

# The Thioredoxin System Protects Ribosomes against Stress-induced Aggregation

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We previously showed that thioredoxins are required for dithiothreitol (DTT) tolerance, suggesting they maintain redox homeostasis in response to both oxidative and reductive stress conditions. In this present study, we screened the complete set of viable deletion strains in *Saccharomyces cerevisiae* for sensitivity to DTT to identify cell functions involved in resistance to reductive stress. We identified 195 mutants, whose gene products are localized throughout the cell. DTT-sensitive mutants were distributed among most major biological processes, but they particularly affected gene expression, metabolism, and the secretory pathway. Strikingly, a mutant lacking *TSA1*, encoding a peroxiredoxin, showed a similar sensitivity to DTT as a thioredoxin mutant. Epistasis analysis indicated that thioredoxins function upstream of *Tsa1* in providing tolerance to DTT. Our data show that the chaperone function of *Tsa1*, rather than its peroxidase function, is required for this activity. Cells lacking *TSA1* were found to accumulate aggregated proteins, and this was exacerbated by exposure to DTT. Analysis of the protein aggregates revealed that they are predominantly composed of ribosomal proteins. Furthermore, aggregation was found to correlate with an inhibition of translation initiation. We propose that *Tsa1* normally functions to chaperone misassembled ribosomal proteins, preventing the toxicity that arises from their aggregation.

## INTRODUCTION

Cells originally evolved in a reducing environment. The accumulation of oxygen in the atmosphere then made possible the efficient energy generation process of respiration. Cells have therefore evolved to survive both oxidative and reductive conditions. Although much is now known regarding the damaging effects of oxidation, little is known regarding the molecular responses to a reducing environment, which is the subject of this present study.

Glutathione is the most abundant low-molecular-weight thiol in eukaryotic cells and has been widely used as an indicator of the cellular redox state (Schafer and Buettner, 2001). The cytoplasm is generally very reducing; for example, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in yeast is in the range of ~70–190:1 (Grant *et al.*, 1998; Garrido and Grant, 2002). In contrast, the endoplasmic reticulum is more oxidizing and the GSH:GSSG ratio has been measured in the range of 1:1–3:1 (Hwang *et al.*, 1992; Bass *et al.*, 2004). Disulfide bonds are essential for the folding and stability of proteins that are secreted or localized through the secretory pathway. In eukaryotic cells, disulfide bonds are formed in nascent proteins within the lumen of the endoplasmic reticulum (ER). Genetic evidence has identified a pathway for disulfide bond formation where oxidizing equivalents are transferred between thiol-containing proteins and secretory proteins (Frandsen *et al.*, 2000; Gross *et al.*, 2002). Thus, oxidative protein folding proceeds primarily by protein–protein-based relays. Glutathione pro-

vides a buffer against hyperoxidizing conditions in the ER and functions in the reductive pathway for the formation of native disulfide bonds (Cuozzo and Kaiser, 1999; Jessop and Bulleid, 2004). However, GSH also competes for oxidizing equivalents in the ER, because elevated GSH disrupts ER function and activates the unfolded protein response (UPR) owing to an accumulation of misfolded proteins (Cuozzo and Kaiser, 1999). Compartmentalization of the oxidative chemistry required for disulfide formation is important to protect cells from being exposed to nonspecific oxidation or reduction events.

DTT is a strong reducing agent that is often used to promote reductive stress. It can cross membranes and prevent disulfide bond formation. For example, DTT reversibly blocks the folding and transport from the ER of vacuolar carboxypeptidase Y (CPY), which contains five disulfides in its native form (Simons *et al.*, 1995). A reductive stress induced by DTT treatment leads to an accumulation of misfolded proteins in the ER, which signals the UPR through the action of the Ire1 transmembrane kinase/nuclease (Cox *et al.*, 1993; Kohno *et al.*, 1993). Microarray analysis has elucidated the transcriptional scope of the UPR and shown that it affects multiple ER and secretory pathway functions that normally function to counteract ER stress (Travers *et al.*, 2000). Interestingly, many genes are up-regulated in response to DTT treatment whose functions seem unrelated to the ER and do not form part of the UPR (Gasch *et al.*, 2000). These gene products are likely to function in protection against reductive stress.

A reductive stress can occur in response to any conditions that shift the redox balance of important biological redox couples, such as the NAD<sup>+</sup>/NADH, NADP<sup>+</sup>/NADPH, and GSSG/2GSH couples, to a more reducing state (Schafer and Buettner, 2001). These couples are often thermodynamically linked, and the redox environment of a cell is a reflection of the state of these couples. There is also increasing evidence

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that alterations in the redox state of protein-sulfhydryl groups can affect many protein functions and activities (Rietsch and Beckwith, 1998). Thus, any reductive or oxidative challenges that alter the cellular redox environment will influence many cellular processes. Not surprisingly, therefore, a strong correlation has been observed between the redox environment of the cell and cellular growth and differentiation; for example, a more reducing environment is linked with the proliferation of some tumor cells (Schafer and Buettner, 2001; Nkabyo *et al.*, 2002; Yeh *et al.*, 2005). Excess reductants may also disrupt signaling pathways that rely on low physiologically relevant concentrations of reactive oxygen species (ROS) as well as redox-sensitive transcription factors (Sen, 2000). A reductive stress can also arise from an imbalance in the levels of key metabolites. For example, the inability to oxidize NAD<sup>+</sup> results in an NADH-reductive stress that impairs yeast cell growth (Valadi *et al.*, 2004). NAD is required for numerous biological processes, and many human diseases are associated with changes in NAD<sup>+</sup> levels and/or the NAD<sup>+</sup>:NADH ratio (Lin and Guarente, 2003).

We have previously tested whether known redox regulatory systems are required for protection against a reductive stress and shown that thioredoxin mutants (*trx1 trx2*) are sensitive to DTT (Trotter and Grant, 2002). Thioredoxins are small, highly conserved oxidoreductases that have been best characterized for their role in protection against ROS (Holmgren, 1989). Sensitivity to a reducing agent is somewhat surprising, given their accepted role as antioxidants. This sensitivity to reducing conditions is not a general property of mutants affected in redox control because mutants lacking components of the GSH/glutaredoxin system are unaffected. Further evidence that thioredoxins form part of the cellular response to a reductive challenge came from the finding that *TRX2* gene expression is induced in response to DTT treatment and does not depend on the presence of *IRE1* (Trotter and Grant, 2002). Loss of thioredoxins is known to result in elevated glutathione levels, indicating a link between the thioredoxin system and glutathione metabolism in the cell (reviewed in Carmel-Harel and Storz, 2000; Wheeler and Grant, 2004). We proposed that the combination of exogenous DTT and high intracellular GSH results in an additive reductive stress that is toxic to thioredoxin mutants (Trotter and Grant, 2002). However, subsequent analysis of the cellular redox potential of a *trx1 trx2* mutant revealed that it is comparable with that of a wild-type strain because the mutant also contains elevated levels of GSSG (Trotter and Grant, 2003). Thus, there does not seem to be a correlation between the cellular redox state and DTT sensitivity.

In this present study, we have performed a genome-wide screen to identify mutants that are sensitive to DTT. This analysis shows that a wide range of gene functions are required to protect against DTT stress, including a number of factors that affect the thioredoxin system. We show that the role of thioredoxins in protection against reductive stress is dependent on their activity as a cofactor for the Tsa1 peroxiredoxin (Prx). In particular, the chaperone activity of Tsa1 is required to protect against DTT-mediated protein aggregation. Surprisingly, ribosomal proteins were found to be particularly susceptible to aggregation in response to reductive stress. This finding has important implications in the regulation of protein synthesis in response to changes in redox status.

## MATERIALS AND METHODS

### Yeast Strains and Plasmids

The *Saccharomyces cerevisiae* strains used in this study were isogenic derivatives of CY4 (*MATa ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100*) (Grant *et al.*, 1996b). Strains deleted for thioredoxins (*trx1::TRP1 trx2::URA3*) and *YAP1* (*yap1::HIS3*) have been described previously (Grant *et al.*, 1996a; Draculic *et al.*, 2000). Strain CY399, which is deleted for *GPX3* (*gpx3::HIS3*); strain CY947, which is deleted for *SKN7* (*skn7::LEU2*); and strain CY954, which is deleted for *TSA1* (*tsa1::LEU2*) were constructed using a one-step PCR amplification protocol (Baudin *et al.*, 1993). Multicopy plasmids containing *TRX1*, *TRX2*, *TSA1* (Garrido and Grant, 2002), *TSA1* containing mutations in the active site cysteine residues (Cys47 and Cys170) (Wong *et al.*, 2004), *ERO1* (Haynes *et al.*, 2004), *HAC1*, and a *HAC1* gene fusion with the activation domain of the Gal4 transcriptional activator [mcGAL4(AD)-*HAC1*] (Mori *et al.*, 2000) have all been described previously.

### Growth Conditions

Strains were grown in rich YEPD medium [2% (wt/vol) glucose, 2% (wt/vol) bacto-peptone, and 1% (wt/vol) yeast extract] or minimal SD medium (0.17% (wt/vol) yeast nitrogen base without amino acids, 5% (wt/vol) ammonium sulfate, 2% (wt/vol) glucose supplemented with appropriate amino acids and bases (Sherman *et al.*, 1974) at 30°C and 180 rpm. Media were solidified by the addition of 2% (wt/vol) agar. SGal plates contained 2% (wt/vol) galactose. For anaerobic growth conditions, media was supplemented with 0.1% (vol/vol) Tween 80 and 30 mg/l ergosterol, and plates were maintained in an anaerobic jar (Oxoid, Basingstoke, Hampshire, England) containing a gas generating kit (Anaerobic system BR38; Oxoid). Stress sensitivity was determined by growing cells to stationary phase and spotting onto agar plates containing various concentrations of DTT, cysteine, H<sub>2</sub>O<sub>2</sub>, or diamide.

