPY*-HA has failed in either *cdc48-10* (Figure 8A) or *ufd1-2* (Figure 8B) strains. Thus, we speculated that we might have identified an ERAD-M-specific component. However, as the evidence accumulated that p*SSZ1* restored ERAD-M by upregulating Cdc48p, the disparate effects of p*SSZ1* on ERAD-M and ERAD-L were puzzling because both pathways converge at the Cdc48p–Ufd1p–Npl4p complex (Ismail and Ng 2006; Nakatsukasa and Brodsky 2008). This conundrum was resolved by our finding that neither p*cdc48-10* (Figure 8C) nor p*CDC48* (Figure 8D) restored the impaired degradation of CPY*-HA in *cdc48-10* or *ufd1-2* strains, respectively. Therefore, the correlation between excess Ssz1p and excess Cdc48p stands firm, suggesting that the ability of Ssz1p to restore ERAD is attributed mainly to its capacity to upregulate Cdc48p.

DISCUSSION

Our genetic screen for suppressors that restore defective ERAD in *cdc48-10* cells identified Ssz1p. Being a member of the Hsp70 family, we considered the possibility that Ssz1p acted as a cytosolic chaperone that functions in ERAD by either activating or replacing the defective Cdc48p. The ability of Ssz1p to partially restore the impaired 6myc-Hmg2 degradation in *ufd1-2* and *npl4-1* mutants could have suggested that Ssz1p replaced the entire Cdc48p–Ufd1p–Npl4p complex in its role in ERAD. However, Ssz1p was not an essential ERAD factor and we could not detect any interaction of Ssz1p with the ERAD-M substrate 6myc-Hmg2. Moreover, Ssz1p is an unusual chaperone (Gautschi *et al.* 2001; Gautschi *et al.* 2002; Hundley *et al.* 2002; Shaner and Morano 2007), and therefore, we attributed its ability to correct ERAD to other functions of this Hsp70 member.

On the basis of the RAC-independent participation of Ssz1p in the PDR network, we examined whether other PDR members might be linked to ERAD. Indeed, Pdr1p and Rpn4p, which operate downstream of Ssz1p along the same PDR activation path, exerted similar suppression of the ERAD defects in the *cdc48-10* cells. Thus, PDR upregulation could compensate for the defective Cdc48p in ERAD. A connection between ERAD and PDR was previously suggested by the observation that the cytosolic Hsp70 Ssa1p, which was implicated in ERAD (Huyer *et al.* 2004), interacted with Pdr3p, repressed its activity, and downregulated the expression of its target gene *PDR5* (Shahi *et al.* 2007). Clearly, Pdr1p and Rpn4p, which are transcriptional activators that operate in the nucleus, are highly unlikely to play a direct role in ERAD. However, our finding that Rpn4p was essential for ERAD suggested that the suppressing effects of Ssz1p and Pdr1p were mediated through Rpn4p.

The involvement of Rpn4p in ERAD-M of 6myc-Hmg2 (Figure 4) and ERAD-L of CPY*-HA (Ng *et al.* 2000) is consistent with its well-established role as a transcriptional activator of proteasome subunit genes. Hence, stabilization of ERAD substrates in the Δ*rpn4* could have resulted from a globally reduced proteasomal degradation, as was shown for N-end rule and ubiquitin fusion degradation (UFD) model substrates (Johnson *et al.* 1995). However, in Δ*rpn4* cells, the Cdc48p-independent proteasomal degradation of the cytosolic ΔssCPY*-GFP proceeded unabated and expression of *CDC48* plasmid partially restored the impaired ERAD-M and alleviated

