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Dissecting functional similarities of ribosome-associated chaperones from *Saccharomyces cerevisiae* and *Escherichia coli*

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Summary

Ribosome-tethered chaperones that interact with nascent polypeptide chains have been identified in both prokaryotic and eukaryotic systems. However, these ribosome-associated chaperones share no sequence similarity: bacterial trigger factors (TF) form an independent protein family while the yeast machinery is Hsp70-based. The absence of any component of the yeast machinery results in slow growth at low temperatures and sensitivity to aminoglycoside protein synthesis inhibitors. After establishing that yeast ribosomal protein Rpl25 is able to recruit TF to ribosomes when expressed in place of its *Escherichia coli* homologue L23, the ribosomal TF tether, we tested whether such divergent ribosome-associated chaperones are functionally interchangeable. *E. coli* TF was expressed in yeast cells that lacked the endogenous ribosome-bound machinery. TF associated with yeast ribosomes, cross-linked to yeast nascent polypeptides and partially complemented the aminoglycoside sensitivity, demonstrating that ribosome-associated chaperones from divergent organisms share common functions, despite their lack of sequence similarity.

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Introduction

Ribosome-associated chaperones, the first members of the cellular folding machinery encountered by newly synthesized proteins, accomplish an intimate coupling between protein synthesis and chaperone-assisted protein folding in the cytosol (Bukau *et al*., 2000; Frydman, 2001; Hartl and Hayer-Hartl, 2002; Craig *et al*., 2003). Trigger factor (TF), the only known ribosome-associated chaperone in bacteria, associates with ribosomes via its N-terminal domain, and this association is a prerequisite for TF interaction with nascent polypeptides (Lill *et al*., 1988; Hesterkamp *et al*., 1997; Kramer *et al*., 2002). TF cooperates with the cytosolic Hsp70 chaperone DnaK in the folding of newly synthesized proteins (Deuerling *et al*., 1999; Teter *et al*., 1999). Neither the absence of TF nor of DnaK causes severe folding defects, however, the combined loss of both chaperones leads to aggregation of more than 300 different proteins and cell death at temperatures above 30°C. In addition to its chaperone activity, TF displays peptidyl-prolyl-isomerase (PPIase) activity (Stoller *et al*., 1995; Hesterkamp *et al*., 1996). However, *in vivo* the PPIase-deficient TF-F198A mutant protein is fully functional, indicating that the catalytic activity of TF is not crucial *in vivo* (Kramer *et al*., 2004a).

Although ribosome-tethered chaperones exist in eukaryotes, they bear no known structural resemblance to TF. The best-characterized eukaryotic ribosomal chaperone system that of the yeast *Saccharomyces cerevisiae* has at its core the Hsp70 Ssb. A DnaJ-related protein Zuotin (Zuo) and a second Hsp70-type protein Ssz complete the ribosome-associated chaperone machinery (Pfund *et al*., 1998; Yan *et al*., 1998; Gautschi *et al*., 2001). In this chaperone triad only Ssb has been shown to directly contact the nascent chain while the Zuo–Ssz complex is believed to be the cochaperone of Ssb and essential for Ssb nascent chain interaction (Pfund *et al*., 1998; Gautschi *et al*., 2002; Hundley *et al*., 2002). Zuo tethers Ssz to the ribosome, whereas Ssb contacts the ribosome autonomously (Pfund *et al*., 1998; Yan *et al*., 1998; Gautschi *et al*., 2001). Inactivation of the triad through deletion of either one or all of its members leads to cold-sensitivity and hypersensitivity to cations, including aminoglycosides such as paromomycin that inhibit protein

synthesis and impair translational fidelity (Yan *et al*., 1998; Gautschi *et al*., 2001; Hundley *et al*., 2002; Rakwalska and Rospert, 2004; Kim and Craig, 2005).

Despite the lack of sequence similarity between TF and yeast chaperones, they share several common features (Hesterkamp and Bukau, 1996; Pfund *et al*., 1998; 2001; Craig *et al*., 2003). TF and Ssb associate with ribosomes stoichiometrically, regardless of whether a nascent chain is present. Both can be cross-linked to radioactively labelled nascent chains that extend only a short distance beyond the polypeptide exit site. The affinity of both TF and Ssb for ribosomes is affected by the presence of a nascent chain, as indicated by increased salt-resistance of the chaperone–ribosome interaction (Hesterkamp and Bukau, 1996; Pfund *et al*., 1998). While the localization of the yeast triad on the ribosome is unknown, TF associates with the ribosome via an interaction with the ribosomal protein L23, which is a universally conserved protein and belongs to a set of proteins surrounding the ribosomal tunnel exit (Kramer *et al*., 2002; Ferbitz *et al*., 2004).

