The Ribosomal Protein Rpl22 Controls Ribosome Composition by Directly Repressing Expression of Its Own Paralog, Rpl22l1

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Abstract

Most yeast ribosomal protein genes are duplicated and their characterization has led to hypotheses regarding the existence of specialized ribosomes with different subunit composition or specifically-tailored functions. In yeast, ribosomal protein genes are generally duplicated and evidence has emerged that paralogs might have specific roles. Unlike yeast, most mammalian ribosomal proteins are thought to be encoded by a single gene copy, raising the possibility that heterogeneous populations of ribosomes are unique to yeast. Here, we examine the roles of the mammalian Rpl22, finding that Rpl22−/− mice have only subtle phenotypes with no significant translation defects. We find that in the Rpl22−/− mouse there is a compensatory increase in Rpl22-like1 (Rpl22l1) expression and incorporation into ribosomes. Consistent with the hypothesis that either ribosomal protein can support translation, knockdown of Rpl22l1 impairs growth of cells lacking Rpl22. Mechanistically, Rpl22 regulates Rpl22l1 directly by binding to an internal hairpin structure and repressing its expression. We propose that ribosome specificity may exist in mammals, providing evidence that one ribosomal protein can influence composition of the ribosome by regulating its own paralog.

Introduction

Protein synthesis is a major energy consuming process involving intricate coordination of translation machinery in response to nutrient availability and stress sensing signals, as well as hormonal and growth factor cues in multi-cellular organisms. The ribosome is comprised of two ribonucleoprotein subunits: the 40S and 60S (‘small’ and ‘large’ subunits, respectively). Together these subunits facilitate peptide bond formation, performing different roles during translation. Ribosome synthesis is a highly controlled process, whereby three distinct RNA polymerases are synchronously coordinated to produce equimolar amounts of four rRNAs and 79 mammalian ribosomal proteins (RPs) [1–4].

A growing number of human diseases have been linked to mutations in genes encoding factors involved in ribosome biogenesis and protein synthesis [5,6]. These include developmental malformations, inherited bone marrow failure syndromes and cancer in a variety of organisms [5,7–9]. In addition, interventions leading to reduced translation, such as dietary restriction and reduced 60S ribosomal protein expression, elicits lifespan extension in yeast, worms and flies [10–13]. Determining the molecular pathology underlying diseases and the role of ribosomes in aging requires a better understanding of ribosome specificity and the functions of individual RPs.

RPs are generally thought to be essential components of the functional ribosome and although they do not play a direct role in catalyzing peptide transfer, they may be critical for both regulatory and structural functions of the ribosome [14,15]. In addition to their role in the ribosome, many RPs, including murine Rpl22, have been shown to have extra-ribosomal functions [16–18]. In particular, as RNA binding proteins, RPs have been found to bind cellular and viral RNAs outside of the context of the ribosome. Some RPs also function to regulate their own expression, such as Rpl30 in yeast [19,20] and human RPS13 [21].
Author Summary

Translation is the process by which proteins are made within a cell. Ribosomes are the main macromolecular complexes involved in this process. Ribosomes are composed of ribosomal RNA and ribosomal proteins. Ribosomal proteins are generally thought to be structural components of the ribosome but recent findings have suggested that they might have a regulatory function as well. A growing number of human diseases have been linked to mutations in genes encoding factors involved in ribosome biogenesis and translation. These include developmental malformations, inherited bone marrow failure syndromes and cancer in a variety of organisms. Here, we describe the role of one ribosomal protein regulating another. We provide evidence that ribosomal proteins can influence the composition of the ribosome, which we hypothesize, may impact the function of the ribosome.

Ribosomes are often essential for viability. For example, embryonic lethality was reported in the first murine knockout of a ribosomal protein (RP, gene, Rpl19 [22]. Rpl24 [23,24], and Rps6 [25–27], also play essential roles. However, two reports have found that mice lacking either Rpl22 or Rpl29 survive without these RP genes [28,29]. In yeast, approximately 85% of the RP genes are duplicated as a result of an ancient genome duplication event [30] and many of these paralogous genes are functionally redundant [31]. Generally, deletion of either paralog, but not both simultaneously, results in viability; however, yeast that lack non-duplicated RP genes or both paralogs of an individual subunit are often, but not always, inviable [32]. Cross-complementation studies in yeast, analyzing defects in growth, have shown that most RP paralogs are functionally redundant [31]; however, several recent studies suggest that some paralogs might have subtle functional differences [33–38]. In the case of Rpl22, however, tetrad analysis indicated that the rpl22aΔ rpl22bΔ double mutant was viable, although slow growing [37,39], while in worms disruption of rpl22 expression is lethal (http://www.shigen.nig.ac.jp/c.elegans/index.jsp).

Rpl22 is an external protein on the 60S ribosomal subunit that is incorporated into the ribosome at later stages of ribosome maturation [40]. An early study suggested that Rpl22 was not required for translation in vitro [41]; however, the protein has been identified as a component of the ribosome [40] and likely plays a role in protein translation. In addition, other activities have been attributed to Rpl22 in mammals, including association with both viral RNAs, like EBER1, and cellular RNAs, such as human telomerase RNA [42–44]. Mice lacking Rpl22 are viable but have a defect in T cell development attributed to p53-dependent arrest of the 2B lineage T cells [28]. Recently, RPL22 has been found to be mutated or downregulated in various cancers, including T-acute lymphoblastic leukemias [45], invasive breast carcinoma [46], and lung adenocarcinoma [47].

Here we report evidence that in mice one ribosomal protein can control composition of the ribosome by regulating expression of its own paralog. Knocking out Rpl22 results in up-regulation of Rpl22-like1 (Rpl22l1), a paralog of Rpl22 whose predicted protein sequence is highly homologous to Rpl22. Rpl22l1 was first identified [although mis-labeled Rpl22] in a screen for 14-3-3ε binding partners in mouse brain and has been identified as a tissue component of ribosomes in mouse liver and mammary gland tissues [49]. We find that Rpl22l1 co-sediments with actively translating ribosomes in Rpl22−/− mice and a compensatory increase in Rpl22l1 expression likely accounts for the lack of translational defects in these animals. Enhanced Rpl22l1 expression also occurs upon acute knockdown of Rpl22 expression, indicating that Rpl22 has an active role in suppressing the synthesis of its paralog. Mechanistically, we find that Rpl22 directly represses expression of Rpl22l1 mRNA by binding to an internal hairpin structure. shRNA-mediated knockdown of Rpl22l1 causes a severe growth defect in cells lacking Rpl22. Accordingly, we demonstrate that the composition of the ribosome is regulated by the novel mechanism of direct repression of one paralog by another, and offer the hypothesis that this is one mechanism by which ribosome specificity is coordinated.

