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The yeast Tsa1 peroxiredoxin is a ribosome-associated antioxidant

Eleanor W. Trotter¹, Jonathan D. Rand², Jill Vickerstaff and Chris M. Grant*

The University of Manchester, Faculty of Life Sciences, Manchester M13 9PT, U.K.

*Corresponding Author: Dr Chris M. Grant,
The University of Manchester,
Faculty of Life Sciences, The Michael Smith Building,
Oxford Road, Manchester M13 9PT, U.K.
Phone: (0161) 306 4192
Fax: (0161) 275 5082
Email: chris.grant@manchester.ac.uk

Running title: Ribosome function of Tsa1 peroxiredoxin

Current address: ¹Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, UK. ²Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK.

SYNOPSIS

The yeast Tsa1 peroxiredoxin, like other 2-Cys peroxiredoxins, has dual activities as a peroxidase and as a molecular chaperone. Its peroxidase function predominates in lower MW forms, whereas, a super-chaperone form predominates in high MW complexes. Loss of *TSA1* results in aggregation of ribosomal proteins indicating that Tsa1 functions to maintain the integrity of the translation apparatus. We report here that Tsa1 functions as an antioxidant on actively translating ribosomes. Its peroxidase activity is required for ribosomal function since mutation of the peroxidatic cysteine residue, which inactivates peroxidase but not chaperone activity, results in sensitivity to translation inhibitors. The peroxidatic Cys residue is also required for a shift from ribosomes to its HMW form in response to peroxide stress. Thus, Tsa1 appears to function predominantly as an antioxidant in protecting both the cytosol and actively translating ribosomes against endogenous ROS, but shifts towards its chaperone function in response to oxidative stress conditions. Analysis of the distribution of Tsa1 in thioredoxin system mutants revealed that the ribosome-associated form of Tsa1 is increased in mutants lacking thioredoxin reductase (*trr1*) and thioredoxins (*trx1 trx2*) in parallel with the general increase in total Tsa1 levels which is observed in these mutants. We show that de-regulation of Tsa1 in the *trr1* mutant specifically promotes translation defects including hypersensitivity to translation inhibitors, increased translational error-rates and ribosomal protein aggregation. These data have important implications for the role of peroxiredoxins in stress and growth control since peroxiredoxins are likely to be deregulated in a similar manner during many different disease states.

KEYWORDS: Peroxiredoxin, ribosome, oxidative stress, thioredoxin, chaperone, yeast

INTRODUCTION

Peroxiredoxins (Prx's) are ubiquitous, thiol-specific proteins that have multiple functions in stress protection. In addition to their peroxidase function, they have proposed roles in diverse cellular processes including, differentiation, proliferation, modulation of intracellular signaling, apoptosis and gene expression [1, 2]. Peroxiredoxins use redox-active cysteine residues to reduce peroxides and have been divided into two categories, the 1-Cys and 2-Cys Prx's, based on the number of cysteine residues directly involved in catalysis [3]. Typical 2-Cys peroxiredoxins are active as a dimer and contain two redox active Cys residues that are directly involved in enzyme activity [4, 5]. During catalysis, the peroxidatic cysteine is oxidized to a sulphenic acid, which condenses with a resolving cysteine (from the other subunit of the dimer) to form a disulphide. This disulphide bond is reduced by thioredoxin to regenerate the active peroxiredoxin. Peroxiredoxins are therefore active in a redox cycle, accepting electrons from NADPH via thioredoxin and thioredoxin reductase.

Three cytoplasmic 2-Cys Prx's (Tsa1, Tsa2, Ahp1) have been described in yeast. They all display thioredoxin peroxidase activity, but their exact intracellular roles and targets remain unclear [5]. Tsa1 is the major peroxiredoxin and has best been characterized as an antioxidant in the detoxification of hydroperoxides [6, 7], but more recently has been shown to act as a molecular chaperone that promotes resistance to heat shock [8]. Its chaperone activity involves a stress-dependant switch from low MW species to high MW complexes [8]. During normal growth conditions, Tsa1 is present as a mixture of low and oligomeric forms which possess dual activities as a peroxidase and a chaperone. In response to a peroxide stress it shifts to the HMW "super-chaperone" form. Thus, the peroxidase function predominates in lower MW forms of Tsa1, whereas, the chaperone function predominates in high MW complexes. Like other molecular chaperones, Tsa1 can suppress thermal aggregation *in vitro* by binding to unfolded proteins via exposed hydrophobic sites [8]. Studies of several typical 2-Cys Prx's have revealed similar changes in oligomeric state that are linked to changes in redox state [3] and human PrxII and *Helicobacter pylori* AhpC have been shown to possess dual functions as peroxidases and as molecular chaperones [9, 10].

Yeast mutants lacking *TSA1* are sensitive to heat shock and accumulate heat-shock induced protein aggregates [8]. The levels of aggregation are also increased in response to oxidative (H_2O_2) or reductive (DTT) stress conditions which may contribute to the sensitivity of this mutant to these stress conditions [11]. We recently identified a number of the proteins that aggregate in a *tsa1* mutant. Remarkably, the most abundant aggregated proteins were all ribosomal proteins, including proteins of both the small and large ribosomal subunits [11]. Disrupting the thioredoxin system, through loss of both thioredoxins (*trx1 trx2*) or thioredoxin reductase (*trr1*), results in a similar pattern of protein aggregation, presumably reflecting the role of the thioredoxin system in maintaining the redox status of Tsa1. Ribosomal protein aggregation was found to correlate with an inhibition of translation initiation suggesting that Tsa1 normally functions to protect against the damaging consequences of disrupting the protein synthetic machinery [11].

