Molecular Microbiology (2004) **53**(1), 335–344 doi:10.1111/j.1365-2958.2004.04134.x

# Activation of pleiotropic drug resistance by the J-protein and Hsp70-related proteins, Zuo1 and Ssz1

**Helene C. Eisenman[†] and Elizabeth A. Craig***
*Program in Cellular and Molecular Biology, and Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706, USA.*

## Summary

**Ssz1 (Pdr13) and Zuo1, members of the Hsp70 and J-protein molecular chaperone families, respectively, form a heterodimer and function on the ribosome with the Hsp70, Ssb, presumably assisting folding of newly synthesized polypeptides. As it has also been reported that Ssz1 induces pleiotropic drug resistance (PDR) when overexpressed, a possible role for Zuo1 in PDR was investigated. The C-terminal domain of Zuo1, which is dispensable for Zuo1's chaperone function on the ribosome, is both necessary and sufficient for PDR induction by Zuo1. A single domain of Ssz1, the N-terminal ATPase domain, is sufficient for PDR induction as well, indicating that Ssz1 does not function as a chaperone in PDR. No role for Ssb was found in PDR; overexpression did not affect PDR, nor was its presence required for Ssz1's or Zuo1's effect on PDR. As our results also indicate that Ssz1 and Zuo1 must be free of ribosomes to induce PDR, we propose that Ssz1's and Zuo1's function in PDR is distinct from their role as ribosome-associated co-chaperones and may be regulatory in nature.**

## Introduction

Ssz1, an Hsp70 homologue of *Saccharomyces cerevisiae*, is a component of the general ribosome-associated chaperone machinery that most probably facilitates folding of newly synthesized polypeptides (Gautschi *et al*., 2002; Hundley *et al*., 2002). Ssz1, along with the J-protein Zuo1, is a subunit of an unusually stable heterodimeric ribosome-associated complex (RAC) (Gautschi *et al*., 2001). Normally all the Ssz1 in the cell is ribosome associated via its interaction with Zuo1, which interacts with ribosomes independently of Ssz1, at least in part through interactions with ribosomal RNA. Another Hsp70, Ssb1/2 (Ssb), also associates with ribosomes (Nelson *et al*., 1992).

Ssb, not Ssz1, is thought to act as a chaperone of newly synthesized ribosome-associated nascent polypeptides. Ssb, the function of which requires its C-terminal peptide-binding domain, as would be expected of an Hsp70 chaperone, can be cross-linked to ribosome-bound nascent polypeptide chains (Pfund *et al*., 1998; 2001). In contrast, Ssz1's C-terminal putative peptide-binding domain is not required for normal growth, nor have cross-links between Ssz1 and nascent chains been observed (Hundley *et al*., 2002). It is proposed that Ssz1 acts as a cofactor, allowing Zuo1 to function as the J-protein partner of Ssb (Gautschi *et al*., 2002; Hundley *et al*., 2002). The suggestion that two Hsp70s and one J-protein function together, with one Hsp70 forming a stable heterodimer with the J-protein, is unusual, as typically the J-domain of a J-protein interacts transiently with the N-terminal ATPase domain of a single Hsp70, stimulating ATP hydrolysis and stabilizing interaction of the C-terminal peptide-binding domain with unfolded protein substrates (Bukau and Horwich, 1998).

Along with its proposed role on the ribosome, Ssz1 has also been identified as an activator of pleiotropic drug resistance (PDR) when overexpressed (Hallstrom *et al*., 1998). PDR, defined as resistance to multiple drugs with unrelated structure or mode of action, occurs by upregulation of expression of ABC transporters causing increased efflux of drugs such as cycloheximide and oligomycin. This upregulation occurs primarily through the activation of the Pdr1 transcription factor that binds to Pdr1/3 response elements (PDRE) in the promoters of a number of genes involved in PDR, including those encoding the ABC transporters Pdr5 and Yor1 (Balzi *et al*., 1994; Balzi and Goffeau, 1995; Katzmann *et al*., 1995; 1996).

The connection between Ssz1's ability to activate PDR and its role as a ribosomal co-chaperone is unclear. We therefore set out to determine the domain(s) of Ssz1 required for PDR activation and whether other components of the ribosome-associated chaperone machinery function in PDR. We conclude that both Ssz1 and Zuo1 induce PDR when not associated with ribosomes and use domains other than those expected to be required for chaperone activity. Thus, many aspects of their function in PDR are distinct from their ribosome-associated chaperone function.

Accepted 18 March, 2004. *For correspondence at the Department of Biochemistry. E-mail ecraig@facstaff.wisc.edu; Tel. (+1) 608 263 7105; Fax (+1) 608 262 3453. [†]Present address: Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA.

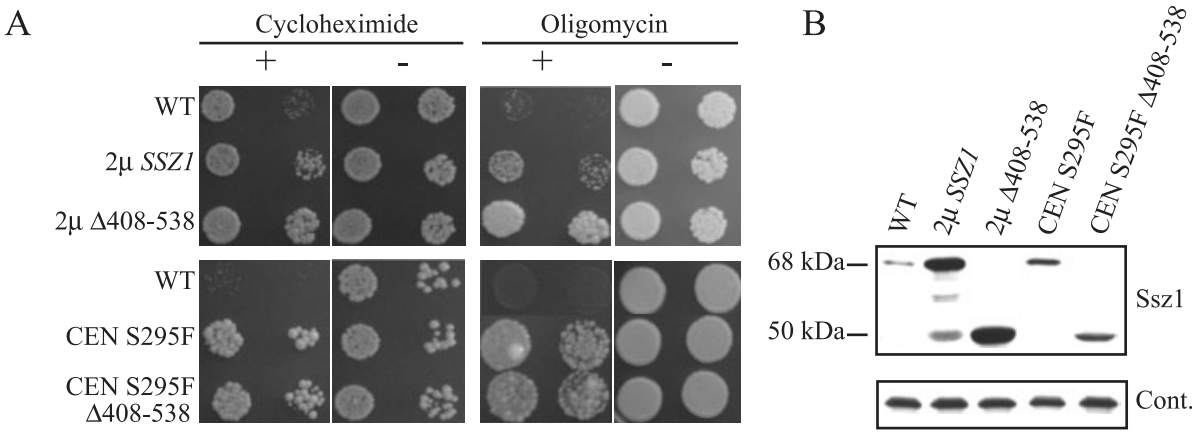


**Fig. 1.** The C-terminal domain of Ssz1 is not required for PDR. Strains used: wild type + vector (WT), Δ*ssz1* + multicopy *SSZ1* (2μ *SSZ1*), Δ*ssz1* + multicopy *SSZ1* Δ408–538 (2μ Δ408–538), Δ*ssz1* + centromeric *SSZ1* S295F (CEN S295F), Δ*ssz1* + centromeric S295F Δ407–538 (CEN S295F Δ407–538).
A. Serial dilutions of yeast cells were spotted on to media with (+) or without (–) cycloheximide or oligomycin and incubated as described in *Experimental procedures*, except for +cycloheximide, which were incubated for 4 days.
B. Whole-cell lysates were subjected to electrophoresis and immunoblotting with a polyclonal antibody recognizing the N-terminal domain of Ssz1 (Ssz1) or the mitochondrial protein, Ssc1, as a control (Cont.).