Results

Rpl22−/− mice are viable

A gene-trapped mouse embryonic stem cell clone harboring a mutation in Rpl22 was obtained from Bay Genomics and used to generate Rpl22 heterozygous mice (Rpl22+/−) (see Text S1). 5′ rapid amplification of cDNA ends (5′ RACE) followed by automated DNA sequencing determined that the gene-trap vector inserted between the third and fourth exons of Rpl22 (Figure S1A). Gene-trap vector disruption of Rpl22 expression was confirmed by PCR and western blot analysis (Figure S1B). Mice heterozygous for the Rpl22 mutation (Rpl22+/−) were interbred to obtain homozygous Rpl22-null (Rpl22−/−) mice. Surprisingly, Mendelian ratios of Rpl22+/+, Rpl22+/−, and Rpl22−/− were found in the resulting progeny. During the construction of our mouse line, Anderson et al. reported the generation of viable Rpl22−/− mice and observed defects in lymphocyte development [28].

Characterization of our Rpl22−/− mice indicated that they have defects in lymphocyte development (Figure S2, S3, S4) similar to that described by Anderson et al. (2007) [28]. B220+ B cells in the bone marrow were also significantly reduced in Rpl22−/− mice (Figure S4A, B). Further analysis indicated that B cell development was interrupted by Rpl22 deficiency, as evidenced by a decrease in the B220+ developing IgM-IgD- and immature IgM+IgD-B cells (Figure S4C, D).

Despite the ubiquitous expression of Rpl22 and its hypothesized role in mRNA translation, disruption of Rpl22 in mice results in a remarkably mild phenotype. Hematologic parameters are normal in these mice [50]. Also, unlike deletion of Rpl22 in yeast [51], Rpl22−/− mice have no substantial difference in growth rate or size relative to Rpl22+/− and Rpl22+/− littermates [Anderson et al. 2007 and our unpublished data]. Surprisingly, no significant differences were observed in the polysome profiles of lystate from Rpl22−/− liver, lung or cultured ear fibroblasts when compared to samples collected from Rpl22+/+ mice (unpublished data), indicating that Rpl22 is not essential for translation efficiency or ribosome biogenesis in the tissues evaluated.

Compensation by Rpl22l1 in Rpl22-null mice

We considered the possibility that another factor might be compensating for lack of Rpl22 in mice. A bioinformatic search identified Rpl22-like1 (Rpl22l1) as a candidate. Rpl22l1 encodes a 122 amino acid protein that is 73% identical to Rpl22 (Figure 1A). The protein sequence of Rpl22l1 is highly conserved from human to zebrafish (Figure S5). To determine if significant levels of Rpl22l1 mRNA exists in tissues from Rpl22+/− mice and whether Rpl22l1 transcript levels increase in Rpl22−/− mice, lung, liver, spleen and kidney were harvested from Rpl22+/− mice and their littermate controls and analyzed by quantitative RT-PCR (qRT-PCR) for Rpl22 and Rpl22l1 expression with Acidic Ribosomal Protein (ARBP) mRNA levels used for normalization (Figure 1B). In Rpl22+/− samples, high Rpl22 expression was detected, while
Rpl22l1 transcripts were less abundant. In samples isolated from Rpl22+/− mice, qRT-PCR revealed a ~3-fold induction of Rpl22l1 mRNA expression relative to littermate controls. Rpl22l1 transcripts were found associated with actively translating ribosomes in both Rpl22+/− and Rpl22−/− mouse ear fibroblast samples (unpublished data), suggesting that the Rpl22l1 mRNA is actively translated into protein. Consistently, equivalent increases in Rpl22l1 protein levels are observed in the absence of Rpl22 in lung, liver, spleen and kidney (Figure 1C). Similar increases in Rpl22l1 mRNA and protein expression were observed in Rpl22−/− skeletal muscle and brain (unpublished data). Additionally, Rpl22l1 was found to be abundantly expressed in both Rpl22+/− and Rpl22−/− pancreas (Figure 1C). These results indicate that Rpl22 negatively regulates, either directly or indirectly, Rpl22l1 expression in a range of mouse tissues.

Rpl22l1 protein co-sediments with actively translating ribosomes

To determine if Rpl22l1 is incorporated into actively translating ribosomes, liver tissue was isolated from Rpl22−/− mice and their littermate controls followed by sedimentation of the lysates on sucrose gradients. Fractions collected from the gradients, were subsequently loaded onto an SDS-page gel for western blot analysis. In Rpl22−/− samples, Rpl22l1 is present in fractions containing 60S ribosome subunits and polysomes, suggesting that it is incorporated into free ribosome subunits and ribosomes actively translating mRNA in the absence of Rpl22 (Figure 2).

Rpl7, a RP that is incorporated into the large subunit of the ribosome, is present in the fractions containing 60S ribosome subunits and polysomes in both Rpl22+/− and Rpl22−/− samples. Rpl22 and Rpl22l1, but not Rpl7, were detected in fractions 1 and 2, representative of the free, non-ribosomal lysate (Figure 2C, D), consistent with the hypothesis that these RPs exist in states within the cell both associated with the ribosome and independent of the ribosome. Additionally, while Rpl22l1 levels are relatively evenly detected in 60S containing fractions of the polysome profile (Figure 2D, fractions 3–7) Rpl22 is detected at higher levels in the fractions containing free 60S subunits and messages loaded with fewer ribosomes (Figure 2C, fractions 3–5 vs 6–7).

To verify that Rpl22l1 was incorporated into 60S subunits and actively translating ribosomes, free 60S subunits and 80S monosomes from actively translating polysomes were isolated from liver lysates of Rpl22+/− (WT) and Rpl22−/− (KO) mice using sucrose density gradient fractionation (Figure 2E, F; see Text S1). Samples were then concentrated and prepared for mass spectrometry analysis using standard methods (see Text S1). In order to measure the relative amounts of Rpl22 and Rpl22l1 in the WT and KO mouse liver lysate samples, we used multiple reaction monitoring (MRM), a targeted mass spectrometry (MS) approach that is highly sensitive. MRM is a targeted type of MS and requires a list of peptide targets and their subsequent fragment targets—known as a transition list—to program the analysis on the instrument (see Text S1). The final MRM analysis consisted of 4 peptide targets for Rpl22 (5 counting uniquely modified targets) and 3 peptide targets for Rpl22l1, and was limited to the top 8 fragment ions per peptide, creating a total of 64 transitions targeted in the analysis.