In this current study we have examined whether Tsa1 is associated with actively translating ribosomes which might explain its role in protecting the translation apparatus. We show that a portion of Tsa1 is associated with actively translating ribosomes and this ribosome-associated pool shifts from ribosomes to the HMW super-chaperone form in response to oxidative stress conditions. A further link with ribosome function comes from the finding that de-regulation of Tsa1 in a thioredoxin reductase mutant promotes hypersensitivity to translation inhibitors, an increased translational error-rate and elevated ribosomal protein aggregation. Thus, Tsa1 is the first peroxiredoxin to be shown to function on the translational apparatus, where we propose it acts to protect ribosomal proteins against oxidative damage and non-specific ribosomal protein aggregation.

EXPERIMENTAL PROCEDURES

Yeast Strains, plasmids and growth conditions. The *Saccharomyces cerevisiae* strains used in this study were isogenic derivatives of W303 (*MATa ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100*). Strains deleted for thioredoxins (*trx1::TRP1 trx2::URA3*), thioredoxin reductase (*trr1::HIS3*) and *TSA1* (*tsa1::LEU2*) have been described previously [6, 11, 12]. Strains CY524 (*trx1::TRP1 trx2::URA3 trr1::HIS3*) and CY1025 (*trr1::HIS3 tsa1::LEU2*) were constructed using standard yeast techniques. The centromeric plasmids pFL-TSA1 and pFL-TSA1^{C47S}, containing wild-type and Cys47 mutant versions of *TSA1*, respectively, have been described previously [13].

Strains were grown in rich YEPD medium (2% w/v glucose, 2% w/v bactopectone, 1% w/v yeast extract) or minimal SD medium (0.17% w/v yeast nitrogen base without amino acids, 5% w/v ammonium sulfate, 2% w/v glucose) supplemented with appropriate amino acids and bases [14] at 30 °C and 180 rpm. Media were solidified by the addition of 2% (w/v) agar. Stress sensitivity was determined by growing cells to stationary phase and spotting diluted cultures ($A_{600} = 1.0, 0.1$) onto agar plates containing various concentrations of inhibitor. Growth was monitored after three days. The chaperone activity of *tsa1* mutants was determined by exposing exponential phase cultures to 47°C for 30 min.

Translation fidelity assays. The levels of termination codon readthrough were measured using a β -galactosidase reporter system [15]. Readthrough was quantified using plasmid pUKC817 (which carries the *lacZ* gene that bears a premature termination codon) and expressed as a proportion of control β -galactosidase levels, measured in transformants carrying the control plasmid pUKC815 (which carries the wild-type *lacZ* gene). The missense error rate was measured using an assay based on the misincorporation of Arg at position 218 in luciferase [16]. The assay is based on the replacement of codon AGA (R218) with AGC (encoding S218) which requires recognition by a near cognate tRNA for luciferase activity.

Protein aggregation and Western Blot Analysis. For Tsa1 analysis, total protein extracts were electrophoresed on 12% reducing SDS-PAGE minigels or on 4-12% native PAGE gels and electroblotted onto PVDF membrane (Amersham Pharmacia Biotech). Bound antibody (α Tsa1) was visualised by chemiluminescence (ECL, Amersham Pharmacia Biotech) following incubation of the blot in donkey anti-rabbit immunoglobulin-HRP conjugate (Amersham Pharmacia Biotech). The aggregation of soluble proteins was analysed as described previously [11]. Aggregated protein extracts were separated by 12% reducing SDS-PAGE and visualized by Silver Staining with the BioRad silver stain plus kit.

Analysis of Ribosome Distribution by Sucrose Density Gradients. Ribosomal fractions were separated from soluble components by centrifugation through sucrose cushions. 0.5 ml cell extracts prepared in CSB buffer (300 mM sorbitol, 20 mM HEPES pH 7.5, 1 mM EGTA, 5 mM MgCl₂, 10 mM KCl, 10% (v/v) glycerol, 10 μ g/ml cycloheximide) were layered onto 0.4 ml 60 % (w/v) sucrose in CSB buffer without sorbitol [17]. Samples were spun at 55,000 rpm in a Beckman MLA-130 rotor for 2.5 hr at 4 °C. For quantitative comparison, supernatant fractions were precipitated in 10 % (w/v) TCA and pellets washed in acetone before resuspension in SDS sample buffer. For polysome analysis, extracts were prepared in the presence of cycloheximide (100 μ g/ml), and layered onto 15 to 50% sucrose gradients. Gradients were sedimented via centrifugation at 40,000 rpm in a Beckman ultracentrifuge for 2.5 h, and the A₂₅₄ measured continuously [11]. Sucrose gradients were fractionated into 1ml fractions and boiled in SDS sample buffer prior to Western blot analysis. EDTA (15 mM) was added to breakage and gradient buffers to dissociate polysomes.

RESULTS

Tsa1 is associated with translating ribosomes. Polysome profiles were examined to determine whether Tsa1 associates with actively translating ribosomes. Cell lysates were analysed by sedimentation through sucrose density gradients and the distribution of ribosomes quantified by measuring absorbance at 254nm (Fig. 1A). Proteins were recovered from fractionated gradients and the presence of Tsa1 and a ribosomal protein (Rps3) detected by Western blot analysis. Tsa1 was most abundant at the top of the gradient, which is where soluble proteins sediment. However, a significant portion fractionated with polysomes, comparable with ribosomal protein Rps3. Dissociation of polysomes by treatment with EDTA shifted Tsa1 into lower molecular weight fractions, confirming that Tsa1 associates with actively translating ribosomes (Fig. 1B). Ribosomal fractions were separated from soluble components by centrifugation through sucrose cushions to enable a quantitative assessment of the relative distribution of Tsa1. This analysis revealed that approximately 5% of Tsa1 is associated with the ribosomal fraction compared with soluble components (Fig. 1C). This is not a general property of Prx's, since the cytoplasmic Ahp1 Prx could not be detected in the ribosomal fraction (data not shown).