## Results

### *The putative peptide-binding domain of Ssz1 is not required for PDR activation*

To determine whether chaperone activity is necessary for induction of PDR caused by increased expression of Ssz1, we tested whether the N-terminal ATPase domain of Ssz1 was sufficient for activation using a growth assay. As expected, based on previously published results (Hallstrom *et al*., 1998), overexpression of Ssz1 from a multicopy plasmid allowed more robust growth on media containing either cycloheximide or oligomycin (Fig. 1A). A previously isolated mutant, *SSZ1*, encoding a single amino acid substitution in the ATPase domain, S295F, was more effective, even when expressed from a centromeric plasmid (Hallstrom *et al*., 1998; Fig. 1A). Overexpression of the ATPase domain of wild-type Ssz1 (Δ408–538) also increased resistance to these two drugs. Similarly, Ssz1 S295F was able to increase drug resistance in the absence of the C-terminal domain (Fig. 1A). Immunoblot analysis of extracts using Ssz1 ATPase domain-specific antibodies showed that both full-length Ssz1 and Ssz1 Δ408–538 were expressed from the multicopy plasmid at a level about fivefold higher than that of Ssz1 expressed from its normal chromosomal location. Ssz1 S295F and Ssz1 S295F Δ408–538 were present at twice the wild-type level (Fig. 1B, compare CEN S295F with WT).

As a second assay for PDR activation, we measured the induction of two Pdr1-dependent reporters, *PDR5-lacZ* and *YOR1-lacZ*, with the *PDR5* and *YOR1* promoters, respectively, fused to the *lacZ* gene. Expression of Ssz1 from a centromeric plasmid resulted in slightly higher β-galactosidase activities in cells carrying the *PDR5-lacZ* and *YOR1-lacZ* reporters compared with cells with a vector control (Table 1). When wild-type Ssz1 or Ssz1 Δ408–538 was overexpressed from a multicopy

**Table 1.** Induction of PDR reporters by Ssz1.

| | β-Galactosidase activity of reporter gene fusions in the presence of *SSZ1* plasmids[a]: | | | | | |
|---|---|---|---|---|---|---|
| | *PDR5-lacZ* | | *YOR1-lacZ* | | *TRP5-lacZ* | |
| *SSZ1* plasmid | Activity ($U/OD_{600}$) | Fold activation | Activity ($U/OD_{600}$) | Fold activation | Activity ($U/OD_{600}$) | Fold activation |
| Vector | 61 ± 16 | 1.0 | 4.8 ± 0.6 | 1.0 | 70 ± 10 | 1.0 |
| Centromeric *SSZ1* | 140 ± 33 | 2.3 | 6.8 ± 2.2 | 1.4 | 65 ± 26 | 0.9 |
| Multicopy *SSZ1* | 350 ± 81 | 5.8 | 15 ± 1.0 | 3.1 | 82 ± 48 | 1.2 |
| Multicopy Δ408–538 | 290 ± 15 | 4.8 | 18 ± 5.6 | 3.8 | 62 ± 14 | 0.9 |
| Centromeric S295F | 500 ± 96 | 5.2 | 29 ± 6.2 | 6.0 | 44 ± 6.9 | 0.6 |
| Centromeric S295F Δ408–538 | 970 ± 260 | 16 | 79 ± 20 | 16 | 63 ± 21 | 0.9 |

**a.** β-Galactosidase activity was measured in a Δ*ssz1* strain co-transformed with the indicated reporter and *SSZ1* plasmids. A minimum of two independent transformants were assayed for activity.

plasmid, greater induction was observed (between 4.8-fold and 5.8-fold for *PDR5-lacZ* and 3.1-fold and 3.8-fold for *YOR1-lacZ*). Likewise, Ssz1 S295F did not require its C-terminus for activation of the Pdr1-dependent reporters. Overexpression of full-length Ssz1 S295F led to between fivefold and sixfold induction of the *PDR5-lacZ* and *YOR1-lacZ* reporters. Ssz1 S295F Δ408–538 resulted in the highest level of induction observed for any construct at 16-fold. As a control, we used a construct with the *TRP5* promoter, which is not regulated by Pdr1, fused to the *lacZ* gene, *TRP5-lacZ*. The activity of *TRP5-lacZ* did not increase in the presence of any of the Ssz1 constructs. From these data, we conclude that neither wild-type Ssz1 nor Ssz1 S295F requires its C-terminal region to activate PDR.

### *Ribosome–Zuo1 interaction inhibits Ssz1 function in PDR*

Under normal growth conditions, nearly all the Ssz1 in cells is ribosome associated via Ssz1's interaction with Zuo1, consistent with the fact that Ssz1 and Zuo1 are expressed at equimolar amounts, form a very stable heterodimer and are equimolar in concentration to ribosomes (Gautschi *et al*., 2001). As expected, when Ssz1 was overexpressed, the majority of protein was not ribosome associated, with most of Ssz1 remaining at the top of a sucrose gradient, rather than co-migrating with ribosomes after centrifugation of a cell extract (Fig. 2A).

This result suggested the possibility that Ssz1 needs to be free of ribosomes to activate PDR. We then tested whether the hyperactive allele *SSZ1* S295F, which induces PDR when expressed from a centromeric plasmid, could induce PDR when associated with ribosomes. We found that, when expressed from a centromeric plasmid, Ssz1 S295F was present at about twice the normal level (Fig. 1B), with about half the protein being free of ribosomes (Fig. 2A). After integration of the *SSZ1* S295F allele into the chromosome, Ssz1 S295F was expressed at normal levels, and all the detectable Ssz1 was ribosome associated (Fig. 2A and B). Consistent with the idea that Ssz1 must be free of ribosomes in order to modulate drug resistance, these cells did not exhibit increased resistance to cycloheximide (Fig. 2C).

To test more directly whether disruption of the interaction of S295F with the ribosome would increase drug resistance, a strain was constructed that contained a disruption of *ZUO1* as well as the integrated *SSZ1* S295F allele. The absence of Zuo1 restored the ability of Ssz1 S295F to increase drug resistance when expressed at normal levels (Fig. 2C). In agreement with the drug sensitivity data, the strain with the integrated *SSZ1* S295F allele had the same level of *PDR5-lacZ* and *YOR1-lacZ* reporter activity as wild type (Fig. 2D). When the integrated *SSZ1* S295F allele was combined with a *ZUO1* deletion, the strain had higher *PDR5-lacZ* and *YOR1-lacZ* reporter activity, similar to that seen in a *ssz1 zuo1* strain with *SSZ1* S295F present on a centromeric plasmid.

These data suggest that lack of association with the ribosome (or perhaps the ribosome and Zuo1) is important for PDR activation by Ssz1. However, a Δ*zuo1* strain with the wild-type *SSZ1* allele is not drug resistant (Fig. 2C; data not shown). Therefore, wild-type SSZ1 must be expressed at higher than normal levels to induce PDR, even in the absence of Zuo1.

### *Zuo1 Δ285–364 functions in PDR*

As deletion of *SSZ1* results in identical growth phenotypes as deletion of *ZUO1* or *SSB* (Gautschi *et al*., 2002; Hundley *et al*., 2002), we tested whether overexpression of either of these two chaperones increased PDR. However, expression of Zuo1 or Ssb from a multicopy plasmid did not increase resistance to cycloheximide (Fig. 3A and C; data not shown).