Summing the integrated MRM peak areas for all transitions from all observed peptides for each protein (Rpl22 and Rpl22l1) yielded the total MRM peak areas plotted for each of the four tested samples (WT60S, WT80S, KO60S, KO80S) and indicated relative amounts of Rpl22 and Rpl22l1 in these samples (Figure 2G, H). These data indicate that Rpl22l1 levels are significantly higher in the 60S and 80S subunits isolated from Rpl22−/− liver than in Rpl22+/− littermate controls (Figure 2H), supporting the hypothesis that Rpl22 regulates Rpl22l1 expression and, as a result, incorporation into ribosomes.

Rpl22 directly regulates Rpl22l1 expression

Why does expression of Rpl22l1 increase in mouse tissues lacking Rpl22? We considered two potential explanations: (1) Rpl22 directly regulates Rpl22l1 expression, or (2) compensation occurs during development in Rpl22−/− mice. To distinguish between these two possibilities, Rpl22 was acutely knocked down in 3T9 fibroblasts using a lentiviral-mediated inducible knockdown system that allows doxycycline-inducible regulation of Rpl22 and changes in Rpl22l1 expression were examined. 3T9 cells were transduced with 2 different tet-on shRNA lentivirus constructs (shRNA 1 and shRNA 2) that target Rpl22 mRNA or a nonspecific control construct. Following 3 days of doxycycline treatment, Rpl22l1 mRNA expression is enhanced 1.8 fold in 3T9 cells with reduced Rpl22 expression (Figure 3A). Western blot analysis confirmed that Rpl22l1 protein levels were elevated by the knockdown of Rpl22, while expression of other RPs, such as Rpl7 remained unchanged (Figure 3B). These results confirm that Rpl22 negatively regulates the expression of Rpl22l1 acutely and raise the possibility that Rpl22-mediated regulation of Rpl22l1 is an active process with biological significance.

Collectively, these data suggest that Rpl22 is regulating expression of Rpl22l1 but the mechanism leading to the increased expression is unknown. To determine whether Rpl22 affects the stability of Rpl22l1 mRNA, cultures of 3T9 cells were treated with actinomycin...
If Rpl22 is involved in mediating Rpl22l1 stability, does Rpl22 bind directly to Rpl22l1 mRNA? Previous studies determined that Rpl22 is associated with Epstein-Barr virus-expressed RNA, EBER1 [43,53] and evaluation of the RNA binding specificity of Rpl22 suggested that Rpl22 recognizes a stem loop (hairpin) structure with a G-C at the neck followed by a U [42,53]. To address whether regulation of Rpl22l1 expression is directly mediated by Rpl22, an algorithm termed M-fold that predicts RNA secondary structure was used to evaluate Rpl22l1 mRNA structure for potential Rpl22 RNA binding motifs [54]. Analysis revealed the presence of a consensus Rpl22 RNA-binding motif within exon 2 of zRpl22l1, suggesting that Rpl22 might interact directly with Rpl22l1 mRNA (Figure 4B).

To test whether Rpl22 can directly bind Rpl22l1 mRNA via the hairpin structure identified in Rpl22l1 mRNA by M-fold analysis, an RNase protection analysis was performed. Recombinant proteins were incubated with radiolabeled RNA, and UV-crosslinked. The RNAs were then digested with RNase A, and samples were run on a protein gel. Proteins bound to radiolabeled RNA were detected by autoradiogram at their expected molecular weight. Unbound proteins are not detected on autoradiogram. Recombinant Rpl22 was found to bind to in vitro transcribed zRpl22l1 mRNA, but not the zRpl22l1 mRNA lacking the hairpin structure (zRpl22l1Δhp) (Figure 4C), suggesting that Rpl22 directly binds to Rpl22l1 mRNA.

To determine if Rpl22 directly regulates expression of Rpl22l1, we employed a biosensor quantification assay using GFP as a fluorescent indicator of effects on expression. Zebrafish embryos were microinjected with mRNAs EGFP-zRpl22, EGFP-Rpl22l1 or a mutant form of Rpl22l1 in which the Rpl22l1 hairpin was modified (EGFP-zRpl22l1mt) in combination with constructs expressing zRpl22 or zRpl22l1. mCherry mRNA was co-injected to allow for quantification of the relative fluorescence intensity. Co-injection of zRpl22 with EGFP-Rpl22l1mt led to a significant decreased fluorescence relative to those embryos co-injected with zRpl22 and EGFP-Rpl22l1mt (Figure 5A–E), suggesting that the hairpin structure within Rpl22l1 mRNA is necessary for Rpl22 to directly regulate its expression. Next, to assess if the presence of the hairpin structure within the Rpl22l1 mRNA is sufficient to regulate expression, the hairpin sequence from zRpl22l1 mRNA was fused to EGFP and evaluated in the biosensor quantification assay. The heterologous reporter mRNA, zRpl22l1-150-EGFP, containing the minimal sequence identified by M-fold to form the hairpin structure, was co-injected with mCherry mRNA (injection control) and (Figure 5G) zRpl22 mRNA or (Figure 5I) Rpl22-Morpholino (Rpl22-MO) into zebrafish embryos. Rpl22 repressed the expression of zRpl22l1-150-EGFP reporter, while knockdown Rpl22 can increase the expression of reporter, suggesting the hairpin structure identified in zRpl22l1 mRNA is sufficient to regulate mRNA abundance.

Rpl22l1 incorporation into ribosomes is associated with increased cell proliferation

In yeast, ribosomal protein paralogs are thought to functionally compensate for one another, each incorporating into the ribosome in the absence of the other. Deletion of both yeast RPL22 paralogs results in viable, but slow growing cells [37]. Recently Rpl22+/+ or Rpl22+/− primary mouse embryonic fibroblasts (MEFs) were found to grow faster and display increased transformation potential relative to MEFs isolated from Rpl22−/− littersmates [45]. To further evaluate the effect of Rpl22 and Rpl22l1 on expression on growth rates, Rpl22+/+ or Rpl22+/− 3T9 fibroblasts were transfected with one of 2 different tet-on shRNA lentivirus constructs (shRNA#1- and shRNA#2-Rpl22l1) that target

D, which blocks transcription by all three eukaryotic polymerases [52]. After actinomycin D treatment, the levels of Rpl22l1 mRNA in Rpl22+/+ 3T9 cells decreased significantly (p<0.01) relative to the untreated control, while in Rpl22−/− 3T9 cells Rpl22l1 levels were maintained and the rate of decay was reduced (Figure 4A). These results suggest that Rpl22 affects the stability of Rpl22l1 mRNA.