These similarities between the bacterial and the yeast chaperones, despite their lack of sequence identity, led us to speculate that they fulfil overlapping or similar cellular functions. We found that TF was able to interact with yeast ribosomes both *in vivo* and *in vitro*, complement the aminoglycoside sensitivity of a yeast strain lacking the three components of the chaperone triad Ssb, Ssz and Zuo, and could be cross-linked to yeast nascent chains produced *in vitro*.

Results

Yeast ribosomal protein Rpl25 complements the lethality of ΔrplW *in* Escherichia coli

Escherichia coli L23 protein and its yeast homologue, Rpl25, are 30% identical. The residue Glu18 of L23, which is essential for TF ribosome association, is conserved in Rpl25 (Glu70), while the amino acids surrounding this residue are only partially conserved: T*SE*T*A*M in yeast Rpl25, compared to V*SE*K*A*S in *E. coli* L23 (Fig. 1A). To

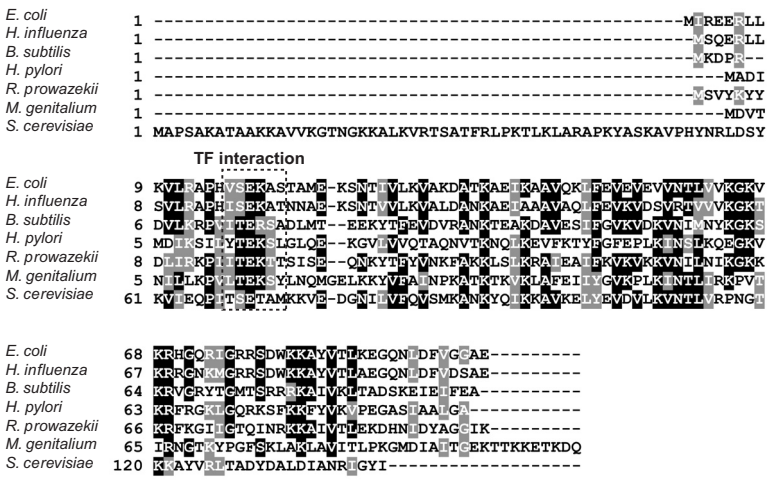

Fig. 1. Yeast Rpl25 complements *E. coli* cells lacking L23.
A. Alignment of bacterial ribosomal L23 proteins with the yeast homologue Rpl25 using ClustalW and Boxshade. The box indicates the region in L23 including the essential Glu residue important for TF binding.
B. Growth analysis of *E. coli* WT and Δ*rplW* cells containing plasmids with *rplW* or yeast *RPL25* under control of an IPTG-inducible promoter. Serial dilutions (10-fold) of overnight cultures grown in the presence of IPTG were spotted on LB-agar plates containing the indicated concentrations of IPTG and incubated at 20, 30, 37 or 42°C overnight. The upper panel shows the inducer-dependent growth of Δ*rplW* cells at 30°C and the lower panel shows the results obtained under 500 μM IPTG at all temperatures tested.

determine whether Rpl25 could substitute for the essential L23, we expressed the yeast *RPL25* gene in *E. coli* under the control of a regulated promoter. In the presence of the inducer IPTG, *rplW*, the gene encoding L23, could be deleted. The growth of the resulting strain *ΔrplW::kan* (pRpL25) was characterized at different temperatures and inducer concentration (Fig. 1B). Between 30 and 42°C *ΔrplW* cells expressing Rpl25 grew in an IPTG-dependent manner comparable to a *ΔrplW* deletion strain expressing *E. coli* L23. Thus, eukaryotic Rpl25 can functionally substitute for bacterial L23 at some temperatures both above and below the optimal growth temperature of 37°C.

Trigger factor binds to hybrid E. coli *Rpl25-ribosomes*

The ability of yeast Rpl25 to functionally replace L23 allowed us to isolate hybrid ribosomes (containing Rpl25 substituted for L23) from *ΔrplW* cells expressing yeast Rpl25 and test whether Rpl25 could recruit TF to ribosomes and nascent chains. TF was found associated with Rpl25 hybrid ribosomes at a level comparable to 50% of the total amount that was bound to wild-type (WT) ribosomes (Fig. 2A).

To test TF binding *in vitro*, ribosomes isolated under high salt conditions were incubated with equimolar, or fivefold molar excess, of purified TF. Ribosomes were separated from soluble protein by centrifugation through sucrose cushions and analysed by SDS-PAGE (Fig. 2B). At equimolar concentrations of TF and ribosomes, ~50% of *E. coli* ribosomes bound TF; under the same conditions 28% of hybrid ribosomes bound TF, consistent with the reduced ribosome association observed *in vivo* (Fig. 2B, compare lines 6 and 12). Occupancy of TF on hybrid ribosomes increased to ~50% upon incubation with a fivefold excess of TF, which was comparable to that of WT ribosomes at equimolar concentrations (Fig. 2B, compare lanes 6 and 14).