Considering the results presented above, the fact that Ssb overexpression did not result in increased PDR is, perhaps, not surprising as Ssb is normally produced at about twice the levels of Zuo1 and Ssz1 and about 50% is free of ribosomes (Nelson *et al*., 1992; Pfund *et al*., 1998; 2001). Because Zuo1 and Ssz1 are normally so tightly associated in the cell, we decided to analyse Zuo1 more closely, asking whether Zuo1 could activate PDR if its association with ribosomes was disrupted. Amino acids 285–364 of Zuo1, which have a high percentage of charged residues, are required for ribosome association, as well as for the general RNA-binding activity of Zuo1 (Yan *et al*., 1998). Zuo1 Δ285–364 does not rescue the growth defects of Δ*zuo1* cells (slow growth and sensitivity to salt and aminoglycosides such as paromomycin) even when overexpressed (Fig. 3D; Yan *et al*., 1998). However, when Zuo1 Δ285–364 was expressed in either a wild-type (Fig. 3A) or a Δ*zuo1* (data not shown) background, resistance to both cycloheximide and oligomycin increased. Furthermore, the β-galactosidase activity of the *PDR5-lacZ* and *YOR1-lacZ* reporters increased approximately sixfold, whereas no increase was observed upon overexpression of wild-type Zuo1 (Fig. 3B). Zuo1 Δ285–364 expression did not affect the activity of the control reporter, *TRP5-lacZ*, indicating that the increase is specific for Pdr1 targets. In addition, the induction by Zuo1 Δ285–364 was not dependent on Ssz1, as the *PDR5-lacZ* reporter increased by eightfold in a *zuo1 ssz1* deletion strain (Fig. 3B). In summary, the outcome of overexpression of Zuo1 Δ285–364 on PDR was similar to that of overexpression of Ssz1.

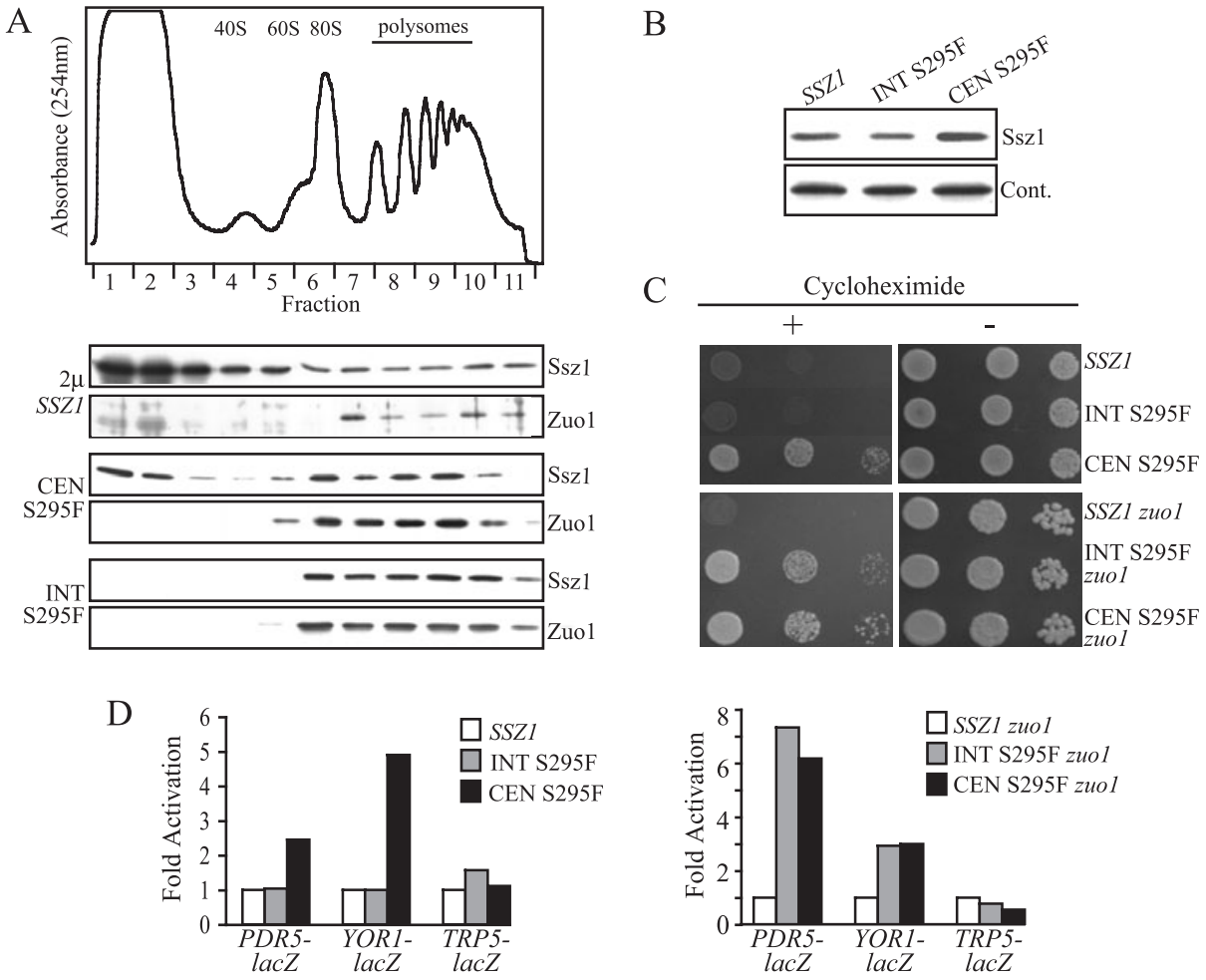


**Fig. 2.** Integrated S295F is not drug resistant. Strains used: wild type + vector (*SSZ1*), *SSZ1* S295F integrated in chromosomal locus (INT S295F), Δ*ssz1* + centromeric *SSZ1* S295F (CEN S295F), Δ*zuo1* + vector (*SSZ1 zuo1*), *zuo1* combined with *SSZ1* S295F integrated in chromosomal locus (INT S295F *zuo1*), Δ*zuo1* Δ*ssz1* + centromeric *SSZ1* S295F (CEN S295F *zuo1*) and wild type + multicopy *SSZ1* (2μ *SSZ1*).
A. Cellular extracts were separated on sucrose gradients, and fractions were collected and subjected to electrophoresis and immunoblotting with polyclonal antibodies recognizing Ssz1 or Zuo1. Migration of ribosomal subunits, monosomes and polysomes in the gradients is shown at the top.
B. Whole-cell lysates were subjected to electrophoresis and immunoblotting with a polyclonal antibody recognizing Ssz1 (Ssz1) or Ssc1 as a control (Cont.).
C. Serial dilutions of yeast cells were spotted on to media with (+) or without (–) cycloheximide and incubated as described in *Experimental procedures*.
D. β-Galactosidase activity of *PDR5-lacZ*, *YOR1-lacZ* or *TRP5-lacZ* reporters was measured as described in *Experimental procedures*. Data are presented as fold activation relative to *SSZ1* (left) or *SSZ1 zuo1* (right).The average β-galactosidase activities of the *PDR5-lacZ*, *YOR1-lacZ* and *TRP5-lacZ* reporters in the *SSZ1* strain were 270 ± 33, 10 ± 2.3 and 55 ± 5.9 units respectively. In the *SSZ1 zuo1* strain, the average activities of the *PDR5-lacZ*, *YOR1-lacZ* and *TRP5-lacZ* reporters were 160 ± 19, 28 ± 5.0 and 120 ± 14 units respectively.

