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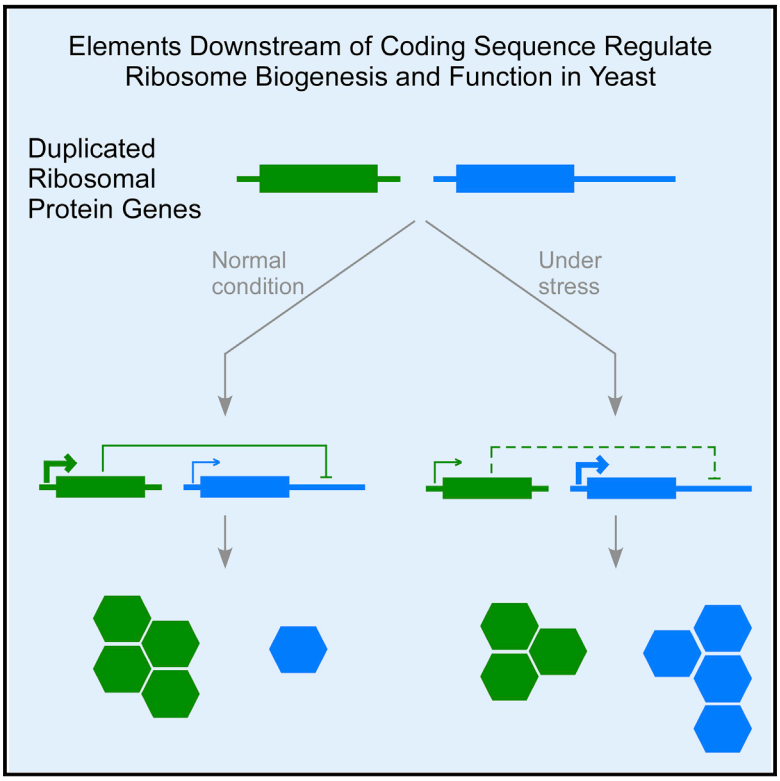
Preservation of Gene Duplication Increases the Regulatory Spectrum of Ribosomal Protein Genes and Enhances Growth under Stress

Graphical Abstract



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In Brief

Parenteau et al. describe the mechanism regulating the expression of non-intron-encoding ribosomal protein genes (RPGs) and explain the basis of the copy-specific function and expression patterns of the duplicated RPGs. Paralog-specific phenotypic effects are generated by differences in expression patterns supporting growth under stress.

Highlights

- Duplicated ribosomal protein genes (RPGs) are asymmetrically expressed and regulated
- Transcription termination regulates the expression of non-intron-containing RPGs
- Differences in expression levels enforce the subfunctionalization of duplicated RPGs
- Duplication of RPGs improves cell fitness and growth under stress

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Preservation of Gene Duplication Increases the Regulatory Spectrum of Ribosomal Protein Genes and Enhances Growth under Stress

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SUMMARY

In baker's yeast, the majority of ribosomal protein genes (RPGs) are duplicated, and it was recently proposed that such duplications are preserved via the functional specialization of the duplicated genes. However, the origin and nature of duplicated RPGs' (dRPGs) functional specificity remain unclear. In this study, we show that differences in dRPG functions are generated by variations in the modality of gene expression and, to a lesser extent, by protein sequence. Analysis of the sequence and expression patterns of non-intron-containing RPGs indicates that each dRPG is controlled by specific regulatory sequences modulating its expression levels in response to changing growth conditions. Homogenization of dRPG sequences reduces cell tolerance to growth under stress without changing the number of expressed genes. Together, the data reveal a model where duplicated genes provide a means for modulating the expression of ribosomal proteins in response to stress.

INTRODUCTION

Ribosomes are traditionally viewed as uniform units of ribonucleoprotein complexes composed of four rRNAs (18S, 5S, 5.8S, and 25S rRNA) and ~80 proteins (Ben-Shem et al., 2010). However, recent studies indicate that eukaryotic cells may produce ribosomes with different compositions and functions (Xue and Barna, 2012). For example, inclusion of the tissue-specific ribosomal protein L38 was shown to facilitate cap-independent translation of mRNA featuring internal ribosome entry site (IRES)-like structures (Xue et al., 2015). Modification of ribosome functions could also be achieved through the association with non-ribosomal proteins like the receptor for activated C kinase 1 (RACK1), which promotes mRNA-specific repression of translation via the recruitment of microRNA (miRNA) (Jannot et al., 2011). Similarly, the stress-induced protein mazEF was shown to modulate the function of bacterial ribosomes by removing the anti-Shine-Dalgarno (aSD) sequence required for the translation of normal mRNAs (Vesper et al., 2011). In yeast, the majority of ribosomal protein genes (RPGs) are duplicated (dRPGs, Figure S1A), and this leads to the generation of ribosomes with different protein configurations (Wapinski et al., 2010). However, the reason behind the preservation of this ribosomal gene duplication and its impact on cell functions remain unclear.