### Genome-Wide Screening for Mutations Causing Sensitivity to DTT

The *S. cerevisiae* strains used in this study were derivatives of BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ura3Δ0*), which are homozygous for the relevant gene deletion. The construction of the yeast genome deletion library by the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF) has been described previously (Winzler, 1999). Cells were grown to stationary phase in YEPD medium in static 96 well-plates. After resuspension, cells were plated onto YEPD containing 32 mM DTT using a 96 pin replicator. Plates were incubated at 30°C for 3–4 d before scoring growth. The screen was repeated twice, and mutants were identified which were reproducibly sensitive to DTT.

### Measurement of Intracellular Oxidation

Oxidized glutathione levels were determined as described previously (Grant *et al.*, 1998). Briefly, cells were grown to exponential phase in YEPD medium, treated with DTT for 1 h, and harvested by centrifugation. Cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, to remove any traces of growth medium and resuspended in ice-cold 8 mM HCl, 1.3% (wt/vol) 5-sulfosalicylic acid. Cells were broken with glass beads using a Minibead beater (Biospec Products, Bartlesville, OK) for 40 s at 4°C. Cell debris and precipitated proteins were pelleted in a microcentrifuge for 15 min (13,000 rpm; 4°C). The collected supernatants were pretreated with 5% (vol/vol) 2-vinylpyridine for 1 h at room temperature before analysis. GSSG levels are expressed as nanomoles per absorbance at 600 nm of cells.

Intracellular ROS were measured using the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). Cells growing exponentially in SD medium were treated with DTT for 15 min. After treatment, cells were harvested, resuspended in PBS containing 10 μM DCFH-DA, and incubated for 1 h at 28°C. Cells were broken with glass beads using a Minibead beater, and fluorescence was measured using a SynergyHT plate reader (Bio-Tek Instruments, Winooski, VT) with excitation and emission wavelengths of 485 and 528 nm, respectively. Extracts from untreated cells that had been incubated without DCFH-DA were used as blanks for background fluorescence.

### Measurement of Protein Aggregation

The aggregation of soluble proteins was analyzed essentially as described by Jang *et al.* (2004). Cells were grown to exponential phase in YEPD medium and treated with DTT for 1 h. Equivalent cell numbers (10 A<sub>600</sub> units) were harvested by centrifugation, washed, and resuspended in 300 μl of lysis buffer [50 mM potassium phosphate buffer, pH 7, 1 mM EDTA, 5% (vol/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, and Complete Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)]. The cells were lysed by freezing and thawing followed by an incubation (30°C for 30 min) with 100 μl of lyticase (9830 U/ml) (Sigma-Aldrich, St. Louis, MO). Cells were disrupted with glass beads using a Minibead beater for 3 × 1 min at 4°C. Each beating was followed by a 1-min cooling at 4°C. Intact cells were removed by centrifugation at 3000 × g for 15 min. The membrane and aggregated proteins were isolated by centrifugation at 15,000 × g for 20 min. The membrane

proteins were removed by washing twice with 320  $\mu$ l of lysis buffer and 80  $\mu$ l of 10% Igepal CA-630 (NP-40) (Sigma-Aldrich), centrifuging at 15,000  $\times$  g for 20 min each time. The final aggregated protein extract was resuspended in 100  $\mu$ l of lysis buffer and analyzed by 12% reducing SDS-PAGE. Proteins were visualized by silver staining with the Bio-Rad (Hercules, CA) silver stain plus kit.

For protein identification, gels were stained using colloidal Coomassie blue (Sigma-Aldrich) and dried using a Bio-Rad Gelair drier. Proteins of interest were excised from gels and peptide mass fingerprints were generated using matrix-assisted laser desorption ionization/time of flight (MALDI-TOF). Proteins were identified using the Mascot mass fingerprinting program ([www.matrixscience.com](http://www.matrixscience.com)) to search the NCBI nr and Swissprot databases.

### Western Blot Analysis

Protein extracts were electrophoresed under reducing conditions on 12% SDS-PAGE minigels ( $\alpha$ Rps3) or 16% tricine Novex minigels ( $\alpha$ Trx1,2) and electroblotted onto polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Bound antibody was visualized by chemiluminescence (GE Healthcare) after incubation of the blot in donkey anti-rabbit immunoglobulin-horseradish peroxidase conjugate (GE Healthcare).

### Measurement of Protein Synthesis Rate

To measure the rate of protein synthesis, exponential phase cells were treated with DTT for 1 h and pulse labeled for the last 5 min with 85  $\mu$ M L-[<sup>35</sup>S]cysteine/methionine. Aliquots of cells were lysed by boiling them in 20% (wt/vol) trichloroacetic acid (TCA) for 10 min and then placing them on ice for 10 min. Proteins were precipitated onto GF/C glass microfiber filters (Whatman, Maidstone, United Kingdom) and washed using cold (4°C) 20% (wt/vol) TCA. Radioactive incorporation was measured by scintillation counting radioactivity retained on the filters and expressed as cpm per minute per absorbance at 600 nm.

### Analysis of Ribosome Distribution on Sucrose Density Gradients

Yeast cultures were grown to exponential phase and treated with DTT as appropriate. Extracts were prepared in 100  $\mu$ g of cycloheximide/ml, and these were layered onto 15–50% sucrose gradients. The gradients were sedimented via centrifugation at 40,000 rpm in a Beckman ultracentrifuge for 2.5 h, and the  $A_{254}$  measured continuously to give the traces shown (Ashe *et al.*, 2000).

## RESULTS

### Identification of DTT-sensitive Strains

The homozygous diploid deletion mutant collection (EURO-SCARF) was screened to identify nonessential gene products that are required for resistance to DTT. Strains were grown in YEPD media in microtiter plates and spotted onto YEPD plates containing 32 mM DTT. This concentration of DTT was chosen because it allowed for normal growth of the wild-type strain but prevented growth of a *hac1* mutant that is known to be DTT sensitive (our unpublished data). The screen was repeated twice with 708 sensitive mutants identified from the first screen and 616 mutants identified from the second screen. Mutants were scored as sensitive if they showed reduced growth after 3 d of incubation on media containing DTT. Common mutants (204) were identified as DTT sensitive in both screens. These mutants were re-screened for DTT sensitivity, resulting in a total of 195 mutants identified as DTT sensitive from the 4711 mutants tested (Table 1).

The reductive stress caused by DTT has primarily been studied because of its effects on ER function and protein processing in the secretory pathway. However, grouping the genes according to their cellular component revealed that DTT-sensitive mutants are localized throughout the cell (Figure 1A). As predicted, a large number of gene products that localize to the ER, Golgi, and vacuole were identified. In addition, gene products that localize to other cellular compartments, including the cytoplasm, mitochondria, nucleus, and cell wall were required for DTT resistance. Gene products were grouped into functional categories according to

the MIPS functional database (<http://mips.gsf.de/genre/proj/yeast/index.jsp>) and *Saccharomyces* Genome Database GO term mapper (<http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermMapper>). DTT-sensitive mutants were distributed among most major biological processes but particularly affected gene expression (transcription and protein synthesis), metabolism, and the secretory pathway (Figure 1B and Table 1). Approximately 30% of the mutants are defective for growth on nonfermentable carbon sources (<http://www.yeastgenome.org/>). However, there does not seem to be any link between respiratory ability and DTT tolerance because a respiratory incompetent petite strain shows a similar level of resistance to DTT as a wild-type strain (our unpublished data).

As expected, a major class of mutants included those that are defective in the secretory pathway, including vacuolar function, glycosylation, and vesicular transport. In addition, a number of mutants predicted to influence ion homeostasis are DTT sensitive, including several mutants in the vacuolar membrane H<sup>+</sup>-ATPase. A diverse range of mutants affecting metabolism are DTT sensitive. These could be subdivided into groups involved in carbon, amino acid, lipid, nucleotide, and energy metabolism. Together, these data indicate that active metabolism may be required to protect against reductive stress. A number of gene functions involved in transcription and translation are required for DTT tolerance, indicating that gene expression may be required in response to a reductive stress. Previous analysis of mutants that are required for general oxidative stress resistance has indicated that a number of similar gene functions are commonly required for protection against ROS (Thorpe *et al.*, 2004). Unique to reductive stress, a number of mutants affecting ribosomal proteins are sensitive to DTT. These include components of the small subunit, large subunit, mitochondrial ribosome, and factors that are involved in ribosome export and biogenesis.

Relatively few mutants affected in cell rescue and defense were identified from this screen. They include genes that are generally required under conditions that generate denatured proteins, confirming that DTT can increase the cellular levels of misfolded proteins. Examples include members of the heat-shock protein (Hsp) 70 family, chaperones, and genes involved in ubiquitin-mediated protein degradation. As might be expected, loss of *IRE1* and *HAC1*, which are essential for the induction of UPR-regulated genes, resulted in DTT sensitivity (Cox *et al.*, 1993; Mori *et al.*, 1993). Interestingly, strains deleted for *GPX3*, encoding a phospholipid hydroperoxide glutathione peroxidase (PHGPx), and *TSA1*, encoding a Prx, were sensitive to DTT. In addition, a strain deleted for *SKN7*, encoding a transcription factor involved in the oxidative stress response was found to be DTT sensitive. Given our original observation that thioredoxins are required for protection against DTT stress (Trotter and Grant, 2002), these mutants were chosen for further investigation.

