Expanded View Appendix

Supplementary Methods

Cultivation of mouse ESC

Mouse embryonic stem cells (E14 10^{th} passage) were grown as adherent cultures in 10 cm plates with 10 ml DMEM/F12 media supplemented with 10 % knockout serum replacement, nonessential amino acids (NEAA supplement), 0.1 mM β-mercapto-ethanol, 1 % penicillin and streptomycin, leukemia inhibitory factor (LIF; 1,000 U LIF/ml), and $2i$ (GSK3β and Mek 1/2 inhibitors). The growth curve in Figure EV1 indicates that the cells grew exponentially at a growth rate of 0.08 per hour, which corresponds to 9 hours doubling time. During the middle of the exponential growth period, the cells were detached from the plate by 2 min incubation with accutase (Millipore) at 37 ^oC. The cells were pelleted by a 2 min centrifugation, and the pellet was frozen immediately in liquid nitrogen.

Cultivation of yeast

All yeast experiments used a prototrophic diploid strain (DBY12007) with a S288c background and wild type HAP1 alleles (Hickman and Winston, 2007). We grew our cultures in a commercial bioreactor (LAMBDA Laboratory Instruments) using minimal media with the composition of yeast nitrogen base (YNB) and supplemented with $2g/L$ D-glucose. Before inoculation, the reactor was filled with 2L of minimal media and warmed up to a working temperature of

30^oC. Then cultures were started by inoculating the media with 100 μl overnight culture from DBY12007. The overnight cultures were prepared by first streaking frozen DBY12007 on YPD plates (YPD; 10 g of Bacto-Yeast extract, 20 g of Bacto-peptone, 20 g of Bacto-agar, and 20 g of glucose in 1000 ml of water) and then growing a single colony in the same minimal media used for the subsequent growth experiment in the bioreactor. The density of the culture used for inoculation was 2×10^7 cells per ml, resulting in an initial density of 10^3 cells/ml for the culture in the reactor. The cultures were grown at 30^oC and continuously stirred to ensure their homogeneity. The culture was aerated with air coming from a compressed gas cylinder (Airgas, AI-B300 breathable air). The incoming flow of air was controlled by a thermal-based mass-flow controller and filtered through a $0.2\mu m$ filter to ensure sterility.

Cell density was measured on Beckman-Coulter Multisizer 4 by counting at least 20,000 single cells (Slavov *et al*, 2011; Slavov and Botstein, 2011). The samples were taken during the first exponential growth phase on glucose carbon source and during the second exponential growth phase on ethanol carbon source (Slavov *et al*, 2014). To take samples without disturbing the cultures, we used a metal tube attached to silicon tubing and a syringe. The metal tube could be inserted in and out of the cultures, and the syringe used to sample the required volume quickly from the homogeneous cultures. The sampling tubing was kept sterile and no culture was left in it after sampling. All samples were immediately filtered, frozen in liquid nitrogen, and processed as described below.

Sucrose gradients and mass spectrometry work flow

Both yeast and mouse embryonic stem cells were lysed by vortexing for 10 min with glass beads in cold PLB (20 mM HEPES-KOH at pH 7.4, 1 % Triton X–100, 2 mM Magnesium Acetate, 100 mM Potassium Acetate, 0.1 mg/ml cycloheximide, and 3 mM DTT). The crude extracts obtained from this lysis procedure were clarified by centrifugation, and the resulting

supernatants were applied to linear 11 ml sucrose gradients ($10\ \% - 50\ \%$) and spun at 35,000 rpm in a Beckman SW41 rotor either for 3 hours (for yeast samples) or for 2.5 hours (for mouse samples). Twelve fractions from each sample were collected using a Gradient Station (BioComp, Cat. # 153-001). The RNA profile across the gradient was measured by Gradient Profiler (BioComp).

The ratio of polysomal to monosomal peaks in mouse ESC (Fig. 1A) is higher than in developing mouse neural tube and somites (Kondrashov *et al*, 2011) but lower than in neuro-progenitor and cancer cell–lines, consistent with previous observations in mouse ESC (Sampath *et al*, 2008).

Sample preparation

The proteins from each sucrose fraction were methanol-chloroform precipitated, and the amount of protein was estimated by the dry weight of the pellet. To ensure full dissociation of the ribosomes, the analyzed volume from each sucrose fraction was mixed with 4 volumes of 8 M guanidinium chloride and vortexed for at least 10 min at 37 ^oC. The proteins from each sucrose fraction were processed via the FASP protocol (Wiśniewski *et al*, 2009), and digested with either lys-C or a mixture of trypsin and lys-C (Promega; # V5073). The digestion with the trypsin/lys-C mix resulted in more identified and quantified peptides. Subsequently each sample was labeled with TMT reagent (Prod # 90061, Thermo Fisher, San Jose, CA) according to the manufacturer's protocol.