### *The C-terminal domain of Zuo1 is necessary and sufficient for PDR activation*

To determine which regions of Zuo1 are required for the activation of PDR, we combined the *zuo1* Δ285–363 mutation with deletions of other regions of *ZUO1*: the N-terminal region (N) of 110 amino acids, the J domain between residues 111 and 165 (J), a middle region between residues 166 and 284 (M) and the C-terminal region containing residues 365–433 (c) (schematic diagrams shown in Fig. 4A). As shown in Fig. 4A, the presence of any construct containing the C-terminal region resulted in increased drug resistance. However, Zuo1 Δ285–433, which lacks the C-terminal region, did not cause drug resistance and did not activate the Pdr1-dependent *lacZ* reporter constructs (Fig. 4B). In contrast, removing the N-terminus, J domain or middle region did not affect Zuo1 Δ285–364's ability to increase cycloheximide resistance (Fig. 4A). Using polyclonal antibodies specific for either the N-terminal or the C-terminal region of Zuo1, we confirmed that each construct was expressed at a level similar to Zuo1 Δ285–365 (Fig. 4C; data not shown).

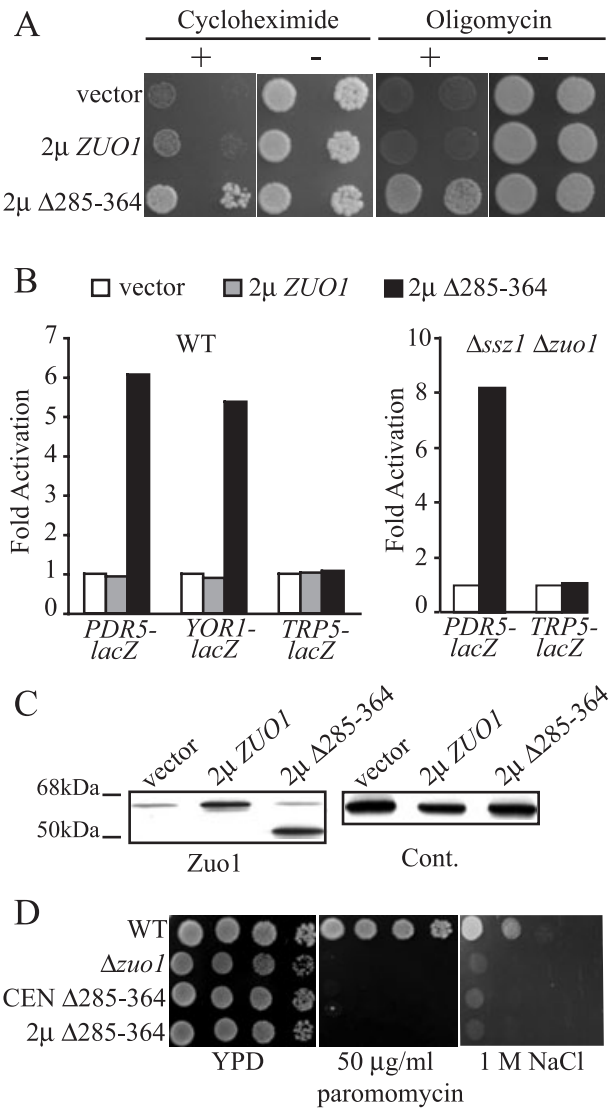


**Fig. 3.** Zuo1 Δ285–364 activates PDR.
A. Strains used: wild type + vector (vector), wild type + multicopy *ZUO1* (2μ *ZUO1*) and wild type + multicopy *ZUO1* Δ285–364 (2μ Δ285–364). Serial dilutions of yeast cells were spotted on to media with (+) or without (–) cycloheximide or oligomycin and incubated as described in *Experimental procedures*.
B. β-Galactosidase activity of PDR reporters was measured in a wild-type (left) or a Δ*ssz1* Δ*zuo1* (right) strain as described in *Experimental procedures*. The results represent fold activation relative to vector control. The average activities for the *PDR5-lacZ*, *YOR1-lacZ* and *TRP5-lacZ* reporters in the presence of vector were 53 ± 24, 4.3 ± 3.0 and 26 ± 8.4 units, respectively, in the wild-type strain. The average activities of the *PDR5-lacZ* and *TRP5-lacZ* reporters in the presence of vector were 21 ± 2 and 17 ± 3, respectively, in the Δ*ssz1* Δ*zuo1* strain.
C. Whole-cell lysates from the strains used in (A) were subjected to electrophoresis and immunoblotting with a polyclonal antibody recognizing the N-terminal domain of Zuo1 (Zuo1) or Ssc1 as a control (Cont.).
D. Serial dilutions of Δ*zuo1* yeast cells transformed with centromeric *ZUO1* (WT), vector (Δ*zuo1*), centromeric *ZUO1* Δ285–364 (CEN Δ285–364) or multicopy *ZUO1* Δ285–364 (2μ Δ285–364) were spotted on to YPD media with the indicated additions and incubated at 30°C for 3 days.

Although these results indicate that the C-terminal region is required for induction of PDR by Zuo1, we could not determine using this approach whether the C-terminal region was sufficient, because the C-terminal fragment by itself was not produced at detectable levels even when expressed from a multicopy plasmid. Therefore, we constructed a chimeric gene that expressed a fusion of the C-terminal amino acids (365–433) of Zuo1 to glutathione-*S*-transferase (GST) under the control of the galactose-inducible promoter *GAL1*. When cells were grown in the presence of galactose, increased cycloheximide resistance and PDR reporter activity were observed, indicating that the C-terminal region of Zuo1 is sufficient for PDR activation (Fig. 4A and B). To ensure that the J domain from wild-type Zuo1 in the background of the strain was not acting *in trans* with Zuo1 Δ285–364 to activate PDR, deletion, mutants lacking this region were tested for cycloheximide resistance in an *ssz1 zuo1* strain. Expression of either -M-C (Fig. 4A) or N-M-C (data not shown) verified that the J domain of Zuo1 is not required for activation of PDR.

### *Ssz1 and Zuo1 can function independently in PDR*

On the ribosome, Ssb, Zuo1 and Ssz1 function together in chaperoning nascent chains (Gautschi *et al.*, 2002; Hundley *et al.*, 2002). The results described above demonstrated that Ssz1 can increase PDR in the absence of Zuo1, and that Zuo1 Δ285–364 can activate PDR in the absence of Ssz1. To analyse the dependence on chaperones more thoroughly, we assessed the ability of Ssz1 or Zuo1 mutant proteins to increase PDR in a strain lacking wild-type Ssb, Zuo1 and Ssz1. Expression of either Ssz1 S295F or Zuo1 Δ285–354 in an *ssz1 zuo1 ssb* background conferred resistance to cycloheximide and oligomycin (Fig. 5A). In addition, an Ssz1 overexpression vector was competent to activate the *YOR1-lacZ* reporter in the absence of Ssb or Zuo1 (Fig. 5B). Similarly, Zuo1 Δ285–364 could activate the *YOR1-lacZ* reporter in the absence of Ssz1 and Ssb (Fig. 5C). We conclude that the activation of PDR can occur by either Ssz1 or Zuo1 Δ285–364 in the absence of other components of the ribosome chaperone machinery.