The duplication of RPGs in *Saccharomyces cerevisiae* is believed to be the consequence of a whole-genome duplication event that occurred before the *Saccharomyces* and *Kluyveromyces* lineages diverged from each other about 150 million years ago (Langkjaer et al., 2003). This presumed polyploidization was followed by substantial losses of duplicated genes through degenerative processes, except for a few gene classes like the RPGs. Approximately 10% of the surviving ohnologs (i.e., paralogs generated by whole-genome duplication events) encode ribosomal proteins (RPs) (Evangelisti and Conant, 2010). The majority of the surviving RPG ohnologs produce proteins with more than 95% sequence identity (Wapinski et al., 2010) due to gene conversion events that maintain similarity between the duplicated genes (Evangelisti and Conant, 2010). Despite this high similarity between protein sequences, deletions of yeast ohnologs result in different phenotypes, suggesting that they may have developed specialized functions responsible for their preservation (Komili et al., 2007; Parenteau et al., 2011). However, the mechanism by which dRPGs might preferentially affect cell function remains largely unexplored. It was proposed that duplicated genes could be preserved through partitioning of ancestral gene functions by qualitative or quantitative subfunctionalization or by neofunctionalization of the duplicated genes (Force et al., 1999; Lynch, 2007). In the first model, the dRPGs in *S. cerevisiae* would be preserved because they complement each other's expression levels or functions, while, in the second, the dRPGs would be preserved because one or both genes developed new functions not found in their ancestral gene but at the expense of ancestral gene functions.

Unlike most genes in *S. cerevisiae*, 81% of dRPGs include introns and require splicing for expression (Parenteau et al., 2011). Analysis of intron-encoding RPGs suggested that the functional specialization of ohnologs may result at least in part from differences in expression patterns (Parenteau et al., 2011). Intron deletions affected the expression of dRPGs in different ways leading to the modification of ohnolog expression

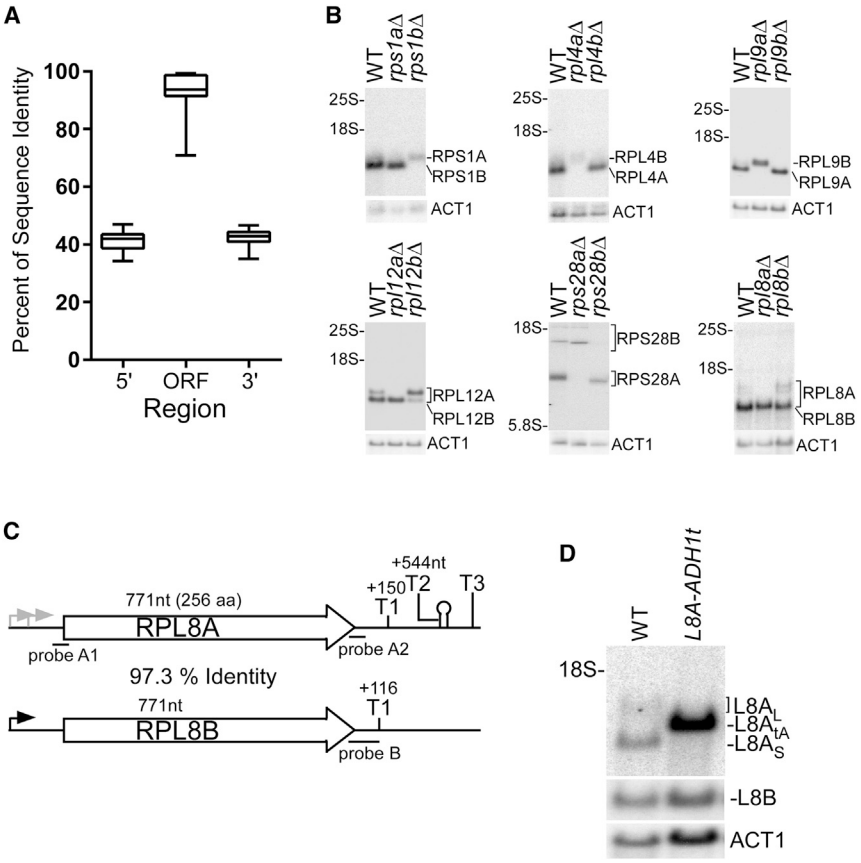

Figure 1. niRPGs Have Ohnolog-Specific Regulatory Sequences and Expression Patterns