Figure 2. Both mouse Rpl22 and Rpl22l1 proteins can be incorporated into ribosomes. Liver tissue was isolated from Rpl22+/+ (A, C) and Rpl22−/− (B, D) mice then, after sedimentation of the lysates on sucrose gradients, fractions were collected and loaded onto an SDS-page gel for western blot analysis (C and D, respectively). Images are representative of 3 independent experiments. Multiple Reaction Monitoring Mass spectrometry (MRM-MS) analysis was performed on free 60S subunits and 80S monosomes from actively translating polysomes. Liver lysates from Rpl22+/+ and Rpl22−/− mice were subjected to a brief treatment with low amounts of RNase A to degrade mRNA between ribosomes in polysomes and release the ribosomes as 80S monomers. After inhibiting the RNase with KCl and degrading mRNA between ribosomes in polysomes and releasing the ribosomes as 80S monomers, polysomes were run on a protein gel. Proteins bound to radiolabeled RNA were detected by autoradiogram at their expected molecular weight. Unbound proteins are not detected on autoradiogram. Recombinant Rpl22 was found to bind to in vitro transcribed zRpl22l1 mRNA, but not the zRpl22l1 mRNA lacking the hairpin structure (zRpl22l1Δhp) (Figure 4C), suggesting that Rpl22 directly binds to Rpl22l1 mRNA.

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Rpl22l1 mRNA to acutely knock-down its expression. Western blot analysis confirmed that Rpl22l1 protein levels were elevated in Rpl22\(^{-/-}\) 3T9 fibroblasts (Figure 6A). No significant difference was observed in the growth rates of Rpl22\(^{+/+}\) or Rpl22\(^{-/-}\) 3T9 fibroblasts (Figure 6B). In doxycycline-treated Rpl22\(^{+/+}\) or Rpl22\(^{-/-}\) 3T9 fibroblasts expressing the shRNA constructs, Rpl22l1 protein levels were confirmed to be reduced by western blot analysis (Figure 6C, E). Knockdown of Rpl22 significantly reduced growth rates of Rpl22\(^{+/+}\) fibroblasts and greatly impaired that of Rpl22\(^{-/-}\) 3T9 fibroblasts (Figure 6D, F and S6), indicating that cells lacking both paralogs have severe growth defects. In contrast, acute knockdown of Rpl22 in Rpl22\(^{+/+}\) 3T9 fibroblasts resulted in no change in the rate of proliferation (Figure S7). In summary, these cell culture studies indicate that expression of at least one paralog of Rpl22 is required for normal growth and suggests that Rpl22l1 may also affect cell proliferation by a mechanism independent of Rpl22.

**Discussion**

Here we report on phenotypes of mice lacking the large subunit ribosomal protein, Rpl22. Surprisingly, Rpl22\(^{-/-}\) mice have no developmental defects, other than previously reported defects in T and B cell development (Anderson et al. 2007 and our data). More generally, Rpl22\(^{-/-}\) mice have no significant defects in translation, as judged by sucrose gradient sedimentation to detect ribosome occupancy on transcripts (unpublished data). This is somewhat surprising since yeast lacking Rpl22 function are slow growing with substantial defects in translation [10,37]. One possible explanation is that the lack of a defect in translation in Rpl22\(^{-/-}\) mice is explained by compensatory increases in expression of another gene, Rpl22l1, which shares a high degree of homology with Rpl22. While Rpl22l1 mRNA is detected at low levels in most tissues of wild-type mice, mRNA and protein levels are dramatically increased in the Rpl22 knockout. It is interesting that Rpl22l1 is expressed at relatively high levels in the pancreas even though Rpl22 is present as well. Future studies will be needed to determine why these two paralogs are jointly expressed in this tissue. When expressed, Rpl22l1 was incorporated into ribosomes and actively translating polysomes. These findings indicate that Rpl22l1 is capable of functioning within ribosomes that are actively translating mRNA. Future studies examining the function of Rpl22l1 will help decipher to what extent Rpl22 and Rpl22l1 may have redundant roles and also determine their independent functions, for which accumulating evidence is emerging [28,45] (O’Leary et al, unpublished data).

Although poorly understood, gene compensation during development is a recurrent phenomenon in mouse knockout studies [55]; therefore, enhanced expression of Rpl22l1 might reflect developmental compensation in Rpl22\(^{-/-}\) mice. Alternatively, Rpl22 could play an active role in the repression of Rpl22l1. To test this, we examined the consequences of acute knockdown of Rpl22 in 3T9 fibroblasts. Our findings indicate that Rpl22l1 mRNA and protein levels are rapidly increased following Rpl22 knockdown and support the conclusion that developmental compensation does not account for the increased Rpl22l1 mRNA and protein levels. Instead, we find that inhibition of transcription in cells lacking Rpl22 results a slower decay of Rpl22l1 mRNA compared to Rpl22\(^{+/+}\) cells, suggesting Rpl22 affects the stability of Rpl22l1 mRNA. It is possible that more than one mechanism is involved in increasing Rpl22l1 expression in the absence of Rpl22. Further studies interrogating other mechanisms are needed to fully understand what regulates Rpl22l1 upon Rpl22 deficiency. We find that Rpl22 binds to a hairpin structure in the Rpl22l1 mRNA. The hairpin motif identified in Rpl22l1 mRNA is necessary and sufficient for regulation of mRNA abundance by Rpl22. These data suggest that Rpl22 might function in an extra-ribosomal capacity to bind and destabilize Rpl22l1 mRNA. Rpl22l1 is yet another RNA demonstrated to interact with Rpl22. The viral RNA EBER1 contains three Rpl22 binding sites and is thought to compete with the 28S rRNA for Rpl22 binding in Epstein-Barr virus-infected cells [42,43,53,56,57]. In yeast, recent studies have revealed regulation of the expression of one paralog in a duplicated RP gene pair by the other. Ribosomal protein S29A (Rps29a), for example, regulates its own expression along with expression of its paralog, RPS29B [58]. Interestingly, Rpl22 has also been ascribed functions independent of its role as a component of the ribosome [17,18]; these
extra-ribosomal functions include regulation of telomerase activity [44] and association with histone H1 [59]. In fact, many ribosomal proteins, including Rpl7, Rpl13a, and Rps5 [60–62] have extra-ribosomal functions, which will have to be considered as mechanistic links are sought to explain diseases associated with RP mutations and possibly age-related phenotypes. Given their ancient nature, it is not surprising that evolution has settled on ways of exploiting these proteins for multiple uses.

