Ribosome-associated Complex Binds to Ribosomes in Close Proximity of Rpl31 at the Exit of the Polypeptide Tunnel in Yeast

Kristin Peisker,^{*†‡} Daniel Braun,^{*†} Tina Wölfle,^{*†} Jendrik Hentschel,^{*†}
Ursula Fünfschilling,[§] Gunter Fischer,[§] Albert Sickmann,^{||} and Sabine Rospert^{*†}

^{*}Institute of Biochemistry and Molecular Biology, ZBMZ, University of Freiburg, D-79104 Freiburg, Germany;

[†]Centre for Biological Signalling Studies (BIOSS), University of Freiburg, D-79104 Freiburg, Germany;

[‡]Fakultät für Biologie, University of Freiburg, D-79104 Freiburg, Germany; [§]Max Planck Research Unit for Enzymology of Protein Folding, D-06120 Halle/Saale, Germany; ^{||}Department of Proteomics, Institute for Analytical Sciences, D-44139 Dortmund, Germany

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Ribosome-associated complex (RAC) consists of the Hsp40 homolog Zuo1 and the Hsp70 homolog Ssz1. The chaperone participates in the biogenesis of newly synthesized polypeptides. Here we have identified yeast Rpl31, a component of the large ribosomal subunit, as a contact point of RAC at the polypeptide tunnel exit. Rpl31 is encoded by *RPL31a* and *RPL31b*, two closely related genes. $\Delta rpl31a\Delta rpl31b$ displayed slow growth and sensitivity to low as well as high temperatures. In addition, $\Delta rpl31a\Delta rpl31b$ was highly sensitive toward aminoglycoside antibiotics and suffered from defects in translational fidelity. With the exception of sensitivity at elevated temperature, the phenotype resembled yeast strains lacking one of the RAC subunits or Rpl39, another protein localized at the tunnel exit. Defects of $\Delta rpl31a\Delta rpl31b\Delta zuo1$ did not exceed that of $\Delta rpl31a\Delta rpl31b$ or $\Delta zuo1$. However, the combined deletion of *RPL31a*, *RPL31b*, and *RPL39* was lethal. Moreover, *RPL39* was a multicopy suppressor, whereas overexpression of RAC failed to rescue growth defects of $\Delta rpl31a\Delta rpl31b$. The findings are consistent with a model in that Rpl31 and Rpl39 independently affect a common ribosome function, whereas Rpl31 and RAC are functionally interdependent. Rpl31, while not essential for binding of RAC to the ribosome, might be involved in proper function of the chaperone complex.

INTRODUCTION

Two Hsp70 family members Ssb1/2 (Ssb1 and Ssb2 differ by only four amino acids) and Ssz1 and one J-domain protein (Zuo1) are abundant components of the translation machinery of *Saccharomyces cerevisiae* (Raue *et al.*, 2007). The three chaperones are genetically linked and form a functional triad. Lack of either *SSB1/2*, *SSZ1*, or *ZUO1* results in slow growth, cold sensitivity, and pronounced hypersensitivity against aminoglycosides such as paromomycin (Gautschi *et al.*, 2002; Hundley *et al.*, 2002). Ssz1 and Zuo1 assemble into a stable heterodimeric complex termed RAC (ribosome-associated complex). RAC acts as a cochaperone for Ssb1/2 and stimulates its ATP hydrolysis. The function requires both RAC subunits (Huang *et al.*, 2005; Conz *et al.*, 2007).

RAC is anchored to the ribosome via Zuo1 (Gautschi *et al.*, 2001). The idea is that positioning of RAC on the ribosome is

required for its interaction with Ssb1/2 (Yan *et al.*, 1998). However, the function of Ssz1 does not strictly depend on stable interaction with Zuo1 or ribosomes (Conz *et al.*, 2007). How exactly Zuo1 anchors RAC is currently unclear. It was proposed that Zuo1 binds to ribosomes, in part, by interaction with rRNA (Yan *et al.*, 1998). However, purified Zuo1 unspecifically interacts with a variety of nucleic acids. Initially, Zuo1 was identified via its ability to interact with Z-DNA (Zhang *et al.*, 1992), it also interacts tightly with tRNA (Wilhelm *et al.*, 1994) and recently was shown to bind to a small inhibitor RNA (Raychaudhuri *et al.*, 2006). The mouse homolog MIDA1 interacts with DNA that forms small stem loop structures (Inoue *et al.*, 2000). The diversity of nucleic acids that interact with Zuo1 raises the question how targeting to a specific binding site on the ribosome is achieved. An attractive hypothesis is that auxiliary interactions with ribosomal protein components mediate specificity (Yan *et al.*, 1998). To what region of the ribosome Zuo1 binds has not been analyzed. The exit region, which has a higher than average concentration of ribosomal proteins (Klein *et al.*, 2004), would be ideally suited to position RAC close to its partner chaperone Ssb1/2. However, direct evidence for binding close to the polypeptide tunnel exit is missing.

In terms of protein composition the archaeal ribosome is a small-scale model of the eukaryotic one (Lecompte *et al.*, 2002). The crystal structure revealed that six proteins Rpl19/L19e, Rpl17/L22, Rpl25/L23, Rpl26/L24, Rpl35/L29, and Rpl31/L31e, form a rim around the polypeptide tunnel exit (Nissen *et al.*, 2000; Klein *et al.*, 2004). Rpl (ribosomal protein

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Address correspondence to: Sabine Rospert (sabine.rosper@biochemie.uni-freiburg.de).

Abbreviations used: NAC, nascent polypeptide associated complex; RAC, ribosome-associated complex; $\Delta 31$ -ribosomes, ribosomes isolated from a $\Delta rpl31a\Delta rpl31b$ strain; $\Delta 39$ -ribosomes, ribosomes isolated from a $\Delta rpl39$ strain; RPB, ribosome-associated protein biogenesis factors; SRP, signal recognition particle.