The peroxidase activity of Tsa1 is required for ribosome function. Tsa1 shifts from its low MW forms to the HMW super-chaperone form in response to peroxide stress [8]. We therefore examined whether oxidative stress affects the ribosome association Tsa1. Following a treatment with 0.2 mM H₂O₂ for 10 min, polysome analysis revealed a significant decrease in the ribosome association of Tsa1 (Fig. 2). Loss of Tsa1 from polysomes is not simply due to inhibition of translation since this peroxide treatment had a relatively minor effect on polysome distribution and did not alter the sedimentation of Rps3.

For comparison, we examined the effect of this peroxide treatment on the oligomeric structure of Tsa1 using native PAGE (Fig. 3A). Wild-type Tsa1 was predominantly detected as low MW species, whereas, a portion of Tsa1 shifted to the HMW form in response to peroxide treatment in agreement with previous observations

[8]. The peroxidatic cysteine residue of Tsa1 is known to be required for the switch to the HMW super-chaperone form [8]. We confirmed that a Tsa1^{C47S} mutant, which lacks the peroxidatic cysteine residue, was unable to shift to HMW complexes in response to H₂O₂ (Fig. 3A). Similarly, no dissociation of this mutant from ribosomes was observed in response to peroxide treatment indicating that the shift from its ribosome-associated form is dependent on oxidation of the peroxidatic cysteine residue (Fig. 2). Thus, Tsa1 is predominantly present in low MW and ribosome-associated forms during normal growth conditions, but shifts to its super-chaperone form in response to oxidative stress conditions.

Sensitivity to translation inhibitors is often taken to indicate a functional link with the translation apparatus. We therefore examined the sensitivity of a *tsa1* mutant to translation inhibitors including hygromycin, paromomycin and cycloheximide. This analysis revealed that a *tsa1* mutant is sensitive to all three inhibitors compared with the wild-type control (Fig. 3B, compare wt v with *tsa1* v). Sensitivity was most pronounced with paromomycin and hygromycin compared to the relatively minor sensitivity observed with cycloheximide. Tsa1 is known to function as a peroxidase in the detoxification of hydroperoxides, and as a molecular chaperone that protects against protein misfolding. To determine which of these activities is important for ribosome function, we examined the sensitivity of the Tsa1^{C47S} mutant to translation inhibitors. The Cys47 residue of Tsa1 is not required for chaperone activity but is essential for peroxidase activity [8]. We first confirmed that the Tsa1^{C47S} mutant lacks peroxidase activity since it does not rescue the hydrogen peroxide sensitivity of a *tsa1* deletion strain, whereas, it is active as a chaperone since it complements the temperature sensitivity of the *tsa1* mutant (Fig. 3B). We reasoned that if the chaperone function of Tsa1 is important for tolerance to translation inhibitors, then the Tsa1^{C47S} mutant should still be able to promote resistance. However, the Tsa1^{C47S} mutant was unable to rescue the sensitivity of the *tsa1* mutant to paromomycin and hygromycin indicating that the peroxidase activity is most likely required for tolerance to aminoglycoside antibiotics. In contrast, the Tsa1^{C47S} mutant was able to increase the resistance of the *tsa1* mutant to cycloheximide. Taken together, our data indicate that under normal growth conditions, Tsa1 functions predominantly as an antioxidant protecting both the cytoplasm and translation apparatus against endogenous

ROS. In response to an oxidative stress, Tsa1 shifts towards a form in which it functions to chaperone stress-denatured proteins.

Ribosome association of Tsa1 in thioredoxin mutants. The redox state of Tsa1 is maintained by the thioredoxin system and so we examined whether thioredoxin reductase (Trr1) or thioredoxins (Trx1/Trx2) influence its ribosome function. This is important since we have previously shown that ribosomal protein aggregation is particularly elevated in a *trr1* mutant. Aggregation is actually greater than that observed in a *tsa1* mutant indicating that it does not simply arise due to loss of Tsa1 activity [11]. Tsa1 was found to be highly elevated in polysome fractions from a *trr1* mutant (Fig. 2 and Fig. 4A) and this elevated ribosome-association was unaffected by loss of the peroxidatic cysteine residue (Fig. 2). Ribosome-associated Tsa1 was also elevated in a *trx1 trx2* mutant, although not to the same extent as in the *trr1* mutant (Fig. 4A). Increased ribosome association presumably arises as a result of the elevated total Tsa1 protein levels which are observed in thioredoxin mutants (Fig. 4B) due to the constitutive Yap1 activation which has been described for these mutants [18, 19].

Analysis of the oligomeric structure of Tsa1 revealed that a significant portion is constitutively shifted to the HMW form in the *trr1* mutant (Fig. 4C). Given that the oxidant-induced switch from LMW species to HMW complexes requires thioredoxins, we examined whether thioredoxins are also required for this shift in oligomeric form. No Tsa1 was present in the HMW form in a *trr1 trx1 trx2* mutant indicating that thioredoxins promote the oligomerization of Tsa1 in the *trr1* mutant (Fig. 4C). In contrast, polysome analysis revealed that loss of thioredoxins did not affect the elevated Tsa1 ribosome-association observed in the *trr1* mutant (Fig. 4A). Thus, thioredoxins are required for the switch to the HMW chaperone form of Tsa1, but not its increased ribosome association in the *trr1* mutant. The *trr1 trx1 trx2* mutant therefore enabled us to examine the phenotypic consequences of the elevated total and ribosomal Tsa1 in a *trr1* mutant in the absence of any complications arising from the shift to the super-chaperone form (see below).