(A) The average percentage sequence identity between *S. cerevisiae* niRPG ohnologs. The percentage identity of the coding sequence (open reading frame [ORF]) and the surrounding 500 nucleotides (5′ and 3′) is shown in the form of a boxplot. The horizontal black line inside the box indicates the median, boxes delineate first and third quartiles, and whiskers delineate data points at or less than 1.5 times the first to third interquartile range.

(B) RNA extracted from wild-type (WT) cells or cells carrying deletions in ohnologs with variable UTR length was visualized using probes complementary to each gene pair. *ACT1* mRNA is shown at the bottom as loading control (see also Figure S1).

(C) Representation of the *RPL8* gene-pair structure. The positions of transcription start (arrow heads) and termination (T1, T2 and T3) sites and probes positions are indicated.

(D) Northern blot analysis using probes specific to each *RPL8* ohnolog. $L8A_L$ and $L8A_S$ indicate the position of the long and short forms of Rpl8A. $L8A_{tA}$ indicates the RNA produced from *RPL8A* gene fused to *ADH1* terminator (*L8A-ADH1t*). The position of the 18S rRNA is shown on the left (see also Figure S2).

ratios and decreased growth under stress. Consistently, growth under stress, including exposure to drugs, modulated the expression of dRPGs in an ohnolog-specific manner and this modulation was suppressed when introns were deleted (Parenteau et al., 2011). This suggests that introns play an important role in defining the expression ratios of the 48 intron-containing RPG-pairs and control the cellular response to stress. However, it remains unclear how the expression of the other 11 non-intron encoding dRPGs (niRPGs) is coordinated and how the differences in the ohnolog-specific phenotypes are generated. In this study, we evaluated the mechanism regulating the expression of niRPGs and monitored their impact on cell growth. Most duplicated genes were differentially expressed and exhibited high variation in both the upstream and downstream regulatory sequences required for gene expression. Changes in ohnolog regulatory elements resulted in the production of multiple RNA isoforms with different sizes, stability, and sensitivity to growth under stress. Expression of two copies of the same gene failed to restore the ohnolog-specific deletion defects and increased sensitivity to drugs, illustrating the specificity of ohnolog function. Homogenization of ohnolog regulatory or coding sequences separately largely restored growth under stress, suggesting that the functional specificity of the duplicated genes stems from the combined differences in both expression pattern and protein functions. Together, our data reveal a new model for ribosome production, where one RPG may provide most RPs needed for growth under normal condition, while the other provides the extra RP amounts and/or function needed for optimum growth in response to a changing environment. This duality increases the regulatory spectrum of RPGs to meet the demands for coordinating the expression of the different ribosome components via autoregulation, while maintaining the capacity for rapid response to stress.

RESULTS

Expression of niRPGs Is Regulated by Ohnolog-Specific Regulatory Sequences

Comparison of niRPG sequences indicates that, while the coding sequence is highly conserved between ohnologs, the adjacent 5′ and 3′ sequence, which include the promoters, UTR, and transcription-termination sequences are highly variable (Figure 1A; Table S1). Indeed, on average only 40% of the regulatory sequence was shared between ohnologs. In order to determine whether or not the detected variations in the UTR primary structure reflects differences in gene regulation, we monitored the expression levels of ohnologs with predicted differences in UTR length using northern blots. We focused on mRNAs with different lengths to permit direct comparison between the ohnologs using a single probe. As shown in Figure 1B, all six gene pairs tested displayed differences in mRNAs size and amount, confirming the differential expression of niRPG ohnologs. The underexpressed copy of all gene pairs (except *RPL9* and *RPS1*) generated two or more mRNA forms, and in most cases