of the large subunit) designates the yeast homologues of ribosomal proteins as in Lecompte *et al.*, (2002); see Figure 1E. Rpl39/L39e, a small protein predominantly localized within the polypeptide tunnel also exposes a small surface at the tunnel exit. This set of proteins is supposed to provide a platform for the interaction of ribosome-associated protein biogenesis factors (RPFs), a set of proteins that reversibly interact with ribosomes and affect nascent polypeptides in multiple ways (Rospert *et al.*, 2005a; Raue *et al.*, 2007). To date, only two of the ribosomal proteins at the tunnel exit, Rpl25/L23 and Rpl35/L29, have been shown to interact with RPFs. At least four RPFs, signal recognition particle (SRP), nascent polypeptide associated complex (NAC), the ER-membrane protein ERj1, and the eubacterial trigger factor, interact with ribosomes via Rpl25/L23. SRP interacts with Rpl25/L23, Rpl35/L29, and rRNA of both ribosomal subunits (Pool *et al.*, 2002; Gu *et al.*, 2003; Ullers *et al.*, 2003; Halic *et al.*, 2004). Trigger factor binds to the ribosome through interactions with Rpl25/L23, Rpl35/L29, and 23S rRNA (Kramer *et al.*, 2002; Blaha *et al.*, 2003; Ferbitz *et al.*, 2004; Baram *et al.*, 2005; Schlünzen *et al.*, 2005). NAC and ERj1 were recently found to interact with Rpl25/L23 (Blau *et al.*, 2005; Wegrzyn *et al.*, 2006). Accordingly, the current idea is that this protein constitutes a general factor binding site. However, evidence was recently presented that Rpl35/L29 is the attachment site for the *N*^α-acetyltransferase NatA (Polevoda *et al.*, 2008), which is bound to ribosomes via its subunit Nat1 (Gautschi *et al.*, 2003).

Here we set out to characterize the interaction of RAC with the ribosome in more detail. To that end, we have used a cross-linking approach that allowed us to identify Rpl31 as a ribosomal protein that directly contacts the Zuo1 subunit of RAC. Although Rpl31 was not essential for Zuo1 binding to the ribosome, genetic evidence is consistent with the possibility of functional coupling between Rpl31 and RAC. Moreover, we found that simultaneous deletion of the genes encoding Rpl31 and Rpl39 resulted in synthetic lethality as expected if the two proteins at the tunnel exit were involved in a common function of the ribosome.

MATERIALS AND METHODS

Media and Culture Conditions

Strains were grown to log phase on 1% yeast extract, 2% peptone, and 2% dextrose (YPD) or in glucose-containing minimal media. Growth defects were analyzed by spotting 10-fold serial dilutions containing the same number of cells to YPD plates. Plates were supplemented with paromomycin at the concentration specified in the figure legends. Plates were incubated at 30°C to analyze the slow growth phenotype, at 20°C to determine cold sensitivity, and at 37°C to screen for temperature sensitivity. Sensitivity to paromomycin was analyzed in liquid cultures as described (Dresios *et al.*, 2000).

Strains and Plasmids

MH272-3f a/α (*ura3/ura3*, *leu2/leu2*, *his3/his3*, *trp1/trp1*, *ade2/ade2*; Heitman *et al.*, 1991) was the parental strain for the mutants used in this study. Strains lacking ZUO1 (IDA1: *Δzuo1*) and ZUO1/SSZ1 (IDA12: *Δzuo1Δssz1*) have been previously described (Gautschi *et al.*, 2001). To generate strains lacking Rpl31a, Rpl31b, and Rpl39 RPL31a, RPL31b, and RPL39 plus 600 base pairs up- and 300 base pairs downstream of the respective *orf* was cloned into pSP65 (Promega, Madison, WI). In the case of RPL31a an internal 638-base pair StyI fragment was replaced with the TRP1 gene. In addition a SpeI/Tth111I fragment was removed in order to delete the start codon of RPL31a plus the following 19 nucleotides of the first exon. In the case of RPL31b an internal BsaAI/Bpu10I fragment of 586 base pairs within the coding region was exchanged for the ADE2 marker. An internal BamHI/BsaBI fragment of RPL39 was replaced for the HIS3 marker. The resulting disruption constructs were used to generate MH272-3fa *rpl31a::TRP1*, MH272-3fb *rpl31b::ADE2* and MH272-3fc *rpl39::HIS3*, respectively. After mating of *rpl31a::TRP1* with *rpl31b::ADE2*, the resulting diploid was sporulated, and tetrad dissection was performed. Deletion of both copies of Rpl31 and of Rpl39 was confirmed via

immunoblotting. *Δrpl31aΔrpl31bΔzuo1* was derived by mating *Δrpl31aΔrpl31b* with *Δzuo1* followed by sporulation and tetrad dissection.

For expression in yeast, Rpl31a, Rpl24a, Rpl17a, Rpl39, and Zuo1 plus 300-base pairs up- and downstream of the *orf* were transferred into pYCPlac33 (CEN, URA3). For overexpression of RAC Zuo1 was cloned into pYEPlac181 (2μ, LEU2) and Ssz1 into pYEPlac195 (2μ, URA3; Gietz and Sugino, 1988). Mutations in pYCPlac33-Zuo1 were generated by QuikChange using the manufacturers protocol (Stratagene, La Jolla, CA). Zuo1-15A contains mutations C(167)A, KEEKKKE(290–296)AAAAAAA, RRK(299–301)AAA, ERE(303–305)AAA, E(313)A, and K(315)A (see Figure 5C). pYCPlac33-Zuo1Δ282-331 was generated by introducing two Afel sites at the positions corresponding to E(282) and K(331) by QuikChange. Subsequently the construct was cut with Afel followed by blunt-end ligation. pYCPlac33-Zuo1-15A was introduced into *Δzuo1* and *Δrpl31aΔrpl31bΔzuo1* deletion strains resulting in strains *Δzuo1* + Zuo1-15A and *Δrpl31aΔrpl31bΔzuo1* + Zuo1-15A. pYCPlac33-Zuo1Δ282-331 was introduced into *Δzuo1* resulting in strain *Δzuo1* + Zuo1Δ282-331. For in vivo read-through experiments MH272-3f-derived strains were transformed with a high copy number plasmid encoding HSP104 (pRS423: 2μ, HIS3; Christianson *et al.*, 1992) to ensure their [*psi*[−]] status (Rakwalska and Rospert, 2004). The *lacZ-luc* chimera (see Figure 2D) were expressed from pYEPlac195 (2μ, URA3; Gietz and Sugino, 1988). Construction of the read-through reporters is based on previously published vectors; however, the stop codon context was altered such that the basal level of read-through was low (Bidou *et al.*, 2000; Rakwalska and Rospert, 2004). The two genes were separated by a short in-frame linker containing the TGA stop codon (*lacZ*-STOP-*luc*) or GCT encoding alanine (*lacZ-luc*; see Figure 2D).

