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The functional role of eL19 and eB12 intersubunit bridge in the eukaryotic ribosome

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Abstract

During translation, the two eukaryotic ribosomal subunits remain associated through 17 intersubunit bridges, five of which are eukaryote-specific. These are mainly localized to the peripheral regions and are believed to stabilise the structure of the ribosome. The functional importance of these bridges remains largely unknown. Here the essentiality of the eukaryote-specific bridge eB12 has been investigated. The main component of this bridge is ribosomal protein eL19 which is composed of an N-terminal globular domain, a middle region and a long C-terminal α -helix. Analysis of deletion mutants demonstrated that the globular domain and middle region of eL19 are essential for cell viability, most likely functioning in ribosome assembly. The eB12 bridge, formed by contacts between the C-terminal α -helix of eL19 and 18S rRNA in concert with additional stabilising interactions involving either eS7 or uS17, is dispensable for viability. Nevertheless, eL19 mutants impaired in eB12 bridge formation displayed slow growth phenotypes, altered sensitivity/resistance to translational inhibitors and enhanced hyperosmotic stress tolerance. Biochemical analyses determined that the eB12 bridge contributes to the stability of ribosome subunit interactions *in vitro*. 60S subunits containing eL19 variants defective in eB12 bridge formation failed to form 80S ribosomes regardless of Mg^{2+} concentration. The reassociation of 40S and mutant 60S subunits was markedly improved in the presence of deacetylated tRNA, emphasising the importance of tRNAs during the subunit association. We propose that the eB12 bridge plays an important role in subunit joining and in optimizing ribosome functionality.

Graphical Abstract

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Keywords

Saccharomyces cerevisiae; ribosome subunit association; eukaryote-specific bridges; ribosome assembly; stress tolerance

INTRODUCTION

Ribosomes consist of two unequal subunits in all three domains of life: 30S and 50S (*Archaea* and *Bacteria*) or 40S and 60S (*Eukarya*). The rotation of subunits relative to one another enables translocation of tRNAs and mRNAs across the ribosome^{1; 2; 3; 4}. The 3.0 Å resolution *Saccharomyces cerevisiae* 80S ribosome crystal structure reveals that rotation of the yeast ribosomal subunits is regulated by 17 intersubunit bridges⁵. Twelve of these are conserved among all three domains of life and contain three types of interactions: rRNA-rRNA, protein-rRNA and protein-protein. The conserved bridges are located close to, and coordinate the activity of the ribosomal functional centres^{5; 6}. The five eukaryote-specific intersubunit bridges are composed of the protein-rRNA and protein-protein type interactions between eukaryote-specific rRNA expansion segments (ES) and eukaryote-specific protein domains. They are localized to peripheral regions and are thought to structurally stabilise the 80S ribosome. Two eukaryote-specific proteins, eL19 and eL24, contain long α -helices extending from the 60S subunit E- and A-site sides, respectively (Fig. 1A). These helices form the distinctive eB12 and eB13 intersubunit bridges. Bridge eB12 is formed by contacts between the C-terminal α -helix of eL19 and eukaryote-specific expansion segment 6 (ES6S) of 18S rRNA (Fig. 1B, 1C). The dynamic structure of eB12 is dependent on conformational changes that occur during ribosomal translocation. In the pre-translocational state, eB12 is formed by the contacts between eL19 and uS17. In contrast, these contacts are replaced by interactions between eL19 and eS7 when the ribosome occupies the post-translocational state⁵. The essential eL19 protein is encoded by two paralogous genes, *RPL19A* and *RPL19B*⁷. Deletion of either *RPL19A* or *RPL19B* is not lethal for yeast cells^{7; 8}. However, both genes are required for optimal growth. Cells lacking *RPL19A* or *RPL19B* are characterised by 1.2- and 1.5-fold increased doubling rates, respectively⁹. Deletion of both *RPL19A* and *RPL19B* causes a synthetic lethality phenotype⁷. The 189 amino acid residue long eL19 proteins encoded by these genes are identical. Yeast eL19 protein exhibits 57.5% homology with mammalian eL19, and 35.8 % homology with archaeal eL19, although there is no eL19 counterpart in bacterial ribosome^{7; 10}. eL19 contains three structural domains: the N-terminal globular domain, the middle region and the long C-terminal α -helix (Fig. 1B).

The eB13 bridge contains contacts between the C-terminal α -helix of eL24 and eS6 with assistance from uL3 in both conformational states⁵. However, an additional interaction

between eL24 and the 18S rRNA helix 10 occurs in the post-translocational state. The main component of this bridge – ribosomal protein eL24 – is also encoded by two paralogous genes *RPL24A* and *RPL24B*¹¹. These genes produce two near identical proteins differing in 5 out of 155 amino acid residues. Similar to eL19, eL24 is present only in archaeal and eukaryotic ribosomes¹⁰. Interestingly, eL24 is one of the few ribosomal proteins that are not essential for viability^{9; 11}. Deletion of the both paralogous genes slightly reduces the growth rate of the cells. Given that eL24 is nonessential, the eB13 bridge is dispensable for viability.

In the current study several eL19 deletion mutants were constructed in order to evaluate the essentiality of the eB12 bridge and of various domains of the eL19. *In vivo* depletion of eL19 demonstrated that eL19 has an important role during pre-rRNA processing and ribosome assembly. Analysis of eL19 mutants deficient in eB12 formation demonstrated that the eB12 bridge is not essential for cell viability. However, our results indicated that eB12 plays a substantial role in shaping cell growth in sub-optimal conditions, while biochemical analyses determined that eB12 contributes to the stability of ribosome subunit interaction *in vitro*. Consequently, although the eB12 intersubunit bridge is dispensable for cell growth *per se*, it plays an important role in subunit joining and in optimizing ribosome functionality.