We next tested whether Rpl25 permitted the interaction of TF with nascent polypeptides generated in an *E. coli*-based *in vitro* transcription/translation system. In the presence of TF, WT or hybrid ribosomes were used to generate radioactively labelled stalled nascent chains of isocitrate dehydrogenase (ICDH). The addition of a chemical cross-linker, disuccinimidyl suberate (DSS), led to the appearance of distinct bands at 80 and 110 kDa, regardless of the type of ribosome used. As expected from previously reported results (Patzelt *et al*., 2001), these bands contained cross-linked TF and radiolabelled nascent polypeptide, as they were immunoprecipitated by TF-specific antibodies. TF could be cross-linked to nascent chains generated with hybrid ribosomes, albeit less efficiently than to nascent chains generated by WT ribosomes (Fig. 2D, compare lines 8 and 11), consistent with our previous observation that TF has a decreased affinity to hybrid ribosomes.

Trigger factor binds to yeast ribosomes in vitro

The binding of TF to hybrid *E. coli* ribosomes suggested that TF might specifically interact with yeast ribosomes. To test this idea we isolated WT yeast ribosomes under high-salt conditions and tested the ability of TF to bind, as described above for *E. coli* ribosomes. At a 1:1 ratio of TF and yeast ribosomes about 25% of TF was found in the ribosome pellet fraction (Fig. 2B). When a fivefold excess of TF was added, binding was increased, but at a level less than the binding of TF to Rpl25 hybrid ribosomes. Thus TF binds yeast ribosomes *in vitro*, albeit less efficiently than *E. coli* ribosomes, perhaps because of local structural differences between yeast and *E. coli* ribosomes. Additionally, we tested whether TF competed with Ssb or Zuo for binding to yeast ribosomes *in vitro*. TF, Ssb and Zuo can associate simultaneously with yeast ribosomes suggesting different ribosomal docking sites (Fig. 2C).

Trigger factor partially substitutes for the yeast triad in vivo

Based on our findings that TF associates with yeast ribosomes *in vitro*, we speculated that TF might be able to at least partially carry out the function of the yeast ribosome-associated chaperone triad. To test this idea *in vivo*, we expressed the TF encoding gene *tig* in a yeast strain lacking the ribosome-associated triad Ssb, Ssz and Zuo (*Δssb,zuo,ssz*). We determined the concentration of TF to be ~50% of the concentration of Ssb in WT yeast cells (Fig. 3A), and therefore present at approximately the same concentration as ribosomes.

We compared the ability of *Δssb,zuo,ssz* cells, with or without ectopic expression of TF or TF variants, to grow at low temperature or in the presence of cations. Expression of TF resulted in increased resistance to the aminoglycoside paromomycin, correlated with an increase in colony forming units of at least two orders of magnitude (Fig. 3B). Rescue of the lithium-sensitivity, another toxic cation, was also found, but was less pronounced. In contrast, rescue of the cold-sensitivity of the *Δssb,zuo,ssz* strain was not consistently observed (Fig. 3B). We therefore conclude that TF can partially fulfil some of the functions of the yeast ribosome-associated triad.

In addition to WT TF, two mutant proteins were tested: (i) TF-F198A, which has less than 1% residual PPIase activity (Kramer *et al*., 2004a) and has been found to be fully functional in *E. coli*; (ii) TF (145–432), lacking the N-terminal ribosome-binding domain, which is essential for TF function in *E. coli* (Kramer *et al*., 2004a). Expression of the PPIase-deficient mutant TF-F198A in *Δssb,zuo,ssz*