Peptide Scan

Peptide libraries were synthesized according to standard protocols (Frank, 1992; Reineke *et al.*, 1996; Kramer and Schneider-Mergener, 1998). The amino acid sequence of Zuo1 was used to generate linear overlapping 15mer peptides shifted by one amino acid. Peptides were C-terminally attached to cellulose via a (β-Ala)₂ spacer. After washing in ddH₂O, cellulose membranes were incubated in buffer A (20 mM HEPES-KOH, pH 7.4, 120 mM KAcetate, 5 mM MgAcetate, 2 mM DTT, 0.5 mM PMSF) for 1 h. Unspecific binding sites were blocked by incubation with 2% milk in buffer A overnight at 25°C. Subsequently, membranes were incubated with high-salt washed yeast ribosomes in buffer A containing 2% milk for 10 h at 24°C. Unbound ribosomes were removed by washes with buffer A and peptide-bound ribosomes were transferred to a nitrocellulose membrane for 10 min at 15 V using a semidry blotting apparatus. Ribosomes were visualized with an antibody directed against ribosomal protein Rpl16.

Cross-linking Experiments

Cross-linking reactions were performed either on ribosomes isolated under low-salt conditions (120 mM KAcetate), on ribosomes isolated under high-salt conditions (800 mM KAcetate) after rebinding of purified RAC or on ribosomes in cell lysates. Note that RAC covers about one-third of ribosomes isolated under low-salt conditions (Raue *et al.*, 2007). RAC is released from ribosomes in the presence of 800 mM KAcetate (Gautschi *et al.*, 2001). When the salt concentration is lowered, RAC can rebind to ribosomes. As purified RAC was added in molar excess to high-salt washed ribosomes, a higher fraction is occupied under these conditions (data not shown). A standard reaction contained 20–60 nM ribosomes in 100–200 μl cross-link buffer (80 mM KAcetate, 20 mM HEPES-KOH, pH 7.4, 2 mM MgAcetate, 2 mM DTT, and 1 mM PMSF). Reactions were supplemented with the amino-reactive cross-linking agent BS³ [bis(sulfosuccinimidyl)suberate; Pierce, Rockford, IL] to a final concentration of 0.8 mM and were incubated at 21°C for 20 min. BS³ cross-linking was stopped by addition of glycyl-glycine to a final concentration of 30 mM followed by incubation for 10 min on ice. Direct interaction between Zuo1 and Rpl31 was tested using the zero-length cross-linker EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, Pierce). In this case, 100 μl reactions in cross-link buffer contained 56 nM ribosomes, which were supplemented with 20 mM EDC. Reactions were incubated at 20°C for 20 min. Cross-link reactions in total cell lysate were performed using cells corresponding to an OD₂₈₀ of 300. Cell lysates in a volume of 100 μl cross-link buffer were supplemented with 6.5 mM EDC. Cross-link reactions were analyzed by SDS-PAGE followed by immunoblotting.

For the identification of Zuo1's cross-link partner immunoprecipitations were performed under denaturing conditions. To this end, high-salt washed ribosomes (20 nM) and purified RAC (40 nM) were preincubated at 21°C for 15 min in cross-link buffer. BS³ was added to a final concentration of 2 mM, and the reaction was incubated for 30 min on ice before cross-linking was terminated by the addition of 15 mM glycyl-glycine. Reactions were precipitated with 5% trichloroacetic acid, and protein pellets were resuspended in immunoprecipitation buffer (200 mM Tris-HCl, pH 7.5, 4% SDS, 10 mM EDTA, 100 μg/ml ovalbumin, 1 mM PMSF, and protease inhibitor mix containing 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastinal, and 5 μg/ml pepstatin A). In a standard reaction, 30 μl of the samples were diluted into 600 μl TNET buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 25 μg/ml ovalbumin, 1% Triton X-100, 0.5 mM PMSF, and protease inhibitor mix). Protein A Sepharose beads, 30 μl,

precoated with antibodies directed against Zuo1 were added, and the reaction was incubated overnight at 4°C on a shaker. Aliquots of supernatant and the material bound to protein A Sepharose beads were analyzed by SDS-PAGE followed by Coomassie G-250 staining (Neuhoff *et al.*, 1988). The band corresponding to the Zuo1 cross-link was excised from the gel and analyzed by mass spectrometry.

Ribosome Profiles

Yeast cells at an OD₆₀₀ of 0.4 were harvested in the presence of 100 µg/ml cycloheximide. Preparation of extracts was carried out by glass bead disruption in 20 mM HEPES-KOH, pH 7.4, 100 mM KAcetate, 2 mM MgAcetate, 100 µg/ml cycloheximide, and 0.5 mM DTT as described (Ashe *et al.*, 2000). Of each lysate, 80–120 µl corresponding to 10 A₂₆₀ units were loaded onto a 15–55% linear sucrose gradient. After centrifugation for 2.5 h at 200,000 × g (TH641, Sorvall Instruments, Newton, CT) gradients were fractionated from top to bottom with a density gradient fractionator monitoring A₂₅₄ (Teledyne Isco, Lincoln, NE).

Preparation of Ribosomes and Ribosome-Binding Assay

A 10-l culture of yeast grown on YPD was harvested at an OD₆₀₀ of 2.0. Cells were washed with ice-cold water and were subsequently resuspended in 200 ml sorbitol buffer (1.4 M sorbitol, 50 mM KAcetate, and 10 mM DTT) at 25°C. To generate spheroblasts Zymolyase 20T was directly dissolved in the cell suspension to a final concentration of 2.5 mg/gram of cells, and the mixture was incubated at 30°C for 40 min with gentle shaking. Spheroblasts were harvested by centrifugation at 4°C for 5 min at 3800 × g, were washed three times with a total of 0.9 l of ice-cold sorbitol buffer containing 5 mM DTT and were subsequently resuspended in lysis buffer (20 mM HEPES-KOH, pH 7.4, 120 mM KAcetate, 2 mM MgAcetate, 5 mM DTT, 1 mM PMSF, and 1× protease inhibitor mix). The suspension was homogenized with 20 strokes in a glass homogenizer (Bellco, Vineland, NJ). Ribosomes were isolated by consecutive centrifugation for 15 min at 26,900 × g (SS34, Sorvall), 30 min at 81,000 × g (T647.5, Sorvall), and 2 h at 207,000 × g (TI70.1, Beckman, Fullerton, CA). The resulting pellet was resuspended in 1–2 ml of lysis buffer and is referred to as low-salt washed ribosomes. High-salt washed ribosomes were prepared by adjusting low-salt washed ribosomes to 800 mM KAcetate followed by centrifugation through a cushion containing 25% sucrose and 800 mM KAcetate in lysis buffer at 247,000 × g (TLA100.2, Beckman). The resulting pellet was resuspended in lysis buffer and is referred to as high-salt washed ribosomes. Aliquots of low- and high-salt washed ribosomes were stored at –80°C.