## Discussion

The results presented in this paper indicate that both Ssz1 and Zuo1 can activate pleiotropic drug resistance. In attempting to characterize this function, we concentrated on answering two questions: Is the activity of Zuo1 and Ssz1 in PDR directly connected to their roles as components of chaperone machinery? Do these proteins perform this PDR function when not bound to the ribosome, their predominant cellular location?

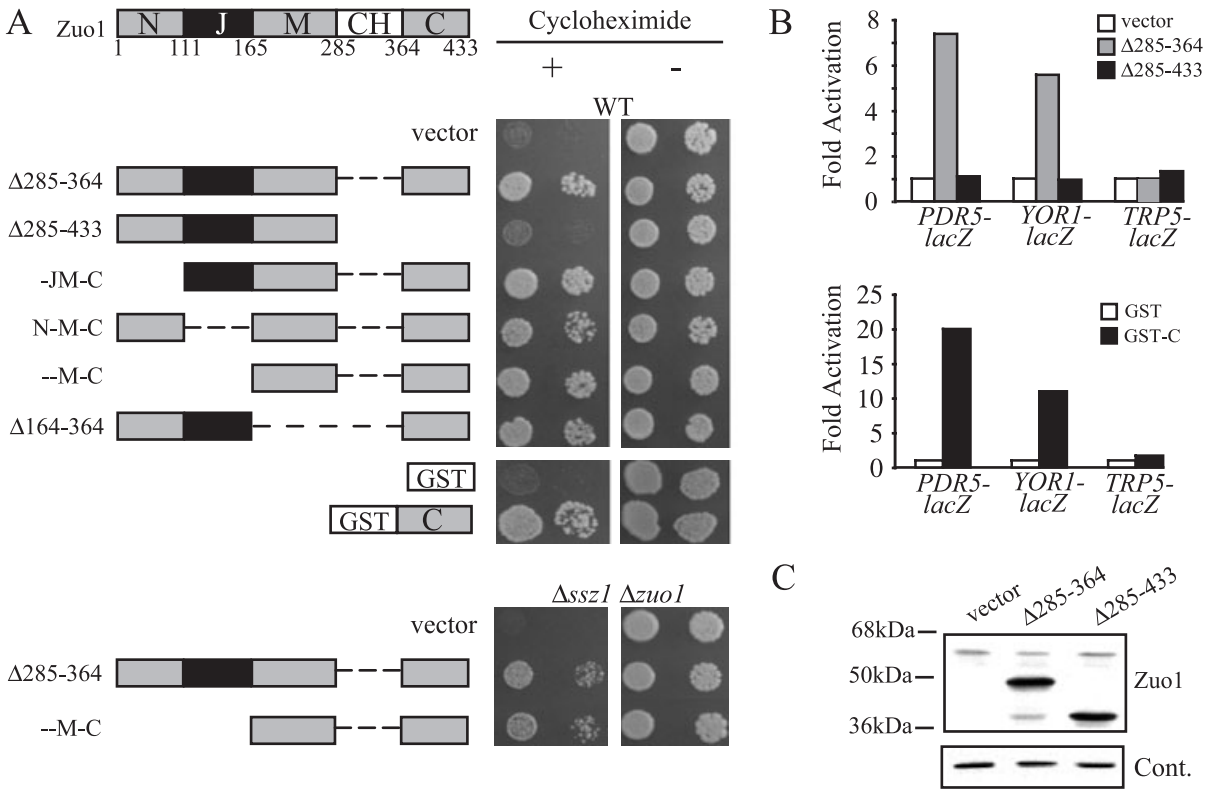


**Fig. 4.** The C-terminal region of Zuo1 activates PDR.
A. Schematic diagrams of *ZUO1* constructs tested for PDR activation are shown on the left. Regions of Zuo1 protein are labelled as follows: N, N-terminal region; J, J domain; M, middle region; CH, charged region; C, C-terminal region. Serial dilutions of wild-type or Δ*ssz1* Δ*zuo1* cells transformed with these *ZUO1* constructs were spotted on to media with (+) or without (–) cycloheximide and incubated as described in *Experimental procedures*. The GST and GST-Zuo1 C-terminal fusion constructs were spotted on minimal galactose media with (+) or without (–) cycloheximide and incubated for 4 days at 30°C.
B. β-Galactosidase activity in wild-type yeast transformed with the indicated PDR reporter and *ZUO1* plasmids was measured as described in *Experimental procedures*, with the exception of the GST and GST-Zuo1 C-terminal constructs, which were grown in minimal galactose media. Data are expressed as fold activation relative to vector. The average β-galactosidase activities of the *PDR5-lacZ*, *YOR1-lacZ* and *TRP5-lacZ* reporters in the presence of vector were 79 ± 31, 8.8 ± 0.69 and 63 ± 13 units, respectively, in glucose media and 95 ± 16, 13 ± 2.4 and 82 ± 51 units, respectively, in galactose media.
C. Whole-cell lysates from wild-type yeast transformed with the indicated Zuo1 constructs were subjected to electrophoresis and immunoblotting with a polyclonal antibody recognizing the N-terminal domain of Zuo1 (Zuo1) or Ssc1 as a control (Cont.).

Our results strongly support the idea that Zuo1 and Ssz1 function in PDR in a manner distinct from their expected role as a component of chaperone machinery. Zuo1 possesses a J domain, a domain found in all J-proteins that interacts with the ATPase domain of Hsp70s, and stimulates ATP hydrolysis (Wall *et al*., 1994; Greene *et al*., 1998). Zuo1's J domain is required for its well-established function on the ribosome (Yan *et al*., 1998; Gautschi *et al*., 2002), but is not required in PDR activation. Rather, the C-terminal domain of Zuo1 is both necessary and sufficient for activation of PDR. Little is known about the function of the C-terminal region of Zuo1, which is not required for its ribosome-associated chaperone activity (Yan *et al*., 1998). However, a precedent for a J domain-independent function of a J-protein has been reported. The mammalian J-protein $P58^{IPK}$ can inhibit certain eIF2α protein kinases, resulting in loss of inhibition of protein synthesis. Alteration of the J domain of $P58^{IPK}$ in a manner that disrupts interactions with Hsp70s did not alter $P58^{IPK}$'s ability to inhibit the kinase (Yan *et al*., 2002).

In a similar vein, the C-terminal region of Hsp70s contains the binding cleft for unfolded proteins that is absolutely critical for their chaperone activity (Tokunaga *et al*., 1998; Pfund *et al*., 2001). However, neither wild-type Ssz1 nor Ssz1 S295F required the C-terminal domain for activation of PDR. Thus, we conclude that Ssz1 does not require an interaction with unfolded substrates, and thus does not act as a molecular chaperone, in its PDR function. Therefore, the mechanism of Ssz1 activation of Pdr1 does not resemble other cases of transcription factor activation by chaperones, such as the activation of steroid receptors that are dependent on molecular chaperone activity (Johnson and Craig, 1997).