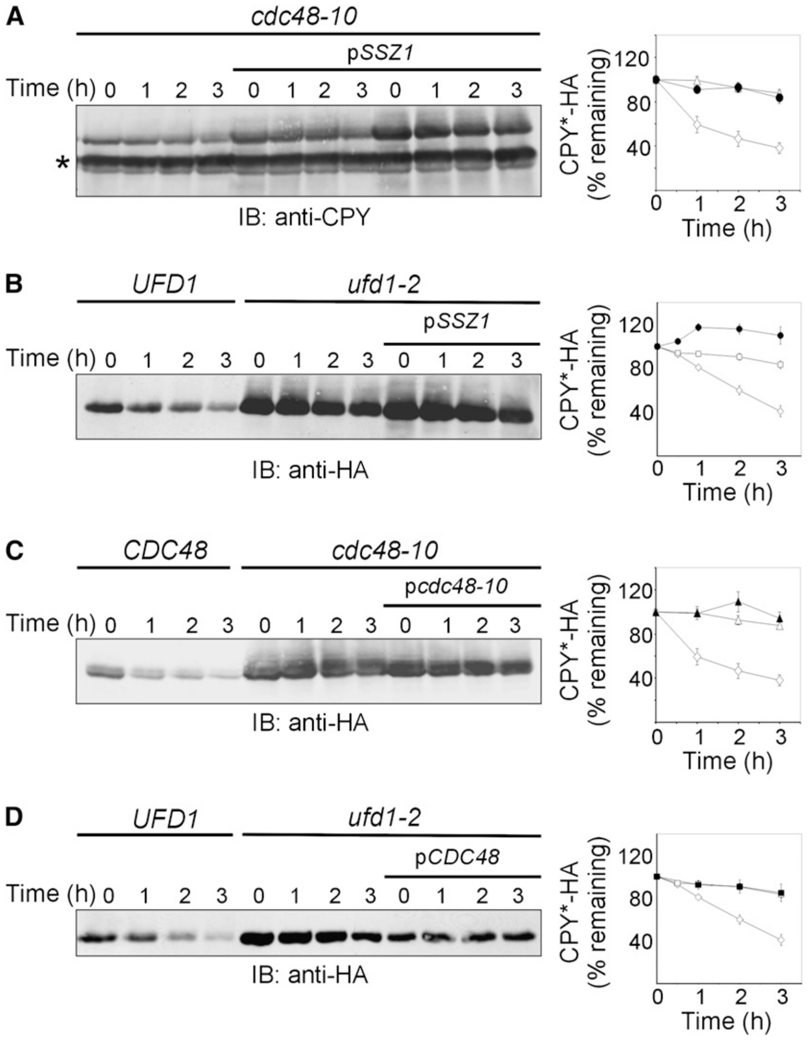

FIGURE 8.—Not p*SSZ1* or p*cdc48-10* or p*CDC48* plasmids restore ERAD-L in either *cdc48-10* or *ufd1-2* strains. Turnover of CPY*-HA was measured at 37° (A and C) in wild-type (C; *CDC48*; open diamonds), *cdc48-10* (A and C; open triangles), *cdc48-10* expressing p*SSZ1* (A; closed circles) or *cdc48-10* expressing p*cdc48-10* (C; closed triangles). Turnover of CPY*-HA was measured at 30° (B and D) in wild-type (*UFD1*; open diamonds), *ufd1-2* (open squares), *ufd1-2* expressing p*SSZ1* (B; closed circles), or *ufd1-2* expressing p*cdc48-10* (D; closed squares). The degradation of CPY*-HA was monitored by the cycloheximide chase, as described in Figure 1A. Cellular proteins resolved by SDS–PAGE were probed (IB) with mouse anti-CPY (A) or anti-HA (B–D) antibodies. Endogenous CPY, marked by an asterisk, indicates protein loads. The remaining CPY*-HA was calculated as described in Figure 1B. Values are means ± SEM of at least three independent experiments.

the hypersensitivity to cadmium (Figure 5), strongly suggesting that Cdc48p rather than the proteasome was a major limiting ERAD factor in Δ*rpn4* cells. Indeed, the level of the Cdc48 mRNA (KARPOV *et al.* 2008) and protein (Figure 5) was similarly diminished in Δ*rpn4* cells. This agrees with the presence of Rpn4p-binding PACE sequence in the *CDC48* promoter and its contribution to the *CDC48* gene expression (MANNHAUPT *et al.* 1999; KAPRANOV *et al.* 2001). Interestingly, the beneficial effects of Cdc48p in Δ*rpn4* cells were manifested mainly in ERAD, since *CDC48* expression in these cells did not correct their cell cycle abnormalities (XIE and VARSHAVSKY 2001).

