Specialized ribosomes and specific ribosomal protein paralogs control translation of mitochondrial proteins

Nadav Segev and Jeffrey E. Gerst

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

Genome duplication in eukaryotes created paralog pairs of ribosomal proteins (RPs) that show high sequence similarity/identity. However, individual paralogs can confer vastly different effects upon cellular processes, e.g., specific yeast paralogs regulate actin organization, bud site selection, and mRNA localization, although how specificity is conferred is unknown. Changes in the RP composition of ribosomes might allow for specialized translation of different subsets of mRNAs, yet it is unclear whether specialized ribosomes exist and if paralog specificity controls translation. Using translatome analyses, we show that the translation of mitochondrial proteins is highly down-regulated in yeast lacking RP paralogs required for normal mitochondrial function (e.g., RPL1b). Although RPL1a and RPL1b encode identical proteins, Rpl1b-containing ribosomes confer more efficient translation of respiration-related proteins. Thus, ribosomes varying in RP composition may confer specialized functions, and RP paralog specificity defines a novel means of translational control.

Introduction

Translation regulation allows cells to rapidly alter their proteome in response to environmental changes and different cellular stimuli (Sonenberg and Hinnebusch, 2009; Spriggs et al., 2010), and it encompasses several layers of control. These include the interaction with ribosome-associated proteins (Fleischer et al., 2006), binding and recognition of mRNA motifs (Wilkie et al., 2003; Xue et al., 2015), mRNA localization (Kraut-Cohen et al., 2013; Buxbaum et al., 2015; Lesnik et al., 2015), and translation initiation (Sonenberg and Hinnebusch, 2009).

Although ribosomes were once conceptualized as uniform, fixed molecular machines that perform translation, it is now known that structural variations generate ribosome heterogeneity. For example, ribosomal RNA and ribosomal proteins (RPs) differ in their modification and composition within ribosomes to affect translation (Byrgazov et al., 2013; Ferretti et al., 2017; Shi et al., 2017). In yeast, 59 of the 79 RPs are encoded by paralog pairs and, although a pair may display high sequence identity, the deletion of a specific paralog can lead to phenotypes in actin regulation (Haarer et al., 2007), sporulation (Enyenihi and Saunders, 2003), bud site selection (Ni and Snyder, 2001), and mRNA localization (Komili et al., 2007). A paralog specificity model called the “ribosomal code” suggests that different RP paralogs may regulate specific physiological processes. Thus, distinct subpopulations of ribosomes (i.e., specialized ribosomes) that vary in RP paralog composition might reside within cells (Komili et al., 2007). These subpopulations might promote the translation of finite sets of mRNA and therefore yield specific translational patterns (Filipovska and Rackham, 2013).

Respiratory-deficient yeast mutants fail to grow on non-fermentable carbon sources (e.g., glycerol) and form small colonies on fermentable carbon sources (e.g., glucose; Day, 2013). Four strains bear mutations in nuclear-encoded RPs: Rpl1b, Rpl2b, Rps11a, and Rps26b (Dimmer et al., 2002; Steinmetz et al., 2002; Merz and Westermann, 2009). Importantly, each paralog has an identical or near-identical copy in the genome that shows no deficiencies in respiration when mutated. Thus, RP paralogs exhibit functional differences in a manner that remains unknown.

To determine whether RP paralogs generate specialized ribosomes that shape the cellular translatome, we characterized the respiratory-deficient RP paralog mutants with respect to translational regulation. We found that specific paralogs (Rpl1b, Rpl2b, and Rps26b) are required for proper mitochondrial morphology and function. Moreover, translatome mapping using a nascent chain sequencing approach (puromycin-associated nascent chain proteomics; PUNCH-P; Aviner et al., 2014) showed that the rpl1bΔ translatome is distinct from respiratory-competent rpl1Δ cells, the former being deficient in the production of mitochondrial and cell wall proteins. This suggests that RP paralogs play a crucial role in translational control and, in the case of Rpl1b, directly affect mitochondrial function and cell wall integrity. The ability of specific RP paralogs to favor...
the translation of a subset of mRNAs reveals a novel layer of translational regulation.

Results and discussion

The pet phenotype is observed in specific RP paralog mutants

Yeast petite (pet) mutants form small colonies on fermentable carbon sources (e.g., glucose) and show little or no growth on nonfermentable carbon sources (e.g., glycerol). Of these, RPL1b, RPL2b, RPS11a, and RPS26b were identified as RP paralog genes necessary for normal respiratory growth. Three paralog pairs (RPL1a/b, RPL2a/b, and RPS11a/b) are encoded by identical ORFs, and the fourth (RPS26a/b) shows a high degree of similarity. To validate the contribution of each paralog to respiratory growth, we created new deletion mutants of RPL1b, RPL2b, RPS11a, and RPS26b as well as their corresponding paralogs. We examined growth of the different RP mutants on glucose-containing yeast peptone dextrose (YPD) and glycerol-containing YPGly medium at 30°C and 35°C and found that three (rpl1bΔ, rpl2bΔ, and rps26bΔ) displayed the pet phenotype (Fig. 1 a). We measured replication times of the deletion mutants in YPD and YPGly at 30°C (Fig. 1 b) and observed that although rpl1aΔ, rpl2aΔ, and rps26aΔ mutant strains grow similar to WT cells, their corresponding paralog mutants showed longer replicative times, especially on glycerol. However, the replication times of RP paralog pair deletions (e.g., rpl1aΔ/rpl2aΔ cells) showed no additive effects upon growth (Fig. S1 a). Likewise, the deletion of two respiration-related paralogs (e.g., rpl1bΔ/rpl2bΔ) grew similar to single mutants (e.g., rpl1bΔ or rpl2aΔ) and did not confer more severity. Thus, the respiration-related RP paralogs act in an epistatic fashion upon the same pathway.