To assess the stability of RAC–ribosome complexes, ribosomes were isolated at increasing salt concentrations as previously described (Gautschi *et al.*, 2001). In brief, aliquots of yeast ribosomes resuspended in lysis buffer were adjusted to the indicated KAcetate concentration in a total of 60 µl. Samples were layered on top of a 90-µl sucrose cushion (25% sucrose, 20 mM HEPES-KOH, pH 7.4, 2 mM MgAcetate, 2 mM DTT, 1 mM PMSF, 1× protease inhibitor mix, and KAcetate as indicated). After centrifugation at 217,000 × g (TLA100.1, Beckman) for 120 min at 4°C, aliquots of supernatant, ribosomal pellet, and a total corresponding to the amount loaded onto the cushion were analyzed by SDS-PAGE followed by immunoblotting.

Miscellaneous

Purification of RAC was performed as described (Conz *et al.*, 2007). A polyclonal rabbit antibody was generated using as an antigen Rpl31a purified after expression of the N-terminally His-tagged protein in *Escherichia coli* (pET28a, Novagen, Madison, WI). Total yeast protein for immunoblot analysis and quantification was prepared as described (Yaffe and Schatz, 1984). Immunoblots were developed using ECL. Quantification of ribosomes and RAC was performed as described (Raue *et al.*, 2007). AIDA Image Analyzer (Raytest, Straubenhardt, Germany) was used for the quantification. Preparation of cell extracts, determination of β-galactosidase and luciferase activity, and calculation of the read-through efficiencies was performed as described (Rakwalska and Rospert, 2004).

RESULTS

RAC Can Be Cross-linked to a Single Ribosomal Protein

To localize RAC on the ribosome and to identify potential ribosomal proteins that interact with RAC we have chosen a cross-linking approach. To that end, purified RAC was allowed to rebind to ribosomal particles stripped of interacting proteins. Subsequently RAC–ribosome complexes were isolated and were treated with the amino-reactive homobifunctional cross-linker BS³. The procedure yielded a prominent cross-link product with molecular mass below 80 kDa that was detectable with Zuo1-specific antibodies (Figure 1A). Immunoprecipitation under denaturing conditions con-

firmed that Zuo1 was contained in the cross-link product (Figure 1A). To identify the protein with which Zuo1 formed the cross-link, the procedure was scaled up, and the cross-link was excised from a Coomassie-stained gel and analyzed by mass spectrometry (Figure 1B). Analysis revealed two peptides corresponding specifically to either Rpl31a or Rpl31b, two 12.8-kDa proteins, which belong to the large group of ribosomal proteins that exist in two nearly identical copies. Rpl31a and Rpl31b differ in only one amino acid and are referred to as Rpl31. The Rpl31 homolog from archaea is one of the proteins localized at the rim surrounding the exit of the polypeptide tunnel (Ban *et al.*, 2000; Figure 1E). Interaction with Rpl31 would position RAC ideally to act on nascent polypeptides. However, the spacer length of BS³ is 11.4 Å, and therefore Rpl31 and Zuo1 must not be in direct contact to be cross-linked by this reagent. To test for direct interaction, we have used the zero-length cross-linker, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), which couples carboxyl groups to primary amines directly. Zuo1 and Rpl31 were efficiently cross-linked also by EDC, indicating that RAC touches Rpl31 when bound to the ribosome (Figure 1C; compare also Figure 4C). The prominent cross-link detected at a molecular mass of ~120 kDa was to the Zuo1 partner Ssz1 as it was detected not only by antibodies directed against Zuo1 but also Ssz1 (Figure 1D).

Δrpl31aΔrpl31b Is Viable But Suffers from Growth Defects Resembling Defects of Δzuo1

To characterize the function of Rpl31, we have generated strains Δrpl31a, Δrpl31b, and the double deletion strain Δrpl31aΔrpl31b. The total level of Rpl31 was only moderately reduced in Δrpl31b, whereas expression in Δrpl31a was significantly lower than in the wild-type strain (Figure 2A). Δrpl31b grew like wild type under the conditions tested, Δrpl31a and Δrpl31aΔrpl31b displayed slow growth at 20, 30, and 37°C. At 37°C the double deletion strain was more severely affected than Δrpl31a (Figure 2A). Thus, growth defects correlated with the expression level of Rpl31. Although slow growth and cold sensitivity is frequently observed in the absence of a nonessential ribosomal component, a defect at elevated temperature is uncommon (Aguilera *et al.*, 2007). As a control, expression of Rpl31a from a plasmid in the Δrpl31aΔrpl31b background rescued all growth defects (Figure 2A).

Next we compared Δrpl31aΔrpl31b and Δzuo1 at different growth conditions. A characteristic of Δzuo1 or Δzuo1Δssz1 strains is hypersensitivity toward aminoglycosides such as paromomycin. Thus, this drug was included in the analysis. Paromomycin increases the frequency of translational errors (Palmer *et al.*, 1979). Sensitivity toward the drug correlates with increased stop codon read-through and effects on programmed –1 ribosomal frameshifting of strains lacking functional RAC in vivo and in vitro (Rakwalska and Rospert, 2004; Muldoon-Jacobs and Dinman, 2006). Δrpl31aΔrpl31b and Δzuo1 displayed similar defects at 20°C or in the presence of paromomycin. At 30°C slow growth was more pronounced in Δrpl31aΔrpl31b compared with Δzuo1 (Figure 2B). The difference at 30°C likely relates to the commencing temperature-sensitive phenotype of Δrpl31aΔrpl31b, which is absent from Δzuo1 (Figure 2B). To test for genetic interaction between RPL31a/b and ZUO1, the triple deletion strain Δzuo1Δrpl31aΔrpl31b was generated. Growth defects did not exceed defects of the individual mutants (Figure 2B). This lack of synthetic effects is consistent with a scenario in that Zuo1 can still bind to ribosomes when