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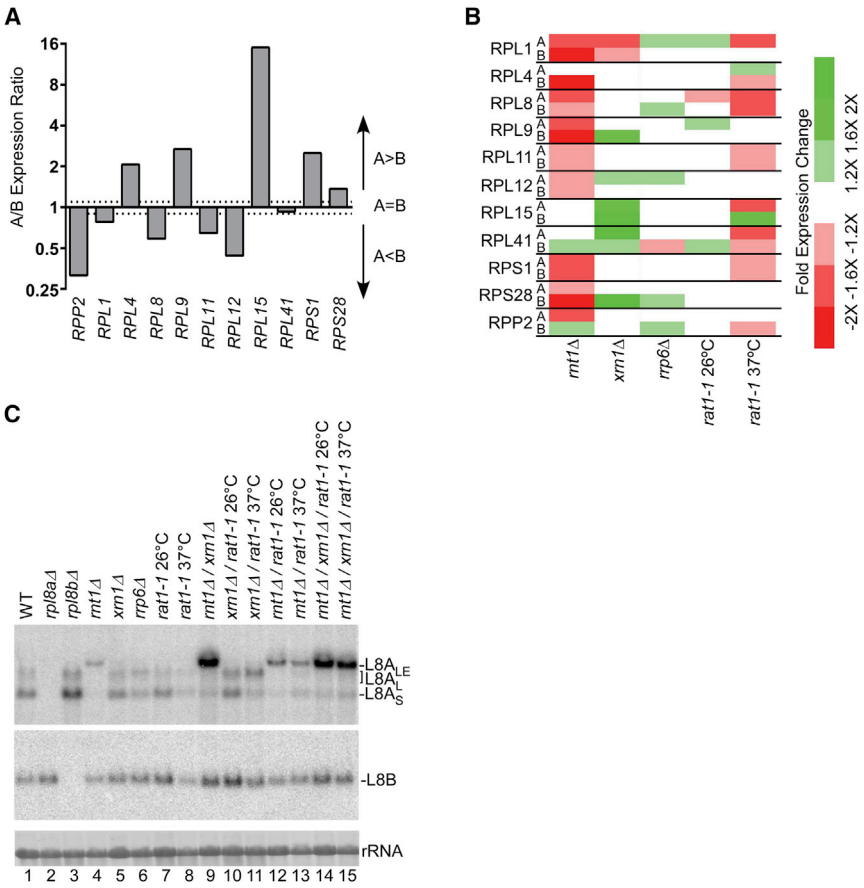

Figure 2. The Ohnologs of niRPGs Are Asymmetrically Expressed and Regulated

(A) Histogram showing the ratio of the duplicated niRPG mRNAs (A/B) as determined by microarray (http://transcriptome.ens.fr/ymgv). The dotted line indicates more than 10% variation in the ohnolog ratio (see also Figure S3).

(B) The relative expression levels of the niRPGs were determined in WT cells or cells carrying deletions in the RNases *RNT1 (rnt1Δ)*, *XRN1 (xrn1Δ)*, or *RRP6* (*rrp6*Δ) genes or cells carrying temperature-sensitive mutations in *RAT1* (*rat1-1*) grown under permissive (*rat1-1* 26°C) and restrictive temperatures (*rat1-1* 37°C) using qRT-PCR and are shown in the form of a heatmap.

(C) The RNases were deleted or inactivated as described in (B), and the expression levels of *RPL8* mRNAs were detected using ohnolog-specific probes. $L8A_{LE}$, $L8A_L$, and $L8A_S$ indicate the position of the extended, long and short forms of *RPL8A*, while L8B indicates the position of the *RPL8B* mRNA. The 18S rRNA is shown as loading control.

the transcript sizes corresponded to the predicted differences in the length of the 3′ UTR (Figure S1B). The largest number of alternative transcripts generated by a single gene was observed using probes complementary to the large subunit protein ohnolog *RPL8A* which is implicated in the processing of 27S rRNA (Jakovljevic et al., 2012) (Figure 1D).