De-regulation of Tsa1 function in a *trr1* mutant promotes translation defects. We examined the sensitivity of the *trr1* mutant to translation inhibitors to determine the

consequences of elevated Tsa1 levels in this mutant. The *trr1* mutant displayed little or no sensitivity to cycloheximide compared with a wild-type strain (Fig. 5A). However, it was hypersensitive to hygromycin and paromomycin. The *trx1 trx2* mutant was also sensitive to hygromycin and paromomycin, but not to the same extent as the *trr1* mutant. To determine whether the sensitivity of the *trr1* mutant to aminoglycoside antibiotics is influenced by Tsa1 activity, we examined the effect of deleting *TSA1* in the *trr1* mutant. Interestingly, loss of *TSA1* partially rescued its sensitivity to hygromycin and paromomycin indicating that Tsa1 promotes sensitivity to these inhibitors in a *trr1* mutant. In comparison, loss of thioredoxins did not affect the sensitivity of the *trr1* mutant to aminoglycosides (Fig. 5A). These data indicate that it is most likely the ribosome-associated form of Tsa1, rather than the increased total Tsa1 levels *per se*, which causes hypersensitivity in this mutant. Similar to the deletion of *TSA1*, mutation of its peroxidatic cysteine residue also rescued the sensitivity of the *trr1* mutant to paromomycin and hygromycin (Fig. 5B). Thus, de-regulation of the active ribosomal form of Tsa1 causes sensitivity to aminoglycoside antibiotics in a *trr1* mutant.

Aminoglycosides have well characterized effects on translational fidelity [20, 21]. They can act on the ribosome to cause mistranslation, and hence mutants which are affected in the accuracy of the decoding process often show enhanced sensitivity. We therefore examined the fidelity of translation in strains lacking *TRR1*. Readthrough of termination codons was quantified using a plasmid which carries the *lacZ* gene with a premature UAA termination codon [15]. The wild-type and *tsa1* mutant displayed a similar level of termination codon readthrough (Table 1). In contrast, readthrough was elevated approximately 15-fold in the *trr1* mutant. This high level of readthrough was completely abrogated in the *trr1 tsa1* mutant consistent with the finding that deletion of *TSA1* rescues the aminoglycoside sensitivity of a *trr1* mutant. In contrast, readthrough was unaffected by loss of thioredoxins and the *trr1 trx1 trx2* mutant still displayed a high translational error-rate. The missense error rate was measured using a luciferase assay which requires misincorporation of Arg (AGC) at an AGA codon (encoding Ser), by a near cognate tRNA, for activity [16]. The missense error-rate was increased by approximately 1.6-fold in the *trr1* mutant and this increased error-rate was again abrogated by loss of *TSA1* (Table 1).

Tsa1 promotes ribosomal protein aggregation in a *trr1* mutant. To determine whether deregulated Tsa1 accounts for the high levels of protein aggregation in a *trr1* mutant, we examined protein aggregation in the *trr1 tsa1* mutant. Significantly, deleting *TSA1* in the *trr1* mutant reduced the high basal levels of aggregated proteins normally detected in the *trr1* mutant (Fig. 5C). In fact, aggregation was somewhat lower in the *trr1 tsa1* than in a *tsa1* mutant, which is surprising given that the *trr1 tsa1* mutant completely lacks functional Tsa1. We presume that other peroxidases may be able to substitute for Tsa1 in a *trr1 tsa1* mutant, and for example, *TSA2* is transcriptionally induced in *trr1* and *trr1 tsa1* mutants (our unpublished data). It is unclear at present whether Tsa2 has any ribosomal function, but it is highly homologous to Tsa1 and possess a similar chaperone activity, although it is normally expressed at significantly lower levels compared with Tsa1 [8]. Immunoblot analysis with an anti-ubiquitin antibody revealed the presence of a number of ubiquitin-modified proteins in the *trr1* mutant, presumably reflecting aberrant proteins marked for degradation (Fig. 5D). Ubiquitination was abrogated in the *trr1 tsa1* mutant confirming that Tsa1 propagates protein damage in the *trr1* mutant.

DISCUSSION

The production of ribosomes is a complex, highly regulated energy-requiring process [22]. 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 [23, 24]. For example, transcriptional co-regulation of genes involved in ribosome biogenesis has been linked to cellular proliferation and ribosome synthesis may serve as a measure for cell cycle progression [24, 25]. Little is known regarding how cells maintain ribosome homeostasis once they have been assembled and are active in cytoplasmic translation. This is surprising since ribosome synthesis represents greater than 50% of total transcription in growing eukaryotic cells [reviewed in [26]]. Our data indicate that Tsa1 functions as an antioxidant on actively translating ribosomes, protecting the protein synthetic machinery against oxidative damage.

Ribosomal proteins are major targets of aggregation in a *tsa1* mutant and aggregation correlates with an inhibition of protein synthesis [11]. We previously attributed ribosomal protein aggregation to a loss of Tsa1 chaperone activity. However, given the functional link with the translation apparatus, it is more likely that the loss of Tsa1 ribosomal activity accounts for ribosomal protein aggregation and the translation effects reported in this current study. Tsa1 was originally identified as a thiol-specific antioxidant which could provide protection against a thiol-containing oxidation system [27]. The purified protein was subsequently shown to possess peroxidase activity towards hydrogen peroxide and alkyl hydroperoxides in the presence of the thioredoxin and thioredoxin reductase reducing system [4]. It is this thioredoxin peroxidase activity that appears to be required for Tsa1 ribosomal function, since the Tsa1 peroxidatic Cys residue is required for resistance to translation inhibitors. These data can be integrated into a model to describe the multiple functions of Tsa1 (Fig. 6). Under normal growth conditions, Tsa1 functions predominantly as an antioxidant protecting both the cytoplasm and translational apparatus against endogenous ROS. Over-oxidation of the peroxidatic cysteine residue causes a shift to the HMW super-chaperone form. Thus, in response to an acute oxidative stress, a portion of Tsa1 is shifted towards its super-chaperone form

which protects against stress-denatured proteins [8]. The Tsa1 peroxidase function appears therefore to be less important during oxidative stress conditions. Presumably, other peroxidases can substitute for Tsa1 during oxidative stress conditions, many of which are induced in response to such conditions [28]. Tsa1 is ideally structured to undergo this kind of functional switch since its peroxidatic cysteine residue, in common with other 2-Cys Prx's, can be readily over-oxidized to cysteine-sulphinic acid following exposure to peroxides [3, 29].