Do Rpl22 and Rpl22l1 have shared or unique functions? The observation that one ribosomal protein represses expression of its own paralog is likely to be of biological significance and determining whether mice lacking Rpl22l1 have specific phenotypes will identify tissues or conditions where Rpl22l1 is the functional RPL22 paralog that participates in translation. We propose that Rpl22 and Rpl22l1 share the ability to participate in protein translation as part of the ribosome, since both paralogs are found incorporated into ribosomes and Rpl22 has an active role in suppressing expression of Rpl22l1. However, unique roles are also well established, since knockout of mouse Rpl22 leads to specific phenotypes in T and B cell development (Anderson et al. 2007 and our data), and data from Zhang et al. indicate that both Rpl22 and Rpl22l1 are essential for T cell development in zebrafish and exhibit antagonistic functions in regulating the emergence of hematopoietic stem cells [63]. Together, these observations lead us to propose that Rpl22 and Rpl22l1 may have overlapping activities, sharing a role in enhancing large subunit ribosome function but also having distinct roles in development of the immune system [63] and, in the case of Rpl22l1, perhaps other tissues.

Based on data from S. cerevisiae, in which genes for most ribosomal proteins are duplicated, Komili et al. proposed the existence of a ribosome code, whereby ribosome subunits composed of different ribosomal proteins would have differential specificity for mRNAs [38]. This proposal was based on an accumulation of data, including differential localization of paralogs and large-scale phenotypic screens, which indicated that specific subsets of ribosomal protein genes were often identified in phenotypic screens of gene deletion strains. This is an exciting hypothesis that, if corroborated, would identify ribosome composition as a new mechanism for regulation of gene expression. Our studies in yeast raise the possibility that

Figure 4. Rpl22 directly binds Rpl22l1 mRNA to regulate its expression levels. (A) In the absence of Rpl22, Rpl22l1 mRNA levels are more stable in the presence of Actinomycin D. Rpl22+/+ or Rpl22−/− 3T9 cells were treated with Actinomycin D (1 μM final concentration) and total RNA was harvested at the time points shown. Levels of Rpl22l1 mRNA were quantitated by qRT-PCR. Results are the average ± SEM of 3 independent experiments and the statistical significance indicated is (*, p<0.01, compared to Rpl22+/+ untreated; ** p<0.001, compared to Rpl22+/+ at each time point). (B) M-fold analysis [54] of zRpl22l1 mRNA reveals the presence of a consensus Rpl22 RNA-binding motif. In green are the residues deleted to remove the hairpin (zRpl22l1hp). In blue are the residues known to be essential for Rpl22 binding. (C) Autoradiogram of ribonuclease protection assay reveals Rpl22 protein binds to Rpl22l1 mRNA and this binding is abrogated upon removal of the hairpin. 32P labeled EBER1 (positive control), EBER2 (negative control), zRpl22l1 or zRpl22l1hp RNAs were incubated in the absence or presence of GST-Rpl22 (41.7 kDa), GST (27 kDa) or m88, a GST-Rpl22 RNA binding mutant (41.6 kDa), as indicated, then UV-cross-linked, digested with RNase A, and run on a SDS protein gel. GST-Rpl22 was detected, hence, bound to EBER1 and zRpl22l1 RNAs but not Rpl22l1hp RNA, indicating Rpl22 binds to Rpl22l1 mRNA and this binding is abrogated upon removal of the hairpin. Numbers indicate molecular weight protein ladder in kDa. doi:10.1371/journal.pgen.1003708.g004
Figure 5. Regulation of Rpl22l1 mRNA expression is mediated by a hairpin structure. (A) Schematic representation of the biosensor quantification assay. (B–D) Stereoimages of zebrafish embryos illustrate that co-injection of zRpl22 repressed fluorescence derived from an EGFP-Rpl22l1 fusion protein upon injecting mRNA for both and assessing fluorescence at 6 hours post fertilization. Rpl22, Rpl22l1 or mutated Rpl22l1 (Rpl22l1mt) coding sequence was fused to EGFP mRNA and co-injected with mCherry mRNA (injection control) along with the corresponding inhibitor mRNAs (zRpl22 or zRpl22l1) into 1-cell stage zebrafish embryos. (F) Schematic representation of the experimental procedure. A zRpl22l1-150h-EGFP heterologous reporter mRNA, containing the minimal sequence identified by mFold to form the hairpin structure, was co-injected with mCherry mRNA (injection control) and (G) Rpl22 mRNA or (I) Rpl22-Morpholino (Rpl22-MO) into 1-cell stage zebrafish embryos. (E, H, J) At 10 hpf, the relative fluorescence intensity was calculated and normalized to control injections (n = 3, each group). Data are shown as mean ± standard deviation (s.d.). doi:10.1371/journal.pgen.1003708.g005
ribosome specificity may occur in yeast aging [10,37]. In a long-lived, slow-growing and translation-compromised \( rpl22a \) background, we find that deletion of the other paralog, \( RPL22B \), causes no significant further reduction in translation, but blocks lifespan extension (Steffen et al., unpublished data). One possible explanation for this result is that the portion of ribosomes containing \( Rpl22b \) is increased in the \( rpl22a \) background, leading to specific changes in translation conducive to enhanced replicative lifespan. Other explanations are possible and further studies will be necessary to test this important hypothesis. Structural studies do not point to an obvious mechanism by which \( Rpl22 \) paralogs would influence the pool of translated RNAs and other models involving specific non-ribosomal functions of \( Rpl22 \) and \( Rpl22l1 \) have to be identified and/or tested.

One limitation to the ribosome specificity hypothesis proposed in \( S. cerevisiae \) is that ribosomal proteins are generally not thought to be duplicated in other organisms. Therefore, if ribosome specificity indeed exists [38], it may be more prominent in yeast than mammals. Recently, Xue and Barna have suggested that specialized ribosomes might also regulate gene expression in mammals [64]. Our findings support the hypothesis, at least in the case of murine \( Rpl22 \), that one ribosomal protein may repress expression of the other, raising the possibility that ribosome specificity may extend to organisms other than yeast. In addition, a recent study of \( Rpl30 \) mutant embryos found that although global translation was unchanged, translation of Hox genes were altered [65], providing additional support for the hypothesis that differential composition of the ribosome might contribute to transcript-specific translational regulation. Cryo-EM studies of the eukaryotic 80S ribosome have demonstrated that \( Rpl38 \) is located on the surface of the ribosome and interacts with a region of the rRNA known as expansion segment 27 (ES27), which has two distinct orientations toward the L1 stalk or toward the exit tunnel [66,67]. The location of \( Rpl30 \) in the ribosome is consistent with its proposed role in regulating transcript-specific translation.