RPL1b, RPL2b, or RPS26b are required for normal mitochondrial function and morphology

To determine whether the slow growth phenotype of rpl1bΔ, rpl2bΔ, and rps26bΔ cells is caused by a specific mitochondrial impairment, we examined mitochondrial morphology and functionality in the mutants. First, we imaged mitochondria using mitochondria-localized RFP (mtsRFP) and observed that their morphology in rpl1bΔ, rpl2bΔ, and rps26bΔ cells appeared aggregated and less tubular, especially on glycerol (Fig. 2 a). 3D reconstruction of the mitochondria illustrates a wide range in the diversity of morphological phenotypes observed between WT and mutant cells (Fig. 2 b). Second, we determined whether RP deletions affect mitochondrial function. We measured mitochondrial inner membrane potential (Δψ) as an indicator of functionality using tetramethylrhodamine ethyl ester (TMRE) and flow cytometry (Ludovico et al., 2001). The measured Δψ was exceptionally lower on glycerol for all three mutants (rpl1bΔ, rpl2bΔ, and rps26bΔ) when compared with their α paralogs of WT cells (Fig. 2 c). Analysis of the data also allowed for quantification of the changes observed in mitochondrial morphology. We calculated the mitochondrial signal/cell size ratio and found that it was lower for the respiration-deficient mutants (significantly in rpl1bΔ and rps26bΔ cells) in comparison with their corresponding paralog deletions or WT cells (Fig. S1 b). Thus, mitochondria are smaller/more aggregated in the respiration-deficient mutants, as observed visually (Fig. 2, a and b). These results indicate acute mitochondrial dysfunction and likely explain the slow growth phenotype of the rpl1bΔ, rpl2bΔ, and rps26bΔ cells.
RP paralogs show functional specificity on a nonfermentable carbon source

Ribosome assembly and biogenesis is coordinated by multiple levels of regulation, including RP expression (Warner, 1999; Woolford and Baserga, 2013). For example, expression may involve different parts of the RP mRNA transcript, such as the UTRs and introns (Parenteau et al., 2011). In parallel, cognate RP paralogs were proposed to regulate the level of the other paralog’s mRNA (Parenteau et al., 2015; Gabunilas and Chanfreau, 2016). However, alterations in the transcription of one paralog caused by deletion of the other do not correlate with changes in the level of RP protein (Parenteau et al., 2015; Liu et al., 2016). More importantly, RP paralogs do not show synonymous functional substitution but rather diversification. Komili et al. (2007) demonstrated that the overexpression of one RP paralog in the deletion mutant of its cognate partner may not fully complement the deletion phenotype. This finding led to the idea of paralog specificity, although there has been no firm evidence connecting paralog selectivity (in ribosomes) to changes in translation.

To verify that the rpl1bΔ, rpl2bΔ, and rps26bΔ phenotype is caused by paralog specificity and not a byproduct of alterations in gene expression, we measured mRNA levels of the paralogs in WT cells grown at 35°C on glucose or glycerol. The change in carbon source did not alter the a/b paralog expression ratio for any of the paralog pairs (Fig. 3 a).

To determine whether mutation of one RP paralog alters expression of the other, we examined the mRNA levels of each paralog upon deletion of its paralogous partner. mRNA levels of the different paralogs did not undergo noticeable changes in the absence of their cognates on YPD at 30°C, whereas increased levels of mRNA were observed for the RPL2 and RPS26 a paralogs in the b paralog deletion strains grown on YPGly at 35°C (Fig. 3 b). Thus, the removal of one paralog may influence expression of the other, but does not necessarily confer normal cellular physiology—hence the respiration-deficient growth observed in Fig. 1.

We determined whether overexpression of a given RP paralog can complement the absence of its cognate partner. We overexpressed a paralogs in b (respiration-deficient) deletion mutants and examined their ability to grow on YPD and YPGly at 30°C and 35°C (Fig. 3 c). Plasmid-based reexpression of the b paralogs fully rescued deletion of their genomic copies (Fig. S2 a). In contrast, overexpression of the corresponding a paralogs in rpl1bΔ, rpl2bΔ, and rps26bΔ cells could not fully compensate for loss of the respective b paralogs on glycerol.
but did confer normal growth on glucose (Fig. S2 b). Thus, the mitochondrial deficiencies observed upon the deletion of RPL1b, RPL2b, and RPS26b cannot be attributed to changes in mRNA levels alone, indicating that the a and b paralogs are not fully redundant.