Tsa1 protein levels are elevated in thioredoxin system mutants (*trr1* and *trx1 trx2*) due to the constitutive activation of the Yap1 transcription factor in these mutants [18, 19]. There is no apparent alteration in the portion of Tsa1 associated with ribosomes as a fraction of the total Tsa1 levels present in thioredoxin mutants compared with the wild-type strain. The elevation in total Tsa1 protein levels presumably accounts for the high levels of ribosomal Tsa1 detected in *trr1* and *trx1 trx2* mutants. We found that thioredoxin mutants are sensitive to aminoglycoside inhibitors and sensitivity is particularly pronounced in a *trr1* mutant. The best evidence that translation defects do not simply arise due to elevated levels of total Tsa1 in the *trr1* mutant comes from a comparison of the various thioredoxin mutants analyzed in this study. Mutants lacking thioredoxin or thioredoxin reductase contain similar elevated levels of total Tsa1, but only the *trr1* mutant is hypersensitive to aminoglycoside antibiotics and displays elevated translational errors. These data indicate that there is not a correlation between elevated Tsa1 levels and translation defects. The HMW super-chaperone form of Tsa1 present in the *trr1* mutant does not appear to effect translation since loss of thioredoxins (*trr1 trx1 trx2*) prevents Tsa1 from shifting to its HMW form in the *trr1* mutant but does not rescue its sensitivity to aminoglycoside antibiotics. Taken together therefore, these data indicate that the de-regulation of Tsa1 ribosomal function most likely causes translation defects.

Many protein synthesis mutants which alter the fidelity of translation are sensitive to aminoglycoside inhibitors such as paromomycin and hygromycin [30]. Similarly, misreading is increased in the *trr1* mutant as detected by the elevated levels of nonsense suppression and misincorporation. Loss of *TSA1* itself, does not affect translational fidelity in a wild-type strain. Rather, it appears that de-regulation of Tsa1 in the *trr1* mutant promotes translation defects including ribosomal protein aggregation, sensitivity

to aminoglycosides and elevated translational error rates. Further studies will be required to determine whether the high translational error rate in the *trr1* mutant promotes aggregation, or alternatively, whether ribosomal protein aggregation physically disrupts the translation process promoting misreading in the *trr1* mutant.

Peroxiredoxins have been implicated in many disease processes [31, 32]. For example, alterations in the levels of 2-Cys Prx's have been reported for many cancers [32-35]. A tumor suppressor role has been proposed for Prdx1 based on the observations that overexpression inhibits c-Myc-mediated transformation and increased malignancies are observed in mice with inactivated Prdx1 [36, 37]. Furthermore, the 2-Cys Prx, Pag, can interact with the oncoproteins c-Abl and c-Myc and affects cell size and apoptosis [38, 39]. Additionally, altered peroxiredoxin expression levels have been observed in different brain regions of patients affected by neurodegenerative disorders [reviewed in [40]]. Up-regulation of peroxiredoxins in these various pathologies may reflect an antioxidant response to oxidative stress. However, our data indicate that rather than serving a simple protective role, deregulation of peroxiredoxins can promote stress sensitivity via effects on protein aggregation and errors in translation. This finding is important since many of the disease processes described above are liable to include elevations in ROS levels which could potentially affect the thioredoxin system and Prx function, in a similar manner to that described here for yeast thioredoxin mutants.

There is increasing evidence that the translational apparatus is a target of oxidative damage. A recent global analysis of protein carbonylation identified a large number of ribosomal proteins which become oxidized in response to hydrogen peroxide stress, representing approximately 80% of possible ribosomal protein targets [41]. Interestingly, many of these ribosomal proteins were found to form protein-RNA cross links which may contribute to ribosomal protein aggregation [42]. Oxidative damage to the protein synthetic machinery has been implicated in the pathogenesis of neurodegenerative disorders, including Alzheimer's disease [43-45]. Furthermore, oxidation of mRNA has been shown to affect translational fidelity, and active translation of oxidized mRNA can result in the production of aberrant proteins [46]. The antioxidant activity of Tsa1 may therefore be important to maintain both the fidelity of translation during potentially error-prone conditions, as well as to protect the translational apparatus

itself, against oxidative damage. It appears that disruption of the translation apparatus may be more widespread than previously recognized, with ribosomal proteins representing important targets that must be protected against the formation of non-native protein structures and aggregates during stress conditions.

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FIGURE LEGENDS

Fig. 1. Tsa1 is associated with translating ribosomes. (A) A polyribosome trace (measured at 254 nm) is shown for the wild-type strain. Peaks containing the small ribosomal subunit (40S), the large ribosomal subunit (60S) both subunits (80S) and polysomes are indicated. Sucrose gradients were fractionated and the distribution of Tsa1 and Rps3 detected by immunoblot analysis. (B) EDTA (15 mM) was added to breakage and gradient buffers to dissociate polysomes. (C) Ribosomal fractions (R) were separated from soluble components (S) by centrifugation through sucrose cushions. Proteins were separated by SDS-PAGE and Tsa1 and Rps3 detected by immunoblot analysis. Representative polysome traces and Western blots are shown from repeat experiments.

Fig. 2. Tsa1 dissociates from ribosomes in response to hydrogen peroxide. Polyribosome traces are shown for the *tsa1* mutant containing wild-type Tsa1 (*pTSA1*) or mutant Tsa1 (*pTSA1^{C47S}*) and the *tsa1 trr1* mutant containing wild-type Tsa1 (*pTSA1*). Where indicated, strains were treated with 0.2 mM hydrogen peroxide for 10 min (+H). The distribution of Tsa1 and Rps3 was detected by immunoblot analysis. Rps3 is shown as a control protein which does not change its ribosome-association in response to hydrogen peroxide. Representative data are shown from repeat experiments.