RESULTS

Loss of the eB12 bridge leads to reduced cell growth

The eukaryote-specific eB12 intersubunit bridge is formed by contacts between the C-terminal α -helix of eL19 and 18S rRNA ES6S (Fig. 1C). Additional stabilising interactions between eL19 and eS7 or uS17 are also involved depending upon the rotational state of the ribosome. Several eL19 mutants deficient in eB12 formation were constructed to analyse the importance of eB12 in terms of ribosome functionality (Fig. 1B, Table 1). In mutant eL19_{1–183}, the C-terminal 6 amino acid residues which interact with ribosomal protein eS7 were deleted. In mutant eL19_{1–154}, the C-terminal 35 amino acid residues were removed, eliminating the bridge forming region in which eL19 interacts with 18S RNA ES6S and ribosomal proteins eS7 and uS17. Mutant eL19_{1–146}, harbouring a deletion of 43 amino acid residues, mimicked the archaeal version of eL19¹². In mutant eL19_{1–133}, the entire C-terminal α -helix (56 amino acid residues) was deleted. Cells expressing only mutant variants of eL19 were selected by the plasmid shuffling method (Fig. 1D). The eL19 mutant deficient in eS7 binding (eL19_{1–183}) displayed wild type growth, while growth was impaired in the mutants deficient in eB12 bridge formation (eL19_{1–154} and eL19_{1–146}). In contrast, deletion of entire α -helix (eL19_{1–133}) was lethal. To study the growth characteristics of the viable eL19 deletion mutants in detail, temperature sensitivity analysis by serial dilutions spot-test on rich medium at different temperatures was performed (Fig. 2A). The eL19 mutant deficient in eS7 binding exhibited nearly wild type growth rates at all temperatures assayed. Loss of the eB12 bridge conferred slow growth at 30°C and 36°C. The slow growth phenotype was enhanced at lower temperatures. Ribosome-polysome profile analysis of mutant cells grown in rich medium at 30°C and 20°C was performed to assess the effects of the eL19 deletions on ribosome biogenesis (Fig. 2B). No significant changes in monosome and polysome levels compared to wild type cells were observed in cells expressing eL19_{1–183}. In contrast, mutants lacking the eB12 bridge displayed considerable increases of

free 60S subunit levels at 20°C. Altogether, these results indicate that loss of interactions between eL19 and eS7 has a minor influence on cell viability. The inability to form the eB12 bridge leads to reduced cell growth and accumulation of free 60S subunits indicating a reduction in ribosome functionality.

The C-terminal domain of eL19 is dispensable for pre-rRNA processing

A conditional null mutant system (*rpl19A rpl19B + [pRS_{Sp}GAL-RPL19A]*), where *RPL19A* was ectopically expressed under the control of the galactose inducible *GAL1* promoter was constructed to enable analysis of the impact of eL19 C-terminal deletions on ribosome assembly and pre-rRNA processing. While growth of this strain was indistinguishable from wild type cells on permissive galactose containing medium, shut-down of eL19 expression on glucose containing medium resulted cell death (Fig. 3A), consistent with previous reports that eL19 is essential for yeast growth¹³. Polysome profile analysis of this strain subsequent to 12 h or 24 h shift to glucose containing medium revealed the dramatic consequences of eL19 depletion: reduction of polysome and monosome levels and accumulation of free 40S subunits (Fig. 3B). This phenotype suggests a defect in 60S subunit assembly. Next, the relative changes in steady-state levels of 60S precursor rRNAs (Fig. 3C) were analysed by primer extension analysis in cells shifted to glucose containing medium up to 24 hours. Examination of pre-rRNA species revealed very slight reductions in levels of 27SA₂ and 27SA₃ pre-rRNAs (Fig. 3D). In contrast, the levels of 27SB_L and 27SB_S pre-rRNAs were significantly increased under eL19 depletion conditions. Unfortunately, this specific primer (Probe F, Fig. 3C) does not enable one to distinguish whether pre-rRNA processing is affected at the level of 27SB, 7S pre-rRNAs, or both during depletion of eL19. Interestingly, enhancement of numerous nonspecific stops in the internal transcribed spacer region 2 (ITS2) close to the C₂ cleavage site was detected (Fig. 3D, shown by asterisks). In agreement with previous analysis, these results indicate that eL19 is important for efficient conversion of 27SB pre-rRNAs¹³. Furthermore, an RNA pulse labelling experiment demonstrated that depletion of eL19 caused accumulation of 25SB pre-rRNAs¹³. Primer extension analysis of pre-rRNA species in eL19 mutant cells grown in rich medium at 30°C and 20°C was performed to investigate whether the truncated variants of the C-terminal α-helix affected pre-rRNA processing (Fig. 3E). The deletion mutants showed no changes in maturation patterns of the 27SA₂, 27SA₃, 27SB_L, 27SB_S and 25.5S pre-rRNAs compared to the wild type. These results indicate that deletion of the eB12 forming region in the C-terminal α-helix of eL19 has no influence on pre-rRNA processing. In contrast, the globular and middle domains of eL19 are essential for cell growth, suggesting that this protein is essential during large subunit assembly.