The tissue specific defects observed in \( Rh22^{+/−} \) and \( Rh29^{+/−} \) mice, along with mice expressing mutated \( Rpl38 \) [63], suggest that temporal and spatial expression of RPs are critical for proper development and tissue patterning. In plants and \( Drosophila \), many RPs paralogs display tissue specific variations and are differentially expressed during development [68–71]. In mammals, recent studies found mRNA expression patterns of RPs vary in different tissues and cell types [65,72]. Together with the findings presented here, these studies illustrate that heterogeneous expression of RPs is tightly regulated; however additional studies will be crucial in confirming the influence of these differential expression patterns on specialized ribosome activity and message specific translation.

Further studies are also required to address the questions that have arisen from this study. Are there tissues or cell types in mice

![Figure 6. Acute knockdown of Rpl22l1 expression impairs cellular growth.](image-url)
where Rpl22l1 and not Rpl22 is the predominant paralog? What is the mechanism by which Rpl22 represses expression of Rpl22l1 through interaction with its mRNA? What are the non-ribosomal functions of Rpl22 paralogs? And most importantly, do ribosomes with Rpl22l1 have different specificity for mRNAs than those with Rpl22? Findings presented in this study provide interesting leads in which to test the hypothesis that specialized ribosomes exist and, furthermore, point to a new level of regulation in ribosome biogenesis, wherein ribosomal paralogs regulate each other’s synthesis to optimally maintain the organism.

Materials and Methods

Generation of Rpl22 null mice

A detailed description of the targeting vector along with the generation and genotyping of the Rpl22−/− mice can be found in the Text S1.

Materials

DMEM tissue culture medium was purchased from Mediatech (Manassas, VA), L-glutamine, penicillin/streptomycin, and trypsin were from Gibco (Carlsbad, CA). Fetal calf serum was from Gemini (West Sacramento, CA). Rpl22 antibody was from BD Biosciences (Franklin Lakes, NJ). Rpl22l1 antibody was from Santa Cruz (Santa Cruz, CA) and the Rpl7 antibody was purchased from Novus Biologicals (Littleton, CO). GAPDH antibody was from Ambion (Austin, TX). HRP-conjugated donkey anti-rabbit and donkey anti-mouse were from Jackson Immunoresearch Laboratories, Inc (West Grove, PA). Rpl22, Rpl22l1, and ARBP primers were from Operon (Huntsville, AL). Lentiviral shRNA constructs V2MM_120192 and V2LHS_131608, directed at Rpl22 (denoted shRNA 1 and 2, respectively), along with V3LMM_473587 and V3LHS_322499, directed at Rpl22l1 (denoted shRNA 1 and 2, respectively), were purchased through Open Biosystems (Huntsville, AL) and. A non-silencing-TRIPZ lentiviral inducible shRNA mir control was also purchased through Open Biosystems.

RNA isolation and real time PCR

RNA was isolated from mouse tissue or 3T9 cells using the RNeasy kit (Qiagen) with a DNaseI treatment following the manufacturer’s protocol. cDNA synthesis was performed using oligo (dT) and reverse transcriptase Superscript Preamplification System (Invitrogen). Quantitative PCR was performed using a Bio-rad iCycler and measuring SYBR green incorporation for product detection. The fold-increase of Rpl22 and Rpl22l1 were calculated by the CT method and normalized to ARBP.

Mass spectrometry

A detailed description of the sample preparation and the experimental procedure can be found in the Text S1.

MEF isolation and 3T9 cell lines

Embryos were harvested at day 13.5dpc and mouse embryonic fibroblasts (MEFs) were harvested as previously described [74]. Following isolation, MEFs were cultured in DMEM (Dulbecco’s modified Eagle medium) containing 10% FBS, 1% Penicillin/Streptomycin and 1% L-Glutamine and subjected to 3T9 (Figure 6) protocol [74,75]. Cells were removed from tissue culture plates with 0.05% trypsin and 0.02% EDTA for 3 min at 37°C, washed with DMEM, and plated overnight prior to experimentation.

Lentiviral transduction

For Rpl22 and Rpl22l1 knockdown, lentiviral pGIPZ shRNA constructs were purchased from Open Biosystems and cloned into inducible pTRIPZ constructs. Briefly, 293T cells were transfected with pTRIPZ constructs bearing shRNA against Rpl22 (shRNA 1 and 2) or a non-silencing control using calcium phosphate transfection. After 48 h, viral supernatant was harvested and used to transduce 3T9 cells. Cells were selected for 5 days in puromycin (5 μg/ml) followed by 3 days of doxycycline (1 μg/ml) treatment to induce shRNA expression before experimentation. For cell proliferation assays, Rpl22−/− or Rpl22l1−/− 3T9 cells were infected with Notropilus cellulose membranes using the Invitrogen (Carlsbad, CA) Nu-Sage system. Proteins in polysome fractions from sucrose density gradients (described above) were TCA precipitated and resuspended in 30 μl of Sample Buffer (1× Laemmli buffer with bromophenol blue and DTT), while for mouse liver polysome fractions, 32 μl of each 1 ml fraction was loaded and proteins were separated and transferrred as described above. Blots were blocked with 5% milk in TBST for 1 h and incubated overnight at 4°C with the following dilutions of primary antibodies: Rpl22 (1:250), Rpl22l1 (1:250), Rpl7 (1:1000), GAPDH (1:50,000). Membranes were incubated for 2 h with HRP-conjugated donkey anti-rabbit or sheep anti-mouse (1:10,000) secondary antibodies and antigen was detected using enhanced chemiluminescence (ECL, Plus Western Blotting Detection System, Amersham).
with control shRNA or 1 of 2 shRNA constructs targeting Rpl22 or Rpl22l1. After 5 days of selection in puromycin, growth rates of cells transduced with each shRNA construct were determined by plating cells in triplicate at a density of 30,000 cells/well in 6-well plate in media with or without doxycycline (1 μg/ml). Every 3 days for the cells were counted and replated at 30,000 cells/well for 15 days. Protein was isolated from 10 cm plates treated in parallel to the cells were counted and replated at 30,000 cells/well for 15 days.

### Bioinformatics

**Protein sequences for murine Rpl22 (NP_033105) and Rpl22l1 (NP_080793)** were obtained from Genbank (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed using ClustalW2 Algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/), mRNA sequences for mRpl22l1 (NM_026517) and zRpl22l1 (NM_001043335) were entered into mFold (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) for prediction of RNA secondary structure.