Fig. 3. The peroxidase activity of Tsa1 is required for ribosome function. (A) Protein extracts were prepared from the indicated strains treated with hydrogen peroxide and separated by native PAGE. Immunoblot analysis confirms that the peroxide-induced shift of Tsa1 to the HMW chaperone form requires the peroxidatic cysteine residue. (B) Stress sensitivity was determined by growing cells to stationary phase and spotting diluted cultures ($A_{600} = 1.0, 0.1$) onto agar plates containing various concentrations of inhibitor. The indicated strains were tested for growth on plates containing no inhibitor (SD), 0.4 mM H_2O_2 , 300 μ g/ml hygromycin (Hyg), 0.1 μ g/ml cycloheximide (CHX) and 5.0 μ g/ml paromomycin (Pm). To test the chaperone function of Tsa1, exponential phase cultures were exposed to 47°C for 30 min. Representative data are shown from repeat experiments.

Fig. 4. Ribosome association of Tsa1 in thioredoxin mutants. (A) The ribosome-association of Tsa1 was examined in the indicated strains by immunoblot analysis of sucrose gradients. A polyribosome trace (measured at 254 nm) and immunoblot analysis of Rps3 is only shown for the wild-type strain, but was similar for all strains tested. (B) Protein extracts were prepared from the indicated strains and separated by SDS-PAGE. Immunoblot analysis reveals that Tsa1 protein levels are elevated in thioredoxin mutant. (C) Protein extracts from the same strains were analyzed by native-PAGE and Tsa1 detected by immunoblot analysis. The amount of protein loaded onto native gels was reduced by 50% compared with Fig. 3A due to the high concentrations of Tsa1 present in thioredoxin mutants causing smearing if loaded at too high a concentration. Representative data are shown from repeat experiments.

Fig. 5. De-regulation of Tsa1 promotes translation defects. (A) Stress sensitivity was determined by growing cells to stationary phase and spotting diluted cultures ($A_{600} = 1.0, 0.1$) onto agar plates containing various concentrations of inhibitor. Growth of the indicated strains was monitored after 3 days incubation at 30°C for plates containing no inhibitor (YEPD), 40 µg/ml hygromycin (Hyg), 0.1 µg/ml cycloheximide (CHX), 200 µg/ml paromomycin (Pm). Please note that Hyg is used at a significantly lower concentration than in Fig. 3B due to the hypersensitivity of the *trr1* mutant to this antibiotic. Pm is less toxic in YEPD media compared with SD media and hence a higher concentration has been used compared with Fig. 3B. (B) The indicated strains were tested for growth on plates containing no inhibitor (SD), 200 µg/ml hygromycin (Hyg), and 1.5 µg/ml paromomycin (Pm). (C) Protein aggregation was detected by examining insoluble proteins in the indicated strains. Proteins were separated by SDS-PAGE and detected by silver staining. (D) Immunoblot analysis (α -ubiquitin) of the insoluble proteins shown in (C). Representative data are shown from repeat experiments.

Fig. 6. Model of multiple Tsa1 functions. Tsa1 functions as an antioxidant during normal growth conditions. The peroxidase activity of Tsa1 is required to protect the cytoplasm and translation machinery against endogenous ROS. In response to an oxidative stress, Tsa1 shifts to a HMW form which primarily functions to chaperone

oxidatively damaged proteins. The shift to the chaperone form requires oxidation of the Tsa1 peroxidatic cysteine residue. The redox status of Tsa1 is controlled by the thioredoxin system (circled), which comprises two thioredoxins (Trx1-2) and thioredoxin reductase (Trr1). The oxidised disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase (Trr1). Oxidized peroxiredoxins, such as Tsa1, are in turn reduced by thioredoxin. For details see text.

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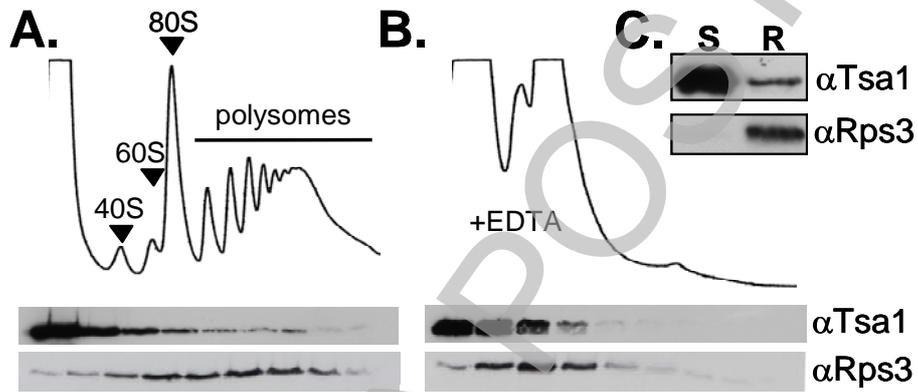
Table 1. Measurements of readthrough and missense error rates.

	Nonsense (%)¹	Missense (%)¹
wild-type	0.054 ± 0.013	0.45 ± 0.19
<i>tsa1</i>	0.042 ± 0.005	0.34 ± 0.07
<i>trr1</i>	0.793 ± 0.113	0.70 ± 0.23
<i>trr1 tsa1</i>	0.057 ± 0.013	0.51 ± 0.04
<i>trx1 trx2</i>	0.046 ± 0.005	n.d.
<i>trr1 trx1 trx2</i>	0.488 ± 0.034	n.d.