### Mutants Affecting the Thioredoxin System Are Sensitive to Reductive Stress

Isogenic haploid deletion strains were constructed to confirm the sensitivity of selected mutants to reductive stress. Strains were grown to stationary phase and spotted onto plates containing various concentrations of DTT (Figure 2A). The *tsa1* mutant was the most sensitive and showed no growth on 16 mM DTT. Yeast contains five thioredoxin peroxidase isoforms, including cytoplasmic (Tsa1, Tsa2, and Ahp1), mitochondrial (Prx1/mTpx), and nuclear (Dot5/nTpx) isoforms (Park *et al.*, 2000). However, only a strain



**Table 1.** Genes required for DTT resistance

Gene	ORF	Function
<b>Protein synthesis</b>		
RPL31A	YDL075W	60S large subunit ribosomal protein L31.e
RPP1B	YDL130W	60S large subunit acidic ribosomal protein L44prime
RPL35A	YDL191W	60S large subunit ribosomal protein
RPL1B	YGL135W	60S large subunit ribosomal protein
RPL27A	YHR010W	60S large subunit ribosomal protein L27.e
RPL39	YJL189W	60S large subunit ribosomal protein L39.e
RPL13B	YMR142C	60S large subunit ribosomal protein
RPS27B	YHR021C	Ribosomal protein S27.e
RPS12	YOR369C	40S small subunit acidic ribosomal protein S12
RPS6A	YPL090C	Ribosomal protein S6.e
MRPL23	YOR150W	Mitochondrial ribosomal protein, large subunit
IMG1	YCR046C	Mitochondrial ribosomal protein, large subunit
MRPS28	YDR337W	Mitochondrial ribosomal protein, small subunit
MSR1	YHR091C	Arginyl-tRNA synthetase, mitochondrial
ARX1	YDR101C	Required for 60S pre-ribosome formation
FUN12	YAL035W	General translation factor, IF2 homolog
SWS2	YNL081C	Mitochondrial ribosomal protein of the small subunit
	YER087W	Putative prolyl-tRNA synthetase
<b>Transcription/RNA processing</b>		
SRB5	YGR104C	DNA-directed RNA polymerase II holoenzyme
GAL11	YOL051W	DNA-directed RNA polymerase II holoenzyme
RPB9	YGL070C	DNA-directed RNA polymerase II, 14.2-kDa subunit
PAF1	YBR279W	DNA-directed RNA polymerase II regulator
RRN10	YBL025W	RNA polymerase I-specific transcription initiation factor
SSN8	YNL025C	DNA-directed RNA polymerase II holoenzyme
NUT1	YGL151W	Negative transcription regulator from artificial reporters
TUP1	YCR084C	General transcription repressor
HFI1	YPL254W	Transcriptional coactivator
CYC8	YBR112C	General repressor of transcription
HHO1	YPL127C	Histone H1 protein
MOT2	YER068W	Transcriptional repressor
NOT5	YPR072W	Component of the NOT protein complex
ROX3	YBL093C	Transcription factor
SPT3	YDR392W	General transcriptional adaptor or coactivator
NAM2	YLR382C	Leucine-tRNA ligase precursor, mitochondrial
CBP2	YHL038C	Apo-cytochrome <i>b</i> pre-mRNA processing protein 2
DBP7	YKR024C	RNA helicase required for 60S ribosomal subunit assembly
MRM2	YGL136C	Mitochondrial rRNA methyltransferase
PRP18	YGR006W	U5 snRNA-associated protein
RAI1	YGL246C	Required for pre-rRNA processing
SIN4	YNL236W	Global regulator protein
HPR1	YDR138W	Hyperrecombination protein related to Top1p
PGD1	YGL025C	Mediator complex subunit
<b>Secretory pathway</b>		
<b>Vacuolar protein sorting</b>		
VPS5	YOR069W	Involved in Golgi retention and vacuolar sorting
VPS16	YPL045W	Vacuolar sorting protein
VPS17	YOR132W	Vacuolar protein sorting-associated protein
SNF7	YLR025W	Class E Vps protein
VPS34	YLR240W	Phosphatidylinositol 3-kinase
VPS20	YMR077C	Vacuolar protein sorting (putative)
VPS33	YLR396C	Vacuolar sorting protein
VPS45	YGL095C	Vacuolar protein sorting-associated protein
VPS54	YDR027C	Subunit of VP51–54 complex
PEP8	YJL053W	Vacuolar protein sorting/targeting protein
PEP3	YLR148W	Vacuolar membrane protein
PEP7	YDR323C	Vacuolar segregation protein
FAB1	YFR019W	Phosphatidylinositol 3-phosphate 5-kinase
VPS27	YNR006W	Vacuolar protein sorting-associated protein
PEP12	YOR036W	Syntaxin (t-SNARE), vacuolar
VPS1	YKR001C	Member of the dynamin family of GTPases
<b>Glycosylation</b>		
ANP1	YEL036C	Required for protein glycosylation in the Golgi
MNN9	YPL050C	Required for complex <i>N</i> -glycosylation
OCH1	YGL038C	$\alpha$ -1,6-Mannosyltransferase
OST3	YOR085W	Oligosaccharyltransferase $\gamma$ subunit

*Continued*

Table 1. *Continued*

Gene	ORF	Function
LDB7	YBL006C	Mannosylphosphorylation of cell wall mannoproteins
Vesicular transport (Golgi network, etc.)		
AKR1	YDR264C	Ankyrin repeat-containing protein
ARF1	YDL192W	Small GTP-binding protein of the ARF family
CHC1	YGL206C	Clathrin heavy chain
CLC1	YGR167W	Clathrin light chain
SWA2	YDR320C	Clathrin-binding protein
GYP1	YOR070C	GTPase activating protein for Ypt1p and Sec4p
TRS33	YOR115C	TRAPP subunit of 33 kDa
SAC6	YDR129C	Actin filament bundling protein, fimbrin
SAC1	YKL212W	Recessive suppressor of secretory defect
SIW14	YNL032W	Protein involved in actin filament organization
YDJ1	YNL064C	Mitochondrial and ER import protein
Cell cycle and differentiation		
TPD3	YAL016W	Ser/Thr protein phosphatase 2A, regulatory chain A
YVH1	YIR026C	Protein tyrosine phosphatase
SLK19	YOR195W	Involved in control of spindle dynamics together with kar3p
SFP1	YLR403W	Zinc finger protein
BUB3	YOR026W	Cell cycle arrest protein
DOC1	YGL240W	Component of the anaphase promoting complex
RAD6	YGL058W	E2 ubiquitin-conjugating enzyme
RAD50	YNL250W	DNA repair protein
PHR1	YOR386W	Deoxyribodipyrimidine photolyase
IWR1	YDL115C	Interacts with RNA polymerase II
GLO3	YER122C	Zinc finger protein
SET3	YKR029C	Meiotic-specific repressor of the sporulation gene program
SPO19	YPL130W	Putative GPI-anchored spore wall protein
HTL1	YCR020W-B	High-temperature lethal, chromosome stability and fertility
SNT1	YCR033W	Component of meiotic-specific repressor of the sporulation
SAC7	YDR389W	Suppressor of actin mutation
FYV6	YNL133C	Protein required for survival upon exposure to K1 killer toxin
BUD19	YJL188C	Questionable protein
BUD22	YMR014W	Protein involved in bud-site selection
BUD23	YCR047C	Protein involved in bud-site selection
BUD25	YER014C-A	Questionable protein
BUD32	YGR262C	Protein involved in bud-site selection
BUD7	YOR299W	Involved in bud-site selection
BUD30	YDL151C	Involved in bipolar bud site selection
BEM1	YBR200W	Bud emergence mediator
Metabolism		
Carbon metabolism		
PFK2	YMR205C	6-Phosphofructokinase
GCR2	YNL199C	Glycolytic genes transcriptional activator
ADH1	YOL086C	Alcohol dehydrogenase
GND1	YHR183W	6-Phosphogluconate dehydrogenase
RPE1	YJL121C	D-Ribulose-5-phosphate 3-epimerase
REG1	YDR028C	Regulatory subunit for protein phosphatase Glc7p
SHP1	YBL058W	Potential regulatory subunit for Glc7p
SNF1	YDR477W	Carbon catabolite derepressing Ser/Thr protein kinase
TPS1	YBR126C	$\alpha,\alpha$ -Trehalose-phosphate synthase
GRR1	YJR090C	Required for glucose repression/glucose and cation transport
RBK1	YCR036W	Ribokinase
Amino acid metabolism		
ARO2	YGL148W	Chorismate synthase
PRO1	YDR300C	Glutamate 5-kinase
PRS3	YHL011C	Ribose-phosphate pyrophosphokinase
AAT2	YLR027C	Aspartate aminotransferase, cytosolic
URE2	YNL229C	Nitrogen catabolite repression regulator
PHO2	YDL106C	Homeodomain protein
NPR2	YEL062W	Nitrogen permease regulator
Lipid metabolism		
ERG2	YMR202W	C-8 sterol isomerase
ERG6	YML008C	S-Adenosyl-methionine delta-24-sterol-c-methyltransferase
ERG24	YNL280C	C-14 sterol reductase
ARV1	YLR242C	Sterol uptake and distribution into the plasma membrane
CRD1	YDL142C	Cardiolipin synthase
Nucleotide metabolism		
NPT1	YOR209C	Nicotinate phosphoribosyltransferase