Loss of the eB12 bridge has no effect on the rotational state of the ribosomes

The transition from the non-rotated to rotated state after peptidyltransfer leads to the changes in the interactions between the ribosomal subunits. Recently the chemical modification profiles for non-rotated and rotated yeast ribosome were determined¹⁴. SHAPE (selective 2'-hydroxyl acylation analysed by primer extension) using 1-methyl-7-nitroisatoic anhydride (1M7) was utilized to map the chemical protection patterns of base pair interactions located in several universally conserved regions. SHAPE analysis of specific 25S rRNA nucleotides that form bridges B7a (A2207) and B2a (U2258, A2262), the sarcin/ricin loop (U3023,

A3027) and the aa-tRNA accommodation corridor (U2860, U2924 and A2926) was performed in order to examine the rotational status of eB12 mutant ribosomes (Fig. S1A). Salt washed, intact ribosomes (80S) were pre-treated with puromycin, chemically probed with 1M7 (or only DMSO as controls) and the base reactivities were assessed by primer extension analysis and quantified. SHAPE reactivities of seven analysed nucleotides (U2258, A2262, U2860, U2924, A2926, U3023, A3027) were similar in ribosomes purified from cells expressing mutant alleles or wild type eL19 (Fig. S1B, S1C, data not shown). In contrast, nucleotide A2207 was less reactive in mutants lacking the eB12 bridge as compared to wild type ribosomes (Fig. S1B, S1C). This nucleotide interacts with 18S rRNA nucleotide G913 in the non-rotated state, but becomes accessible to modification by 1M7 in the rotated state (Fig. S1A)¹⁴. The ribosomes in the non-rotated state are also characterised by reactive nucleotides in the aa-tRNA accommodation corridor (U2924, A2926)¹⁴. SHAPE analysis did not show the strong chemical reactivity of these nucleotides (data not shown). Therefore we conclude that ribosomes with defective eB12 bridges are free to transit between the non-rotated and rotated states in the absence of ligands as has been shown for the vacant wild type ribosomes^{14; 15}.

The eB12 bridge is essential for stable 40S and 60S subunit reassociation *in vitro*

Translation initiation in eukaryotes is a multi-step process that involves several initiation factors, mRNA and methionyl initiator tRNA in addition to the 40S and 60S subunits. Therefore, deficiencies in the intrinsic contacts between the subunits can be compensated by trans-acting factors. Wild type 40S subunits and 60S subunits containing the wild type or mutant versions of eL19 were isolated using the high salt treatment of purified monosomes to enable more direct assessment of the role of eB12 in ribosome functionality. *In vitro* formation of 80S ribosomes at 4.5 – 25 mM Mg²⁺ concentrations was examined by sucrose gradient sedimentation (Fig. 4, data not shown). No differences in the ability of 60S subunits containing either eL19₁₋₁₈₃ variant or wild type eL19 to associate with 40S subunits were observed in all conditions analysed. In contrast, 60S subunits containing either eL19₁₋₁₅₄ or eL19₁₋₁₄₆ variants failed to form 80S ribosomes, regardless of Mg²⁺ concentration. However, their reassociation activities were markedly improved in the presence of 20 mM Mg²⁺ and saturating amounts of deacylated tRNA indicating that the purified 60S subunits were functional and not damaged. This behaviour of the eL19₁₋₁₅₄ and eL19₁₋₁₄₆ mutants can be explained by the loss of contacts between the eL19 C-terminal α -helix and eS7, uS17 and 18S rRNA ES6S. We conclude that bridge eB12 is essential for the formation of 80S ribosome *in vitro*.

Absence of the eB12 bridge affects stress tolerance

Serial dilutions spot-test analysis of sensitivity/resistance was performed in the presence of several translational inhibitors that affect different steps in protein synthesis to assess the response of eL19 mutant cells to environmental stress conditions (Fig. 5A). Two aminoglycosides, paromomycin and neomycin, bind to the A-site decoding region of the 40S subunit^{16; 17}. Their binding inhibits ribosome translocation and compromises translational fidelity. The glutarimide inhibitor, cycloheximide, binds in the E-site on the 60S subunit¹⁸. It competes with the E-site tRNA and inhibits eEF2-mediated tRNA translocation¹⁹. Anisomycin is a macrolide that binds to the A-site in the 60S subunit and

competitively inhibits the peptidyl transferase reaction thereby inhibiting translation elongation^{20; 21}.

The eL19 mutant cells deficient in eS7 binding (eL19₁₋₁₈₃) exhibited similar phenotypes in response to all of the antibiotics compared to cells expressing wild type eL19 (Fig. 5A). In contrast, mutants impaired in eB12 bridge formation (eL19₁₋₁₅₄ and eL19₁₋₁₄₆) displayed hypersensitivity to both of the aminoglycosides, *i.e.* paromomycin and neomycin. Increased sensitivity of these mutants to cycloheximide in comparison to the cells expressing wild type eL19 was observed only at 25°C. Surprisingly, these mutants conferred increased resistance to anisomycin at both temperatures as compared to the cells expressing wild type eL19. These results suggest that the eB12 bridge may affect overall ribosomal function rather than specific steps of the protein translation cycle.