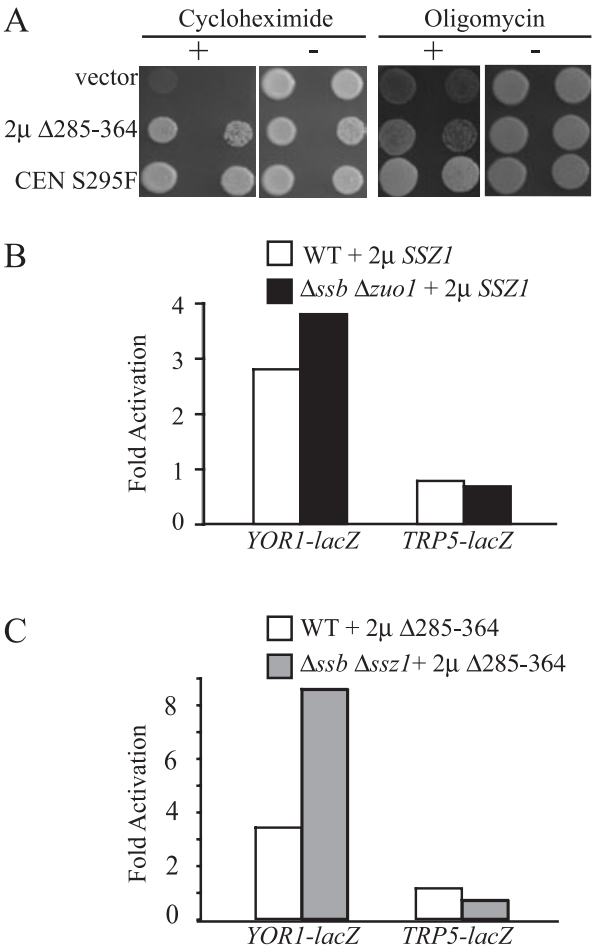


**Fig. 5.** Ssz1 and Zuo1 Δ285–364 activate PDR independently.
A. Serial dilutions of *ssb ssz1 zuo1* yeast expressing Zuo1 Δ285–364 or Ssz1 S295F were spotted on to media with (+) or without (–) cycloheximide or oligomycin and incubated at 30°C for 4 days (without cycloheximide) or 6 days (all others).
B. *YOR1-lacZ* and *TRP5-lacZ* reporter activation by multicopy *SSZ1* was measured in wild-type (WT + 2μ *SSZ1*) and Δ*ssb1* Δ*ssb2* Δ*zuo1* (Δ*ssb* Δ*zuo1* + 2μ *SSZ1*) strains. Activation is expressed as fold increase in the presence of multicopy *SSZ1*. The β-galactosidase activities of the *YOR1-lacZ* and *TRP5-lacZ* reporters in the presence of vector were 6.9 ± 2.3 and 64 ± 11 units, respectively, in the wild-type strain and 3.8 ± 0.1 and 52 ± 16 units, respectively, in the Δ*ssb* Δ*zuo1* strain.
C. *YOR1-lacZ* and *TRP5-lacZ* reporter activation by multicopy *ZUO1* Δ285–364 was measured in wild-type (WT + 2μ Δ285–364) and Δ*ssb1* Δ*ssb2* Δ*ssz1* (Δ*ssb* Δ*ssz1* + 2μ Δ285–364) strains. Activation is expressed as fold increase in the presence of multicopy Δ285–364. The β-galactosidase activities of the *YOR1-lacZ* and *TRP5-lacZ* reporters in the presence of vector were 27 ± 6.0 and 92 ± 13 units, respectively, in the wild-type strain and 26 ± 6.4, and 160 ± 58 units, respectively, in the Δ*ssb1* Δ*ssb2* Δ*ssz1* strain.

Ssb has been found to participate with the chaperonin TRiC in early folding events (Siegers *et al*., 2003); Ssz1 and Zuo1 are thought to work as co-chaperones with Ssb on the ribosome. Loss of any one of these three proteins results in the same phenotype with regard to growth rate or sensitivity to either aminoglycosides or salt. Furthermore, lack of all three chaperones has no additive phenotypic effect, indicating that, in this system, no single chaperone can function independently of the other (Gautschi *et al*., 2002; Hundley *et al*., 2002). The opposite was found to be true in PDR. Not only can Ssz1 and Zuo1 activate PDR independently of each other, they can activate PDR in the absence of Ssb as well. We have no evidence that Ssb is involved in the activation of PDR.

The results presented here also support the idea that Ssz1 and Zuo1 must be detached from ribosomes in order to function in PDR. Overexpression of Ssz1 led to an accumulation of Ssz1 that was not ribosome associated. Even the gain-of-function protein Ssz1 S295F, which can activate PDR when expressed from a centromeric plasmid, could only activate PDR when expressed at levels high enough that a small amount was free of ribosomes, or when expressed at the normal level in the absence of Zuo1, its ribosome tether. Similarly, Zuo1 only activates PDR when it does not contain the domain required for ribosome binding. But our data do not rule out the possibility that wild-type Ssz1 must be free of Zuo1, rather than, or in addition to, being free of ribosomes, to activate PDR. Unfortunately, problems were encountered when attempting to test this possibility critically, as we were unable to overexpress Zuo1 and Ssz1 at precisely the same levels.

Although ribosome detachment appears to be required for Ssz1 and Zuo1 activation of PDR, our results indicate that it is not sufficient. We think that an additional component may be involved in this regulation, in part because overexpression of wild-type Zuo1 does not induce PDR, even though it is not ribosome bound. But substantial induction is seen when normal levels of Zuo1 lacking its RNA-binding region are expressed. Wild-type Zuo1 is known to bind non-specifically to RNA (Wilhelm *et al*., 1994; Yan *et al*., 1998). Such interactions, which might sequester Zuo1 when it is ectopically overexpressed, may be masked when the co-operating factor is present. The requirement for an additional co-operating factor is also suggested by the fact that a *zuo1* deletion is not drug resistant, a situation in which Ssz1 is not ribosome associated (Gautschi *et al*., 2001). Ssz1 must also be present at high levels or contain the S295F alteration. It is possible that the substitution of phenylalanine for serine promotes an interaction required for activation of PDR. Interestingly, modelling of the Ssz1 ATPase domain structure predicts S295 to be exposed on the exterior of the protein, and therefore capable of being part of an interaction surface.

Our data demonstrate that Ssz1 and Zuo1 can function to enhance PDR independently. However, as Ssz1 and Zuo1 are normally found tightly associated in the cell, it seems likely that they normally function together. Perhaps both Zuo1 and Ssz1 directly contact a target protein, but interaction of either alone, if present at adequate levels, is sufficient for observable activation. The S295F alter-

ation may also enhance an interaction in the absence of Zuo1. Thus, we propose that, normally, the Ssz1–Zuo1 complex (RAC) functions, perhaps with another component, to regulate the activation of PDR.