**Statistical analysis**

Statistical comparisons were made between groups by a 2-tailed Mann-Whitney U test (Figures 4, S2, S3, S4), or a 1-way ANOVA (Figure 3). Significant differences between groups are indicated in figure legends.

### Zebrafish embryos

AB wild-type zebrafish strain was bred and maintained at 28.5°C under standard aquaculture conditions. Embryos were staged as described previously [76].

### mRNAs, morpholino microinjection and biosensor quantification assay in zebrafish embryos

Full-length coding sequence for EGFP, mCherry, zebrafish Rpl22 (zRpl22) and Rpl22l1 (zRpl22l1) were cloned into pCS2+. Full-length cDNA sequences encoding EGFP-zRpl22 and EGFP-zRpl22l1 were subcloned in the pCS2+. The zRpl22l1mut, mutant form of zRpl22l1 in which hairpin GATGGGATTCTC-GATT was mutated to GACGGTATCTTAGACT, was generated from plasmid zRpl22l1-pCS2+ and EGFP-zRpl22l1-pCS2+, respectively, with deletion of the hairpin TGGG-ATTCTCGA using GeneTailor mutagenesis kit according to manufacturer’s instruction [Invitrogen, Carlsbad, CA].

**Plasmids**

The coding regions of EBER1 and EBER2 were amplified by PCR from the cDNA of EBV-infected African Burkitt lymphoma KabIM cells (kindly provided by Jeffrey Sample, Penn State Hershey College of Medicine, Hershey, PA), adding a T7 promoter, and cloned into pCR2.1 plasmid (Invitrogen, Carlsbad, CA). The primers used for PCR were: forward 5’-TAATA CGACCTCACTATAGGCAACCCCTCACGGCCTACCGT, TAATACGACTCAGATATAGGTACTTCGGAATAC and reverse 5’-GAACCTGCGGGATAATGATGCA, AAGCCGAAATCC TTTCTCCAG for EBER 1 and EBER2 respectively. Then the EGFP-zRpl22l1mut were generated from plasmid zRpl22l1-pCS2+ and EGFP-zRpl22l1-pCS2+, respectively, with deletion of the hairpin TGGG-ATTCTCGA using GeneTailor mutagenesis kit according to manufacturer’s instruction [Invitrogen, Carlsbad, CA].

**RNAse protection assay**

The plasmids used for RPA assay were EBER 1, EBER 2, zRpl22l1 and zRpl22l1-Hap. The plasmids were linearized using EcoRI for EBERs and NotI for zRpl22l1s (enzymes from New England Biolab, Ipswich, MA), and radioactive probes were prepared by in vitro transcription with T7 (EBERs) or SP6 (zRpl22l1s) RNA polymerases at 37°C (Maxiscript, Ambion, TX), in presence of 10 μCi of 3ZP-UTP (Perkin Elmer, Boston, MA), and purified by G-50 columns (Illustra Probe-Quant, GE Healthcare, Buckinghamshire, UK). The radiolabeled RNAs were renatured for 2 min at 95°C and kept on ice for 5 min. Radiolabeled RNAs and protein were incubated on ice for 12 min, in presence of 5 μg of tRNA (Roche) in RPA buffer (10 mM Tris pH 8.0, 50 mM NaCl, 0.75 mM MgCl2, 0.8 mM DTT and 2.5% glycerol), and the mixtures were cross-linked on ice pack for 30 min (using a UV Stratalinker 1800). After cross-linking, the mixture were digested by RNase A (Qiagen, Valencia, CA, 7,500 U/μl) diluted to 3,750 U/μl in RNase A buffer (20 mM Tris pH 7.0, 2 mM MgCl2, and 0.2 mM KCl) for 20 min at 37°C, then 2 μl of denaturing dye were added before denaturation for 2 min at 90°C. Nine μl of the reaction mixtures were loaded on 4–12% Bis-Tris gel, 1.5 mm (Invitrogen, Carlsbad, CA). The gels were dried and expose to BioMax MR films (Kodak, Rochester, NY) or to a phosphoimager plate read using a Fuji BAS-2500 reader.

### Protein expression and purification

E. coli strain BL21DE(3) pLys (Promega, Madison, WI) was used to produce recombinant GST, GST-hRpl22 and GST- hRpl22l1 from plasmids pGEX-3X, pGEX-hRpl22 and pGEX-hRplm88. Isopropyl-β-D-thiogalactopyranoside ( IPTG) at a final concentration of 100 μM was added to 2× YT media at 30°C after 3 h of incubation. The cells were harvested by centrifugation, washed with 0.9% NaCl, and resuspended in 20 mM Tris pH 7.0, 200 mM NaCl, 20 mM MgCl2, 0.5 mM DTT and 2.5% glycerol. The cells were then ultrasonicated on ice and centrifuged at 20,000 g for 45 min. The supernatant was then loaded on a 50 ml column of 5% Bio-Gel P-100 (Bio-Rad) and washed with 20 mM Tris pH 7.0, 200 mM NaCl, 20 mM MgCl2, 0.5 mM DTT and 2.5% glycerol. The GST-hRplm88 protein was eluted with 20 mM Tris pH 7.0, 200 mM NaCl, 20 mM MgCl2, 0.5 mM DTT and 2.5% glycerol.
optical density A600 = 0.4, and incubation was maintained for 4 hours at 37°C. The cells were harvested and resuspended at 0.25 mg/ml in 1 x PBS (2.6 mM KCl, 1.7 mM KH2PO4, 137 mM NaCl, 11 mM Na2HPO4, pH 7.4) and lysed by 3 passages in a M-110L Pneumatic (Microfluidics, Newton, MA), in presence of Complete mini without EDTA tablets (Roche, Mannheim, Germany). The cell extracts were loaded onto glutathione sepharose beads for batch purification (GE Healthcare, Uppsala, Sweden) at 2 ml/batch of culture. The beads, 0.5 ml, were washed with 1 ml of wash buffer (50 mM Tris, pH 9.5, 150 mM NaCl), then washed with 1 ml of lysis buffer (50 mM Tris pH 9.5, 150 mM NaCl, 1 mM reduced GSH). The proteins were eluted with 3 fractions of 1 ml elution buffer (50 mM Tris pH 9.5, 150 mM NaCl, 10 mM reduced GSH). The protein purity was assessed by SDS gel electrophoresis, and the concentration measured using Coomassie Plus (Bradford) Protein Assay (Pierce, Rockford, IL). The proteins were stored in 10% glycerol in 20 μl aliquots at −80°C.

Ethics statement
Appropriate protocols of all work on mice have been approved by the IACUC committees at the Institution where the work was performed and is in accord with accepted national guidelines.