The sensitivity to translational inhibitors inducing misreading (paromomycin and neomycin) of the eL19 mutant strains lacking the eB12 bridge suggests that these mutant ribosomes are more prone to make translational errors than the wild type ribosomes. Decreased translational fidelity in the eB12 bridge mutants may activate the general stress response. Several genes containing stress-responsive elements (STRE) in their promoter regions are transcriptionally induced during the general stress response program²². STREs are found in the promoters of *HSP12* and *CTT1*^{23; 24; 25}. *HSP12* encodes a small heat shock protein and its expression is up-regulated during different stress conditions^{23; 26; 27; 28; 29}. *CTT1* encodes the cytosolic catalase T and its expression is also induced under various stresses^{30; 31}. The levels of *HSP12* and *CTT1* mRNAs in cells expressing wild type and mutant alleles of eL19 grown in rich medium at 30 °C were analysed by quantitative reverse transcriptase PCR (Fig. 5B). No differences in *HSP12* and *CTT1* transcript levels were detected between the eL19 mutant cells deficient in eS7 binding (eL19₁₋₁₈₃) and wild type cells (Fig. 5B). However, *HSP12* mRNA levels were elevated 1.6- and 2.1-fold in cells expressing the eL19₁₋₁₅₄ and eL19₁₋₁₄₆ mutants respectively compared to the cells expressing wild type eL19. Similarly, the *CTT1* transcript level was 1.3-fold higher in eB12 bridge mutant cells relative to cells expressing wild type eL19. From these results we conclude that general stress response is activated in eB12 mutants in response to increased translational errors.

Several reports indicate that mistranslation enhances tolerance to different stress conditions^{32; 33; 34}. For this reason the sensitivity to hyperosmotic stress of cells expressing wild type eL19 was compared to that of cells expressing mutant alleles of eL19. Actively growing cells were exposed to 0.7 M NaCl stress for 2 hours and the viability of cells was measured by determining the colony forming ability of stressed cells (Fig. 5C). There was no difference in sensitivity to NaCl between cells expressing wild type eL19 and eL19 variant deficient in eS7 binding (eL19₁₋₁₈₃) (Fig. 5D). In contrast, mutants lacking eB12 were less sensitive to salt stress than cells containing wild type eL19 (Fig. 5D). We propose that increased misreading by mutant ribosomes lacking eB12 induces the stress response, therefore promoting acquired stress tolerance.

DISCUSSION

In the work presented here, we have evaluated the functional importance of the eukaryote-specific eB12 bridge at the biological, biochemical and structural levels. The core component of this bridge is the eukaryote-specific protein eL19⁵. eL19 is composed of an N-terminal globular domain, a middle region and a long C-terminal α -helix (Fig. 1B). The C-terminal α -helical domain forms the eB12 intersubunit bridge through its interactions with 18S rRNA ES6S, eS7 and uS17 (Fig. 1C). The N-terminal globular domain and middle region are embedded in domain III of 25S rRNA. eL19 is an essential protein⁷. A previous report indicates that eL19 has an essential role in pre-rRNA processing¹³. Expression shut down of eL19 leads to a delay in the endonucleolytic cleavage of 27SB pre-rRNA in the ITS2 region at site C₂, which produces 7S pre-rRNA and 25.5S pre-rRNA. Our results show that depletion of eL19 in its entirety is lethal, and that this impairs pre-rRNA processing and leads to a ribosome 60S subunit assembly defect (Fig. 3A–D). Specifically, primer extension analysis revealed accumulation of 27SB pre-rRNAs and increased the frequency of nonspecific cleavage events in the ITS2 region. Our results also show that deletion of the eB12 moiety of eL19 is dispensable for viability although this affected cellular growth rates (Fig. 2A). Analysis of pre-rRNA processing in these mutants demonstrated that the region responsible for eB12 formation is not required for ribosome biogenesis (Fig. 3E). In contrast, a deletion mutant lacking the entire C-terminal α -helix was inviable (Fig. 1D), indicating that the first 13 amino acids of this helix are required for ribosome functionality. According to the X-ray structure of the yeast 80S ribosome⁵, the beginning of the C-terminal α -helix of eL19 resides inside the large subunit and is probably required for correct eL19 folding or for its association with 25S rRNA. Thus, eL19 has two specific roles. The essential one, carried by the N-terminal globular domain and middle region, is in ribosome biogenesis. The second, provided by the C-terminal α -helical domain, involves structural support for ribosomal subunit joining.

Examination of phenotypes conferred by the eL19 mutant alleles revealed several eB12 bridge specific effects, *i.e.* slow growth, altered sensitivity/resistance to translational inhibitors and enhanced hyperosmotic stress tolerance (Fig. 2A, Fig. 5A, 5D). Loss of the eB12 bridge had a stronger impact on A-site specific functions, *i.e.* the mutants were sensitive to aminoglycosides and resistant to anisomycin. The aminoglycosides paromomycin and neomycin bind to the A-site decoding region of the small subunit. The canonical aminoglycoside binding site is located within the internal loop of helix 44 of 18S rRNA and contains the universally conserved nucleotides A1755 and A1756. Two nonconserved nucleotides (G1645 in yeast, A1408 in bacteria and A1754 in yeast, G1491 in bacteria) have been shown to be responsible for the greater resistance of eukaryotes to aminoglycosides³⁵. Mutational analyses have shown that yeast strain carrying G1645A and A1754G mutations exhibited increased sensitivity to neomycin and paromomycin. Consistent with these findings, replacement of the bacterial A-site helix 44 with its counterpart from eukaryotes conferred aminoglycoside resistance of these mutants comparable to eukaryotic ribosomes³⁶. Recent structural analysis of translational inhibitor binding sites in the yeast ribosome explained this difference between prokaryotes and eukaryotes¹⁸. In the yeast ribosome, nucleotides G1645 and A1754 act as a barrier by