Lastly, we asked whether the deletion of one paralog leads to a reduction in the total protein for a given RP, which might cause ribosome misassembly (McIntosh et al., 2011). Rpl1 protein levels in rpl1aΔ and rpl1bΔ cells grown on YPD or YPGly at 35°C were measured by Western blotting and compared with WT cells (Figs. 3 d and S2 c). A similar decrease in total Rpl1 levels was detected in either the rpl1aΔ or rpl1bΔ mutant (Fig. 3 d). Thus, the deficiencies in mitochondrial function and respiration seen in rpl1bΔ cells cannot be explained by a general reduction in Rpl1 protein.

**Translatome profiling of RP gene deletions reveals paralog specificity**

We hypothesized that specific paralogs might favor the translation of mRNAs encoding mitochondrial proteins (mMPs). Therefore, we adapted PUNCH-P, a technique that allows for translatome mapping by polysome purification and nascent polypeptide chain sequencing (Aviner et al., 2013, 2014), for use in yeast. PUNCH-P incorporates biotinylated puromycin into newly synthesized polypeptides in purified polysomes, which can be isolated using immobilized streptavidin.
and the peptides analyzed by mass spectrometry (MS). We isolated translating polysomes from the rpl1aΔ and rpl1bΔ deletion mutants as well as WT controls grown at 35°C and then mapped their translatomes by MS. We identified >500 translation products from cells grown on glucose and >1,900 proteins from cells grown on glycerol (Table S1, Sheets 1 and 4, respectively).

The translatomes of the different RP paralog deletion mutants were compared by hierarchal clustering (Fig. 4 a). Although the translatomes of rpl1aΔ and WT cells cocluster, they are completely separate from those of rpl1bΔ cells or the control sample (i.e., no added biotin-puromycin; p-values <0.05). Differential clustering of the translation profiles is also seen using principle components analysis (PCA), which groups the WT and rpl1aΔ samples while grouping rpl1bΔ and the control separately (Fig. S3 a). Correlation scores for the biological repeats (Fig. S3 b: WT, r = 0.83 ± 0.03; rpl1aΔ, r = 0.88 ± 0.03; and rpl1bΔ, r = 0.84 ± 0.04) also indicate the reproducibility of PUNCH-P. Thus, the translatome profiles are highly distinctive as a result of major changes in translation caused by the absence of a specific RP paralog (rpl1bΔ).

The rpl1bΔ translatome profile lacks mitochondria-related proteins. rpl1bΔ cells show a severe mitochondrial dysfunction phenotype (Figs. 1 and 2) and exhibit a significantly different trans-
latome profile from *rpl1aΔ* or WT cells (Fig. 4 a). To determine whether Rpl1b promotes translation of a specific subset of mRNAs (e.g., mMPs), we refined the translatome profiles from each growth condition (YPD or YPGly) to list proteins significantly missing from the *rpl1bΔ* translatome profile (Table S1; Lists 2 and 5, respectively; e.g., fold-change ≥2; P ≤ 0.05; for YPD, n = 54/517 of the total proteins identified [TPI] in all three strains; for YPGly, n = 124/1,931 TPI). More than 90% of proteins identified in the YPD translatome profile (n = 472/517 TPI; Table S1, List 1) were also detected in the YPGly profile (Table S1, List 4), indicating that PUNCH-P identifies proteins in a nonbiased fashion independently of growth conditions. More importantly, the refined *rpl1bΔ* translatome profile was lacking in mitochondrial proteins (Fig. 4 and Table S1, Lists 2 and 5), especially in cells grown on glycerol (Fig. 4 b, right, and Table S1, List 5). Gene ontology (GO) enrichment analysis (Huang et al., 2009) shows that ~40% of the proteins depleted from the *rpl1bΔ* translatome in cells grown on glucose are functionally related to mitochondria (n = 21/54 depleted proteins; P = 2.9 E-02; Table S1, List 2). Application of the same threshold (fold change ≥2; P ≤ 0.05) for the translatome profile of cells grown on glycerol revealed a more extensive depletion of mitochondrial proteins in *rpl1bΔ* cells (n = 45/124; P = 3.3 E-03; Table S1, List 5). These included components of major mitochondrial pathways: tricarboxylic acid cycle (e.g., Aco1, Pck1, and Kgd1; n = 9/124 depleted proteins; P = 1.8 E-02); NAD/NADH cycle (e.g., Ndi1, Fox2, and Idh1; n = 10/124; P = 3.5 E-03); and the NADP pathway (e.g., Hmg1, Fas1, and Fas2; n = 11/124; P = 2.6 E-03), along with proteins having critical mitochondrial roles such as Puf3 (mRNA localization), Hsp60 (protein folding), and Pori1 (porin structure; Fig. 4 b, right). Thus, the deletion of *RPL1b* results in significant changes in the translation of mMPs.

Deletion of a specific RP paralog can lead to alterations in mRNA levels (Komili et al., 2007). To determine whether changes in the translatome result from altered mMP transcription, we compared the mRNA levels of proteins that show decreased translation in the *rpl1bΔ* cells relative to *rpl1aΔ* cells. We measured the levels of various mMPs in cells grown on and glyc erol by quantitative real-time PCR (qRT-PCR), but we saw no major differences in their levels of expression between the paralog mutants (Fig. S3, c and d). Accordingly, proteomic differences observed between the paralog mutants cannot be explained merely by changes in mRNA levels, but they can be attributed to differences in translation.