Inasmuch as the critical outcome of *SSZ1* expression is the Rpn4p-dependent upregulation of Cdc48p but not the proteasome, it is puzzling that Pdr1p and Ssz1p are not essential for ERAD, since Rpn4p itself is a short-lived substrate of the proteasome (XIE and VARSHAVSKY 2001) that needs to be continuously replenished. This can be explained by the multitude of stress-related factors that regulate *RPN4* gene transcription (OWSIANIK *et al.* 2002; HAHN *et al.* 2006; DOHMEN *et al.* 2007; GULSHAN and MOYE-ROWLEY 2007; HANNA and FINLEY 2007). These include Pdr1p and its inducible homolog Pdr3p (GULSHAN and MOYE-ROWLEY 2007), Yap1p, a key transcriptional regulator of oxidative stress response (RODRIGUES-POUSADA *et al.* 2004) and cadmium tolerance (WU *et al.* 1993) that contains PACE (FLEMING *et al.* 2002) and requires Rpn4p for its full activation (TEIXEIRA *et al.* 2008), and the heat shock transcription factor HSF, which regulates *PDR3*, *RPN4*, and Rpn4p target genes in response to heat shock (HAHN *et al.* 2006).

The notion that Ssz1 restores ERAD-M in *cdc48-10*, *ufd1-2*, and *npl4-1* cells as a result of Rpn4p-dependent upregulation of Cdc48p was supported by our findings that the levels of the cdc48-10p protein indeed increased upon expression of the p*SSZ1*, p*PDR1-3*, and p*RPN4* plasmids, and by the partially restored degradation of 6myc-Hmg2 in the *cdc48-10* mutant upon a twofold increase in cdc48-10p level when expressed from p*cdc48-10* plasmid (Figure 6). This indicates that an excess of this mutant protein can compensate for its sluggish activity and somewhat reduced levels in the *cdc48-10* strain, possibly driving hexamerization of the *cdc48-10* (P257L/T413R) mutant. Likewise, the p*CDC48* plasmid partially restored the impaired ERAD-M in the *ufd1-2* and *npl4-1* mutant strains (Figure 7), indicating that excess wild-type Cdc48p protein can compensate for weak interactions with its hypomorphic partners in forming the Cdc48p–Ufd1p–Npl4p complex.

Finally, we show that p*SSZ1* can restore ERAD-M but not ERAD-L, as 6myc-Hmg2 is classified as an ERAD-M substrate (Hampton *et al.* 1996; Sato and Hampton 2006). Although ERAD-L, ERAD-M, and ERAD-C converge at the p97/Cdc48p–Ufd1p–Npl4p complex (Carvalho *et al.* 2006; Ismail and Ng 2006; Christianson *et al.* 2008; Nakatsukasa and Brodsky 2008; Wang and Ng 2008), and Ssz1p restores ERAD-M by upregulating a key component of this common complex, its inability to restore ERAD-L may suggest a quantitative difference. Namely, larger amounts of Cdc48p are required for ERAD-L as compared to ERAD-M. This possibility is supported by the effect of p97 on ERAD-M of cystic fibrosis transmembrane conductance regulator (CFTR) in a reconstituted cell-free system, where p97 augmented degradation but was not obligatorily required (Carlson *et al.* 2006). We found that neither excess cdc48-10p in *cdc48-10* strain nor excess Cdc48p in *ufd1-2* strain could restore ERAD-L (Figure 8). Nonetheless, the correlation between p*SSZ1* expression, excess Cdc48p protein, and restored ERAD-M remains. Interestingly, a unique role for Rpn4p in tolerating ERAD-M substrates was recently reported (Metzger and Michaelis 2009). Among the 67 genes induced by the misfolded membrane Ste6p* (Metzger and Michaelis 2009), 23 are regulated by Rpn4p (Mannhaupt *et al.* 1999; Fleming *et al.* 2002; Beyer *et al.* 2006), and many of them encode for proteasome subunits and additional putative members of the ubiquitin–proteasome related pathways (Metzger and Michaelis 2009). By showing that Δ*rpn4* cells are not sensitive to tunicamycin, these authors concluded that only misfolded membrane proteins require Rpn4p, further suggesting that the particular sensitivity of Δ*rpn4* cells to ER stress is due to the limiting proteasome (Metzger and Michaelis 2009). Our results propose that the inability of Δ*rpn4* cells to handle ERAD-M and to tolerate cadmium or defective Cdc48p is due to the role of Rpn4p in regulating Cdc48p. Thus, misfolded membrane proteins may provide a particular challenge to cells with impaired or limiting Cdc48p, which can be restored by Ssz1p, whereas ERAD-L requires even higher levels of Cdc48p that cannot be provided by Ssz1p.