*Continued*

Table 1. *Continued*

Gene	ORF	Function
RIB1	YBL033C	GTP cyclohydrolase II
ADK1	YDR226W	Adenylate kinase, cytosolic
IRA2	YOL081W	GTPase-activating protein for RAS proteins
RNR4	YGR180C	Ribonucleotide reductase small subunit
RNR1	YER070W	Ribonucleoside-diphosphate reductase, large subunit
Energy		
ATP2	YJR121W	F1F0-ATPase complex, F1 $\beta$ subunit
ATP12	YJL180C	F1F0-ATPase complex assembly protein
COX14	YML129C	Cytochrome <i>c</i> oxidase assembly protein
Cell rescue and defense		
PKR1	YMR123W	Resistance against <i>Pichia farinosa</i> killer toxin (SMK toxin)
HYR1 (GPX3)	YIR037W	Glutathione peroxidase
GON7	YJL184W	Hypothetical protein
TSA1	YML028W	Thiol-specific antioxidant
SSE1	YPL106C	Heat-shock protein of Hsp70 family
HIT1	YJR055W	Required for growth at high temperature
ARR4	YDL100C	Similarity to <i>Escherichia coli</i> arsenical pump-driving ATPase
SKN7	YHR206W	Transcription factor with similarity to Hsf1p
CRZ1	YNL027W	Calcineurin-responsive zinc finger
SSQ1	YLR369W	Mitochondrial Hsp70
UBI4	YLL039C	Ubiquitin
DOA1	YKL213C	Involved in ubiquitin-dependent proteolysis
DOA4	YDR069C	Ubiquitin-specific protease
MAP1	YLR244C	Methionine aminopeptidase
IRE1	YHR079C	Protein kinase
HAC1	YFL031W	Transcription factor
Ion homeostasis		
CUP5	YEL027W	H <sup>+</sup> -ATPase V0 domain 17-kDa subunit, vacuolar
PPA1	YHR026W	H <sup>+</sup> -ATPase 23-kDa subunit, vacuolar
VMA8	YEL051W	H <sup>+</sup> -ATP synthase V1 domain 32-kDa subunit, vacuolar
VMA7	YGR020C	H <sup>+</sup> -ATPase V1 domain 14-kDa subunit, vacuolar
VMA13	YPR036W	H <sup>+</sup> -ATPase V1 domain 54-kDa subunit, vacuolar
VMA2	YBR127C	H <sup>+</sup> -ATPase V1 domain 60-kDa subunit, vacuolar
VMA10	YHR039C-B	H <sup>+</sup> -transporting ATPase V0 domain 13-kDa subunit, vacuolar
VMA22	YHR060W	Vacuolar ATPase assembly protein
VPH2	YKL119C	H <sup>+</sup> -ATPase assembly protein
CSG2	YBR036C	Calcium-dependent regulatory protein
VMA21	YGR105W	ATPase assembly integral membrane protein
PHO88	YBR106W	Involved in phosphate transport
CNB1	YKL190W	Calcineurin B, regulatory subunit
PER1	YCR044C	Involved in manganese homeostasis
KSP1	YHR082C	Ser/Thr protein kinase
IMP2'	YIL154C	Involved in maintenance of ion homeostasis
SPF1	YEL031W	P-type ATPase
Cellular transport		
SSH1	YBR283C	Involved in cotranslational pathway of protein transport
TOM70	YNL121C	Mitochondrial outer membrane specialized import receptor
NUP84	YDL116W	Nuclear pore protein
NUP133	YKR082W	Nuclear pore protein
MON2	YNL297C	Role in endocytosis and vacuole integrity
FEN2	YCR028C	Pantothenate permease
Miscellaneous/unclassified		
MDM39	YGL020C	Protein involved in determination of mitochondrial structure
APQ13	YJL075C	Questionable protein
IES6	YEL044W	INO80 chromatin remodeling complex subunit 6
ARP8	YOR141C	Actin-related protein
KRE25	YNL296W	Questionable ORF
FYV5	YCL058C	Required for survival upon exposure to K1 killer toxin
	YLR193C	Protein of unknown function localized to mitochondria
	YLR358C	Protein of unknown function
	YLR426W	Putative 3-oxoacyl-[acyl-carrier-protein] reductase
	YML036W	Similarity to <i>Caenorhabditis elegans</i> hypothetical protein CELW03F8
	YDR048C	Questionable ORF
	YDR049W	Found in mitochondrial proteome
	YDR532C	Protein of unknown function localized to spindle pole body
	YEL033W	Questionable protein
	YFL032W	Questionable ORF
	YHR100C	Hypothetical ORF

*Continued*

Table 1. Continued

Gene	ORF	Function
	YJL028W	Hypothetical protein
	YJL120W	Questionable ORF
	YKL118W	Questionable ORF
	YNL140C	Protein of unknown function
	YNL115C	Protein of unknown function localized to vacuole
	YOR304C-A	Protein of unknown function localized to cytoplasm and bud
	YOR331C	Questionable protein
	YBR025C	Protein of unknown function localized to cytoplasm
	YJL070C	Putative AMP deaminases

ORF, open reading frame.

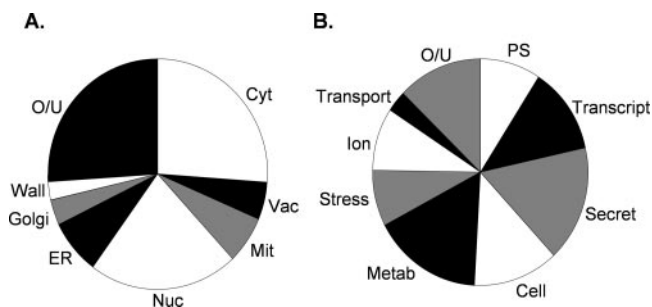
deleted for the *TSA1* isoform is sensitive to DTT (our unpublished data). Strains deleted for *SKN7* were also sensitive to 16 mM DTT. Faster growing revertants were generated at this concentration of DTT, whereas no growth was detected on 24 mM DTT. The *gpx3* mutant was the least sensitive and was able to grow on plates containing 24 mM DTT. Growth of the *gpx3* mutant could be prevented by 32 mM DTT (our unpublished data). Yeast contains three PHGPxs encoded by *GPX1-3* (Inoue *et al.*, 1999; Avery and Avery, 2001). However, only a strain deleted for the *GPX3* isoform is sensitive to DTT (our unpublished data). We next examined the sensitivity of these mutants to a reductive stress induced by the naturally occurring low-molecular-weight thiol cysteine (Figure 2A). The *trx1 trx2* and *tsa1* mutants were sensitive and showed poor growth on plates containing 16 mM cysteine.

We examined the DTT sensitivity of a *yap1* mutant because Gpx3, Skn7, and Tsa1 are all known to affect the activity of the Yap1 transcription factor. Yap1 is a redox-sensitive bZip-transcription factor that regulates the expression of many antioxidant genes, including *TRX2* and *TRR1* (Toledano *et al.*, 2004). The *yap1* mutant was sensitive and showed poor growth on 16 mM DTT (Figure 2A). Given that Gpx3, Skn7, Tsa1, and Yap1 can all affect thioredoxin gene expression, we next examined the levels of thioredoxins in

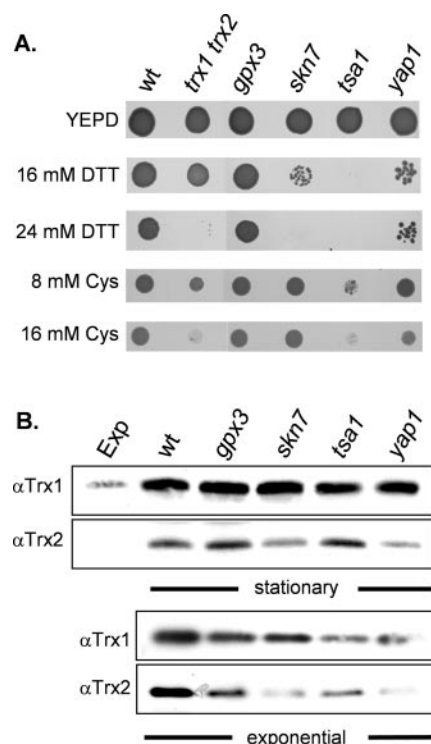
these mutants to determine whether DTT sensitivity could be explained by effects on thioredoxin gene expression.