Supporting Information

Figure S1 Generation of Rpl22−/− mice. (A) Schematic of Rpl22 targeting strategy; (B) Gel shows PCR of mouse tail DNA from Rpl22+/−, Rpl22−/− and Rpl22−/+ mice. (C) Western blot with α-RPL22 antibody antibodies isolated from Rpl22+/+ and Rpl22−/− mice. (TIF)

Figure S2 Rpl22−/− mice have impaired thymocyte development. Thymocytes from Rpl22−/− mice were analyzed to confirm that Rpl22 deficiency resulted in altered T cell development. Thymocytes were stained for CD4, CD8, CD44, CD25, panTCRβ, and TCRγδ surface expression. (A) Representative flow cytometry plots show CD4/CD8 analysis of live-gated thymocytes. Charts show the absolute number of each indicated population. The thymic compartment in Rpl22−/− mice was comprised mostly of CD4−CD8− double negative (DN) thymocytes, while CD4+CD8+ double-positive (DP) and CD4 and CD8 single-positive (SP) populations were clearly diminished. Thymic cellularity was significantly reduced at the DN, DP, and SP stages. (B) CD4/CD8/CD25 plots of CD4−CD8− DN thymocytes and chart showing the absolute number of each DN population (DN1: CD4+CD25−, DN2: CD4+CD25+, DN3: CD4−CD25+, DN4: CD4−CD25−). DN thymocytes had an apparent blockade of development between the DN3 (CD4+CD25+) to DN4 (CD4−CD25−) stages, as evidenced by an increased frequency of DN3 cells, and significantly reduced frequency and number of DN4 cells. (C) Representative flow cytometry plots of total thymocytes analyzed for TCRγδ and panTCRβ surface expression. Chart shows the absolute number of TCRγδ+ thymocytes. γδ T cell development was unimpaired, as evidenced by the similar number of TCR γδ + cells in the thymus of Rpl22−/− mice. Data are compiled from 3 independent experiments (Rpl22+/+ N = 4, Rpl22−/− N = 4). Numbers in plots represent the percentage of each gated population. Charts show the mean percent or number with error bars indicating the standard deviation. P values were calculated using a two-tailed Student’s t test (* p<0.05, ** p<0.001, *** p<0.0001). (TIF)

Figure S3 2B T cell and B cell numbers are reduced in the lymphoid periphery of Rpl22−/− mice. Splenocytes were surface stained for CD4, CD8, CD19, panTCRβ, and TCRγδ. (A) Representative flow cytometry plots and chart show the percent of CD4+ B cells and CD4+ and CD8+ T cells. (B) Absolute number of total splenocytes and CD19+, CD4+ and CD8+ cells per spleen. (C) Rpl22−/− mice also had reduced frequencies and numbers of splenic CD4+ and CD8+ T cells and reduced B cell numbers. (C) Representative plots and chart showing percent of TCRβ+ and TCRγδ+ cells per spleen. (D) Absolute number of TCRβ+ and TCRγδ+ cells per spleen. The frequency of splenic γδ T cells was significantly increased (C), while the γδ T cell number remained similar to that of wild-type mice (D). Data are compiled from 2 independent experiments (Rpl22+/+ N = 3, Rpl22−/− N = 3). Numbers in plots represent the percentage of each gated population. Charts show the mean percent or number with error bars indicating the standard deviation. P values were calculated using a two-tailed Student’s t test (* p<0.05, ** p<0.001, *** p<0.0001). (TIF)

Figure S4 B cell development in Rpl22−/− mice is impaired. Bone marrow single-cell suspensions were stained for surface B220, IgM, and IgD. (A) Representative histograms show the percent of live-gated cells that are B220+. (B) Absolute number of total bone marrow cells and B220− and B220+ populations. (C) Representative IgM/IgD plots of B220+ gated bone marrow cells. (D) Absolute number of B220+ bone marrow cells that are IgM−/IgD−, IgMH+IgD− (immature B cells), and IgM+IgD+ (mature B cells). Data are compiled from 2 independent experiments (Rpl22+/+ N = 3, Rpl22−/− N = 3). Numbers in plots represent the percentage of each gated population. Charts show the mean percent or number with error bars indicating the standard deviation. P values were calculated using a two-tailed Student’s t test (** p<0.001). (TIF)

Figure S5 Alignment of mouse, human and zebrafish Rpl22l1 protein sequences. Identical amino acids are indicated by an * beneath the alignment. (TIF)

Figure S6 Knockdown of Rpl22l1 expression with a second shRNA impairs cellular growth. Rpl22−/− 3T9 cells were transduced with a second shRNA construct directed at Rpl22l1 (shRNA-Rpl22l1 and shRNA-Rpl22l2) and selected with puromycin for at least 5 days. Growth rates of cells transduced with the shRNA construct were determined by plating cells in triplicate at a density of 30,000 cells/well in 6-well plate in media with or without doxycycline (1 μg/ml). Every 3 days for the cells were counted and replated at 30,000 cells/well for 15 days. (A) Growth of Rpl22−/− 3T9 cells transduced with the shRNA2−/−2-Rpl22l1 construct was determined. Knockdown of Rpl22l1 by shRNA2−/−2-Rpl22l1 represses growth in Rpl22−/− cells. (B) Levels of Rpl22l1 were analyzed by Western blot analysis to confirm that the Rpl22l1-shRNA knocked down levels of Rpl22l1 in doxycycline-treated Rpl22−/− 3T9 cells transduced with the shRNA2−/−2-Rpl22l1 construct. Results are representative of 2 independent experiments with error bars representative of ±SD. Statistical significance is indicated (*, p<0.05 compared to untreated control). (TIF)

Figure S7 Acute knockdown of Rpl22l1 expression has no significant effect on cellular growth. Rpl22−/− 3T9 cells were transduced with doxycycline-inducible shRNA lentiviral constructs directed at Rpl22l1 (shRNA1-Rpl22l1 or shRNA2-Rpl22l1) or a non-
specific shRNA construct (shRNA-NS) and selected with puromycin for at least 5 days. Growth rates of cells transfected with the shRNA construct were determined by plating cells in triplicate at a density of 30,000 cells/well in 6-well plate in media with or without doxycycline (1 μg/ml). Every 3 days for the cells were counted and replated at 30,000 cells/well for 15 days. Growth of Rpl22+/−/− 3T9 cells transfected with each shRNA construct was determined. Knockdown of Rpl22 by (A) shRNA1-Rpl22 or (B) shRNA2-Rpl22 does not repress growth in Rpl22+/−/− cells. Results are representative of 2 independent experiments with error bars representative of ±SD.

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