The heterogeneity of the *RPL8* mRNAs observed in Figure 1D could be explained by variation in the site of transcription termination. Genome-wide analysis of the 5′ and the 3′ ends of yeast mRNAs indicates that while *RPL8B* has one major 5′ and 3′ end, *RPL8A* has at least two clearly distinguishable 3′ ends (Nagalakshmi et al., 2008; Yassour et al., 2009). As summarized in Figure 1C, the first 3′ end of the *RPL8A* (T1) appears to be generated by canonical polyadenylation-dependent transcription termination, while the other (T2) is generated through cleavage by the double-stranded RNA (dsRNA)-dependent RNase III (Rnt1p), which is less efficient in producing polyadenylated transcripts and mostly leads to RNA degradation (Ghazal et al., 2009; Rondón et al., 2009). Deletion of Rnt1p leads to the generation of longer transcripts, that terminate at yet another transcription termination site dubbed T3. To confirm the origin of the *RPL8A* heterogeneity, we mapped the 5′ end of the two ohnologs and monitored the effects of the transcription termination sequences on transcript length (Figures 1D and S2). As expected, only one major transcript, with a 5′ end starting at position −24, was detected for *RPL8B*, while *RPL8A* exhibited 6 different 5′ ends between positions −8 and −21. These variations in the transcription initiation site cannot explain the differences in the *RPL8A* transcripts detected in Figure 1B. Therefore, the heterogeneity of *RPL8* transcripts appears to be generated mainly by alternative transcription termination of *RPL8A*. To directly examine the validity of this conclusion, we replaced the transcription termination sequence downstream of the *RPL8A* coding sequence with that of the alcohol dehydrogenase gene *ADH1*. As indicated in Figure 1D, the substitution of the termination sequence (*L8A-ADH1t*) increased the expression and abolished the heterogeneity of the *RPL8A* consistent with the inhibition of the transcriptional readthrough and its associated RNA degradation (Ghazal et al., 2009). This clearly indicates that the different forms of *RPL8A* and its reduced expression level are mediated by weak alternative transcription-termination sites. We conclude that ohnolog-specific expression is not restricted to intron-encoding genes but extends to niRPGs, where differences in the expression may be mediated by regulatory elements positioned downstream of the coding sequence.

Differential Expression and Degradation of niRPGs

Comparison of the expression levels (Marc et al., 2001; Figure 1B) indicates that the majority of niRPG ohnologs are asymmetrically expressed (Figures 2A and S3). Indeed, all RP coding mRNAs (except Rpl41) were generated from one primary ohnolog assisted by a secondary underexpressed copy (Figure 2A; Table S1). The biggest difference in expression was observed between gene pairs coding for the non-conserved protein L15 (Simoff et al., 2009) and the ribosomal stalk heterodimer protein Rpp2A/B (Grela et al., 2014). To determine the reason behind the variation in the expression levels of the duplicated niRPGs, we compared the transcriptional activities and RNA levels of each

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gene pair. Surprisingly, we found that the RNA levels generated by the RPGs are not linked to their transcription levels as indicated by the RNAPII chromatin immunoprecipitation sequencing (ChIP) sequencing data (Bonnet et al., 2014) (Table S1). This suggests that transcription is not the major determinant of the expression hierarchy or dominance of the dRPGs.

Alternatively, we hypothesized that the expression pattern of the niRPG ohnologs is determined at least in part by differential RNA degradation. To test this hypothesis, we monitored the ohnolog expression levels after the deletion or inactivation of the four most studied RNases in yeast cells. As indicated in Figure 2B, the majority of the ohnologs displayed different sensitivity to one or more RNases. The deletion of the 3′-5′ exoribonuclease *RRP6*, which is required for the nuclear surveillance of defective RNA (Hilleren et al., 2001), or the cytoplasmic 5′-3′ cytoplasmic exoribonuclease Xrn1p, required for the degradation of uncapped RNA (Long and McNally, 2003), selectively increased the expression of six RPGs without affecting the expression of their ohnologs (Figure 2B). In contrast, deletion or inactivation of RNases required for RNA maturation and transcription termination like the nuclear endoribonuclease Rnt1p (Catala et al., 2004), and the 5′-3′ exoribonuclease Rat1p (Jimeno-González et al., 2010) selectively inhibited one copy of eight of 11 gene pairs tested. We conclude that the ratio of niRPG ohnologs is controlled by selective RNA degradation.