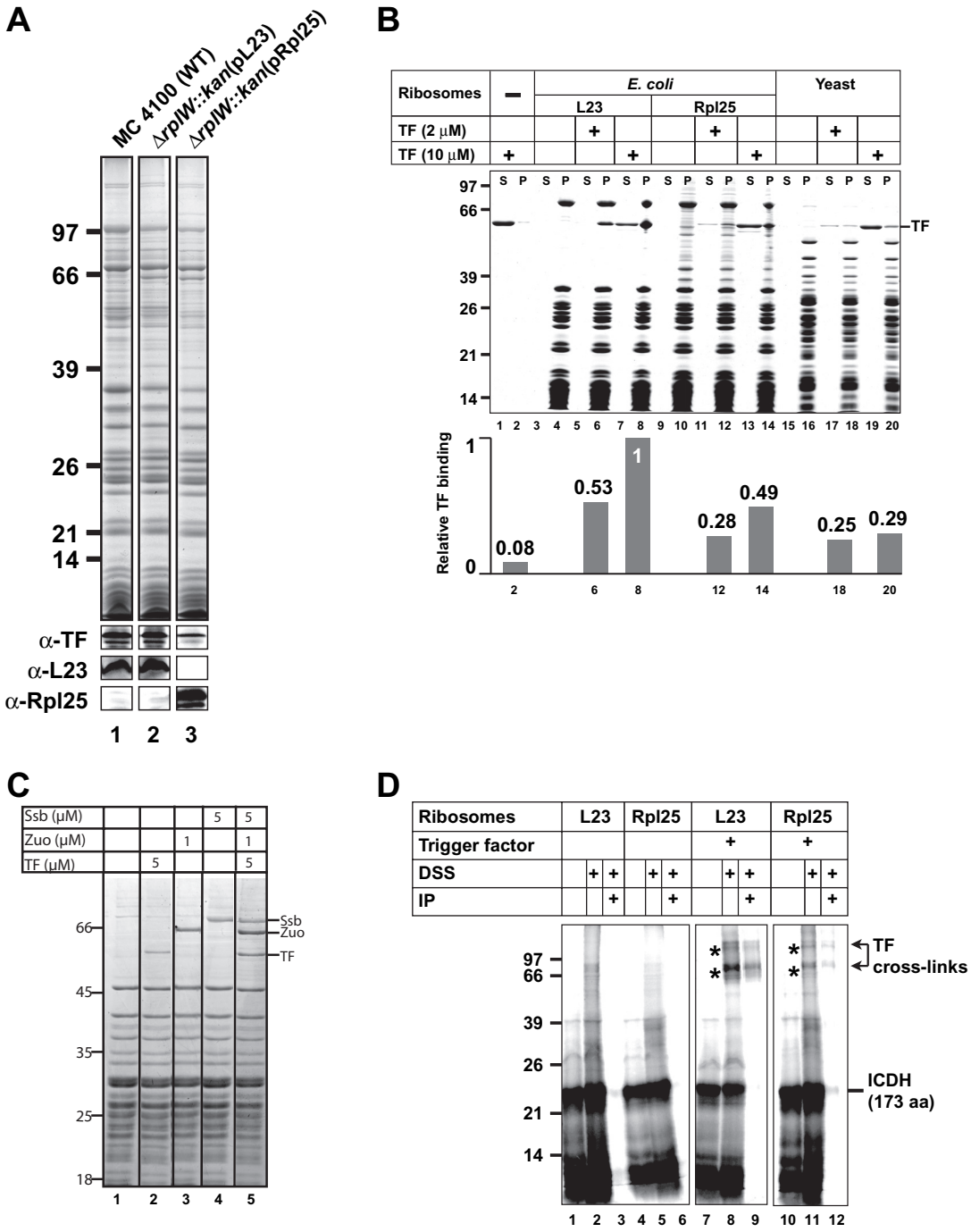

Fig. 2. TF interacts with Rpl25 ribosomes.
A. Equivalent amounts of ribosomes purified from indicated strains were tested for TF, L23, Rpl25 content by immunoblotting (lower panel). The upper panel shows a corresponding Coomassie-stained SDS-PAGE of the analysed ribosome preparations for control.
B. Rebinding of TF to purified ribosomes (upper panel) and quantification of bound TF by MacBAS V2.5 program (lower panel). TF was incubated with ribosomes for 30 min at 30°C and complexes (P) were separated from uncomplexed TF (S) by sucrose cushion centrifugation and subsequently analysed by SDS-PAGE and Coomassie staining. Please note that only one-sixth of total supernatant was loaded per lane. The rebinding efficiency of TF to WT ribosomes (2 μM) under saturating conditions (10 μM) (Patzelt *et al.*, 2002) was set as 1.
C. Competition experiment: Zuotin, TF and Ssb (at indicated concentrations) were simultaneously or individually added to 1 μM yeast ribosomes. Ribosome complexes were re-isolated as described in B and analysed by SDS-PAGE and Coomassie staining.
D. Cross-linking of TF to ^{35}S-labelled nascent ICDH (isocitrate dehydrogenase) using an *E. coli*-based transcription/translation extract (Kramer *et al.*, 2002). TF (600 nM) and ribosomes (200 nM) were added exogenously to a translation extract derived from *Δtig* cells. After translation of radioactive ICDH, samples were chemically cross-linked with DSS and subsequently TF cross-linking products (indicated by asterisks) were immunoprecipitated (IP) with antibody directed against TF. Radioactive products were detected by autoradiography.