#### Loss of *SKN7* and *YAP1* Affects the Basal Levels of Trx2

We examined the concentrations of Trx1 and Trx2 in the various mutants by Western blot analysis with the use of specific antibodies. Yeast contains two gene pairs encoding cytoplasmic thioredoxins (*TRX1*, *TRX2*), which seem to have

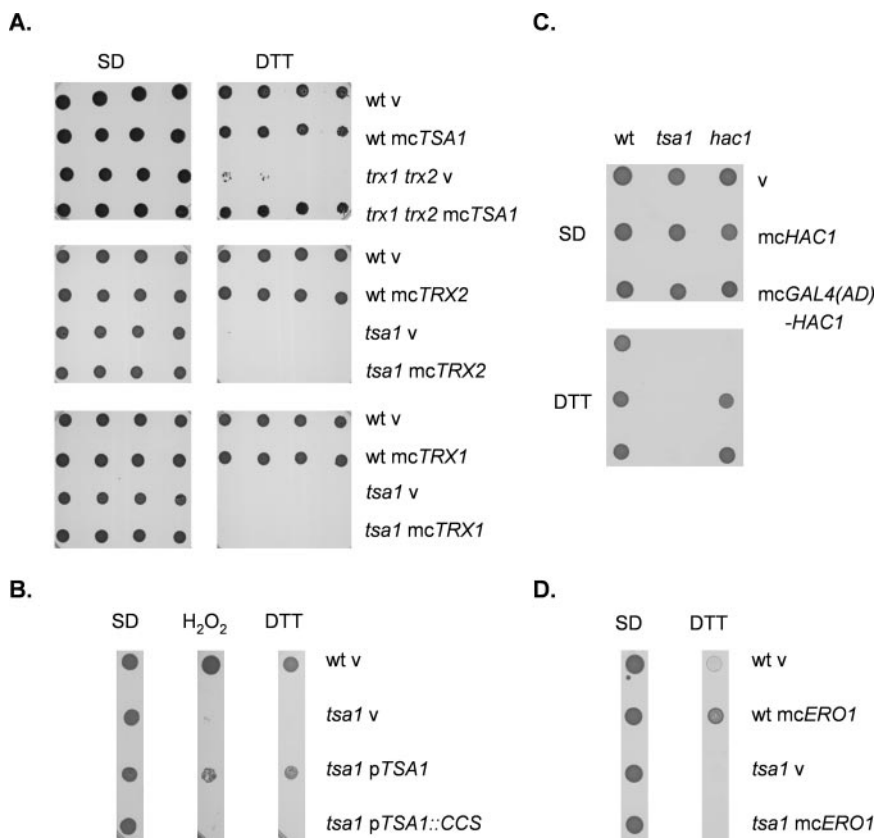


**Figure 1.** Functional grouping of deletion mutant sensitivity data. (A) Localization was assigned based on the "Component" term of the *Saccharomyces* Genome Database GO term mapper. Cyt, cytoplasm; Vac, vacuole; Mit, mitochondrion; Nuc, nucleus; Golgi, Golgi apparatus; Wall, cell wall; O/U, other/unknown. (B) Gene products were grouped into functional categories according to the MIPS functional database and *Saccharomyces* Genome Database, combined with visual inspection. More detailed functional data are available in Table 1. PS, protein synthesis; Transcript, transcription; Secret, secretory pathway; Cell, cell cycle and differentiation; Metab, metabolism; Stress, cell rescue and defense; ion, ion homeostasis; Transport, cellular transport; O/U, other/unknown.



**Figure 2.** Mutants affecting the thioredoxin system are sensitive to reductive stress. (A) Stress sensitivity was determined by spotting strains onto YEPD plates containing DTT or SD plates containing cysteine (Cys). Cultures of wild-type, *trx1 trx2*, *gpx3*, *skn7*, *tsa1*, and *yap1* strains were grown into stationary phase and adjusted to an  $A_{600}$  of 1.0 before spotting onto appropriate plates. Plates were incubated at 30°C for 3 d before scoring growth. (B) Western blot analysis of thioredoxin protein levels. Thioredoxin protein concentrations were measured in wild-type, *trx1 trx2*, *gpx3*, *skn7*, *tsa1*, and *yap1* mutant strains grown to exponential or stationary phases in YEPD media. Blots were analyzed with antibodies specific for Trx1 or Trx2 as indicated.





**Figure 3.** Role of Trx1–2, Tsa1, and the UPR in DTT tolerance. (A) Overexpression of *TSA1* increases the resistance of thioredoxin mutants to DTT, whereas overexpression of thioredoxins does not affect the *tsa1* mutant. The wild-type, *trx1 trx2* and *tsa1* mutant strains containing empty vector (V), mcTRX1, mcTRX2, or mcTSA1, as indicated, were grown into stationary phase and adjusted to an  $A_{600}$  of 1.0, 0.5, 0.25, or 0.125 before spotting onto SD plates containing 2–4 mM DTT. (B) The active site cysteine residues of Tsa1 are required for resistance to DTT. The wild-type and *tsa1* mutant strains containing empty vector (V), pTSA1 expressing wild-type Tsa1 or pTSA1::CCS expressing the active-site mutant of Tsa1p, in which cysteine 47 and 170 have been replaced by serines, were grown into stationary phase and adjusted to an  $A_{600}$  of 1.0 before spotting onto SGal plates containing 1 mM H<sub>2</sub>O<sub>2</sub> or 8 mM DTT. (C) Overexpression of *HAC1* does not rescue the DTT sensitivity of a *tsa1* mutant. The wild-type, *tsa1*, and *hac1* mutant strains containing empty vector (v), mcHAC1, or mcGAL4(AD)-HAC1 were grown into stationary phase and adjusted to an  $A_{600}$  of 1.0 before spotting onto SD plates containing 2 mM DTT. (D) Overexpression of *ERO1* does not rescue the DTT sensitivity of a *tsa1* mutant. The wild-type and *tsa1* mutant strains containing empty vector (V) or mcERO1 were grown into stationary phase and adjusted to an  $A_{600}$  of 1.0 before spotting onto SD plates containing 4 mM DTT. Plates were incubated at 30°C for 3 d before scoring growth.

redundant activities as antioxidants (Garrido and Grant, 2002). TRX2 plays the predominant role and its expression is regulated by both oxidative and reductive stress conditions. The cellular concentrations of Trx1 and Trx2 were elevated in stationary phase compared with exponential growth (Figure 2B) in agreement with previous transcriptional studies (Garrido and Grant, 2002). The amount of stationary phase Trx1 was unaffected by the loss of *GPX3*, *SKN7*, *TSA1*, or *YAP1* compared with the wild-type strain. In contrast, the levels of Trx2 were somewhat lower in the *skn7* and *yap1* mutants compared with the wild-type, *gpx3*, and *tsa1* mutant strains. Similarly, the concentrations of Trx2 in exponential phase cells were lower in the *skn7* and *yap1* mutants compared with the other strains. Lowered levels of Trx2 may therefore account for the DTT sensitivity of *skn7* and *yap1* mutants. In addition, all the mutants contained somewhat lower levels of Trx1 and Trx2 compared with the wild-type strain in exponential phase cells. Thus, there does not seem to be any strong correlation between the basal levels of Trx1–2 in these mutants and DTT sensitivity. The *tsa1* mutant did not significantly affect thioredoxin expression, yet showed the greatest sensitivity to DTT, and so it was chosen for further investigation.

#### Thioredoxins Require Tsa1 for Reductive Stress Resistance

Tsa1 may influence thioredoxin activity through an effect on thioredoxin gene expression as described above, or as a thioredoxin-dependent enzyme. To differentiate between these two possibilities, we examined the epistasis of *TSA1* and *TRX1–2* in the response to reductive stress. Overexpression of *TRX1* or *TRX2* was unable to rescue the DTT sensitivity of a *tsa1* mutant, indicating that thioredoxins require Tsa1 to promote DTT tolerance (Figure 3A). We have previ-

ously confirmed that thioredoxins are overexpressed in these strains and can promote resistance to hydrogen peroxide (Garrido and Grant, 2002). In contrast, overexpression of *TSA1* rescued the growth of a *trx1 trx2* mutant on DTT media, indicating that Tsa1 does not require thioredoxins to promote DTT tolerance. Thus, Tsa1 can act independently of Trx1–2 and must use an alternative source of reducing equivalents under these conditions. In agreement with this idea, previous studies have shown that GSH and DTT can act as alternative electron donors for peroxiredoxins (Park *et al.*, 2000). Together, these data indicate that thioredoxins act upstream of Tsa1 in the thioredoxin-dependent DTT resistance pathway.

In common with all typical Prxs, Tsa1 contains two redox active Cys residues that are directly involved in enzyme activity (Wood *et al.*, 2003). We therefore determined whether an active site mutant of Tsa1, in which Cys47 and Cys170 were substituted with serines (Wong *et al.*, 2004), could promote resistance to DTT. Tsa1 lacking the active site cysteine residues was unable to rescue the DTT sensitivity of a *tsa1* mutant, indicating that peroxiredoxin enzyme activity is required (Figure 3B). Tsa1 has mainly been characterized as an antioxidant in the detoxification of hydroperoxides, but more recently it has been shown to act as molecular chaperone that promotes resistance to heat shock (Jang *et al.*, 2004). The active site Cys residues are required for these activities and Tsa1 may therefore protect against reductive stress via its peroxidase or chaperone activities.