Translatome analysis also revealed proteins whose translation is elevated in *rpl1bΔ* cells in comparison with *rpl1aΔ* and WT cells. GO analysis of the elevated proteins showed an enrichment in proteins related to the ER (e.g., Get1, Sbh2, and Yop1; n = 20/85 up-regulated proteins; P = 5.8 E-04) and mitochondrial oxidative phosphorylation (e.g., Cox7, Tim11, and Atp15; n = 7/85; P = 7.6 E-03) in cells grown on glycerol (Fig. 4 b and Table S1, List 6). RPs (e.g., Rpl40a, Rpl29b, and Rpl42a) were found to be elevated in the *rpl1bΔ* translatome in cells grown on glycerol and, more significantly, when grown on glucose (e.g., Rps15, Rpo9a, and Rps20; n = 19/27 up-regulated proteins; P = 9.8 E-12; Table S1, List 3). The up-regulation of these proteins may hint at a cellular attempt to compensate for the loss of the Rpl1b paralog and may perhaps increase mitochondrial membrane potential and organelle function.

Key missing components of the *rpl1bΔ* translatome include mMPs that display respiration-deficient phenotypes when mutated, including those involved in ATP synthesis (Atp1), protein import (Scc1), tricarboxylic acid cycle (Kgd1), and other processes (Turner et al., 2000; Poirier et al., 2002; Merz and Westermann, 2009; Goswami et al., 2012). Improper translation of any or all of these proteins caused by the absence of Rpl1b likely leads to the respiration deficiency and mitochondrial defects observed. Because the partial mislocalization of a single mMP can affect mitochondrial function (Zabezhinsky et al., 2016), it is not surprising that the altered translatome of *rpl1bΔ* cells disrupts mitochondrial function and leads to the *pet* phenotype.

As the levels of *RPL1* mRNA (Fig. 3 b), mRNA translation (Table S1), and protein production (Fig. 3 d) are similar between the *rpl1aΔ* and *rpl1bΔ* mutants, changes in the translatome cannot result from a lack of Rpl1. Instead, these changes highlight differences in the functional specificity of Rpl1a and Rpl1b, proving that specific paralogs promote the translation of distinct subsets of mRNAs. Thus, not only does the RP composition of ribosomes constitute an additional layer of translational regulation (Ferretti et al., 2017; Shi et al., 2017), but paralog selectivity does as well.

Translatome profiling identifies other cellular processes governed by RP paralog specificity

Analysis of the GO profile of the *rpl1bΔ* translatome revealed reductions in other proteins not related in function to mitochondria. A significantly depleted group included cell wall components (e.g., Hsp150 and Pir1). We hypothesized that *rpl1bΔ* mutant cells might exhibit a sensitivity to cell wall stress in comparison with *rpl1aΔ* and WT cells because of the lack of translation of these proteins. We assessed the growth of yeast exposed to calcofluor, an inducer of cell wall stress, and although WT and *rpl1aΔ* cells grew similarly, *rpl1bΔ* cells demonstrated limited growth and extreme sensitivity (Fig. 4 c). Thus, examination of the *rpl1bΔ* translatome profile led us to predict additional RP paralog-specific processes.

Identification of RNA motifs in proteins depleted in the *rpl1bΔ* translatome

Translation is mediated by different sequence elements in mRNAs (Spriggs et al., 2010; Kuersten et al., 2013). We performed computational analysis of the transcripts encoding proteins depleted in the *rpl1bΔ* translatome in an attempt to reveal motifs or similarities in sequence that might favor translation by paralog-specific ribosomes. A comparison of the codon usage in these transcripts to that of the yeast genome revealed a significant reduction in the usage of CGA and CGG codons for arginine that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source. This information that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source. This information that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source. This information that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source. This information that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source. This information that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source. This information that was observed on both glucose and glycerol (e.g., fold change ≥2; P ≤ 7.81 E-04; adjusted with Bonferroni correction; Fig. S3 e). In contrast, a moderate enrichment in GGT and GCT codons, which encode glycine and alanine, respectively, was noted in transcripts encoding proteins depleted on glucose (Fig. S3 e). Yet, no overall differences in amino acid usage in the *rpl1bΔ* translatome were noted on either carbon source.
completed proteins (Fig. S3 f) that might potentially mediate paralog-specific interactions.

Because RPL1a and RPL1b encode the same protein at the genomic level, how might Rpl1b promote translation of a specific subset of mRNAs? One possible answer is that their different UTRs lead to differential processing of the transcripts (i.e., RNA editing) or posttranslational modifications that create distinct Rpl1 paralogs that confer altered translation. Future studies aim to resolve the potential role of the UTRs in Rpl1 sequence identity and processing. How does Rpl1b confer altered translational control of mMPs and cell wall proteins? Although unclear, it likely acts at the different levels of translational regulation (i.e., initiation and elongation). For example, minor differences in ribosome assembly observed in rpl1aΔ and rpl1bΔ mutants suggest that Rpl1 influences translation initiation by affecting the interaction of ribosome subunits or translation factors with the translation machinery (Petitjean et al., 1995). That said, we could not identify a unique motif in the 5′ UTRs that might serve as an initiation recognition site in transcripts from the rpl1bΔ translatome despite the recent demonstration of an RP that recognizes a particular RNA motif (Xue et al., 2015). However, specific patterns of codon usage within the coding regions (Fig. S3 d) as well as an A-rich motif in the 3′ UTR of transcripts encoding proteins depleted from the rpl1bΔ translatome (Fig. S3 e) were identified. These factors might promote the translation of specific transcripts.