We are indebted to W. S. Moye-Rowley, R. Hampton, E. Craig, J. Brodsky, J. Frydman, Y. Xie, M. Glickman, and K. U. Fröhlich for the gifts of plasmids, strains, and antibodies. We are grateful to E. Grinboim, Y. Manaster, F. Yerusalemski, M. Shaked, G. Alalouf, H. Nelson, Y. Mazor, and G. Bosis for their excellent technical help. We thank J. Roitelman, N. Nelson, E. Nachliel, and members of our laboratory for critical reading of the manuscript. Initial stages of this study were supported (in part) by grants from the Israel Science Foundation (to E.R.) and the United States–Israel Binational Science Foundation (to S.B.) and the Chief Scientist Office of the Ministry of Health, Israel (grant no. 3-3211 to S.B.).

LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

Balzi, E., W. Chen, S. Ulaszewski, E. Capieaux and A. Goffeau, 1987 The multidrug resistance gene PDR1 from Saccharomyces cerevisiae. J. Biol. Chem. **262:** 16871–16879.

Bar-Nun, S., 2005 The role of p97/Cdc48p in endoplasmic reticulum-associated degradation: from the immune system to yeast. Curr. Top. Microbiol. Immunol. **300:** 95–125.

Bays, N. W., S. K. Wilhovsky, A. Goradia, K. Hodgkiss-Harlow and R. Y. Hampton, 2001 HRD4/NPL4 Is Required for the Proteasomal Processing of Ubiquitinated ER Proteins. Mol. Biol. Cell **12:** 4114–4128.

Beyer, A., C. Workman, J. Hollunder, D. Radke, U. Moller *et al.*, 2006 Integrated assessment and prediction of transcription factor binding. PLoS Comput. Biol. **2:** e70.

Bonifacino, J. S., and A. M. Weissman, 1998 Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu. Rev. Cell. Dev. Biol. **14:** 19–57.

Braun, S., K. Matuschewski, M. Rape, S. Thoms and S. Jentsch, 2002 Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J. **21:** 615–621.

Carlson, E. J., D. Pitonzo and W. R. Skach, 2006 p97 functions as an auxiliary factor to facilitate TM domain extraction during CFTR ER-associated degradation. EMBO J. **25:** 4557–4566.

Carvajal, E., H. B. Van Den Hazel, A. Cybularz-Kolaczkowska, E. Balzi and A. Goffeau, 1997 Molecular and phenotypic characterization of yeast PDR1 mutants that show hyperactive transcription of various ABC multidrug transporter genes. Mol. Gen. Genet. **256:** 406–415.

Carvalho, P., V. Goder and T. A. Rapoport, 2006 Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell **126:** 361–373.

Christianson, J. C., T. A. Shaler, R. E. Tyler and R. R. Kopito, 2008 OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1–SEL1L ubiquitin ligase complex for ERAD. Nat. Cell Biol. **10:** 272–282.