Figure 1. The zuotin subunit of RAC binds to ribosomes in close proximity of Rpl31. (A) Cross-linking of high-salt washed ribosomes after rebinding of purified RAC using the homobifunctional, amino-reactive cross-linker BS³ (+). As a control an aliquot was incubated without addition of BS³ (−). Aliquots with (+) or without (−) BS³-treatment were applied to immunoprecipitation reactions under denaturing conditions using protein A Sepharose beads coated with α-Zuo1. Zuo1-X, cross-link detected with α-Zuo1; U, material unbound; B, material bound to beads coated with α-Zuo1 after immunoprecipitation. Immunoblots were decorated with α-Zuo1. (B) Scale-up of the experiment shown in A. Coomassie-stained gel of the material bound to beads coated with α-Zuo1. The band labeled as Zuo1-Rpl31 was detected only in the sample treated with BS³. (C) Cross-linking of low-salt washed ribosomes with the zero-length cross-linker EDC (+). A mock reaction without addition of EDC is shown as a control (−). Zuo1-Rpl31 indicates the cross-link between Zuo1 and Rpl31. Immunoblots were developed with α-Zuo1 or α-Rpl31, as indicated. For details compare *Materials and Methods*. (D) Cross-linking of cell lysate with (+) or without (−) the cross-linker EDC. Zuo1-Ssz1 indicates the cross-link between Zuo1 and its partner subunit Ssz1 detected with α-Zuo1 and α-Ssz1. (E) Crystal structure of the large ribosomal subunit of the archaea *Haloarcula marismortui* (PDB: 1S72 and 1JJ2; Ban *et al.*, 2000; Klein *et al.*, 2001). rRNA in yellow, ribosomal proteins in magenta, ribosomal proteins of the platform around the tunnel exit (E) in violet. The nomenclature of the ribosomal proteins is according to Lecompte

Rpl31 is missing, however, does not properly function. Consistent with such a model, overexpression of RAC in a $\Delta rpl31a\Delta rpl31b$ strain failed to suppress any of the growth defects (Figure 2B).

$\Delta rpl31a\Delta rpl31b$ Displays Defects in Translational Fidelity

Growth of wild type, $\Delta rpl31a\Delta rpl31b$, and $\Delta zuo1\Delta ssz1$ was compared in the presence of increasing concentrations of paromomycin. As a result, $\Delta rpl31a\Delta rpl31b$ and $\Delta zuo1\Delta ssz1$ displayed similar sensitivity with half-maximal inhibition at app. 5 μ M paromomycin (Figure 2C). Translational fidelity in the absence of Rpl31 was directly tested using an in vivo dual reporter construct in which β -galactosidase and luciferase are separated by a single in-frame stop codon (Figure 2D). If the stop codon is recognized β -galactosidase is produced, however, luciferase is not. If an amino acid is incorporated erroneously at the position of the stop codon, the read-through event results in the expression of a β -galactosidase-luciferase fusion protein. Based on this assay, the basal read-through level of $\Delta rpl31a\Delta rpl31b$ was increased (Figure 2E). Read-through was enhanced further when $\Delta rpl31a\Delta rpl31b$ was grown in the presence of paromomycin, whereas no such effect was detected for the wild-type strain (Figure 2E). The combined data indicate that Rpl31, like Zuo1 (Rakwalska and Rospert, 2004), affects the fidelity of translation.

$\Delta rpl31a\Delta rpl31b$ Displays Defects in the Assembly of Ribosomal Subunits

Ribosome profiles of $\Delta rpl31a\Delta rpl31b$ showed a typical halfmer polysome pattern, indicative of inefficient association of large subunits with small subunit translation initiation complexes (Figure 3A). Consistently, the relative level of free small ribosomal subunits was increased in $\Delta rpl31a\Delta rpl31b$ compared with wild type (Figure 3A). The profiles also revealed that the total concentration of ribosomes in $\Delta rpl31a\Delta rpl31b$ was significantly reduced. To determine the decrease of large ribosomal subunits more precisely the concentration of ribosomal protein Rpl17a/b was determined by quantitative immunoblotting using purified Rpl17a as a standard (Raue *et al.*, 2007; Figure 3B). Rpl17 was reduced to ~40% of the wild-type level in $\Delta rpl31a\Delta rpl31b$ (Figure 3, B and C). The combined data indicate that $\Delta rpl31a\Delta rpl31b$ suffers from a deficiency in the concentration of ribosomes and from inefficient subunit assembly.

It is not obvious how Rpl31 at the tunnel exit of the large ribosomal subunit affects decoding at the small ribosomal subunit from which it is ~100 Å away (Rospert *et al.*, 2005b). However, long-range intraribosomal communication has been observed also between the decoding and the GTPase center of the ribosome, which are separated by ~70 Å (Steitz, 2008). Moreover, the absence of yeast Rpl39 at the tunnel exit was previously reported to cause translational errors and cause a halfmer polysome pattern (Dresios *et al.*, 2000; Figure 3E). Based on this behavior, it has been suggested that Rpl39 may allosterically transmit an effect along the polypeptide tunnel to the decoding center (Dresios *et al.*,

(Lecompte *et al.*, 2002). The appendix “e” indicates ribosomal proteins confined to archaea and eukaryotes. *H. marismortui* proteins in close proximity to the tunnel exit correspond to yeast proteins: L24 is the homolog of Rpl26, L29 of Rpl35, L39e of Rpl39, L23 of Rpl25, L19e of Rpl19, L31e of Rpl31, and L22 of Rpl17. The structural representation was prepared with Pymol (<http://pymol.sourceforge.net/>).