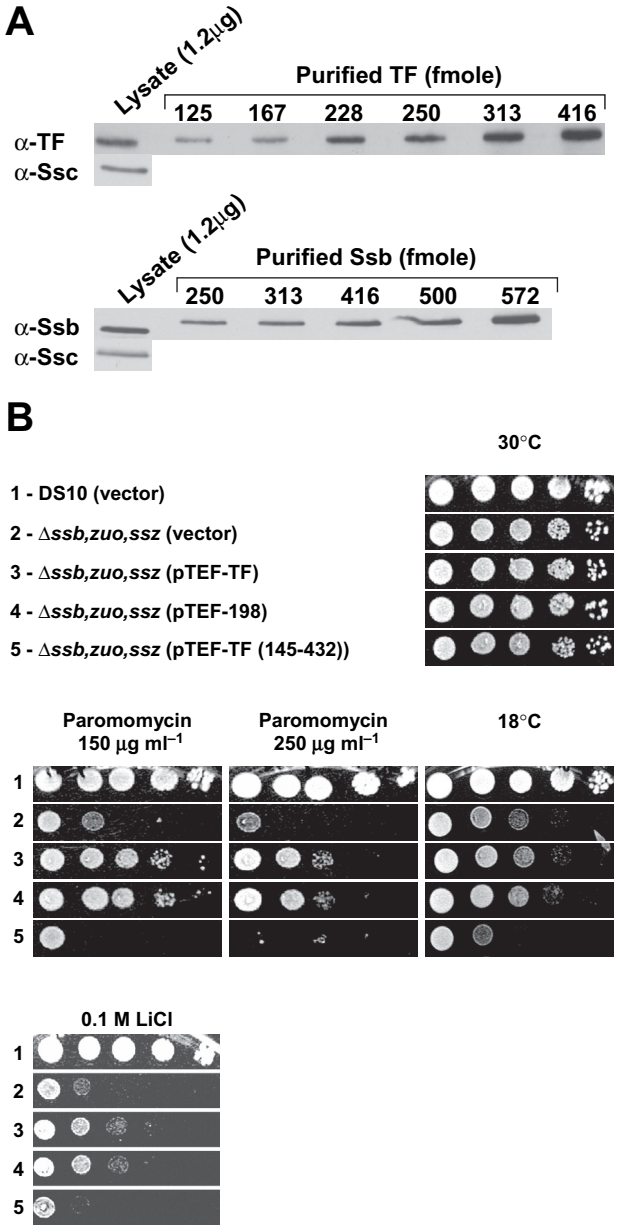

Fig. 3. TF partially complements yeast cells lacking the chaperone triad.
A. Quantification of TF and Ssb expressed in yeast cells. 1.2 μg lysate of yeast WT or *Δssb,zuo,ssz* cells expressing TF, and the indicated amounts of purified Ssb or TF were separated by SDS-PAGE; proteins were detected by immunoblot analysis using antibodies-specific for TF, Ssb and as a loading control for the lysates, Ssc.
B. Growth analysis of yeast cells expressing TF. Serial dilutions of yeast WT and the *Δssb,zuo,ssz* cells harbouring either a control vector or plasmids expressing the indicated TF variants were spotted on plates supplemented with the indicated compounds, and incubated at 30°C for 2 days or 18°C for 5 days.

cells rescued the cation hypersensitivity as efficiently as WT TF (Fig. 3B). In contrast, the expression of TF (145–432) lacking the ribosome binding domain was not able to rescue the *Δssb,zuo,ssz* cells.

Trigger factor binds to yeast translating ribosomes in vivo

The fact that TF can only fulfil its chaperone function in *E. coli* when associated with the ribosome (Kramer *et al.*, 2002) suggested that TF may function on the yeast ribosome *in vivo*. To test this idea, we isolated ribosomes from TF-expressing *Δssb,zuo,ssz* cells and compared the presence of TF in the ribosomal pellet with the amount of soluble TF in the supernatant (Fig. 4A). A substantial portion of WT TF cosedimented with ribosomes. To rule out the possibility that TF was found in the ribosomal pellet because of protein aggregation or unspecific association with membrane fragments, we separated polysomes from the *Δssb,zuo,ssz* cells expressing WT TF on sucrose gradients. TF comigrated with the polysomes. Mild treatment with RNaseA, which digested the mRNA between the ribosomes, resulted in migration of nearly all the ribosomes and TF to the 80S monosome position (Fig. 4B). We conclude that TF specifically associates with yeast ribosomes.

In addition to testing the ribosome association of WT TF, we also characterized the ribosome association of both TF mutants described above. The amount of TF-F198A present in the ribosome-containing pellet (Fig. 4A) was similar to WT TF. Therefore, as both the ribosome association and growth of TF-F198A are similar to WT TF, we suggest that similar to *E. coli*, the function of TF in yeast does not require the PPIase catalytic activity. In contrast to WT and TF-F198A, TF (145–432) was present exclusively in the soluble fraction of the sucrose cushion (Fig. 4A). Thus, similar to TF binding to *E. coli* ribosomes, the N-terminal domain of TF is required for the association of TF with yeast ribosomes.