Translation is also regulated by elongation (Richer and Coller, 2015), and structural models place Rpl1 at the ribosome E site (Fei et al., 2008), which is responsible for release of the uncharged tRNAs and selectivity at the A site of tRNA entry (Wilson and nierhaus, 2006). Importantly, the rpl1bΔ deletion relieves ribosome stalling at CGA arginine codons (Letzring et al., 2014) and the 3′ and 5′ UTR of transcripts encoding proteins depleted from the rpl1bΔ translatome (Fig. S3 e). Thus, Rpl1b may connect the recognition of certain charged tRNA species (by ribosomes) to chain elongation and translational control.

In this study, we show that RP paralog specificity leads to changes in translational control (Fig. 4 d), and although the mechanism remains unclear, all three paralog-related RP paralog genes (RPL1b, RPL2b, and RPS26a) act epistatically (Fig. S1 a) even though they encode different subunits. Ancestoral genome duplication gave rise to a large number of RP paralog gene pairs, each paralog conferring a different function from its cognate partner, despite being either identical or highly similar in amino acid sequence (Komili et al., 2007). How they act jointly to create a specialized ribosome and thus shape the translatome under differing conditions remains to be shown.

Importantly, mutated forms of ribosomal components are associated with cancer (Goudarzi and Lindström, 2016) and Diamond-Blackfan anemia, which is linked to Rps26 (Drapchinskaia et al., 1999; ferretti et al., 2017). Future studies into the connection of RP-mediated translation of mMPs and the broader role of paralog specificity may provide a novel perspective on specialized ribosomes and translational control in human diseases.

Materials and methods

Yeast strains, genomic manipulations, and growth conditions

Cultures were grown at 30°C or 35°C in fermentable carbon source-containing media: either YPD (1% bacto-yeast extract, 2% bacto-peptone, and 2% glucose) or the nonfermentable carbon source-containing media YPGly (1% bacto-yeast extract, 2% bacto-peptone, and 3% glycerol). Synthetic selective media contained 0.67% yeast nitrogen base supplemented with ammonium sulfate, the appropriate amino acid supplements for plasmid selection, and either 2% glucose or 3% glycerol. Yeast strains used in this study are based on the BY4741 laboratory strain. Gene deletions were performed by homologous recombination using standard PCR-based amplification of deletion cassettes (Longtime et al., 1998). Standard LiOAc-based protocols were used for transformations of plasmids and PCR products into yeast.

Yeast strains included: WT cells (BY4741), MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0; rpl1aΔ, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1aΔ::HIS3; rpl1bΔ, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1bΔ::HIS3; rps26Δa, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rps26Δa::HIS3; rps26Δb, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rps26Δb::HIS3; rpl1Δa rpl2aΔ, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1aΔ rpl2aΔ::LEU2; rpl1aΔ rpl2Δa, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1aΔ rpl2Δa::LEU2; rpl1bΔ rpl2aΔ, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1bΔ rpl2aΔ::LEU2; rpl1bΔ rpl2Δa, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1bΔ rpl2Δa::LEU2; rps26Δa::LEU2; rps26Δb, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rps26Δb::LEU2; rpl1Δa rpl2aΔ, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rpl1Δa rpl2aΔ::LEU2; rps26Δa::LEU2; rps26Δb, MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 rps26Δb::LEU2; rps26Δa::LEU2.

Growth tests

For drop tests, serial dilutions (10-fold) of the different strains in growth medium were performed before plating by drops onto YPD and YPGly or selective minimal media containing 2% glucose or 3% glycerol at 30°C or 35°C. Plates containing the fermentable or nonfermentable carbon source were photodocumented after 2–3 d or 3–5 d, respectively. For growth curves, doubling times were calculated based on measured growth of the different strains in liquid YPD or YPGly at 30°C. Growth was assessed by OD600 measurements every 45 min until stationary phase. Each strain was measured for growth in triplicate and scored in three biological experiments.

Plasmids

Plasmids created for this study were constructed by restriction-free cloning (unger et al., 2010) and verified by DNA sequencing. RP genes were cloned into pUG316 CEN plasmids, which contain the URA3 selection marker, and included the coding regions (and introns in the cases of RPL2 and RPS26) along with both the 5′ and 3′ UTRs. UTR regions were based according to kertesz et al. (2010). RP plasmids included pUG36-RPL1A, pUG36-RPL1B, pUG36-RPL2A, pUG36-RPS26A, and pUG36-RPS26B. A multiplicity URA3 expression plasmid, pRS416-MTS-RFP, expressing MTS-RFP was a gift from M. Schulderer (Weizmann Institute of Sciences, Rehovot, Israel).

Fluorescence microscopy

Yeast strains expressing mtsRFP were grown to mid-log phase (OD600 = 0.6–0.8) and harvested. Selective synthetic media containing fermentable (glucose) or nonfermentable (glycerol) carbon source was
used to maintain cells during photodocumentation. Cells were analyzed by fluorescence confocal microscopy. Represented images were acquired at 26°C using an LSM710 confocal microscope and a Plan Apochromat 100x 1.40 NA oil objective (ZEISS). Wavelengths for excitation at 545 nm and emission at 560–580 nm were used for the visualization of RFP in mitochondria. Image acquisition was accomplished by using the software provided by the manufacturer. Z stack projection of the fluorescence signals (Fig. 2a) and 3D reconstructions (Fig. 2b) were obtained using the Fiji/ImageJ (National Institutes of Health) and Volocity (PerkinElmer) programs, respectively.