Figure 2. Yeast strains lacking Rpl31 display slow growth, sensitivity to high- and low-growth temperatures and suffer from defects in translational fidelity. (A) Phenotypic comparison of wild type, $\Delta rpl31a$, $\Delta rpl31b$, $\Delta rpl31a\Delta rpl31b$, and $\Delta rpl31a\Delta rpl31b$ expressing *RPL31a* from a low copy number plasmid. Haploid yeast strains were grown to early log phase at 30°C on minimal glucose medium. Serial 10-fold dilutions containing the same number of cells were spotted onto YPD plates and were incubated as indicated. (B) Phenotypic comparison of wild type, $\Delta rpl31a\Delta rpl31b$, $\Delta zuo1$, $\Delta rpl31a\Delta rpl31b\Delta zuo1$, and $\Delta rpl31a\Delta rpl31b$ expressing *ZUO1* and *SSZ1* from high copy number plasmids. Strains were grown as in A. Paro corresponds to 50 μ g/ml paromomycin. Total cell extracts of strains shown in A and B were analyzed by immunoblotting using antibodies specifically recognizing Rpl31, and the RAC subunits, Ssz1 and Zuo1, as indicated. (C) Wild-type, $\Delta rpl31a\Delta rpl31b$, and $\Delta zuo1\Delta ssz1$ strains were inoculated to the same OD₆₀₀ on YPD or into the same medium containing paromomycin as indicated. Cultures were grown until the control without paromomycin had reached OD₆₀₀ = 1.0, which was set to 100%. (D) Reporter construct for the determination of the frequency of stop codon read-through in vivo. An in-frame fusion between β -galactosidase and luciferase (*lacZ-luc*) served to determine the relative enzymatic activities upon equimolar expression of the two enzymes. Read-through efficiency was determined using the *lacZ-STOP-luc* construct in which the two enzymes were separated by a stop codon (for details compare Results and Materials and Methods). (E) Efficiency of stop codon read-through in wild-type and $\Delta rpl31a\Delta rpl31b$ strains in the presence of increasing concentrations of paromomycin. Enzymatic activities were determined in extracts derived from cells grown to an OD₆₀₀ of 0.4–1 on YPD or YPD supplemented with paromomycin as indicated. Read-through frequency is expressed as the ratio of luciferase/ β -galactosidase activity ([RLU]/[A₄₂₀]) obtained with the *lacZ-STOP-luc* construct normalized to the ratio obtained with *lacZ-luc* as described in Rakwalska and Rospert (2004). Experiments were performed at least in triplicate; error bars, SD.

2001). Prompted by the phenotypic similarities, we tested for genetic interaction between *RPL31a/b* and *RPL39*. Dissection and tetrad analysis of a diploid strain in which each one allele of *RPL31a*, *RPL31b*, and *RPL39* was deleted revealed that none of the viable haploids harbored deletions of all 3 genes (data not shown). The result indicated that a combination of $\Delta rpl31a\Delta rpl31b$ and $\Delta rpl39$ in a haploid strain was synthetically lethal. Moreover, an extra copy of *RPL39* on a centromeric plasmid partly suppressed growth defects of $\Delta rpl31a\Delta rpl31b$ (Figure 3D). Vice versa growth defects of $\Delta rpl39$ were not suppressed by increasing the copy number of *RPL31a*. Suppression of defects in $\Delta rpl31a\Delta rpl31b$ went along with a moderate increase of the Rpl39 expression level (Figure 3D). As ribosomal proteins that do not assemble into ribosomal

particles normally do not accumulate but are rapidly degraded (Fromont-Racine *et al.*, 2003), the result suggests that Rpl39 is inefficiently incorporated into ribosomes lacking Rpl31 ($\Delta 31$ -ribosomes), and higher levels of Rpl39 result in more efficient incorporation. As a control we have also increased the copy number of Rpl17a and Rpl24a, two other proteins of the large ribosomal subunit. Neither *RPL17a* nor *RPL24a* affected growth of $\Delta rpl31a\Delta rpl31b$, suggesting a specific coupling between Rpl31 and Rpl39 (Figure 3D). Higher level of *RPL39* in the $\Delta rpl31a\Delta rpl31b$ background did not result in an attenuation of the abnormal ribosome profile (Figure 3E). This suggests that suppression of $\Delta rpl31a\Delta rpl31b$ by *RPL39* was neither primarily via an effect on $\Delta 31$ -ribosome assembly nor on subunit joining (Figure 3D).

Figure 3. Phenotypic defects of yeast strains lacking Rpl31 can be partly suppressed by high levels of Rpl39. (A) Ribosome profiles of logarithmically growing wild-type and $\Delta rpl31a\Delta rpl31b$ strains. Fractionation of the gradients was monitored at 254 nm. Asterisks indicate the position of halfmer ribosomes. (B) Analysis of large subunit concentration in wild type and $\Delta rpl31a\Delta rpl31b$. Total cell extract corresponding to 0.6×10^7 cells of logarithmically growing wild type and $\Delta rpl31a\Delta rpl31b$ was separated on 10% TRIS-Tricine gels. Purified Rpl17 was applied to the same gel and was analyzed by immunoblotting using α -Rpl17 as a large ribosomal subunit marker. The presence of untagged Rpl17a in the standard is due to the addition of total extract as a carrier (Raue *et al.*, 2007). Note that the purified, His₆-tagged standard protein has a slightly higher molecular mass. (C) Quantification of ribosomes in wild type (white) and $\Delta rpl31a\Delta rpl31b$ (gray). Densitometric analysis was performed to determine the range of linearity and to quantify protein concentrations in the total cell extracts. Error bars, SD. (D) Wild type, $\Delta rpl31a\Delta rpl31b$, $\Delta rpl31a\Delta rpl31b$ harboring the empty vector, or expressing either Rpl17a, Rpl24a, or Rpl39, $\Delta rpl39$, and $\Delta rpl39$ expressing Rpl31 were grown and analyzed as in Figure 2A. Paro corresponds to 50 μ g/ml paromomycin. Total cell extracts were analyzed by immunoblotting using antibodies specifically recognizing Sse1 (as a loading control), Rpl17, Rpl31, Rps9, Rpl24, and Rpl39. (E) Ribosome profiles of logarithmically growing $\Delta rpl31a\Delta rpl31b$, $\Delta rpl39$, and $\Delta rpl31a\Delta rpl31b$ expressing Rpl39 from a low copy plasmid were performed as described in A.

Zuo1 Touches Rpl31, However, the Interaction Is Not Essential for Association of RAC with Ribosomes

Localization revealed that the bulk of RAC cofractionated with $\Delta 31$ -ribosomes on sucrose gradients (Figure 4A). The occupation of $\Delta 31$ -ribosomes (RAC bound to 55% of $\Delta 31$ -ribosomes) was even higher than that of wild-type ribosomes (RAC bound to 33% of ribosomes; Figure 4B). The data are consistent with the relatively constant concentration of RAC but decreased concentration of ribosomes in $\Delta rpl31a\Delta rpl31b$ compared with wild type (Figures 2B and 3C and data not shown). Cross-linking experiments with $\Delta 31$ -ribosomes confirmed that the cross-link of Zuo1 to Rpl31 could no longer be formed (Figure 4C). Moreover, no significant bands corresponding to a molecular mass compatible with a cross-link between Zuo1 (49 kDa) and other core ribosomal proteins (<40 kDa) emerged on $\Delta 31$ -ribosomes. This suggests that Zuo1 did not employ an alternative ribosomal protein as a binding site in the absence of Rpl31. We conclude that Rpl31 is not essential for stable interaction of RAC with ribosomes and that growth defects observed in $\Delta rpl31a\Delta rpl31b$ are not related to a general loss of RAC binding to ribosomes.