#### Activating the UPR in a *tsa1* Mutant Does Not Rescue DTT Sensitivity

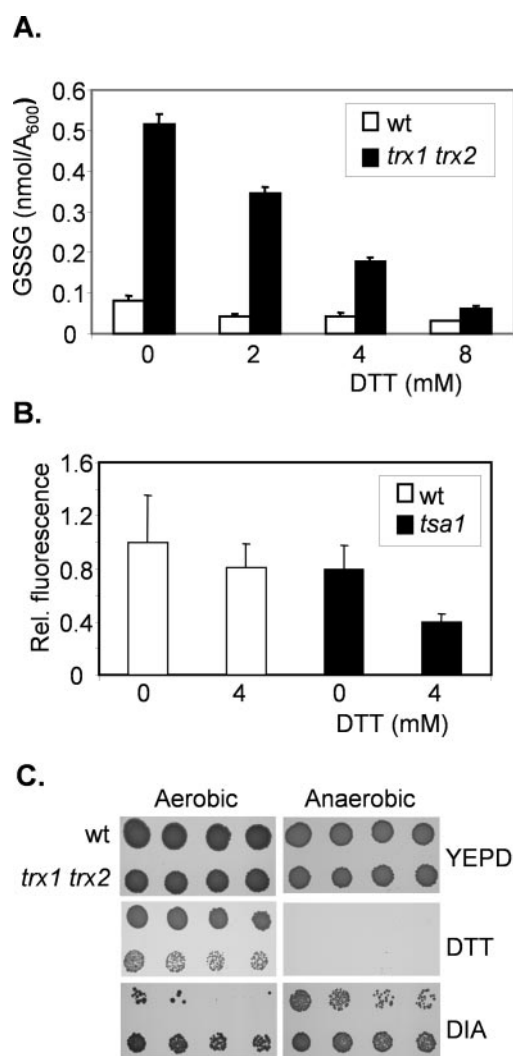
Because DTT can disrupt disulfide bond formation, most studies of DTT stress have focused on protein folding in the



ER and the UPR. We therefore tested whether there is any link between the DTT sensitivity of the *tsa1* mutant and the UPR. The magnitude of the UPR, measured using a reporter construct under the control of the UPR element (Sidrauski *et al.*, 1996), is comparable in the wild-type and *tsa1* mutant strains (our unpublished data). When unfolded proteins accumulate in the ER, the UPR is activated and the Hac1 transcription factor recognizes the UPR element present in target genes. We therefore tested whether overexpression of *HAC1* could rescue the growth of a *tsa1* mutant on DTT. *HAC1* overexpression was achieved using a multicopy vector containing wild-type *HAC1* (YEp-*HAC1*) or *HAC1* fused to the Gal4 activation domain at its N terminus [YEp-*GAL4(AD)-HAC1*] (Mori *et al.*, 2000). An mRNA-splicing event is normally required to fuse the *HAC1* DNA-binding domain to its activation domain, and the presence of the Gal4 activation domain results in constitutively active Hac1 in this construct. However, overexpression of Hac1 or the Gal4-fusion construct was unable to rescue DTT sensitivity of the *tsa1* mutant (Figure 3C). In comparison, both constructs could rescue the DTT sensitivity of a *hac1* mutant. Ero1 is a flavoenzyme that is required for oxidative protein folding in the ER. It is a target of the UPR and overexpression has been shown to confer DTT resistance (Frand and Kaiser, 1998; Pollard *et al.*, 1998). We therefore tested whether overexpression of *ERO1* could rescue the DTT sensitivity of the *tsa1* mutant. *ERO1* increased the tolerance of the wild-type strain to DTT, but it was unable to rescue the DTT sensitivity of the *tsa1* mutant (Figure 3D). Together, these data indicate that the DTT sensitivity of the *tsa1* mutant does not seem to result from an inability to prevent protein misfolding in the ER and is not related to the UPR.

#### DTT Toxicity Is Not Mediated by Reactive Oxygen Species

Although DTT is well known as an ER reducing agent, it is possible that it causes cellular oxidation through an indirect mechanism. For example, recent reports have shown that ER stress is linked with the generation of ROS (Harding *et al.*, 2003; Haynes *et al.*, 2004; Tu and Weissman, 2004). We therefore tested whether DTT exposure generates ROS using two different approaches. First, we determined whether the levels of GSSG are increased in response to DTT. Glutathione is the major cellular redox buffer and the redox state of the glutathione system is often taken as an indicator of the cellular redox environment (Meister and Anderson, 1983; Schafer and Buettner, 2001). Indeed, increases in yeast GSSG of sixfold are observed during conditions promoting ROS formation (Grant *et al.*, 1998). The cellular concentrations of GSSG can therefore be used as an indicator of ROS formation. Exposure of wild-type cells to DTT resulted in lowered levels of GSSG (Figure 4A). This effect was most apparent in a *trx1 trx2* mutant, which contains higher basal levels of GSSG (Muller, 1996; Garrido and Grant, 2002). DTT resulted in a dose-dependent decrease in GSSG levels in the *trx1 trx2* mutant (Figure 4A). Second, to more directly examine ROS levels, we used DCFH-DA. This is a cell-permeable fluorogenic probe that is widely used to estimate ROS formation (Davidson *et al.*, 1996; Izawa *et al.*, 1999). Fluorescence was decreased upon exposure to 4 mM DTT in both a wild-type and *tsa1* mutant (Figure 4B). These data indicate that rather than generating oxidizing conditions, DTT shifts the intracellular redox environment to a more reducing state. As a further test of whether DTT might act through an oxidative mechanism, we examined DTT toxicity under anaerobic conditions (Figure 4C). Anaerobic growth exacerbated the reductive stress and prevented the growth of a wild-type and

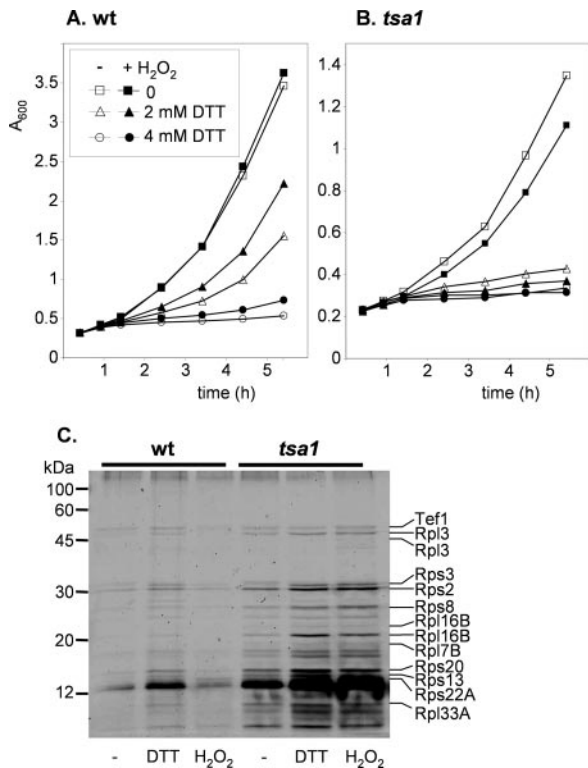


**Figure 4.** DTT toxicity is not caused by the formation of reactive oxygen species. (A) DTT treatment lowers the cellular levels of oxidized GSSG. The wild-type and thioredoxin mutant (*trx1 trx2*) were grown to exponential phase ( $A_{600} = 1.0$ ) in YEPD media and treated with DTT for 1 h. GSSG concentrations shown are the means of at least three independent determinations and are given in nanomoles per milliliter per  $A_{600}$ . (B) DTT treatment lowers intracellular ROS levels. ROS were measured in the wild-type and *tsa1* mutant strain grown in SD media using the cell-permeable fluorogenic probe DCFH-DA after treatment with 4 mM DTT for 15 min. Data are means  $\pm$  SD from three independent experiments. (C) Anaerobic growth conditions do not prevent DTT toxicity. The wild-type and thioredoxin mutant strains (*trx1 trx2*) were spotted onto YEPD plates containing DTT or diamide (DIA) and grown under aerobic or anaerobic conditions.

*trx1 trx2* mutant on plates containing DTT. In comparison, anaerobic conditions rescued the growth of a wild-type strain on the thiol oxidant diamide. Thus, DTT toxicity does not arise through a mechanism that involves ROS formation, making it unlikely that the peroxidase activity of Tsa1 is required to protect against DTT.

#### DTT Induces Protein Aggregation in Thioredoxin Mutants

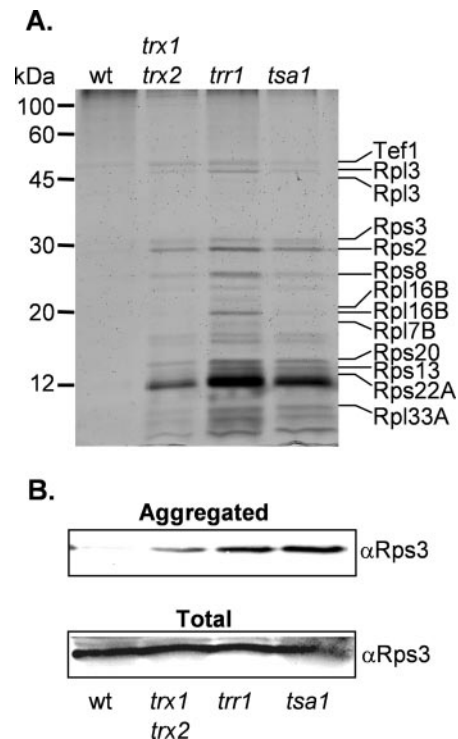
Oxidative stress conditions induced by exposure to low levels of  $H_2O_2$  have been shown to convert Tsa1 to a high-



**Figure 5.** DTT stress induces protein aggregation. Induction of DTT resistance by oxidative stress. Wild-type (A) and *tsa1* (B) mutant cells grown in YEPD media were pretreated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 1 h before growth in the presence of 2 or 4 mM DTT. Growth was monitored by absorbance at 600 nm. (C) Aggregation of ribosomal proteins in the *tsa1* mutant and in response to DTT. Insoluble proteins were extracted from the wild-type and *tsa1* mutant strains grown in YEPD media after treatment with 16 DTT or 4 mM H<sub>2</sub>O<sub>2</sub> for 1 h. Proteins were separated by SDS-PAGE and detected by silver staining. The indicated proteins were identified via MALDI-TOF mass spectrometry.

molecular-weight complex chaperone form (Jang *et al.*, 2004). This chaperone form can enhance yeast resistance to heat shock, and so we determined whether it could also protect against a reductive stress. Wild-type cells were pretreated with 50 mM H<sub>2</sub>O<sub>2</sub> before exposure to toxic levels of DTT. Treatments with 2 or 4 mM DTT decreased the growth rate of wild-type cells, but growth could be improved by pretreatment with H<sub>2</sub>O<sub>2</sub> (Figure 5A). This adaptive response requires Tsa1 because it is absent in the *tsa1* mutant (Figure 5B).