¹Values shown are the means of the triplicate determinations.

n.d. = not determined

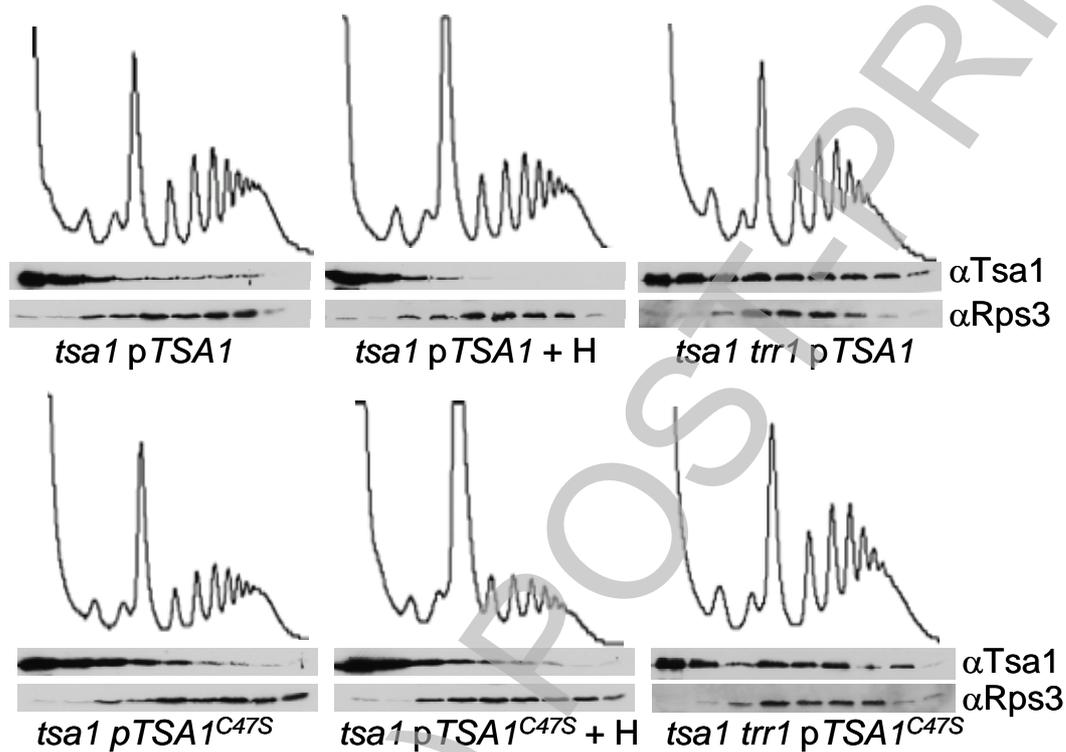
Fig. 1. Trotter *et al*



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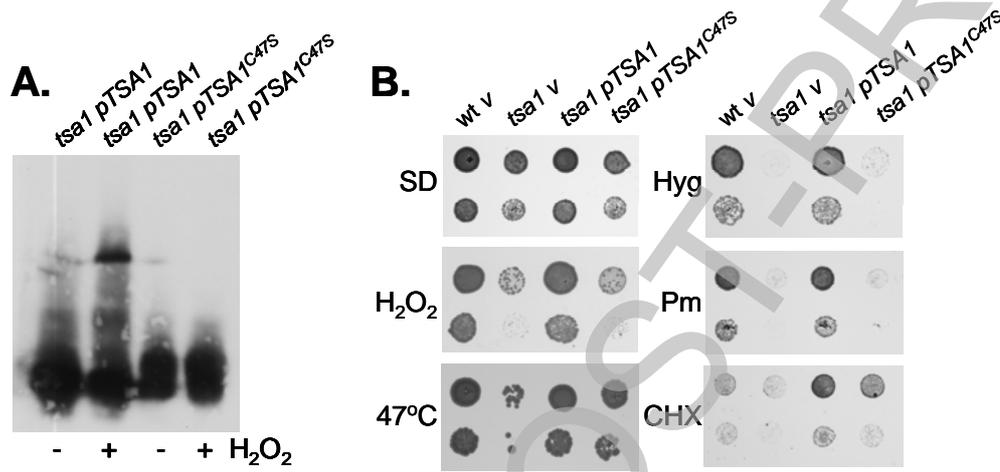
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Fig. 2. Trotter *et al*



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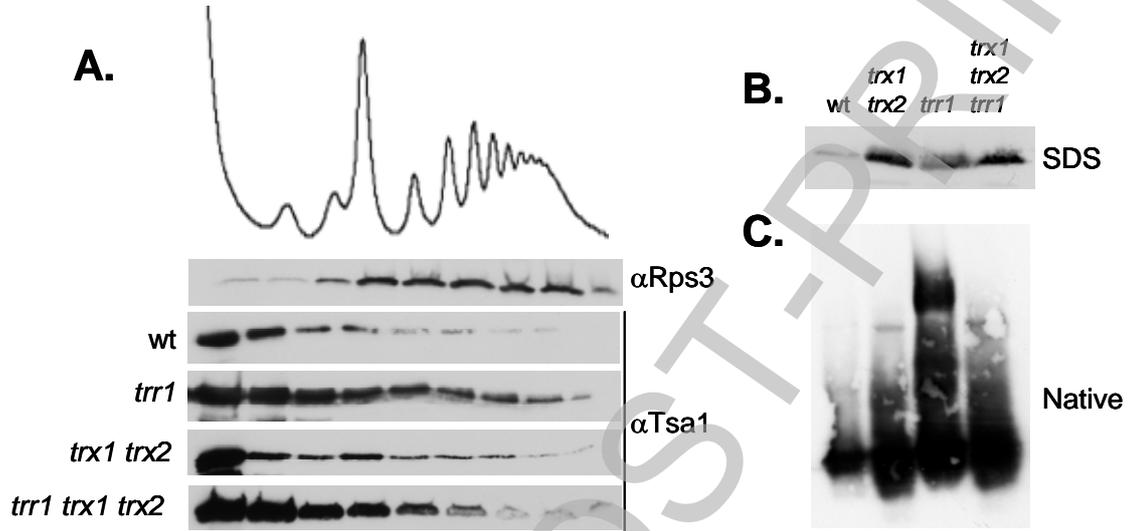
Fig. 3. Trotter *et al*