It is tempting to speculate that Ssz1 and Zuo1 may in some way couple either protein folding or the translational status of the cell to signal transduction pathways regulating the cell's response to stress. Indeed, connections between PDR and the stress response have been found. On one hand, gain-of-function *PDR1* mutations lead to increased expression of genes that are known to be induced by certain types of cell stress (DeRisi *et al*., 2000). On the other hand, stress-activated transcription factors such as Yap1 are also involved in PDR activation (reviewed by Bauer *et al*., 1999), including the activation of *PDR5* (Miyahara *et al*., 1996). Thus, Ssz1 and Zuo1 may be involved in such 'cross-talk' between such different regulatory pathways. Interestingly, Hsp70 in mammalian cells has been implicated in stress signalling. Upregulation of Hsp70 during heat shock was found to reduce the growth rate of cells by binding and sequestering regulatory factors (Song *et al*., 2001). One can envisage a mechanism for Ssz1 and Zuo1 in which changes in their ribosome localization, perhaps mediated by cell stress, lead to binding to unknown effectors of PDR.

## Experimental procedures

### *Yeast strains*

All yeast strains used are isogenic with DS10 (Pfund *et al*., 2001) and contain the following mutations, *his3-11,15 leu2-3112 lys1 lys2* Δ*trp1 ura3-52*, in addition to mutations in *SSZ1*, *ZUO1* and/or *SSB1* and *SSB2*. The strains used were: HE14, Δ*ssz1*::*LYS2*; HE12, Δ*ssz1*::*LYS2* Δ*zuo1*::*HIS3*; HE9, Δ*ssz1*::*LYS2*, Δ*ssb1*::*HIS3*, Δ*ssb2*::*LEU2*; HE15, Δ*ssz1*::*LYS2*, *zuo1*::*URA3*, *ssb1*::*HIS3*, *ssb2*::*LEU2* (Hundley *et al*., 2002); and WY17, Δ*zuo1*::*HIS3* (Yan *et al*., 1998). HH8 was derived from NL164 (Pfund *et al*., 2001) and WY17 and contains the following mutations, Δ*ssb1*::*HIS3*, Δ*ssb2*::*LEU2* Δ*zuo1*::*HIS3*. HE19, *SSZ1* S295F, containing the integrated *SSZ1* S295F allele, was created by transforming HE14 with a 2.4 kb *Spe*I–*Not*I fragment of *SSZ1* containing the S295F mutation and selecting for growth on YPD media with 50 μg ml$^{-1}$ paromomycin. The presence of *SSZ1* S295F at the correct locus was confirmed by Lys$^-$ phenotype, Ssz1 protein detectable by immunoblot and DNA sequencing. HE28, *SSZ1* S295F *zuo1*::*URA3*, was derived from HE19 and WW1 (Yan *et al*., 1998).

### *Plasmid construction*

The *PDR5-lacZ*, *YOR1-lacZ* and *TRP5-lacZ* fusions as well as pRS424-*SSZ1* (pTH87) and pRS314-*SSZ1* S295F were generously provided by W. S. Moye-Rowley (University of Iowa) (Hallstrom *et al*., 1998). All constructs were created using the pRS314/5/6 (Sikorski and Hieter, 1989), pRS317 (Sikorski and Boeke, 1991) and pRS424/5/6 vectors (Christianson *et al*., 1992). pRS424-*SSZ1* Δ408–538 was constructed by cloning a 2 kb *Sal*I–*Not*I fragment of Ssz1 from pRS316-*SSZ1* Δ408–538 (Hundley *et al*., 2002) into pRS424. pRS316-*SSZ1* Δ408–538 S295F was made by cloning a 1 kb *Hin*dIII–*Hin*dIII fragment containing the S295F mutation into pRS316-*SSZ1* Δ408–538. Both *SSZ1* Δ408–538 constructs express amino acids 1–407 of Ssz1 with an additional Phe at the C-terminus. The following additional plasmids were created for the purpose of changing markers. For pRS316-*SSZ1* S295F, a 2.6 kb *Sal*I–*Not*I fragment of *SSZ1* containing the S295F mutation was cloned into pRS316. *PDR5-lacZ* and *YOR1-lacZ* were cloned as 4 kb *Sal*I–*Eco*RI fragments into pRS314 and pRS317. *TRP5-lacZ* was cloned as a 5 kb *Sal*I–*Nru*I fragment into pRS314 and pRS317. pRS425-*ZUO1* contains a 3.1 kb fragment of *ZUO1* cloned into the *Sma*I and *Xho*I sites of pRS425.

The *ZUO1* deletion mutants were created using polymerase chain reaction (PCR) and/or restriction endonuclease cloning into 2μ-based multicopy vectors (pRS424/5/6). *ZUO1* Δ285–364 expresses Zuo1 lacking amino acids 285–364 with an extra Arg-Pro-Lys inserted at the deletion site (Yan *et al*., 1998). *ZUO1* Δ285–433 expresses amino acids 1–284 with an extra Arg-Pro-Lys-Gly inserted at the C-terminus (Yan *et al*., 1998). *ZUO1* Δ164–364 expresses Zuo1 lacking amino acids 164–364. *ZUO1*-M-C expresses Zuo1 lacking amino acids 1–164 and 285–364 with an extra Arg-Pro-Lys inserted as in *ZUO1* Δ285–364 and an initiating Met inserted before amino acid 164. *ZUO1*-JM-C expresses Zuo1 lacking amino acids 1–101 and 285–364 with an extra Arg-Pro-Lys inserted as in *ZUO1* Δ285–364. *ZUO1* N-M-C expresses Zuo1 lacking amino acids 111–165 and 285–364 with an extra Arg-Pro-Lys inserted as in *ZUO1* Δ285–364. pRS316-*GAL1-GST-ZUO1*-C was created by PCR and cloning into a pRS316 vector containing the *GAL1* promoter fused to GST and expresses GST fused to amino acids 365–433 of Zuo1 with an extra Leu-Val-Pro-Arg-Gly-Ser inserted at the fusion point.

### *Yeast growth and media*

To assay drug resistance, approximately equal numbers of cells were subjected to 10-fold serial dilutions and spotted on selective minimal glucose media (0.67% yeast nitrogen base with ammonium sulphate and without amino acids, 2% dextrose, supplemented with all amino acids except those needed for selection) with or without 0.7 μg ml$^{-1}$ cycloheximide or YPGE media (1% yeast extract, 2% peptone, 3% glycerol and 2% ethanol) with or without 0.3 μg ml$^{-1}$ oligomycin and incubated at 30°C for 2–3 days (minimal), 4 days (YPGE), 7–11 days (oligomycin) and 2–3 days (cycloheximide) before photographing, unless noted otherwise.

### *SDS-PAGE and immunoblotting*

Lysates were prepared from cells grown at 30°C in selective minimal media. Approximately 1.0 $OD_{600}$ unit of yeast cells was harvested by brief centrifugation. Cells were resuspended in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 2% β-mercaptoethanol and 0.01% bro-

mophenol blue). Glass beads were added, and the samples were vortexed at 4°C for 1 min, then boiled for 5 min. Approximately 0.1 $OD_{600}$ unit of lysate was subjected to SDS-PAGE, transferred to nitrocellulose membranes (Osmonics) and analysed by immunoblot using chemiluminescence (Perkin-Elmer Life Sciences) for detection of proteins. The level of an unrelated protein, Ssc1, was used to control for equal loading of lysate. Antibodies specific to the N-terminus of Zuo1 (Yan *et al*., 1998), Ssc1 (Liu *et al*., 2003), the C-terminus of Ssz1 (Hundley *et al*., 2002) and the N-terminus of Ssz1, amino acids 1–407 (H. Hundley, unpublished results) were used.