Trigger factor cross-links to yeast nascent chains in vitro

As TF associates with yeast ribosomes, we asked if TF was in close proximity to nascent chains. We used a WT yeast extract to generate ribosome–radiolabelled nascent chain complexes, by addition of a mRNA lacking a stop codon, which encoded the N-terminal 96 amino acids of pCoxIV. The chemical cross-linker DSS was added and cross-linked chaperone–nascent chain complexes were identified by coimmunoprecipitation and subsequent SDS-PAGE and autoradiography. As a control cross-linking of the endogenous yeast ribosome-associated chaperone Ssb was tested. As expected (Pfund *et al.*, 1998, Hundley *et al.*, 2002) Ssb cross-linked efficiently to nascent chains. Cross-linking of TF to nascent chains was observed when it was added to the translation extract at a TF : ribosome ratio of 10:1 (Fig. 4C). While we cannot exclude the possibility that soluble TF cross-linked to the nascent chain, such a occurrence would be surprising as in *E. coli* soluble TF, even when present in excess over ribosomes, was not

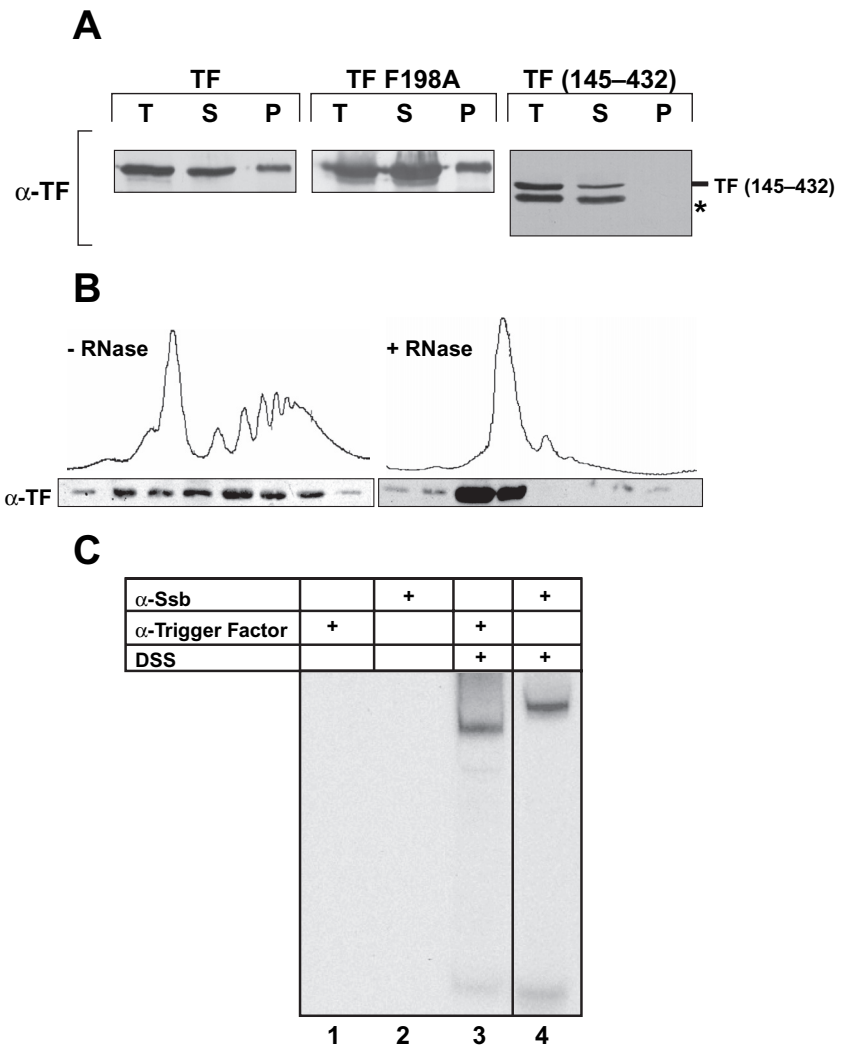

Fig. 4. TF associates with yeast ribosomes and cross-links to nascent chains.
A. Lysates of *Δssb,zuo,ssz* yeast cells expressing TF variants were centrifuged through sucrose cushions, separating the ribosome-containing pellet (P) from the soluble supernatant (S). An equivalent amount of unfractionated lysate (T) served as the loading control. The asterisk indicates a degradation product of TF (145–432).
B. Pelleted ribosomes from *Δssb,zuo,ssz* cells expressing TF were incubated in the presence (+) or absence (–) of RNase, and then subjected to centrifugation through a sucrose gradient. OD_{260} was monitored (top panel); fractions were collected and subjected to immunoblot analysis using TF-specific antibody (bottom panel).
C. Radiolabelled yeast ribosome–pCoxIV nascent chain complexes were generated in a yeast WT translation extract with and without the addition of TF, and cross-linking was initiated with the addition of DSS. After ultracentrifugation, chaperone-nascent chain cross-links were identified by coimmunoprecipitation with Ssb or TF specific polyclonal antibodies, followed by autoradiography. For lanes 1 and 3, exogenous TF was added to the lysate before translation. Exposure: lanes 1–3, 72 h; lane 4, 16 h.

cross-linked to ribosome-associated nascent chains (Kramer *et al*., 2002).