ImageStreamX analysis

Yeast were grown to mid-log phase (OD600 = 0.6–0.8) in liquid YPD or YPGly medium at 35°C, followed by incubation with TMRE (200 nM final concentration; Biotium) for 30 min. Cells were washed once with fresh media before flow cytometry analysis. Quantitative analysis of fluorescence signals was performed by using the ImageStreamX imaging flow cytometer (Amnis; Zuba-Surma et al., 2007). Statistical analysis of ImageStreamX-collected data was performed with the IDEAS software (Amnis). Fluorescence signal intensity and area were measured for each cell examined in addition to cell size based upon differential interference contrast scattered light, which allowed for the calculation of signal intensity/area to cell size.

RNA isolation and reverse transcription (RT)/qRT-PCR analysis

RNA was extracted from each yeast strain using the MasterPure yeast RNA purification kit (Epicenter Biotechnologies). Isolated RNA was treated with DNase (Promega) for 1.5 h at 37°C to remove contaminating DNA. RT was executed by using Moloney murine leukemia virus RT RTase (Promega) under conditions suggested by the manufacturer. RT reactions were performed using 1 µg RNA for each of the samples. All primer pairs produced a single amplification product (80–120 bp) when tested by melting-curve analysis. qRT-PCR was performed using a LightCycler 480 device and CYBR Green I Master reagent (Roche). All primer pairs produced a single amplification product (80–120 bp) when tested by melting-curve analysis. qRT-PCR was performed using a LightCycler 480 device and CYBR Green I Master reagent (Roche). The following primers were used for the detection of the different genes: qRT_RPL1a forward, 5'-CAGTCAACAGTCTGTCACAA-3'; qRT_RPL1b forward, 5'-TGGTGCGGCGATTCGATGT-3'; qRT_RPL2 forward, 5'-CGATGCTCAGGTATGCTATCT-3'; qRT_RPL2b forward, 5'-CTTGATGCGGTGATGCTATCT-3'; qRT_RPL3 forward, 5'-CAATGGCGGCTGATGCTATCT-3'; qRT_RPL1 reverse, 5'-GACAGTCCCGGATGCTATCT-3'; qRT_RPS26a reverse, 5'-ATCGGATGAGGTTGATGCTATCT-3'; qRT_RPS26b reverse, 5'-TTGATGAGGTTGATGCTATCT-3'; qRT_RPS26c reverse, 5'-CAGTCGAGGTTGATGCTATCT-3'; qRT_RPL2 reverse, 5'-CAATGGCGGCTGATGCTATCT-3'; qRT_RPL3 reverse, 5'-GACAGTCCCGGATGCTATCT-3'; qRT_RPS26a forward, 5'-ATCGGATGAGGTTGATGCTATCT-3'; qRT_RPS26b forward, 5'-TTGATGAGGTTGATGCTATCT-3'; qRT_RPS26c forward, 5'-CAGTCGAGGTTGATGCTATCT-3'.

RNA isolation and reverse transcription (RT)/qRT-PCR analysis

RNA was extracted from each yeast strain using the MasterPure yeast RNA purification kit (Epicenter Biotechnologies). Isolated RNA was treated with DNase (Promega) for 1.5 h at 37°C to remove contaminating DNA. RT was executed by using Moloney murine leukemia virus RT RTase (Promega) under conditions suggested by the manufacturer. RT reactions were performed using 1 µg RNA for each of the samples. All primer pairs produced a single amplification product (80–120 bp) when tested by melting-curve analysis. qRT-PCR was performed using a LightCycler 480 device and CYBR Green I Master reagent (Roche). The following primers were used for the detection of the different genes: qRT_RPL1a forward, 5'-CAGTCAACAGTCTGTCACAA-3'; qRT_RPL1b forward, 5'-TGGTGCGGCGATTCGATGT-3'; qRT_RPL2 forward, 5'-CGATGCTCAGGTATGCTATCT-3'; qRT_RPL2b forward, 5'-CTTGATGCGGTGATGCTATCT-3'; qRT_RPL3 forward, 5'-CAATGGCGGCTGATGCTATCT-3'; qRT_RPS26a reverse, 5'-ATCGGATGAGGTTGATGCTATCT-3'; qRT_RPS26b reverse, 5'-TTGATGAGGTTGATGCTATCT-3'; qRT_RPS26c reverse, 5'-CAGTCGAGGTTGATGCTATCT-3'; qRT_RPL2 reverse, 5'-CAATGGCGGCTGATGCTATCT-3'; qRT_RPL3 reverse, 5'-GACAGTCCCGGATGCTATCT-3'; qRT_RPS26a forward, 5'-ATCGGATGAGGTTGATGCTATCT-3'; qRT_RPS26b forward, 5'-TTGATGAGGTTGATGCTATCT-3'; qRT_RPS26c forward, 5'-CAGTCGAGGTTGATGCTATCT-3'.