Exposure to heat stress has been shown to promote protein aggregation in the *tsa1* mutant, and so we examined whether DTT has a similar effect. Protein aggregation was measured using a technique that solubilizes membrane proteins and separates them from aggregated proteins, effectively reducing the background in aggregate fractions (Tomoyasu *et al.*, 2001; Jang *et al.*, 2004). Relatively few aggregated proteins were detected in the wild-type strain during normal growth conditions (Figure 5C). Exposure to DTT increased the levels of several proteins in the aggregate fraction. The *tsa1* mutant contained high basal levels of protein aggregates, comparable with the levels detected in the wild-type strain after DTT stress. Interestingly, the concentrations of these aggregated proteins increased further after exposure to DTT. These data indicate that the sensitiv-



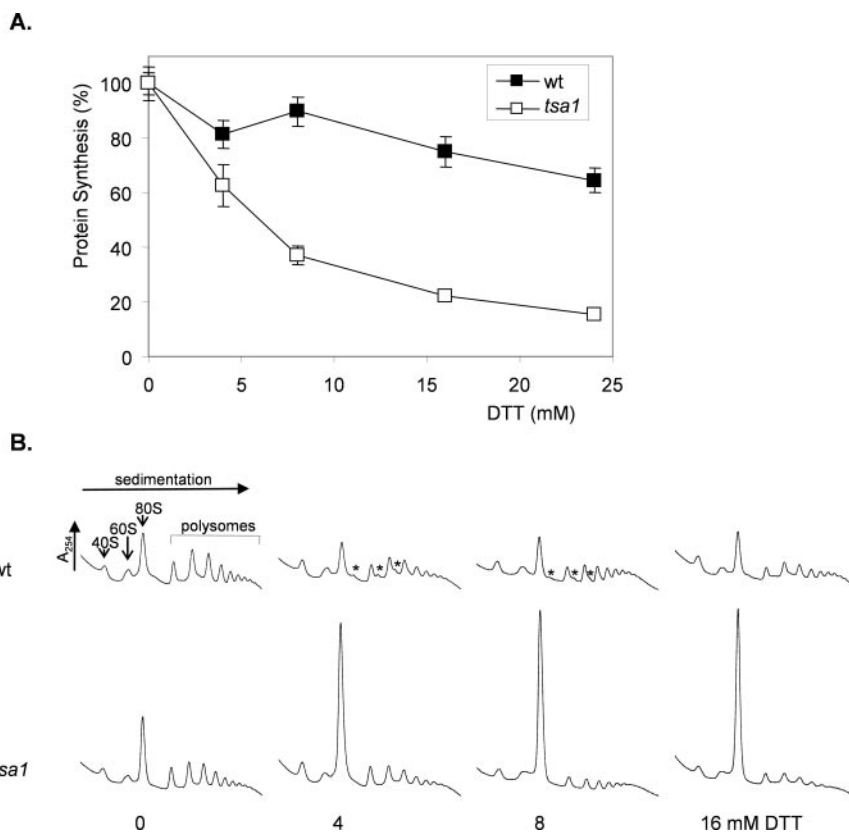
**Figure 6.** Thioredoxin system mutants contain elevated levels of aggregated ribosomal proteins. (A) Analysis of insoluble proteins from the wild-type, *trx1* *trx2*, *trr1*, and *tsa1* mutant strains as for Figure 5B. (B) Western blot analysis of Rps3 in the aggregated protein fraction and in a total cell extract from the wild-type, *trx1* *trx2*, *trr1*, and *tsa1* mutant strains.

ity of a *tsa1* mutant to reductive stress may be caused by an inability to protect against the toxic effects of protein aggregates. We compared the levels of aggregated proteins formed following a reductive stress with those formed during an oxidative stress generated by exposure to hydrogen peroxide. Whereas H<sub>2</sub>O<sub>2</sub> had relatively little effect on the wild-type strain, it increased protein aggregation in the *tsa1* mutant to a similar level to that caused by DTT (Figure 5C).

#### Thioredoxin Mutants Contain Elevated Levels of Aggregated Ribosomal Proteins

Given that protein aggregation may account for the toxicity of DTT, we analyzed the pattern of proteins that aggregate in a *tsa1* mutant. The major proteins were identified by mass spectrometry and are indicated on Figure 5C. Remarkably, the identified proteins were all ribosomal proteins and a ribosome associated protein (Tef1). These include proteins of both the small and large ribosomal subunits. We examined whether other thioredoxin pathway mutants affect ribosomal protein aggregation. High basal levels of aggregated proteins were detected in the *trx1* *trx2* and *trr1* mutants, and were particularly elevated in the *trr1* mutant (Figure 6A).

To check that there are no differences in the basal levels of ribosomal proteins in thioredoxin mutants, Western blot analysis was used to examine the levels of Rps3. Similar levels of Rps3 were detected in total cell extracts prepared from thioredoxin mutant strains (Figure 6B). In contrast, Rps3 is elevated in the aggregated protein preparations from the *tsa1*, *trr1*, and *trx1* *trx2* mutants compared with the wild-type strain (Figure 6B). Probing the Western blots with



**Figure 7.** Reductive stress inhibits protein synthesis. (A) Cultures of the wild-type and *tsa1* mutant strain were grown to exponential phase in minimal SD media. Aliquots of cell culture were used to measure the rate of protein synthesis by pulse labeling with [<sup>35</sup>S]cysteine/methionine for 5 min. Data are shown for untreated cultures (100%) and after treatment with DTT for 1 h. (B) DTT treatment specifically inhibits translation initiation. Polyribosome traces are shown from the wild-type and *tsa1* mutant strains. Yeast were grown in SD medium and treated with the indicated concentrations of DTT for 1 h. Polyribosomes were analyzed as described in *Materials and Methods*. The peaks that contain the small ribosomal subunit (40S), the large ribosomal subunit (60S), and both subunits (80S) are indicated by arrows. The polysome peaks generated by 2, 3, 4, 5, etc., 80S ribosomes on a single mRNA are bracketed. Asterisks indicate an accumulation of halfmer polysomes.

an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a cytoplasmic enzyme, confirmed that no GAPDH is present in the aggregate fraction, whereas it is present in total cell extracts (our unpublished data). These data indicate that a fraction of the ribosomal proteins present in thioredoxin mutants form insoluble aggregates.

#### Reductive Stress Inhibits Protein Synthesis in a *tsa1* Mutant

Given that reductive stress particularly affects ribosomal proteins, we examined whether there is any effect on protein synthesis. The rate of protein synthesis was measured as the incorporation of [<sup>35</sup>S]cysteine/methionine into proteins during a 5-min labeling period. DTT inhibited protein synthesis in the wild-type strain with a treatment of 24 mM DTT for 1 h reducing the rate of protein synthesis by ~35% (Figure 7A). Protein synthesis was much more sensitive to DTT in the *tsa1* mutant; for example, 4 mM DTT reduced the rate of protein synthesis by a similar amount to that seen for 24 mM DTT in the wild-type strain.

The inhibition of protein synthesis caused by DTT prompted us to analyze translational activity by examining the distribution of polysomes. Polysomes are ribosomes that are actively translating mRNAs, and they can be separated on sucrose density gradient and quantified by measuring absorbance at 254 nm. Extracts prepared from the wild-type and *tsa1* mutant strains exhibited normal profiles, including peaks corresponding to 40S and 60S ribosomal subunits, monosomes (80S ribosomes), and polysomes (Figure 7B). Interestingly, treatment of the wild-type strain with lower concentrations of DTT (4 and 8 mM) caused an accumulation of halfmer polysomes. This effect was lost at a higher con-

centration of 16 mM DTT. In the *tsa1* mutant, there was a dramatic shift of ribosomes from the polysomal region into the monosome or 80S peak after treatment with DTT (4–16 mM). The accumulation of ribosomes in the 80S peak of a sucrose gradient is indicative of decreased translation initiation. These data indicate that a reductive stress caused by DTT affects translation initiation in wild-type and *tsa1* mutant strains.

#### DISCUSSION

We previously showed that thioredoxins are required for DTT tolerance suggesting that thioredoxins maintain redox homeostasis in response to both oxidative and reductive stress conditions (Trotter and Grant, 2002). The data in the current study indicate that the requirement for thioredoxins during DTT stress is dependent on the chaperone activity of Tsa1. Tsa1 is a typical 2-Cys peroxiredoxin that has primarily been characterized as an antioxidant (Chae *et al.*, 1994; Park *et al.*, 2000). More recently, a chaperone function has been demonstrated for Tsa1, which enhances yeast resistance to heat shock (Jang *et al.*, 2004). Studies of several typical 2-Cys Prxs have revealed changes in oligomeric state that are linked to changes in redox state (Wood *et al.*, 2003). In the yeast Tsa1 protein, the chaperone function has been shown to predominate in the higher molecular weight complex forms (Jang *et al.*, 2004). The switch between thioredoxin-dependent peroxidase and chaperone functions is linked to oxidation of the peroxidatic Cys47 residue. In this current study, we have shown that Tsa1 is required to protect against DTT-induced protein aggregation. This activity is enhanced by prior treatment with hydrogen peroxide, which shifts the



pool of Tsa1 to the higher molecular weight complex chaperone form (Jang *et al.*, 2004). Analysis of the proteins that aggregate in a *tsa1* mutant identified ribosomal proteins as likely substrates for Tsa1 chaperone function. The resulting defect in protein synthesis seems to underlie the toxicity of reductive stress in thioredoxin mutants.