Tandem Mass Tags (TMT) mass spectrometry

The labeled set–sample was injected from an auto–sampler into the trapping column ($75\ \mu m$ column ID, 5 cm packed with $5\ \mu m$ beads on 20 nm pores, from Michrom Bioresources, Inc.) and washed for 15 min; the sample was eluted to analytic column with a gradient from 2 to 32

% of buffer B (0.1 % formic acid in ACN) over $180\ min$ gradient and fed into LTQ Orbitrap Elite (Thermo Fisher, San Jose, CA). The instrument was set to run in TOP 20 MS/MS mode method with dynamic exclusion. After MS1 scan in Orbitrap with 60K resolving power, each ion was submitted to an HCD MS/MS with 15K or 30K resolving power and to CID MS/MS scan subsequently. All quantification data were derived from HCD spectra.

Analysis of mass spectrometry spectra

Mass/charge spectra where analyzed by MaxQuant (Cox and Mann, 2008) (version 1.4.1.2), SEQUEST HT (Eng *et al*, 1994) and Mascot (Cottrell and London, 1999) (Version 2.4.1) run via the Proteome Discover (64bit version 1.4.0.288, Thermo), and standalone Mascot. All searches were run on a Windows server 2008 64 bit operating system with 64 CPU blades and 256 GB of RAM with the following general parameters. Parent ion mass tolerance was set to 20 ppm, mass tolerance for MS/MS ions was set to 0.02 Da for HCD and to 0.6 Da for CID spectra. For all searches, minimal peptide length was specified as 6 amino acids and maximal peptide length as 50 amino acids. The peptide charge state was limited to +7 for searches with MaxQuant. Searches were performed against either the yeast or the mouse uniprot database and common contaminants that were added to the database. Searches had trypsin or lys-C enzyme specificity, allowing 2 missed cleavages. Asn and Gln deamidation and Met oxidation were included as variable modifications in the search parameters.

The search results from all search engines were filtered at 1 % false discovery rate (FDR) on both protein and on peptide levels using the Percolator (Version 2.05 Build Date May 6 2013). The results exported for further analysis included all peptide spectrum matches (PSM) that were assigned to one or more proteins and passed the statistical significance filter. These results were outputted in the "Evidence File" for MaxQuant and in a peptide–level–results text file for Proteome Discover. The Proteome Discover files are provided as supplementary datasets.

Western blots

The variable RP stoichiometry indicated by our MS data is rather surprising given that for decades the ribosome has been considered the preeminent example of a large RNA–protein complex with a fixed stoichiometry among the constituent core RPs. Thus, we sought to use an independent experimental method, Western blots, to test out findings. While Western blots have lower sensitivity, specificity, and accuracy than MS (Aebersold *et al*, 2013), they also quantify proteins based on an orthogonal method to MS and are thus an excellent method for further testing the variability of the RP stoichiometry.

Because of the lower sensitivity of Western blots, the low–passage E14 ESCs used for the MS measurements did not provide enough protein material for reliable quantification of the polysomes by Western blots. Thus, we used higher passage–number E14 strain that grows faster and provides enough protein for reliable quantification. We estimated that $5\ \mu l$ of the monosomal fraction and $20\ \mu l$ of the polysomal fraction having $7-10$ ribosomes per mRNAs have about equal amounts of total ribosomal protein, and we validated that by using Rpl32 as the loading control.

Samples of the monosomes and polysomes were run on 16% Tris–Glycine Mini Protein Gels (Lifetechnologies catalog number: EC6498BOX) for 100 min using the instructions of the manufacturer. The proteins were blotted using the semi-dry method and CAPS buffer at pH 11 on PVDF membrane. The RPs were detected with antibodies from Santa Cruz Biotechnology, Inc, Catalog Numbers: sc-25931 for Rpl11, sc-133977 for Rpl32, sc-68873 for Rps14, and sc-133962 for Rps29. After incubation with secondary antibodies and visualization with Super-Signal West Femto Chemiluminescent Substrate (Thermo Scientific, catalog number 34095), all antibodies resulted in a single band at the molecular weight corresponding to the cognate RP. The blots were imaged with AlphaImager System, and the images were quantified with Image Studio Lite, version 4.0. Consistent with the MS data (Figure EV4A), the Western Blots data (Figure EV4B) indicate that Rps29 and Rps14 are enriched in polysomes, Rpl11 is enriched in

monosomes, and Rpl32 does not change (loading control).

Correlation between relative RP levels and fitness

To explore the physiological significance (if any) of the altered RP stoichiometry, we computed the correlation between the fitness of yeast strains with single RP-gene deletions (Qian *et al*, 2012) and the corresponding relative RP levels that we measured (Fig. 3). In yeast, 21 pairs of RP-genes encode proteins with identical amino acid sequences within a pair. Since the RPs within these 21 pairs cannot be distinguished by MS, these 21 RPs were excluded from our analysis and not used to compute correlations between RP levels and fitness of RP-delete strains. Furthermore, some RPs that we quantified did not have RP-deletion fitness data and thus could not be included in our correlation analysis.