DeLaBarre, B., J. C. Christianson, R. R. Kopito and A. T. Brunger, 2006 Central Pore Residues Mediate the p97/VCP Activity Required for ERAD. Mol. Cell. **22:** 451–462.

DeRisi, J., H. B. Van Den, P. Marc, E. Balzi, P. Brown *et al.*, 2000 Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. FEBS Lett. **470:** 156–160.

Devaux, F., P. Marc, C. Bouchoux, T. Delaveau, I. Hikkel *et al.*, 2001 An artificial transcription activator mimics the genome-wide properties of the yeast Pdr1 transcription factor. EMBO Rep. **2:** 493–498.

Dohmen, R. J., I. Willers and A. J. Marques, 2007 Biting the hand that feeds: Rpn4-dependent feedback regulation of proteasome function. Biochim. Biophys. Acta **1773:** 1599–1604.

Eisenman, H. C., and E. A. Craig, 2004 Activation of pleiotropic drug resistance by the J-protein and Hsp70-related proteins, Zuo1 and Ssz1. Mol. Microbiol. **53:** 335–344.

Elkabetz, Y., I. Shapira, E. Rabinovich and S. Bar-Nun, 2004 Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplamic reticulum-bound p97/Cdc48p and proteasome. J. Biol. Chem. **279:** 3980–3989.

Finley, D., E. Ozkaynak and A. Varshavsky, 1987 The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell **48:** 1035–1046.

Fleming, J. A., E. S. Lightcap, S. Sadis, V. Thoroddsen, C. E. Bulawa *et al.*, 2002 Complementary whole-genome technologies reveal the cellular response to proteasome inhibition by PS-341. Proc. Natl. Acad. Sci. USA **99:** 1461–1466.

Frohlich, K. U., H. W. Fries, M. Rudiger, R. Erdmann, D. Botstein *et al.*, 1991 Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. J. Cell Biol. **114:** 443–453.

Gautschi, M., H. Lilie, U. Funfschilling, A. Mun, S. Ross *et al.*, 2001 RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin. Proc. Natl. Acad. Sci. USA **98:** 3762–3767.

Gautschi, M., A. Mun, S. Ross and S. Rospert, 2002 A functional chaperone triad on the yeast ribosome. Proc. Natl. Acad. Sci. USA **99:** 4209–4214.

Ghislain, M., R. J. Dohmen, F. Levy and A. Varshavsky, 1996 Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin- mediated proteolysis in Saccharomyces cerevisiae. EMBO J. **15:** 4884–4899.

Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles *et al.*, 2002 Functional profiling of the Saccharomyces cerevisiae genome. Nature **418:** 387–391.

Gulshan, K., and W. S. Moye-Rowley, 2007 Multidrug resistance in fungi. Eukaryot. Cell **6:** 1933–1942.

Hahn, J. S., D. W. Neef and D. J. Thiele, 2006 A stress regulatory network for co-ordinated activation of proteasome expression mediated by yeast heat shock transcription factor. Mol. Microbiol. **60:** 240–251.

Hallstrom, T. C., D. J. Katzmann, R. J. Torres, W. J. Sharp and W. S. Moye-Rowley, 1998 Regulation of transcription factor Pdr1p function by an Hsp70 protein in Saccharomyces cerevisiae. Mol. Cell. Biol. **18:** 1147–1155.

Hampton, R. Y., R. G. Gardner and J. Rine, 1996 Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3- methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol. Biol. Cell **7:** 2029–2044.

Hanna, J., and D. Finley, 2007 A proteasome for all occasions. FEBS Lett. **581:** 2854–2861.

Hundley, H., H. Eisenman, W. Walter, T. Evans, Y. Hotokezaka *et al.*, 2002 The in vivo function of the ribosome-associated Hsp70, Ssz1, does not require its putative peptide-binding domain. Proc. Natl. Acad. Sci. USA **99:** 4203–4208.