Zuo1 Contains an Extended Interface for Ribosome Interaction

An internal, highly charged region localized between amino acids 285 and 364 of Zuo1 is important for Zuo1's interaction with the ribosome as well as for its ability to interact with nucleic acids (Yan *et al.*, 1998). We have generated a slightly shorter deletion mutant lacking amino acids 282–331. Zuo1 $\Delta 282$ –331 formed a complex with Ssz1 but the complex

was not bound to ribosomes under physiological conditions (data not shown). Consistent with previous results (Yan *et al.*, 1998), failure to interact with ribosomes correlated with a loss of function phenotype and Zuo1 $\Delta 282$ –331 was unable to complement growth defects of a $\Delta zuo1$ strain (Figure 5A). To more precisely define the interaction surface on Zuo1, we have performed a peptide scan and probed it for interaction with ribosomes. The strongest interaction was detected for a segment of approximately 10 amino acids located between position 296 and 305 of Zuo1 (Figure 5B, box 4). Other possible interacting segments mapped outside of the region between amino acids 282 and 331 that was required for Zuo1 ribosome interaction (Figure 5B). To test the contribution of amino acids 296–305 for interaction, we have replaced within and in close proximity of this segment a total of 15 lysines, asparagines, and glutamates with alanines (Figure 5C). However, the resulting mutant, Zuo1-15A was able to interact with ribosomes (Figure 5D, $\Delta zuo1$ + Zuo1-15A). Only when expressed in the triple knockout background $\Delta rpl31a\Delta rpl31b\Delta zuo1$, Zuo1-15A displayed a defect in ribosome binding, with the bulk of the protein recovered in the cytosolic fractions of a ribosome profile (Figure 5D, $\Delta rpl31a\Delta rpl31b\Delta zuo1$ + Zuo1-15A). Lastly, we have determined relative stabilities of different RAC-ribosome complexes by probing RAC-release under conditions of increasing ionic strength. The concentration of KAcetate resulting in 50% RAC release was ~340 mM for RAC-ribosome, 300 mM for RAC- $\Delta 31$ -ribosome, 260 mM for RAC-15A-ribosome, and 220 mM for RAC-15A- $\Delta 31$ -ribosome complexes (Figure 5E). The data suggest that, while the segment between 296 and 305 of Zuo1 contributes to ribosome binding, other

Figure 4. Rpl31 is not essential for binding of RAC to ribosomes. (A) Ribosome profiles were run as described in Figure 3A. After fractionation aliquots were analyzed by immunoblotting using α -Ssz1/ α -Zuo1 (RAC), α -Rps9, and α -Rpl24 as indicated. One-twentieth of the material loaded onto the sucrose gradient was analyzed as a total (T). (B) Quantification of Zuo1 in wild type and $\Delta rpl31a\Delta rpl31b$. Ribosomes were isolated under low-salt conditions. Analysis of isolated ribosomes was then performed as described in Figure 3, B and C using purified RAC and Rps9a as a standard for the quantifications. The experiments were performed in triplicate. The occupation of ribosomes with Zuo1 is given in percent (Raue *et al.*, 2007). (C) Zuo1 does not form an alternative cross-link to a ribosomal protein in the absence of Rpl31. Cross-linking with BS³ was performed on low-salt washed ribosomes as described in *Materials and Methods*. Immunoblots were analyzed with α -Zuo1 and with α -Rpl17 as a control.

interaction surfaces, probably localized outside the charged region, must exist.

DISCUSSION

Zuo1 Interaction with Ribosomes

We find that ribosomal protein Rpl31 interacts with Zuo1 physically; however, this interaction is not crucial to anchor Zuo1 to ribosomes. The mild decrease in salt resistance of RAC- Δ 31-ribosome complexes may reflect this protein-protein interaction. Alternatively, the lack of Rpl31 may cause structural changes within the ribosome that in turn affect Zuo1 binding. This may resemble the situation of RAC binding to Δ 39-ribosomes, which we found to be destabilized to a similar extent (data not shown). In the case of Rpl39 it is unlikely that the destabilization is due to direct interaction with RAC. Rpl39 is only marginally exposed on the surface of the ribosome and we were unable to detect cross-links to Zuo1 (data not shown). We cannot exclude that our cross-linking approach failed to uncover an additional ribosomal protein that interacts with Zuo1. However, our data rather support the previous hypothesis (Yan *et al.*, 1998) that stable Zuo1 ribosome binding is predominantly achieved via interaction with rRNA. Genetic evidence suggests relevance of the Zuo1 and Rpl31 contact in respect to function. The deletion mutants $\Delta rpl31a\Delta rpl31b$

Figure 5. A highly charged segment within Zuo1 affects the affinity for ribosomes. (A) Wild type, $\Delta zuo1$, $\Delta zuo1$ expressing Zuo1-15A, or $\Delta zuo1$ expressing Zuo1 Δ 282-331 were grown as in Figure 2A. paro, 200 μ g/ml paromomycin. (B) Scan of a Zuo1 peptide library for segments interacting with ribosomes. Zuo1-15mer peptides on the cellulose membrane are shifted by one amino acid per spot. The cellulose membrane was incubated with high-salt washed ribosomes and subsequently ribosomes were transferred to nitrocellulose and analyzed by immunoblotting using α -Rpl16 as a ribosomal marker. Box1 corresponds to amino acids 119–130, box2 to amino acids 243–253, box3 to

and $\Delta zuo1$ share growth defects like slow growth, cold sensitivity, and paromomycin sensitivity. If these phenotypes of $\Delta rpl31a\Delta rpl31b$ and $\Delta zuo1$ would be due to defects in independent functional entities, one would expect $\Delta rpl31a\Delta rpl31b\Delta zuo1$ to suffer from enhanced growth defects. This is not the case, but interestingly, it is exactly what we observed for the combination of $\Delta rpl31a\Delta rpl31b$ and $\Delta rpl39$, which results in synthetic lethality. It is tempting to speculate that RAC and Rpl31, similar to RAC and Ssb1/2 (Gautschi *et al.*, 2002; Hundley *et al.*, 2002), form a unit in which proper RAC function requires the presence of Rpl31. In contrast, Rpl31 and Rpl39 seemingly exert independent effects on the same ribosomal function.