Supplementary Discussion

Evaluation of factors that may affect the measured RP levels

The estimated RP levels (Fig. 1-3) appear to vary significantly between monosomes and polysomes and across the growth conditions. However, this variation might reflect not only stoichiometry changes among the RPs but also other factors and artifacts, such as noise in the MS measurements, a differential distribution of nascent RP polypeptides among monosomes and polysomes, posttranslational modifications (PTMs) of the RPs, and the presence of 90S ribosomal biogenesis particles. In the subsections below we describe our investigation of such potential artifacts. The results of this investigation (Figure EV2 and Figure EV3), indicate that such potential artifacts are unlikely to contribute significantly to the estimated RP levels (Fig. 1-3), suggesting that the stoichiometry among the RPs can change across polyribosomes and physiological con-

ditions in the absence of genetic perturbations.

Noise, coisolation interference and posttranslational modifications (PTMs)

From most RPs, we quantify multiple unique peptides (whose amino acid sequence is found only in one RP and no other protein in the proteome) both in mouse (Figure EV2A, C) and in yeast (Figure EV2E). In the absence of measurement noise, post-translational modifications (PTMs), or partial peptides (such as nascent poly-peptide chains), the fold-changes of an RP should equal the fold-changes of each unique peptide coming from this RP. Thus the similarity between the fold changes of unique peptides for the same RP, as quantified by the coefficient of variation (CV; the ratio of the standard deviation to the mean), reflects the degree to which the estimated fold–changes for an RP are influenced by post-translational modification, by noise, and by partial protein products. To evaluate the contribution of all these factors to our RP quantification (Fig. 1-3), we computed the distributions of CV values for mouse (Figure EV2B, D) and for yeast (Figure EV2F) for all RPs having multiple quantified unique peptides per RP. These distributions indicate a median CV < 0.25 and thus suggest that PTMs, measurement noise and partial RPs are not dominant factors in the quantification of most RPs. A few fold-changes, however, have larger CVs that might reflect either PTMs or larger noise in the peptide quantification.

Coisolation interference in the quantification of the reporter ions results in underestimation of the fold changes (Bantscheff *et al*, 2007). To reduce the influence of coisolation interference, we filtered out the quantified peptides with large coisolation interference.

Differential distribution of nascent RP polypeptides among monosomes and polysomes

In principle, a differential distribution of nascent RP polypeptides among monosomes and polysomes could contribute to the measured RP changes (Fig. 1-3). As discussed above, the low CVs for protein fold-changes quantified from different unique peptides (Figure EV2) make this possibility unlikely.

We sought to test the possibility that nascent RP polypeptides contribute to our estimates of RP levels even more directly. First, if nascent RPs contribute significant numbers of peptides to the variation in RP levels in Fig. 1-3, the MS1 precursor-area (integrated area under the MS1 spectrum of the precursor ions that reflects peptide abundance) of N-terminal peptides would be higher compared to the MS1 precursor-area of C-terminal peptides. We compared the distributions of MS1 precursor-areas for N-terminal peptides and for C-terminal peptides and found that the two distributions are statistically identical both for yeast and for mouse. This result suggests that nascent RPs do not contribute significantly to the measured changes in the RP stoichiometry. Second, our Western blots for Rpl11, Rps29, Rps14, and Rps32 showed only one band at the expected molecular weight and no lower molecular weight bands that would correspond to growing nascent chains. If present at a significant level in the sucrose fractions, such growing nascent chains should be detected by the antibodies recognizing N-terminal epitops. Indeed, even in the most extreme case when all mRNAs translated by the ribosomes code for RPs, peptides contributed by the nascent chains are less than $1/80 = 1.2\ \%$ of all RP peptides from a digested fraction. Third, some very short RPs, such as Rps29, which is only 56 animo acids long (6.6 kDa), are highly enriched in the mouse fractions having 7-10 ribosomes per mRNA. The mRNA coding for Rps29 cannot physically fit 7-10 ribosomes, and thus the polysomal enrichment of Rps29 and other short RPs cannot possibly be explained by the on-going translation of nascent poly-peptides in the mouse polysomes. More generally, if

nascent proteins contribute to the measured variability, our estimates should indicate that longer RPs are enriched in sucrose fractions having more ribosomes per mRNA since longer mRNA are translated by more ribosomes (Arava *et al*, 2003). We find no such enrichment, suggesting that our measurements are not significantly affected by nascent polypeptide chains. This is particularly clear in the mouse dataset where we quantified sucrose fractions corresponding to 7-10 ribosomes per mRNA.