Huyer, G., W. F. Piluek, Z. Fansler, S. G. Kreft, M. Hochstrasser *et al.*, 2004 Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. J. Biol. Chem. **279:** 38369–38378.

Ismail, N., and D. T. Ng, 2006 Have you HRD? Understanding ERAD is DOAble! Cell **126:** 237–239.

Jarosch, E., C. Taxis, C. Volkwein, J. Bordallo, D. Finley *et al.*, 2002 Protein dislocation from the ER requires polyubiquitination and the AAA- ATPase Cdc48. Nat. Cell Biol. **4:** 134–139.

Jelinsky, S. A., P. Estep, G. M. Church and L. D. Samson, 2000 Regulatory networks revealed by transcriptional profiling of damaged Saccharomyces cerevisiae cells: Rpn4 links base excision repair with proteasomes. Mol. Cell. Biol. **20:** 8157–8167.

Johnson, E. S., P. C. Ma, I. M. Ota and A. Varshavsky, 1995 A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. **270:** 17442–17456.

Ju, D., L. Wang, X. Mao and Y. Xie, 2004 Homeostatic regulation of the proteasome via an Rpn4-dependent feedback circuit. Biochem. Biophys. Res. Commun. **321:** 51–57.

Jungmann, J., H. A. Reins, C. Schobert and S. Jentsch, 1993 Resistance to cadmium mediated by ubiquitin-dependent proteolysis. Nature **361:** 369–371.

Kapranov, A. B., M. V. Kuriatova, O. V. Preobrazhenskaia, V. V. Tiutiaeva, R. Shtuka *et al.*, 2001 Isolation and identification of PACE-binding protein rpn4–a new transcription activator, participating in regulation of 26S proteosome and other genes. Mol. Biol. **35:** 420–431.

Karpov, D. S., S. A. Osipov, O. V. Preobrazhenskaya and V. L. Karpov, 2008 Rpn4p is a positive and negative transcriptional regulator of the ubiquitin–proteasome system. Mol. Biol. (Mosk) **42:** 456–462.

Kim, D. Y., W. Y. Song, Y. Y. Yang and Y. Lee, 2001 The role of PDR13 in tolerance to high copper stress in budding yeast. FEBS Lett. **508:** 99–102.

Lipson, C., G. Alalouf, M. Bajorek, E. Rabinovich, A. Atir-Lande *et al.*, 2008 A proteasomal ATPase contributes to dislocation of endoplasmic reticulum-associated degradation (ERAD) substrates. J. Biol. Chem. **283:** 7166–7175.

Lupas, A. N., and J. Martin, 2002 AAA proteins. Curr. Opin. Struct. Biol. **12:** 746–753.

Mamnun, Y. M., R. Pandjaitan, Y. Mahe, A. Delahodde and K. Kuchler, 2002 The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. Mol. Microbiol. **46:** 1429–1440.

Mannhaupt, G., R. Schnall, V. Karpov, I. Vetter and H. Feldmann, 1999 Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett. **450:** 27–34.

Medicherla, B., Z. Kostova, A. Schaefer and D. H. Wolf, 2004 A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. EMBO Rep. **5:** 692–697.

Metzger, M. B., and S. Michaelis, 2009 Analysis of quality control substrates in distinct cellular compartments reveals a unique role for Rpn4p in tolerating misfolded membrane proteins. Mol. Biol. Cell **20:** 1006–1019.

Moir, D., S. E. Stewart, B. C. Osmond and D. Botstein, 1982 Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. Genetics **100:** 547–563.

Nakatsukasa, K., and J. L. Brodsky, 2008 The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum. Traffic **9:** 861–870.

Nakatsukasa, K., G. Huyer, S. Michaelis and J. L. Brodsky, 2008 Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. Cell **132:** 101–112.

Ng, D. T., E. D. Spear and P. Walter, 2000 The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control. J. Cell Biol. **150:** 77–88.