Genome-wide studies have indicated that there is a common regulatory program, called the environmental stress response (ESR), that is activated in response to diverse environmental stress conditions (Gasch *et al.*, 2000; Causton *et al.*, 2001). In addition to these general responses, each particular stress can invoke a unique pattern of transcriptional regulation that seems to be specialized for the specific conditions. The initial response to DTT includes the induction of ER chaperones consistent with the idea that DTT disrupts protein folding in the ER (Gasch *et al.*, 2000; Travers *et al.*, 2000). Prolonged exposure to DTT activates the ESR, along with the induction of genes that are involved in the response to cell wall damage, indicating that cell wall perturbation may eventually trigger the ESR in response to DTT (Gasch *et al.*, 2000). However, the analysis of genome-wide responses to DNA-damaging agents and oxidants has indicated that relatively few of the genes induced after exposure to these agents are necessary to protect the viability of the cell against these agents (Birrell *et al.*, 2002; Thorpe *et al.*, 2004). Similarly, of the 195 deletion mutants identified in this study as being sensitive to DTT, only 28 are induced within 60 min after DTT stress (Travers *et al.*, 2000). The induced genes are distributed throughout the cell and do not cluster into any particular functional category (our unpublished data). The remaining genes presumably represent constitutive functions that are required for DTT tolerance.

Extensive analysis of gene functions that are required during exposure to different ROS has revealed that distinct gene classes maintain protection against particular oxidants (Thorpe *et al.*, 2004). Additionally, core gene functions are required for tolerance against all forms of ROS, including gene expression, vacuolar protein sorting, and cell wall biosynthesis and maintenance. These core functional classes are also required for DTT tolerance and may represent gene functions that are generally required during stress conditions. Mutants affecting the secretory pathway are overrepresented in the DTT-sensitive strains, compared with ROS-sensitive strains, presumably reflecting the effect of DTT on protein folding and secretion. A strikingly large class of DTT-sensitive mutants are defective in ribosomal proteins and ribosome export and processing. This may indicate that perturbing ribosome production results in DTT sensitivity, consistent with the idea that aggregation of ribosomal proteins is the cause of DTT toxicity in thioredoxin mutants.

Yeast, like most eukaryotes, contains a complete cytoplasmic thioredoxin system, including two thioredoxins (*TRX1-2*) and a thioredoxin reductase (*TRR1*), which functions in protection against oxidative stress (reviewed in Carmel-Harel and Storz, 2000; Wheeler and Grant, 2004). Trx1 and Trx2 are active as antioxidants and play key roles in protection against oxidative stress induced by various ROS. Trx2 seems to play the predominant role because mutants lacking *TRX2* are hypersensitive to hydroperoxides and mutants containing *TRX2*, in the absence of *TRX1*, are resistant to these oxidants (Garrido and Grant, 2002). Additionally, five thioredoxin peroxidases have been identified in yeast, although their exact intracellular roles and targets remain unclear (Park *et al.*, 2000). Components of the thioredoxin system are simi-

larly regulated, with the expression of *TRX2*, *TRR1*, *TSA1*, *TSA2*, and *PRX1* regulated by the cooperative action of the Yap1 and Skn7 transcription factors (Charizanis *et al.*, 1999; Lee *et al.*, 1999; Park *et al.*, 2000). Tsa1 is essential for the transcriptional induction of *TRX2* and *TRR1*, and Gpx3 plays the role of a hydroperoxide sensor and transducer that signals the activation of Yap1 (Kuge and Jones, 1994; Morgan *et al.*, 1997; Ross *et al.*, 2000; Delaunay *et al.*, 2002). Strains lacking *TSA1*, *SKN7*, *GPX3* or *YAP1* were all identified as being DTT sensitive in this study, which may therefore result from lowered levels of thioredoxins in these mutants. Although this seemed to be the case for *skn7* and *yap1* mutants, alterations in thioredoxin levels do not seem to account for the DTT sensitivity of the *gpx3* mutant. We have previously shown that *TRX2* expression is regulated by reductive stress (Trotter and Grant, 2002), and it is possible that *GPX3*, or the other genes identified in this study, are required for this regulation. Because Tsa1 represents the most downstream component of the thioredoxin system identified in this screen, we focused on characterizing the DTT sensitivity of a *tsa1* mutant in this study.

DTT stress was found to disrupt ribosome function and to inhibit translation initiation. Eukaryotic translation initiation is a complex highly regulated process involving more than 30 polypeptide factors interacting with ribosomal subunits, Met-tRNA<sub>met</sub>, and mRNAs (McCarthy, 1998). The initiation phase of protein synthesis is rate limiting and is a target of regulation. Four mammalian protein kinases (HRI, PERK, PKR, and GCN2) have been identified that regulate translation initiation by phosphorylating eukaryotic initiation factor-2 (eIF2) in response to cellular stress (Wek, 1994; Dever, 2002). In yeast, Gcn2 is the sole eIF2 kinase and phosphorylates eIF2 $\alpha$  in response to diverse stress conditions, including amino acid, purine, and glucose starvation, and sodium and rapamycin exposure. This causes a global inhibition of protein synthesis as well as gene-specific translational activation of *GCN4*, which encodes a transcriptional activator protein (Hinnebusch, 1994). More recently, Gcn4 has been found to play an essential role in the UPR: Gcn4 and Gcn2 are required for the induction many UPR target genes during ER stress. Both Hac1 and Gcn4 bind target gene promoters to stimulate transcriptional induction (Patil *et al.*, 2004).

PERK has been found in all multicellular eukaryotes and is a component of the unfolded protein response. Consistent with its central role in the ER stress response, cells lacking PERK fail to phosphorylate eIF2 $\alpha$  and do not down-regulate protein synthesis during ER stress conditions (Kaufman, 1999; Bertolotti *et al.*, 2000). Yeast does not contain PERK and hence DTT stress has not previously been shown to inhibit protein synthesis. Our data show that DTT-stress causes an accumulation of halfmers in a wild-type strain, which are polyribosomes that have an extra 40S or 43S ribosomal subunit attached to the mRNA (Tuite *et al.*, 1999). They have previously been described for yeast strains deficient in translation factors, including Rpl16 and Gcd2, and are thought to reflect a reduced ability of the 60S subunit to join the 43S complex on mRNA (Rotenberg *et al.*, 1988; Foiani *et al.*, 1991). DTT resulted in a more pronounced defect in translation initiation in the *tsa1* mutant, caused ribosome aggregation and strongly inhibited protein synthesis. This block in protein synthesis correlated with the aggregation of ribosomal proteins caused by DTT exposure. These data indicate that some aspect of ribosomal production/function is sensitive to reductive stress conditions.

The production of ribosomes is a complex, highly regulated energy-requiring process (Warner, 1999). It must be



able to respond to environmental conditions and several conserved signaling pathways (TOR, RAS/protein kinase A, and protein kinase C) are known to regulate ribosome biosynthesis (Jorgensen *et al.*, 2004; Schawalder *et al.*, 2004). As yet, it is unclear whether DTT disrupts existing ribosomes or affects the assembly and processing of new ribosomes. Interestingly, *ARX1*, *DBP7*, *SFP1*, and *SSQ1*, which are all implicated in ribosome export and biogenesis were identified in the screen for DTT-sensitive mutants (Table 1). *Sfp1* is a transcription factor that regulates ribosomal protein gene expression in response to nutrients and stress conditions (Fingerman *et al.*, 2003; Marion *et al.*, 2004). In response to diverse environmental conditions, including DTT-stress, it down-regulates transcription of ribosomal protein genes (Marion *et al.*, 2004). *Arx1* has been identified as a protein associated with the ribosomal export complex (Nissan *et al.*, 2002). *Dbp7* is a putative ATP-dependent RNA helicase that is required for 60S ribosomal subunit assembly (Daugeron and Linder, 1998). *Ssq1* is a mitochondrial Hsp70-type molecular chaperone required for the assembly of iron/sulfur clusters into proteins (Muhlenhoff *et al.*, 2003). It may have an indirect effect on ribosomal processing and production because the biogenesis of ribosomes requires the essential iron/sulfur protein *Rli1* (Kispal *et al.*, 2005; Yarunin *et al.*, 2005). The finding that several mutants affected in ribosome production as well as mutants lacking individual ribosomal proteins are DTT sensitive may indicate that it is the production of new ribosomes that is DTT sensitive. Identification of the exact step targeted by DTT will require further experimentation.

Like other molecular chaperons, *Tsa1* has been shown to suppress thermal aggregation *in vitro* by binding to unfolded proteins via hydrophobic protein sites (Jang *et al.*, 2004). Additionally, the absence of *TSA1* led to an accumulation of heat-shock-induced protein aggregates in yeast cells, although the aggregated proteins were not identified (Jang *et al.*, 2004). We have now identified ribosomal proteins as a major target for *Tsa1* chaperone activity. Intact ribosomes require numerous protein-protein and protein-RNA interactions, and so it is perhaps, not surprising, that disrupting their normal structure results in the formation of nonspecific aggregates. Binding of any misassembled ribosomal proteins by *Tsa1* would prevent their aggregation, before recycling or degradation. Given the dramatic effect on protein synthesis of disrupting this process it seems to represent a previously unrecognized stress-protective mechanism.

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