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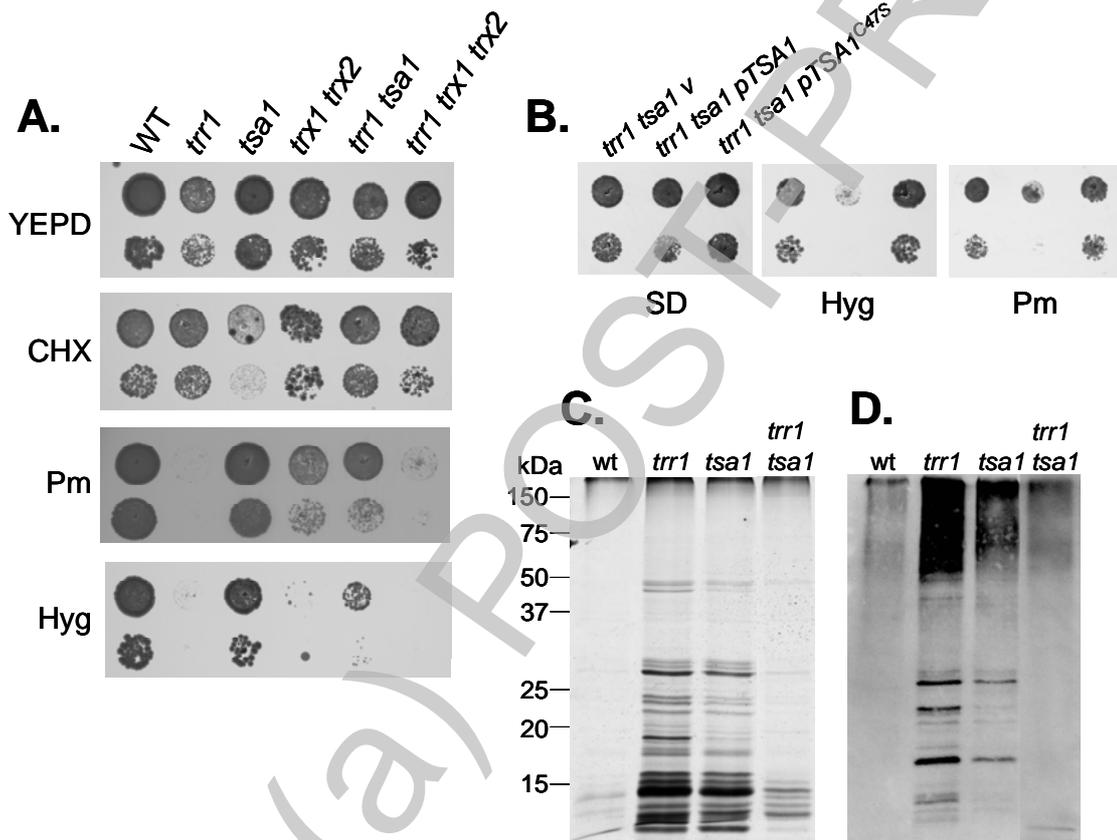
Fig. 4. Trotter *et al*



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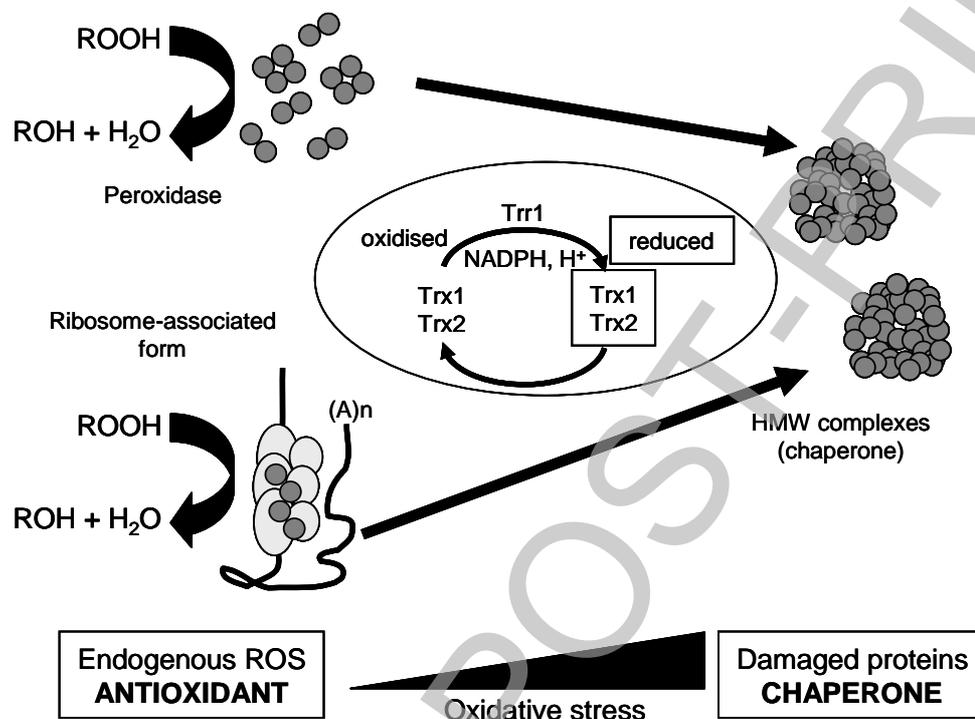
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Fig. 5. Trotter *et al*



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Fig. 6. Trotter *et al*



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