Owsianik, G., I. L. Balzi and M. Ghislain, 2002 Control of 26S proteasome expression by transcription factors regulating multidrug resistance in Saccharomyces cerevisiae. Mol. Microbiol. **43:** 1295–1308.

Patel, S., and M. Latterich, 1998 The AAA team: related ATPases with diverse functions. Trends Cell Biol. **8:** 65–71.

Rabinovich, E., A. Kerem, K. U. Frohlich, N. Diamant and S. Bar-Nun, 2002 AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol. Cell. Biol. **22:** 626–634.

Rodrigues-Pousada, C. A., T. Nevitt, R. Menezes, D. Azevedo, J. Pereira *et al.*, 2004 Yeast activator proteins and stress response: an overview. FEBS Lett. **567:** 80–85.

Rouiller, I., B. DeLaBarre, A. P. May, W. I. Weis, A. T. Brunger *et al.*, 2002 Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle. Nat. Struct. Biol. **9:** 950–957.

Sato, B. K., and R. Y. Hampton, 2006 Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis. Yeast **23:** 1053–1064.

Shahi, P., K. Gulshan and W. S. Moye-Rowley, 2007 Negative transcriptional regulation of multidrug resistance gene expression by an Hsp70 protein. J. Biol. Chem. **282:** 26822–26831.

Shaner, L., and K. A. Morano, 2007 All in the family: atypical Hsp70 chaperones are conserved modulators of Hsp70 activity. Cell Stress Chaperones **12:** 1–8.

Stolc, V., and S. Altman, 1997 Rpp1, an essential protein subunit of nuclear RNase P required for processing of precursor tRNA and 35S precursor rRNA in Saccharomyces cerevisiae. Genes Dev. **11:** 2926–2937.

Teixeira, M. C., P. J. Dias, T. Simoes and I. Sa-Correia, 2008 Yeast adaptation to mancozeb involves the up-regulation of FLR1 under the coordinate control of Yap1, Rpn4, Pdr3, and Yrr1. Biochem. Biophys. Res. Commun. **367:** 249–255.

Urano, F., M. Calfon, T. Yoneda, C. Yun, M. Kiraly *et al.*, 2002 A survival pathway for Caenorhabditis elegans with a blocked unfolded protein response. J. Cell Biol. **158:** 639–646.

Vale, R. D, 2000 AAA Proteins. Lords of the ring. J. Cell Biol. **150:** F13–F20.

Vido, K., D. Spector, G. Lagniel, S. Lopez, M. B. Toledano *et al.*, 2001 A proteome analysis of the cadmium response in Saccharomyces cerevisiae. J. Biol. Chem. **276:** 8469–8474.

Wang, Q., and A. Chang, 2003 Substrate recognition in ER-associated degradation mediated by Eps1, a member of the protein disulfide isomerase family. EMBO J. **22:** 3792–3802.

Wang, Q., C. Song, X. Yang and C. C. H. Li, 2003 D1 ring is stable and nucleotide-independent, whereas D2 ring undergoes major conformational changes during the ATPase cycle of p97-VCP. J. Biol. Chem. **278:** 32784–32793.

Wang, S., and D. T. Ng, 2008 Lectins sweet-talk proteins into ERAD. Nat. Cell Biol. **10:** 251–253.

White, W. H., P. L. Gunyuzlu and J. H. Toyn, 2001 Saccharomyces cerevisiae is capable of de Novo pantothenic acid biosynthesis involving a novel pathway of beta-alanine production from spermine. J. Biol. Chem. **276:** 10794–10800.

Wu, A., J. A. Wemmie, N. P. Edgington, M. Goebl, J. L. Guevara *et al.*, 1993 Yeast bZip proteins mediate pleiotropic drug and metal resistance. J. Biol. Chem. **268:** 18850–18858.

Xie, Y., and A. Varshavsky, 2001 RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc. Natl. Acad. Sci. USA **98:** 3056–3061.

Ye, Y., H. H. Meyer and T. A. Rapoport, 2001 The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature **414:** 652–656.

Communicating editor: M. D. Rose