The 90S ribosomal biogenesis particles

Sucrose gradients separate not only mature ribosomes but also other cellular organelles of comparable size, such as the immature 90S ribosomal biogenesis particles (Granneman and Baserga, 2004; Sykes and Williamson, 2009; Sykes *et al*, 2010; Chen and Williamson, 2013). The 90S particles should have unequal distribution across the gradient, localizing closely to the 80S monosomal peak and decreasing toward the higher sedimentation-velocity region of the polysomes. Thus if the amount of 90S particles is comparable to the amount of ribosomes, 90S particles could contribute significantly to the changes in the RP stoichiometry in Fig. 1-3. However, in exponentially growing cells, the 90S ribosomal biogenesis particles are less abundant than the mature ribosomes (Granneman and Baserga, 2004; Sykes and Williamson, 2009; Sykes *et al*, 2010; Chen and Williamson, 2013), and thus unlikely to contribute substantially to the RP peptides that we quantified.

We used our data to evaluate the extent to which immature 90S particles contribute to our estimates of variability among the RPs. Two key factors that determine this contribution are (i) the level and (ii) the sucrose-gradient localization of the 90S. To estimate these two factors, we used the ribosome-biogenesis proteins that are known to be associated with the 90S particle but not with the mature ribosomes (Granneman and Baserga, 2004). These ribosome-biogenesis proteins provide a solid basis for estimating the abundance of the 90S particles relative to the mature ribosomes and the distribution of the 90S particles across the sucrose fractions.

First, we estimated the abundance of the 90S particle relative to the mature ribosomes. From the 180 proteins annotated by the gene ontology (GO:0042254) term "ribosome biogenesis," we have quantified unique peptides for only 14 proteins that are not core structural RPs. These 14 ribosome-biogenesis proteins are represented in our data by very few peptides (9 proteins are represented by a single peptide), which likely reflects the low abundance of these proteins (relative to the RPs) in our sucrose fractions. This conclusion is strongly supported by the low MS1 precursor-area (integrated area under the MS1 spectrum of the precursor ions) of ribosome-biogenesis peptides compared to the MS1 precursor-area of RP peptides. To obtain a more quantitative estimate for the abundance of 90S relative to the mature ribosomes, we computed and compared the iBAQ scores (Schwanhäusser *et al*, 2011) (a popular measure for absolute protein levels) for the RPs and for the ribosome biogenesis proteins. The iBAQ scores indicate that the detected ribosome biogenesis proteins and thus the 90S particles are about 100 times less abundant than the RPs and thus the mature ribosomes (Figure EV3), consistent with previous observations (Sykes *et al*, 2010). Therefore, the proteins derived from the 90S can contribute about $1\ \%$ to the RP fold-changes. Since many of the measured RP fold-changes exceed $100\ \%$, 90S particles are unlikely to contribute significantly to the variation in the ribosome stoichiometry that we measured.

Second, as expected, the 90S ribosome biogenesis peptides are localized to the 80S monosomal fraction and their levels are even lower and constant across the rest of the sucrose gradient. Thus their small contribution is limited to the monosomes and cannot account for the relative RP changes across polysomes that we observed both in yeast and in mouse (Fig. 1-3).

Extra-ribosomal complexes of RPs

A few RPs have been reported to perform extra-ribosomal functions (Mazumder *et al*, 2003; Wool, 1996; Warner and McIntosh, 2009). Some of these extra-ribosomal functions of RPs are performed by small complexes containing RPs. For example a Rpl5/Rpl11/5S-rRNA preribo-

somal complex is involved in the regulation of p53 (Donati *et al*, 2013). Such extra-ribosomal complexes containing RPs are smaller than the mature ribosomes and should not co-sediment in the sucrose fractions that we analyzed (Donati *et al*, 2013; Warner and McIntosh, 2009). This expectation is strongly supported by our MS data: the non ribosomal proteins identified by our MS analysis included translation factors and protein-folding chaperones, but not proteins known to be involved in extra-ribosomal complexes of RPs. Thus, the levels of extra-ribosomal complexes of RPs that may be present in the analyzed sucrose fractions were too low to be detected by and to affect our analysis.

Stoichiometry among RPs

Our data show that while the levels of some RPs increase, the levels of other RPs decrease. These opposite trends indicate that the ratios (stoichiometries) among RPs making up monosomes and polysomes vary. However, our population–average measurements do not indicate the number of distinct ribosomes in each sucrose fraction or the exact RP composition of such distinct ribosomes.

RPs of the small (40S) and the large (60S) subunits are about equally represented among the subset of RPs that increase or decrease across monosomes and polysomes, Fig. 1-3. Therefore, preferential enrichment of 40S or 60S in some fractions is very unlikely to contribute to the variation among RPs that we observe.

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