Polysonome purification was adapted from a previously described protocol (Lesnik et al., 2014). In brief, yeast strains were grown in 800 ml of YPD or YPGly at 35°C to mid-log phase (OD600 = 0.6–0.8). Cells were then washed once in ice-cold double-distilled water, pelleted, and frozen. Yeast pellets were suspended in polysome extraction buffer (PEB; 20 mM Tris·HCl, pH 7.4, 10 mM MgCl2, 140 mM KCl, 0.5 mM DTT, 1.4 µg/ml pepstatin, 2 µg/ml leupeptin, 40 U/ml RNasin, 0.2 mg/ml heparin [Sigma-Aldrich]), EDTA-free complete protease inhibitor mixture (Roche), and lysed using glass beads (0.4–0.6 mm). Samples were centrifuged at 17,400 g for 30 min at 4°C to remove cell debris before loading the 6 ml supernatant on top of 2.5 ml 70% sucrose cushion. Samples were then centrifuged in a 70.1 Ti ultracentrifuge rotor (Beckman Coulter) at 330,000 g for 105 min at 4°C. The pellet fractions were resuspended in 90 µl PEB (without detergent) and frozen in liquid nitrogen immediately. Purycromycin–bionin labeling of polypeptide nascent chains and purification of the newly synthesized polypeptides from yeast polysomes was performed using an adaptation of the method used by Aviner et al. (2014) for mammalian polysomes. Yeast polysome extraction was determined by measuring RNA absorption at 254 nm. For each sample, 15 OD254 U of polysomes were incubated with 100 pmol of purycromycin–bionin (Jena Bioscience) per OD254 U for 15 min at 37°C. Next, the samples were incubated in an overnight incubation with 5 µl of streptavidin beads (GE Healthcare) per OD254 U at 23°C. Beads were then resuspended with SDS–urea buffer (50 mM Tris·HCl, pH 7.5, 8 M urea, 2% [v/v] SDS, and 200 mM NaCl), incubated for 30 min at 23°C, and then washed five
times with 1 ml SDS–urea buffer. After this, samples were incubated with 1 M NaCl for 30 min at 23°C before washing five times in ultrapure water. Samples from three biological replicate experiments derived from each strain were subjected to MS, and peptide quantification was performed using label-free proteomics using MS1-based peptide intensity (Shalit et al., 2015). Protein quantification was inferred using the Hi-3 method (Silva et al., 2006), and the sum of the three most abundant peptides was used to calculate protein intensity in each sample.

Statistical analyses

Principle components, hierarchical clustering, and three-way ANOVA tests were performed and plotted using Partek software (Partek Inc.). Hierarchical clustering p-values were computed with multiscale bootstrap resampling using the pvclust R computing package (Suzuki and Shimodaira, 2006). ANOVA test p-values were adjusted for the false discovery rate (step-up p-value; Benjamini and Hochberg, 1995).

GO enrichment representation was analyzed by using the Database for Annotation Visualization and Integrated Discovery (DAVID; Huang et al., 2009). A Perl-based script was used to perform a permutation test for amino acid and codon usage in the genes of depleted proteins from the rpl1bΔ translome. RNA motif search and identification was performed by MEME suites (Bailey et al., 2009).

Online supplemental material

Fig. S1 a shows the doubling times of the different RP paralog single- and double-deletion mutants grown on different carbon sources, and Fig. S1 b shows the quantification of mitochondrial morphology by flow cytometry. Fig. S2 a shows the growth of the b paralog mutants overexpressing their corresponding a paralog; Fig. S2 b shows the growth of the b paralog mutants overexpressing the b paralog as control; and Fig. S2 c shows the detection of Rap1 in WT, rpl1aΔ, and rpl1bΔ cells by Western blotting. Fig. S3 a shows the similarity and grouping of the WT, rpl1aΔ, and rpl1bΔ PUNCH-P translome profiles by PCA; Fig. S3 b shows the correlation of biological repeats obtained using PUNCH-P. Fig. S3 c (c and d) show the mRNA levels of various genes coding for proteins depleted in the rpl1aΔ and rpl1bΔ translomes relative to WT cells; Fig. S3 e shows the coding frequency of proteins depleted from the rpl1bΔ translome relative to WT cells; Fig. S3 f lists genes encoding proteins depleted from the rpl1bΔ translome relative to WT cells that contain an A-rich RNA motif in their 3’UTR. Table S1 provides PUNCH-P translome profiles from each growth condition (YPD, List 1; and YP Gly, List 4) for the WT, rpl1aΔ, and rpl1bΔ strains, lists of proteins significantly missing from the rpl1bΔ translome profile (YPD, List 2; and YP Gly, List 5), and lists of proteins which are significantly up-regulated in the rpl1bΔ translome profile (YPD, List 3; and YP Gly, List 6).

Acknowledgments

We thank O. Elroy-Stein and R. Aviner for advice with PUNCH-P, Y. Levin and the Israel National Center for Personalized Medicine for MS, Z. Poral for ImageStreamX, T. Olender for bioinformatics, S. Chuartzman and M. Schuldiner for advice, and F. Lacroute for anti-L1 antibodies. This was supported by grants from the German-Israel Foundation for Scientific Research and Development (I-1190-96.13/2012), the Minerva Foundation—Germany (71130), and Kohn Center for Systems Biology, Weizmann Institute of Science. J.E. Gerst holds the Besen-Breender Chair in Microbiology and Parasitology.