### *β-Galactosidase assays*

Yeast cells were transformed with a reporter and test plasmid. Cells were grown overnight in 5 ml of selective minimal media, diluted into fresh media to an $OD_{600}$ between 0.1 and 0.2 and allowed to grow for two or three generations. Between 0.1 and 1 ml of cells was harvested for each assay. Cells were permeabilized by the addition of SDS and chloroform. β-Galactosidase activity was determined as described by Miller (1972). Unless noted otherwise, a minimum of three independent transformants were tested and the average activity determined. In some cases, cell pellets were frozen in liquid nitrogen and stored at −80°C before measuring the activity.

### *Analysis of ribosome association*

Cell lysates were prepared as described previously (Nelson *et al*., 1992) from cells grown at 30°C in selective minimal media to an $OD_{600}$ of between 0.5 and 1.0. RNAguard (Amersham Pharmacia) was added to lysates at a dilution of 1:1000. Approximately 20 $OD_{260}$ units of lysate was loaded on to a 4.4 ml 10–50% RNase-free sucrose gradient in CB buffer (20 mM Hepes, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol and 2 mM β-mercaptoethanol). Gradients were centrifuged for 90 min at 4°C at 50 000 r.p.m. (237 020 *g*) in an SW55Ti rotor (Beckman). Fractions (400 μl) were collected and analysed by electrophoresis and immunoblotting to detect the presence of Ssz1 and Zuo1. Gradients were monitored at $OD_{254}$ to determine the migration of monosomes and polysomes.

## Acknowledgements

We thank W. S. Moye-Rowley for helpful advice and suggestions and for many of the plasmids used in these studies. We also thank Peggy Huang, Heather Hundley and Robert Seiser for critical reading of the manuscript and helpful suggestions. This work was supported by the National Institutes of Health grants R37GM31107 (E.A.C.) and T32GM08349 (H.C.E.).

## References

Balzi, E., and Goffeau, A. (1995) Yeast multidrug resistance: the *PDR* network. *J Bioenerg Biomembr* **27:** 71–76.

Balzi, E., Wang, M., Leterme, S., Van Dyck, L., and Goffeau, A. (1994) PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. *J Biol Chem* **269:** 2206–2214.

Bauer, B.E., Wolfger, H., and Kuchler, K. (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim Biophys Acta* **1461:** 217–236.

Bukau, B., and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* **92:** 351–366.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110:** 119–122.

DeRisi, J., van den Hazel, B., Marc, P., Balzi, E., Brown, P., Jacq, C., and Goffeau, A. (2000) Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. *FEBS Lett* **470:** 156–160.

Gautschi, M., Lilie, H., Funfschilling, U., Mun, A., Ross, S., Lithgow, T., *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., Mun, A., Ross, S., and Rospert, S. (2002) A functional chaperone triad on the yeast ribosome. *Proc Natl Acad Sci USA* **99:** 4209–4214.

Greene, M., Maskos, K., and Landry, S. (1998) Role of the J-domain in the cooperation of Hsp40 with Hsp70. *Proc Natl Acad Sci USA* **95:** 6108–6113.

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

Hundley, H., Eisenman, H., Walter, W., Evans, T., Hotokezaka, Y., Wiedmann, M., and Craig, E. (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.

Johnson, J.L., and Craig, E.A. (1997) Protein folding *in vivo*: unraveling complex pathways. *Cell* **90:** 201–204.

Katzmann, D.J., Hallstrom, T.C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Moye-Rowley, W.S. (1995) Expression of an ATP-binding cassette transporter-encoding gene (YOR1) is required for oligomycin resistance in *Saccharomyces cerevisiae*. *Mol Cell Biol* **15:** 6875–6883.

Katzmann, D.J., Hallstrom, T.C., Mahe, Y., and Moye-Rowley, W.S. (1996) Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP binding cassette transporter protein-encoding gene PDR5. *J Biol Chem* **271:** 23049–23054.

Liu, Q., D'Silva, P., Walter, W., Marszalek, J., and Craig, E.A. (2003) Regulated cycling of mitochondrial Hsp70 at the protein import channel. *Science* **300:** 139–141.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miyahara, K., Hirata, D., and Miyakawa, T. (1996) yAP-1- and yAP-2-mediated, heat shock-induced transcriptional activation of the multidrug resistance ABC transporter genes in *Saccharomyces cerevisiae*. *Curr Genet* **29:** 103–105.

Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E.A. (1992) The translation machinery and seventy kilodalton heat shock protein cooperate in protein synthesis. *Cell* **71:** 97–105.

Pfund, C., Lopez-Hoyo, N., Ziegelhoffer, T., Schilke, B.A., Lopez-Buesa, P., Walter, W.A., *et al.* (1998) The molecular chaperone *SSB* from *S. cerevisiae* is a component of the ribosome-nascent chain complex. *EMBO J* **17:** 3981–3989.

Pfund, C., Huang, P., Lopez-Hoyo, N., and Craig, E. (2001) Divergent functional properties of the ribosome-associated molecular chaperone Ssb compared to other Hsp70s. *Mol Biol Cell* **12:** 3773–3782.

Siegers, K., Bölter, B., Schwarz, J.P., Böttcher, U., Guha, S., and Hartl, F.-U. (2003) TRiC/CCT cooperates with different upstream chaperones in the folding of distinct protein classes. *EMBO J* **22:** 5230–5240.

Sikorski, R.S., and Boeke, J.D. (1991) *In vitro* mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. In *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, Vol. 194. Guthrie, C., and Fink, G.R. (eds). San Diego: Academic Press, pp. 302–318.

Sikorski, R.S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122:** 19–27.

Song, J., Takeda, M., and Morimoto, R. (2001) Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nature Cell Biol* **3:** 276–282.

Tokunaga, M., Kato, S., Kawamura-Watabe, A., Tanaka, R., and Tokunaga, H. (1998) Characterization of deletion mutations in the carboxy-terminal peptide-binding domain of the Kar2 protein in *Saccharomyces cerevisiae*. *Yeast* **14:** 1285–1295.

Wall, D., Zylicz, M., and Georgopoulos, C. (1994) The NH2-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication. *J Biol Chem* **269:** 5446–5451.

Wilhelm, M.L., Reinholt, J., Gangloff, J., Dirheimer, G., and Wilhelm, F.X. (1994) Transfer RNA binding protein in the nucleus of *Saccharomyces cerevisiae*. *FEBS Lett* **349:** 260–264.

Yan, W., Schilke, B., Pfund, C., Walter, W., Kim, S., and Craig, E.A. (1998) Zuotin, a ribosome-associated DnaJ molecular chaperone. *EMBO J* **17:** 4809–4817.

Yan, W., Gale, M.J., Jr, Tan, S.L., and Katze, M.G. (2002) Inactivation of the PKR protein kinase and stimulation of mRNA translation by the cellular co-chaperone P58 (IPK) does not require J domain function. *Biochemistry* **41:** 4938–4945.