preventing the accommodation of aminoglycosides such as neomycin and paromomycin. It is tempting to speculate that loss of the eB12 in mutant ribosomes changes the conformation of the G1645 and A1754 nucleotides, allowing binding of the aminoglycosides, and thus causing the observed increased sensitivity to neomycin and paromomycin. Additionally, the absence of the eB12 intersubunit bridge may result in conformational changes affecting the structure of the A-site, perhaps leading to increased affinity for aa-tRNA. In support of this, a correlation between increased affinity for aa-tRNA at the A-site and decrease in translational accuracy has been previously demonstrated³⁷. Yeast ribosomes lacking eL39 displayed increased affinity for A-site tRNA and showed a 4-fold increase in the error frequency compared to the wild type ribosomes³⁷. Paromomycin also induces translational misreading errors in yeast. In yeast cell free extracts the misreading of poly(U) was stimulated^{38; 39}. Furthermore, the phenotypic suppression of both nonsense and missense mutations was induced^{38; 39}. Both mechanisms lead to the translational misreading and this cumulative effect may cause the sensitivity to aminoglycosides. Similarly, the resistance to anisomycin can result from conformational changes in the ribosome. Anisomycin competes with the amino acid side chains of aa-tRNAs for binding to the A-site cleft of the large subunit²¹. It is possible that the absence of eB12 bridge may affect the structure of the A-site to favour aa-tRNA binding over the antibiotic.

Interestingly, cells containing mutant ribosomes that are not able to form the eB12 bridge exhibited higher tolerance to hyperosmotic stress when compared with wild type cells. This observation is suggestive of a phenomenon called cross-protection, in which adaptation to one form of stress often helps cells to survive other stress conditions. It is possible that compromise of ribosome function due to lack of the eB12 bridge leads to activation of general stress response. This activation may provide protection against various environmental stress conditions in the eB12 intersubunit bridge mutants.

Little is known about the functional importance of the eukaryote-specific intersubunit bridges. All five of these bridges are composed of protein-rRNA and protein-protein type of contacts⁵. The eB8 intersubunit bridge is formed by contacts between eS1 and 25S rRNA ES31L and is located proximal to the mRNA exit tunnel. The eB11 bridge is mainly formed by interactions between eS8 and 25S rRNA ES41L. The eB14 bridge is the only eukaryote-specific intersubunit bridge that is located in the centre of the ribosome. This bridge is formed by eL41 and 18S rRNA helix 44. The 25 amino acid long eL41 is highly positively charged and forms a single α -helix. Interestingly, eL41 is also a nonessential ribosomal protein, indicating that eB14 is dispensable for viability⁴⁰. However, its deletion results in changes in peptidyltransferase activity and translational fidelity^{41; 42}.

Structural studies of the human 80S ribosome describe large conformational changes in the eB12 and eB13 bridges during the transition from pre- to post-translocation states^{43; 44}. In the case of eB12, the C-terminal helix of eL19 undergoes a structural transition from a straight (post-translocation state) to a bent (pre-translocation state) protein helix. Similarly, the C-terminal region of eL24 is relocalised and interacts with an entirely different region of the 18S rRNA. Given that eB12 is not essential for viability and that yeast cells survive loss of eL24 and consequently the eB13 bridge^{9; 11}, it is possible that the dynamic nature of these bridges is not required for regulation of the information flow between subunits as previously

hypothesised^{5; 45; 46}, but rather that may be required for additional stabilisation during 40S subunit rotation. However, analysis of 80S ribosome formation *in vitro* revealed that 60S subunits devoid of the eL19 C-terminal region which is required for eB12 formation are strongly defective in subunit reassociation in the absence of additional factors. These results lead us to propose that tRNA molecules associated with 80S ribosomes may function as structural elements, helping to stabilise the dynamic subunit interactions in translating ribosomes. We speculate that stabilisation of ribosome subunit association by tRNA helps to ensure translational processivity.

MATERIALS AND METHODS

Yeast strains and media

All *S. cerevisiae* strains used in this study were isogenic derivatives of strain S288C⁴⁷ and are listed in Supplemental Table S1.

Cells were grown in either YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose), YPGal (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose) or synthetic complete medium (0.67% Bacto yeast nitrogen base without amino acids, 2% glucose or 2% galactose) supplemented with the appropriate amino acids and bases⁴⁸. Agar (2%) was added to solid media. When required, the following concentrations of antibiotics were used: 200 µg/ml geneticin, 3 mg/ml paromomycin, 5 mg/ml neomycin, 0.15 µg/ml cycloheximide or 20 µg/ml anisomycin.

Strains TYSC207 and TYSC227 were generated by one-step PCR-based gene disruption⁴⁹ of the *RPL19A* and *RPL19B* genes, respectively. Deletion cassettes containing *kanMX4* were PCR amplified from the genomic DNA isolated from respective EUROSCARF deletion mutants⁸ and transformed into diploid TYSC184 followed by sporulation and tetrad dissection. Strain TYSC237 was constructed by crossing TYSC207 and TYSC227. The resulting diploid was transformed with *pRS316-RPL19* plasmid, sporulated and tetrads were dissected. Strains TYSC278, TYSC280, TYSC282, and TYSC283 were constructed using plasmid shuffling on glucose containing SC-Leu plates supplemented with 1 g/l 5-fluoroorotic acid (5FOA) at 30°C after TYSC237 transformation with plasmids *pRS315-RPL19*, *pRS315-rpl19₁₋₁₈₃*, *pRS315-rpl19₁₋₁₅₄*, or *pRS315-rpl19₁₋₁₄₆*, respectively. Strain TYSC291 was constructed using plasmid shuffling on galactose containing SC-Leu plates with 5FOA after TYSC237 transformation with plasmid *pGAL-RPL19*.