The authors declare no competing financial interests.

Author contributions: N. Segev performed the experiments, data analysis, figure preparation, and writing of the manuscript; J.E. Gerst performed data analysis, figure preparation, and writing of the manuscript.

Submitted: 11 June 2017
Revised: 19 September 2017
Accepted: 17 October 2017

References

Aviner, R., T. Geiger, and O. Elroy-Stein. 2013. Novel proteomic approach (PUNCH-P) reveals cell cycle-specific fluctuations in mRNA translation. Genes Dev. 27:1834–1844. https://doi.org/10.1101/gad.219105.113

Aviner, R., T. Geiger, and O. Elroy-Stein. 2014. Genome-wide identification and quantification of protein synthesis in cultured cells and whole tissues by puromycin-associated nascent chain proteomics (PUNCH-P). Nat. Protoc. 9:751–760. https://doi.org/10.1038/nprot.2014.051

Bailey, T.L., M. Boden, F.A. Buske, M. Frith, C.E. Grant, L. Clementi, J. Ren, W.W. Li, and W.S. Noble. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37(Web Server):W202–W208. https://doi.org/10.1093/nar/gkp335

Benjamini, Y., and Y. Hochberg. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing on JSTOR. J. R. Statist. Soc. 57:289–300.

Berkovits, B.D., and C. Mayr. 2015. Alternative 3’UTRs act as scaffolds to regulate membrane protein localization. Nature. 522:363–367. https://doi.org/10.1038/nature14321

Buxbaum, A.R., G. Hainovich, and R.H. Singer. 2015. In the right place at the right time: visualizing and understanding mRNA localization. Nat. Rev. Mol. Cell Biol. 16:95–109. https://doi.org/10.1038/nrm3918

Byrgazov, K., O. Vesper, and I. Moll. 2013. Ribosome heterogeneity: another level of complexity in bacterial translation regulation. Curr. Opin. Microbiol. 16:133–139. https://doi.org/10.1016/j.mib.2013.01.009

Day, M. 2013. Yeast petals and small colony variants: for everything there is a season. Adv. Appl. Microbiol. 85:1–41. https://doi.org/10.1016/B978-0-12-407672-3.00001-0

Dobzinski, N., S.G. Chuartzman, R. Kama, M. Schuldiner, and J.E. Gerst. 2015. Starvation-Dependent Regulation of Golgi Quality Control Links the TOR Signaling and Vacular Protein Sorting Pathways. Cell Reports. 12:1876–1886. https://doi.org/10.1016/J Celrep.2015.08.026

Draphtsinikaia, N., P. Gustavsson, B. Andersson, M. Pettersson, T.N. Willig, I. Dianzani, S. Bull, G. Tchernia, J. Klar, H. Matsson, et al. 1999. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nat. Genet. 21:169–175. https://doi.org/10.1038/nrg5951

Enyenihi, A.H., and W.S. Saunders. 2003. Large-scale functional genomic analysis of sporation and meiosis in Saccharomyces cerevisiae. Genetics. 163:47–54.

Fei, J., P. Kouris, D.D. MacDougall, and R.L. Gonzalez Jr. 2008. Coupling of ribosomal L1 stalk and tRNA dynamics during translation elongation. Mol. Cell. 30:348–359. https://doi.org/10.1016/j.molcel.2008.03.012

Ferretti, M.B., H. Ghalei, E.A. Ward, E.L. Potts, and K. Karbstein. 2017. Rps26 directs mRNA-specific translation by recognition of Kozak sequence elements. Nat. Struct. Mol. Biol. 24:700–707. https://doi.org/10.1038/nsmb.3442

Filipovska, A., and O. Rackham. 2013. Specialization from synthesis: how ribosome diversity can customize protein function. FEBS Lett. 587:1189–1197. https://doi.org/10.1016/j.febslet.2013.02.052

Fleischer, T.C., C.M. Weaver, K.J. McAfee, J.L. Jennings, and A.J. Link. 2006. Systematic identification and functional screens of uncharacterized proteins associated with eukaryotic ribosomal complexes. Genes Dev. 20:1294–1307. https://doi.org/10.1101/gad.1422006

Gabunilas, J., and G. Chanfreau. 2016. Splicing-Mediated Autoregulation Modulates Rpl22p Expression in Saccharomyces cerevisiae. PLoS Genet. 12:e1005999. https://doi.org/10.1371/journal.pgen.1005999

Goswami, A.V., M. Samaddar, D. Sinha, J. Purushotham, and P.D’Silva. 2012. Enhanced J-protein interaction and compromised protein stability of nmtHsp70 variants lead to mitochondrial dysfunction in Parkinson’s disease. Hum. Mol. Genet. 21:3317–3323. https://doi.org/10.1093/hmg/ddl162

Goudarzi, K.M., and M.S. Lindstrom. 2016. Role of ribosomal protein mutations in tumor development (Review). Int. J. Oncol. 48:1313–1324.

Haarer, B., S. Viggiano, M.A. Hibbs, O.G. Troyanskaya, and D.C. Amberg. 2007. Modeling complex genetic interactions in a simple eukaryotic genome: actin displays a rich spectrum of complex haploinsufficiencies. Genes Dev. 21:148–159. https://doi.org/10.1101/gad.1477507