Within Zuo1 we have identified a segment of 10 amino acids that showed strong interaction with ribosomes in a peptide scan. This segment localized to the highly charged region, a deletion of which abolishes interaction between Zuo1 and ribosomes (Yan *et al.*, 1998). It was thus quite unexpected that severe mutations within this segment showed only minor effects on Zuo1-ribosome interaction. Because 296–305 was the only segment within Zuo1's charged region that displayed significant binding to ribosomes, it seems possible that important contacts are made by other domains of Zuo1. Recent analysis of protein modules mediating RNA binding also point in this direction. Although such modules are diverse, a general theme is strong enrichment of arginines and lysines combined with a significant underrepresentation of glutamates and aspartates (Weiss and Narayana, 1998; Chen and Varani, 2005; Terribilini *et al.*, 2006). Interestingly, the charged region between amino acids 282 and 331, the deletion of which leads to loss of ribosome binding (Figure 5, A and C) contains 5 arginines and 11 lysines, but also contains 14 glutamates and 1 aspartate. Possibly, loss of binding of Zuo1 Δ 282–331 reflects structural alterations within Zuo1 rather than loss of its primary binding surface. Independent of the question whether or not the charged region of Zuo1 provides the prime ribosome interaction surface, the mode of Zuo1's interaction seems to differ from RPBs previously characterized. Eubacterial trigger factor and eukaryotic NAC contain well-defined, rather small binding surfaces (Kramer *et al.*, 2002; Wegrzyn *et al.*, 2006) and ERj1, a membrane-bound homolog of Zuo1 also requires only a short and well-defined motif for stable interaction (Dudek *et al.*, 2002, 2005; Blau *et al.*, 2005). Binding of SRP might be more complex in that it involves several domains (Pool *et al.*, 2002; Gu *et al.*, 2003; Ullers *et al.*, 2003; Halic *et al.*, 2004, 2006).

Figure 5 (cont). amino acids 257–269, box4 to amino acids 296–305. (C) Amino acid sequence of Zuo1. Interacting segments identified in B are indicated. Amino acids 282–331 are shaded in gray. Mutations introduced in box4, and adjacent amino acids are shown in the bottom panel. (D) Ribosome profiles of $\Delta zuo1$ expressing Zuo1-15A and of $\Delta zuo1\Delta rpl31a\Delta rpl31b$ expressing Zuo1-15A were performed as described in Figure 3A. After fractionation aliquots were analyzed by immunoblotting using α -Ssz1/ α -Zuo1 (RAC), α -Rps9, and α -Rpl24. (E) Zuo1-15A binding to ribosomes is destabilized in the presence of high concentrations of salt. Salt-dependent release of RAC from ribosomes derived from wild type, $\Delta rpl31a\Delta rpl31b$, $\Delta zuo1$ + Zuo1-15A, and $\Delta zuo1\Delta rpl31a\Delta rpl31b$ + Zuo1-15A. Separation of extracts (tot) into a postribosomal supernatant (S) and a ribosomal pellet (P) was performed at increasing concentrations of KAcetate as indicated (mM KAc). Aliquots were separated on 10% TRIS-Tricine gels and subsequently analyzed by immunoblotting using α -Ssz1/ α -Zuo1 (RAC), and α -Rpl17 as a ribosomal marker. Boxed is the concentration of KAcetate that resulted in ~50% of RAC release.

Function of Ribosomal Protein Rpl31

Rpl31 belongs to the eukaryotic ribosomal proteins which are not conserved in eubacteria but possess an archaeobacterial homolog. In eubacteria an unrelated protein, L17 occupies the location of Rpl31 at the exit tunnel platform (Harms *et al.*, 2001). It has been suggested that proteins not conserved between eubacteria and archaea/eukaryotes have arrived by convergent evolution with the main purpose to fill the cracks between rRNA helices and stabilize the structure (Klein *et al.*, 2004). However, in the case of Rpl31, which exposes large parts to the exterior of the ribosome (Figure 1E) this seems an unlikely scenario. Possibly, Rpl31 has coevolved to function with RPBs that are restricted to the eukaryotic system. Besides RAC, possible candidates would be the Hsp70 homolog Ssb1/2 or the methionine aminopeptidases for which up to now no binding sites have been identified. However, our analysis suggests that none of the known RPBs was severely affected with respect to ribosome association in the absence of Rpl31 (data not shown). Only in the case of NAC we have observed that a significant fraction was recovered in the cytosol and not attached to ribosomes in a $\Delta rpl31a\Delta rpl31b$ strain. This increase in unbound NAC, however, was due to the shortfall in Δ 31-ribosomes compared with wild-type ribosomes and NAC covered all available Δ 31-ribosomes (data not shown).

It was previously reported that yeast deletion strains lacking either *RPL31a* or *RPL31b* are viable (Winzeler *et al.*, 1999; Enyenihi and Saunders, 2003). Deletion of the gene encoding *RPL31a*, but not *RPL31b*, increases replicative life span in yeast (Kaeberlein *et al.*, 2005; Steffen *et al.*, 2008). Here we show that the double deletion strain $\Delta rpl31a\Delta rpl31b$ is viable but displays severe growth defects. Rpl31 thus belongs to the group of ribosomal proteins with nonessential functions. To date, at the tunnel platform the only other protein dispensable for the life of yeast is Rpl39 (Dresios *et al.*, 2000). Rpl19 (Song *et al.*, 1996), Rpl17 (Winzeler *et al.*, 1999), and Rpl25 are essential components of the yeast ribosome. In the case of Rpl26 and Rpl35, encoded each by two closely related genes, the phenotypes of the respective double deletion strains have so far not been reported (Dresios *et al.*, 2006). We here report synthetic lethality of $\Delta rpl31a\Delta rpl31b\Delta rpl39$. According to the crystal structure of the archaeal ribosome Rpl31 does not directly contact any ribosomal protein but contacts domains III, IV, and VI of the 25S rRNA, whereas Rpl39 contacts rRNA of domains I and III (Ban *et al.*, 2000; Nissen *et al.*, 2000). Interconnection of Rpl31 and Rpl39 via domain III of 25S rRNA may cause, or at least contribute, to the synthetic effect. Such a scenario is supported by the observation that Rpl31 as well as Rpl39 (Dresios *et al.*, 2000) affect translational fidelity. As previously discussed Rpl39 forms part of the polypeptide exit and is thus ideally positioned to allosterically transmit effects along the tunnel to the decoding center (Dresios *et al.*, 2001; Rospert, 2004). How this may function on a molecular level and whether Rpl31 and RAC contribute to this intraribosomal signaling cascade awaits further investigation.

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