Plasmids

Plasmids used in this study are listed in Supplemental Table S2. The *RPL19A* coding region with its 5' upstream (799 bp) and 3' downstream (682 bp) regions was amplified with PCR from genomic DNA and cloned between *Bam*HI and *Cfr*42I restriction sites into the *pRS316* or *pRS315* vector⁴⁷. The resulting plasmids were named *pRS316-RPL19* and *pRS315-RPL19*, respectively. To generate *pRS315-rpl19₁₋₁₈₃*, *pRS315-rpl19₁₋₁₅₄*, *pRS315-rpl19₁₋₁₄₆* and *pRS315-rpl19₁₋₁₃₃* the site-directed mutagenesis was performed by overlap-extension PCR to delete C-terminal coding region for 6, 35, 43 and 56 amino acid residues, respectively. For construction *pGAL-RPL19*, the *RPL19A* coding region with its 3'

downstream (235 bp) region was amplified with PCR and cloned between *Bam*HI and *Sal*I restriction sites into the *pRS415-GAL1* vector⁵⁰. All cloned DNA fragments generated by PCR amplification were verified by sequencing.

Temperature and antibiotic sensitivity/resistance assays

Yeast strains were grown in YPD or YPGal media at 30°C to mid-exponential phase. Cultures were serially diluted, spotted on YPD or YPGal plates and incubated at 16°C, 20°C, 25°C, 30°C and 36°C for 2–6 days. To analyse the sensitivity/resistance to antibiotics the serially diluted cultures were spotted on YPD plates supplemented with appropriate antibiotics and incubated at 25°C and 30°C for 3–5 days.

Salt stress assay

Triplicate samples of yeast cultures grown overnight in YPD at 30°C were diluted into fresh medium and were grown to early exponential phase (OD₆₀₀ of 0.4). Cells were exposed to 0.7 M NaCl stress for 2 hours at 30°C. To determine colony-forming units the cells were plated on YPD agar plates and incubated at 30°C for 3 days. Viability was expressed as percentage of cells forming colonies after salt stress relative to the number of colonies in the untreated samples. The average and standard errors were calculated from three independent experiments. Statistical significance was evaluated with the unpaired *t* test.

In vivo depletion of eL19

The TYSC291 strain was grown in YPGal medium at 30°C until mid-exponential phase. Cells were washed and transferred to YPD medium. At different time points, samples were collected to perform ribosome-polysome profile analysis and RNA extraction.

Ribosome-polysome profile analysis

Ribosome-polysome profile analysis was performed as described previously^{51; 52} with modifications. Briefly, 100 ml of yeast cells was grown in YPD at 30°C or 20°C to mid-exponential phase. Cycloheximide was added at a final concentration of 100 µg/ml 15 min before the cells were harvested and washed with ice-cold breaking buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 30 mM MgCl₂) supplemented with cycloheximide. Cells were disrupted by the glass beads in Precellys 24 homogenizer (Bertin Technologies) (program: 6000 rpm, 4°C, 3×1 min, pause 1 min). Obtained extract was centrifuged at 16060xg for 15 min at 4°C and further clarified using 20 µm pore size filter (Masherey Nagel). Thirty A260 units of extract was layered onto 7%–47% sucrose gradient containing 50 mM Tris-HCl (pH 7.5), 50 mM NH₄Cl, 12 mM MgCl₂, 1mM dithiothreitol and centrifuged at 4°C in SW28 rotor (Beckmann) at $\omega^2t=1.38\times10^{11}$. Gradients were monitored at 260 nm.

RNA extraction and primer extension analysis

RNA was extracted from samples corresponding to 10 OD₆₀₀ units of exponentially grown cells by SDS-acidic phenol-chloroform treatment⁵³. Primer extension reactions were performed as described previously⁵⁴ using primers (Probe F: 5'-GGCCAGCAATTTCAGTTA-3' and probe G: 5'-GAACATTGTTGCCTAGA-3' from⁵⁵)

complementary to a region immediately upstream and downstream of the C₂ cleavage site in ITS2 (Fig. 3C).

Quantitative PCR analysis

Total RNA was isolated by SDS-acidic phenol-chloroform treatment⁵³ from triplicate cultures (15 ml) grown overnight in YPD at 30°C, diluted into fresh medium and grown to exponential phase (OD₆₀₀ of 0.8). DNase I treatment, cDNA synthesis and quantitative PCR were performed as described earlier⁵⁶. Oligonucleotide primers used are listed in Supplemental Table S3. For normalisation of mRNA levels, the geometric mean of two housekeeping genes (*ARP6* and *UBC6*) was used. The average and standard deviations were calculated from two independent experiments.

Preparation of salt-washed ribosomal subunits

400 ml of yeast cells was grown in YPD at 30°C until mid-exponential phase. Cells were harvested, washed with ice-cold buffer A (30 mM Hepes-KOH [pH 7.5], 100 mM KCl, 10 mM Mg(OAc)₂, 2 mM DTT, 0.5 mM EDTA), and disrupted by the glass beads in Precellys 24 homogenizer. The extract was centrifuged at 16060xg for 15 min at 4°C and filtered using 20 µm pore size filter (Masherey Nagel). One hundred and fifty A260 units of extract was layered onto 10%–30% sucrose gradient in buffer A and centrifuged at 4°C in SW28 rotor at $\omega^2t=2.4\times10^{11}$. Gradients were monitored at 260 nm. 80S ribosomes were fractionated and centrifuged at 4°C in Ti50 rotor (Beckmann) at $\omega^2t=1.0\times10^{12}$. The pellet was resuspended in buffer B (30 mM Hepes-KOH [pH 7.5], 500 mM KCl, 5 mM Mg(OAc)₂, 2 mM DTT, 0.5 mM EDTA) and dialysed for 1 hour against buffer B. Forty A260 units of sample was layered onto 10%–25% sucrose gradient in buffer B and centrifuged at 4°C in SW28 rotor at $\omega^2t=2.8\times10^{11}$. Dissociated 60S and 40S subunits were fractionated and concentrated with 100 kDa Centricon Plus-70 filter (Millipore) in buffer C (30 mM Hepes-KOH [pH 7.5], 100 mM KCl, 5 mM Mg(OAc)₂, 2 mM DTT, 0.5 mM EDTA). Obtained salt-washed ribosomal subunits were stored at –80°C.