Discussion

Investigating the degree of functional analogy between ribosome-associated chaperones from *E. coli* and *S. cerevisiae*, we demonstrated that the bacterial-specific ribosome-associated chaperone TF associates with yeast ribosomes and partially complements for the absence of the yeast ribosome-associated chaperone triad of Ssb, Zuo and Ssz *in vivo*. To our knowledge this is the first evidence that a structurally unrelated chaperone from one kingdom can functionally replace a chaperone from another kingdom.

Several lines of evidence suggest that the complementation by bacterial TF of the aminoglycoside and lithium hypersensitivity resulting from the absence of the yeast chaperones is both specific and dependent on TF's association with the ribosome. Aminoglycoside sensitivity can be caused by a number of mutations in yeast in addition to those causing alterations in ribosomal RNA or ribosomal proteins (Alksne *et al*., 1993), particularly those causing alterations in transporters in the plasma membrane (Mulet *et al*., 1999; Kim and Craig, 2005). However, we observed no complementation of the aminoglycoside sensitivity of such transporter mutants by TF expression (data not shown), indicating a chaperone-specific effect of TF. In addition, a similar beneficial effect of overexpression of any other chaperone, including the other major yeast cytosolic Hsp70, Ssa (Craig and Jacobsen, 1985), which is known to facilitate folding of newly synthesized proteins (Kim *et al*., 1998), has never been reported. The importance of ribosome binding for TF's ability to functionally replace the yeast chaperones is also suggested by the findings that TF associates with ribosomes in yeast and cross-links to short, ribosome-associated nascent polypeptide chains, as does Ssb, but not Ssa (Pfund *et al*., 1998). The fact that a truncated TF, which is unable to stably bind to the *E. coli* ribosome, neither binds to yeast

ribosomes, nor complements the *Δssb,zuo,ssz* strain, is consistent with this idea as well. However, as recent structural data (Ferbitz *et al*., 2004) suggest that sequences in the N-terminus are involved in substrate binding and no mutant TF exists that has been shown to be solely defective in ribosome association *in vivo* (Kramer *et al*., 2002; Genevaux *et al*., 2004), we were unable to rigorously test whether ribosome binding is required for TF function in yeast.

Trigger factor does not completely functionally replace the endogenous ribosome-associated chaperone machinery. The partial rescue of the aminoglycoside sensitivity and the lack of significant suppression of the cold-sensitivity could be attributed to the fact that TF is not stoichiometrically associated with, or exactly appropriately positioned on, the yeast ribosome. Consistent with this hypothesis, it was recently reported that although the globular TF binding domain of L23 is similar in archea and bacteria, there are significant differences in the structure of the portion of L23 that extends into the peptide exit tunnel of the two types of ribosomes (Baram and Yonath, 2005). On the other hand, it is also possible that the complete lack of any detectable suppression of the cold-sensitivity may be attributed to a more fundamental difference in functionality between TF and the yeast triad, such as an incomplete overlap in substrate specificity. The phenotypes of aminoglycoside sensitivity and slow growth at low temperatures have been separated genetically through analysis of chimeras of Ssa and Ssb Hsp70s with rescue of the aminoglycoside sensitivity correlated with ribosome association (James *et al*., 1997). This separation suggests that the triad may participate in more than one function, although the exact functions remain ill-defined. It would be of interest to know whether the yeast triad could complement the synthetic lethality and protein aggregation of *E. coli* cells lacking TF and DnaK. Unfortunately we could not carry out this test, as all triad proteins are required for its function and we were unable to efficiently express Ssb in *E. coli* (data not shown).

Taken together, our data suggest a functional analogy between the bacterial ribosome-associated TF and the yeast ribosome-associated triad, indicating that TF and the yeast triad have independently developed overlapping functions during evolution. We propose that all organisms possess a chaperone system tethered to the ribosome, although they may have evolved by convergent rather than divergent evolution to carry out universal chaperone roles at the polypeptide exit site.