In vitro reassociation of 80S

Two A260 units of wild type 40S was mixed with two A260 units of 60S subunits containing wild type or mutant eL19 in 300 µl of buffer R (30 mM Hepes-KOH [pH 7.5], 100 mM KCl, 1 mM DTT) and incubated for 20 min at 30°C. The assays with five different Mg-acetate concentrations (R buffer with 4.5 mM, 10 mM, 15 mM, 20 mM (with and without 0.08 mg of total *E. coli* deacylated tRNA) and 25 mM Mg-acetate) were performed. Samples were diluted to the final volume of 800 µl with ice-cold buffer R, layered onto 10%–30% sucrose gradient with corresponded Mg-acetate concentration and centrifuged at 4°C in SW28 rotor at $\omega^2t=2.4\times10^{11}$. Gradients were monitored at 260 nm.

SHAPE structural analysis

1-methyl-7-nitroisatoic anhydride (1M7) was synthesized according to the method described by Turner and co-authors⁵⁷. Empty puromycin treated salt-washed ribosomes were prepared as described previously⁵⁸. The ribosomes were treated with 1M7 at 30°C for 20 min^{59; 60}. Ribosomes were precipitated, pelleted and RNA was isolated by SDS-acidic phenol-

chloroform treatment⁵³. Primer extension reactions were performed as described previously⁶¹. Oligonucleotide primers used for the primer extension analysis are listed in Supplemental Table S4. All assays were performed with two independent biological replicates. SHAPE reactivities were quantified using Image Quant TL v2005 software (GE Healthcare). Reactivities for the analysed nucleotides were normalized by dividing by the average reactivity of all nucleotides. Absolute reactivities were calculated by subtracting the non-reagent intensities from the 1M7-modified intensities. The reactivities that were less than zero were reset to zero. Representative results of SHAPE analysis are shown in Figure S2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

1M7	1-methyl-7-nitroisatoic anhydride
eB12	eukaryote-specific ribosome intersubunit bridge 12
eL19	eukaryotic ribosomal large subunit protein 19
ES6S	eukaryote-specific rRNA expansion segment 6
eS7	eukaryotic ribosomal small subunit protein 7
ITS2	internal transcribed spacer region 2
uS17	universal ribosomal small subunit protein 17

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HIGHLIGHTS

- Seventeen bridges are formed during association of eukaryotic ribosome subunits
- The main component of the eukaryote-specific eB12 bridge is C-terminal α -helix of eL19
- The globular domain and middle region of eL19 are essential during 60S assembly
- Ribosomes lacking eB12 can support growth and are active in translation
- eB12 has a role in subunit joining and in optimizing ribosome functionality

Figure 1. Functional analysis of eL19 deletion mutants *in vivo*

A. *S. cerevisiae* 80S ribosome. The large subunit (60S) is shown in blue and the small subunit (40S) is grey. Proteins eL19 and eL24 forming the eB12 and eB13 bridges, respectively, are shown in red and orange.

B. Domain structure of eL19. The C-terminal α -helix of eL19 (dark blue) is shown in detail. Amino acid residues forming contacts with eukaryote-specific expansion segment 6 (ES6S) of 18S rRNA and 40S subunit proteins (eS7, uS17) are coloured green and yellow, respectively. Arrows indicate the positions of the last amino acids of the respective eL19 deletion alleles.

C. Close up view of the eB12 intersubunit bridge. Detailed views of contacts formed between α -helix of eL19 (dark blue) and uS17 (red), 18S rRNA ES6S (orange) and eS7 (purple) are shown. Ribosomal structures were generated by PyMol using coordinates from⁵.

D. Analysis of the functionality of eL19 deletion mutants using plasmid shuffle assay. An *rlp19A rlp19B* strain (TYSC237) containing *RPL19A/URA3/CEN* plasmid was transformed with either an empty *LEU2/CEN* plasmid (indicated as vector) or plasmids harbouring wild type eL19 or indicated eL19 mutant alleles. Growth of the transformants was tested on control plates (SC-Leu) or on plates containing 5-fluoroorotic acid (SC-Leu +5FOA) which selects for yeast cells that have lost the *RPL19A/URA3/CEN* plasmid. Plates were incubated at 30°C for 3 days.

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Figure 2. Phenotypic analysis of eL19 deletion mutants

A. Growth phenotypes of *eL19* mutants. Serial dilutions of wild type (WT, TYSC144) and *rpl19A rpl19B* strains carrying *eL19* wild type (*eL19*, TYSC278) or mutant alleles (*eL19*₁₋₁₈₃, TYSC280; *eL19*₁₋₁₅₄, TYSC282; *eL19*₁₋₁₄₆, TYSC283) were spotted onto YPD medium and incubated at the indicated temperatures for 2–4 days.