Experimental procedure

Escherichia coli *strains and plasmids*

Escherichia coli strains (MC4100 derivatives) were grown in LB supplemented with 40 μg ml^{-1} kanamycin, 100 μg ml^{-1} ampicillin and varying concentrations of IPTG if appropriate. P1 transduction, disruption of the *rplW* gene and pL23 have been described (Kramer *et al*., 2002). The *RPL25* gene was amplified as an intron-free open reading frame using yeast RNA as template and the First Strand cDNA Kit (Roche) following the instructions of the manufacturer. The primers created an *Nco*I site at the 5′ end and a *BamH*I site at the 3′ end of the insert, which were cloned into pTrc99B under the control of an IPTG-regulated promoter. DNA inserts were sequenced for verification.

Yeast strains and plasmids

The strain lacking the yeast chaperone machinery HH6 (*his3-11, 15 leu2-3, 112 lys1 lys2 Δtrp1 ura3-52 Δssb1::HIS3 Δssb2::LEU2 Δzuo1::HIS3 Δssz1::LYS2*) is isogenic with DS10 and referred to as *Δssb,zuo,ssz* throughout (Pfund *et al*., 2001; Hundley *et al*., 2002). Absence of expected proteins was confirmed by immunoblot analysis. Strains were grown on minimal (0.67% yeast nitrogen base without amino acids, 2% dextrose, supplemented with appropriate bases and amino acids) media. *tig* genes encoding WT TF, TF F198A or TF (145–432) were polymerase chain reaction (PCR)-amplified from plasmids pTrc-TF, pTrc-F198A and pTrc-PC, respectively (Kramer *et al*., 2004a, b), introducing a *BamH*I site at the 5′ end and a *Sal*I site at the 3′ end. All inserts encoding TF variants were placed into pBluescript KS+ and sequenced.

Yeast strains containing *tig* genes were obtained by 'gap repair' (Ma *et al*., 1987). Both the pRS414-*ADH/TEF* vectors (Mumberg *et al*., 1995) containing base pairs 1–27 and 1109–1299 of *tig* were linearized and cotransformed into yeast with *Xba*I-*Xho*I DNA fragments removed from pBluescript TF vectors. Candidates were selected on medium lacking tryptophan and checked for TF expression by immunoblotting. TF was expressed from the *ADH* promoter at slightly lower levels than from the *TEF* promoter, although, rescue of the *Δssb,zuo,ssz* strain was similar.

Trigger factor binding to ribosomes

Escherichia coli ribosomes isolated under high-salt conditions were tested for TF binding *in vitro* as described (Hesterkamp *et al*., 1997). TF was purified as described (Kramer *et al*., 2004a). DS10 yeast cells, suspended in CSB (300 mM sorbitol, 20 mM HEPES pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 2 mM β–mercaptoethanol), were lysed using a French press. The lysate was cleared by centrifugation (30 000 *g*) and ribosomes were pelleted by ultracentrifugation at 150 000 *g*. After suspension in CSB, samples were adjusted to 1 M KAc and centrifuged through 20% sucrose cushions containing CSB buffer plus 1 M KAc. The high-salt washed ribosome pellets were resuspended in CSB buffer.

Preparation of yeast cell lysates and analysis of ribosomes

Cell lysates, prepared from *Δssb,zuo,ssz* cells expressing WT TF and variants under the control of the *ADH* promoter, were

subjected to sucrose cushion analysis as previously described (Hundley *et al*., 2002). For polysome analysis, pelleted ribosomes (OD_{260} of ~2) were resuspended in CSB after the sucrose cushion step and incubated in the presence or absence of 40 μg ml^{-1} RNase A for 30 min on ice. The resuspended ribosomes were then layered onto a 5–50% linear sucrose gradient containing CSB buffer and subjected to centrifugation at 55 000 r.p.m. for 65 min in a SW55 rotor at 4°C. Gradients were monitored at 260 nm to detect nucleic acids and 0.4 ml fractions were collected for SDS-PAGE and immunobloting as described above. Ssb and Zuo were purified from yeast as described (Pfund *et al*., 2001; Hundley *et al*., 2005).

Trigger factor cross-linking to nascent chains

Trigger factor cross-linking to nascent chains using an *E. coli*-based *in vitro* transcription/translation system was performed as described (Schaffitzel *et al*., 2001; Kramer *et al*., 2002). *In vitro* transcription, translation and cross-linking procedures using yeast lysates were performed as described (Pfund *et al*., 1998; Hundley *et al*., 2002) with the following modifications. A 10-fold molar excess of TF to ribosomes was added to the WT translation lysate along with the mRNA encoding 96 amino acid N-terminal fragment of pCoxIV lacking a stop codon. After translation, the chemical cross-linker DSS (Disuccinimidylsuberate) was added at a final concentration of 2.5 mM. The ribosome–nascent chain complexes were recovered by centrifugation and subjected to immunoprecipitation with antibody directed against TF. The Ssb control cross-linking experiment was performed as described above, except that TF was not added to the lysate and Ssb antibody was used for the immunoprecipitation. Radioactive bands were visualized by autoradiography.

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