B. Analysis of ribosome-polysome profiles by sucrose density gradient centrifugation. The *rpl19A rpl19B* strains carrying *eL19* wild type or mutant alleles were grown in YPD medium at indicated temperatures. The whole cell extracts were prepared from cycloheximide treated cells and analysed in 7%–47% sucrose gradients. The absorbance at 260 nm (A_{260nm}) was recorded. Sedimentation is from left to right. The peaks of free 60S ribosomal subunits, monosomes (80S) and polysomes are indicated.

Figure 3. Processing of pre-rRNAs is not perturbed in eL19 mutants

A. Serial dilutions of wild type (WT, TYSC144) and *rpl19A rlp19B* strain carrying *pGAL-RPL19* plasmid (P_{GAL1}::RPL19, TYSC291) were spotted onto YPGal (galactose) or YPD (glucose) medium and incubated at 30°C for 3 days.

B. Analysis of ribosome-polysome profiles of cells depleted for the eL19 by sucrose density gradient centrifugation. Cells were cultured in YPGal and shifted to YPD for 12 or 24 hours. Whole cell extracts were prepared from cycloheximide treated cells and analysed in 7%–47% sucrose gradients. The absorbance at 260 nm (A_{260nm}) was recorded. Sedimentation is from left to right. The peaks of free 40S ribosomal subunits, monosomes (80S) and polysomes are indicated.

C. Structure and processing sites of the 35S pre-rRNA. The mature 18S, 5.8S and 25S rRNAs are shown as boxes. Two internal transcribed spacer sequences (ITS1 and ITS2) and

two external transcribed spacer sequences (5' ETS and 3' ETS) are shown as lines. The cleavage sites and positions of the oligonucleotides (F and G) used for the primer extension analysis are indicated.

D. Analysis of pre-rRNA processing in cells depleted for eL19 by primer extension. Total RNA was extracted from cells grown at 30°C in YPGal or shifted to YPD for up to 24 hours. Samples were subjected to primer extension analysis of 5' ends of 27S and 25.5S pre-rRNAs. Asterisks indicate the nonspecific cleavage products of 27S pre-rRNA. The origin of the pre-rRNAs analysed by primer extension are shown.

E. Analysis of pre-rRNA processing in eL19 mutants by primer extension. Total RNA was extracted from wild type (WT, TYSC144) and *rlp19A rlp19B* strains carrying eL19 wild type (eL19, TYSC278) or mutant alleles (eL19₁₋₁₈₃, TYSC280; eL19₁₋₁₅₄, TYSC282; eL19₁₋₁₄₆, TYSC283) grown in YPD medium at 30°C or 20 °C.

Figure 4. The eL19 intersubunit bridge is required for 80S formation *in vitro*

Salt-washed 40S and 60S subunits were purified from *rpl19A rlp19B* strains carrying eL19 wild type (eL19, TYSC278) or mutant alleles (eL19₁₋₁₈₃, TYSC280; eL19₁₋₁₅₄, TYSC282; eL19₁₋₁₄₆, TYSC283) grown in YPD medium at 30°C. Two A₂₆₀ units of wild type 40S subunits were co-incubated with two A₂₆₀ units of 60S subunits containing wild type or mutant eL19 in the presence of indicated magnesium acetate concentrations for 20 min at 30°C. Reactions at 20 mM magnesium acetate were also performed in the presence of saturating concentration of deacylated tRNA (20 mM + tRNA) to stimulate 80S formation. Ribosomal subunit association was analysed in 10%–30% sucrose gradients with appropriate

magnesium acetate concentrations. Sedimentation is from left to right. The peaks of free 40S and 60S ribosomal subunits and monosomes (80S) are indicated.

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Figure 5. Physiological responses of eL19 deletion mutants to different stress conditions

A. Drug resistance/sensitivity phenotypes of eL19 mutants. Serial dilutions of *rp119A* *rlp19B* strains carrying eL19 wild type (eL19, TYSC278) or mutant alleles (eL19₁₋₁₈₃, TYSC280; eL19₁₋₁₅₄, TYSC282; eL19₁₋₁₄₆, TYSC283) were spotted onto YPD medium without or with indicated antibiotics and incubated at the indicated temperatures for 3–4 days.

B. Analysis of *HSP12* and *CTT1* transcript levels with quantitative PCR in eL19 mutants. Total RNA was extracted from *rp119A* *rlp19B* strains carrying eL19 wild type or mutant alleles grown in YPD medium at 30°C. The results were normalised to the geometric average of two housekeeping genes (*UBC6* and *ARP6*) and expressed relative to the strain expressing wild type eL19. Error bars represent standard deviations of two independent measurements.

C. Design of the analysis of NaCl stress survival. CFU, colony-forming unit.

D. Sensitivity to salt stress of eL19 mutants. Yeast cells were grown to early exponential phase in YPD at 30°C and exposed to 0.7 M NaCl stress for 2 hours. Cell viability was measured by counting colony-forming cells on YPD plates after the salt stress relative to

untreated cells. The error bars represent the standard errors of three independent experiments. Statistical significance was evaluated with the unpaired t test (* $P<0.001$).

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Table 1

Description of eL19 variants analysed in this study.

eL19 variant	Length of eL19 (aa)	Deleted contacts/region	Viability
eL19	189	–	+
eL19 _{1–183}	183	eS7	+
eL19 _{1–154}	154	eS7, ES6S, uS17	+
eL19 _{1–146}	146	eS7, ES6S, uS17	+
eL19 _{1–133}	133	entire α -helix	–