Northern blot analysis of the Rpl8 mRNAs indicates that RNase deletions do not only alter the expression levels of the RPGs but may also modify the sizes of the different mRNA forms produced from each gene. Most RNases altered both the levels and length of *RPL8A* isoforms without affecting *RPL8B* mRNA (Figure 2C). Deletion of *RNT1* (Figure 2C, lane 4) reduced the expression of the *RPL8A* transcripts detected in wild-type (WT, Figure 2C, lane 1) cells and led to the accumulation of a new long form of *RPL8A* ($L8A_{LE}$). This is consistent with earlier studies suggesting that Rnt1p function as a failsafe transcription termination mechanism for RNAs with weak polyadenylation sites (Ghazal et al., 2009; Rondón et al., 2009). Deletion of *XRN1* (Figure 2C, lane 5) had little effect on either *RPL8A* or *B*, while *RRP6* deletion (Figure 2C, lane 6) modestly increased the expression of *RPL8B* and selectively inhibited the expression of the short form of *RPL8A* ($L8A_S$). In contrast, inactivation of a temperature-sensitive allele of *RAT1* (Figure 2C, lane 8) preferentially inhibited the expression of the short form of *RPL8* ($L8A_S$), which is believed to be generated by the canonical polyadenylation-dependent transcription termination machinery, with little effects on the long form ($L8A_L$) believed to be generated through Rnt1p cleavage. Double deletions of *RNT1* and *XRN1* increased the levels of $L8A_{LE}$, indicating that failure of Rnt1p cleavage leads to the export and degradation of at least a portion of the extended mRNA in cytoplasm. Deletion of *XRN1* and inactivation of Rat1p in the same cell (Figure 2C, lane 11) produced a phenotype similar to that observed after Rat1p inactivation (Figure 2C, lane 8). Similarly the double and triple mutants *rnt1Δ /rat1-1* and *rnt1Δ/xrnt1Δ/rat1-1* (Figure 2C, lanes 13 and 15) had no further effects on the expression of Rpl8 mRNA. Together these observations suggest that the ratio of the *RPL8* ohnologs is defined by differences in the mechanism of transcription termination that favors the expression of *RPL8B*.

The mRNA Levels of niRPGs Are Determined by an Ohnolog-Specific Negative Feedback Loop

If dRPGs are fully redundant copies required for maintaining constant dose of RP, then we expect the loss of one copy to be compensated by an equivalent increase in the expression of the other. As indicated in Figures 3A and 3B, complete reciprocal compensation was not detected for the majority of the tested gene pairs. Instead, gene deletions (Figures S1B and S1C) either did not increase the expression levels of the remaining copy (e.g., *RPP2*, *RPL41*, *RPS1*, and *RPL4*) or resulted in a non-reciprocal increase in expression (e.g., *RPL8*, *RPL9*, *RPL12*, and *RPS28*). Surprisingly, four out of 11 ohnolog deletions (e.g., *RPL1B*, *RPL8A*, *RPL12A*, and *RPS28A*) reduced the expression of the remaining copy (Figure 3A; Table S1). Indeed, the majority of the ohnolog deletions resulted in non-correlated or even opposite effects on ohnologs expression (Figures 3A and S1C). This implies that having two copies of a single gene does not necessarily maintain constant production of RPs. Nevertheless, we found that deletions of niRPGs often increase the expression of only one of the two dRPG copies, presumably due to directional negative feedback loops (Table S1). There is no obvious correlation between expression level of the dRPGs and this feedback loop suggesting that the difference in ohnolog expression levels is not due to changes in their response to the expression of their RP products. For example, in the case of *RPL8*, the deletion of the primary gene *RPL8B*, which produces the majority of the mRNAs, induces the expression of the under-expressed ohnolog *RPL8A*, while in the case of *RPL11*, it is the deletion of the under-expressed copy that increases the expression of its ohnolog (Figure 3C). Therefore, it is the identity of the ohnolog (e.g., differences in sequence) and not its expression level that determines its reaction to changes in the expression levels of RPGs.

To understand the source of the different expression patterns of the ohnologs, we monitored the impact of the promoter and terminator sequence on the expression of the gene pairs coding for the L8 proteins. As indicated in Figure 3D, replacement of the *RPL8A* promoter with the constitutive promoter of the housekeeping gene coding for actin (Gallwitz and Seidel, 1980) (*ACT1p-L8A*) did not change the relative expression of either ohnolog, while the replacement of the terminator sequence (*L8A-ADH1t*) resulted in a substantial increase in the expression level of *RPL8A* without affecting the expression of *RPL8B* mRNA. Deletion of *RPL8B* in *ACT1p-L8A* strain (*ACT1p-L8A rpl8bΔ*) inhibited cell growth, while the same deletion in *L8A-ADH1t* strain (*L8A-ADHt rpl8bΔ*) had no effect on growth when compared to the *L8A-ADH1t* strain (Figure 4C; Table S2). Therefore, while the *RPL8A* promoter is needed for growth in the absence of *RPL8B*, it is the terminator sequence of *RPL8A that* is required for repressing *RPL8A* expression and also for its response to changes in *RPL8* dose. Substitution of the *RPL8B* promoter did not significantly change the expression of *RPL8B*; however, it increased the expression of *RPL8A.* Changing the terminator sequence of *RPL8B* decreased the expression of *RPL8B* and increased the expression of *RPL8A*. Together these data indicate that while the terminator sequence acts as a negative auto-regulator of *RPL8A* its *RPL8B* equivalent promotes the expression of its own gene (Figure S1D).

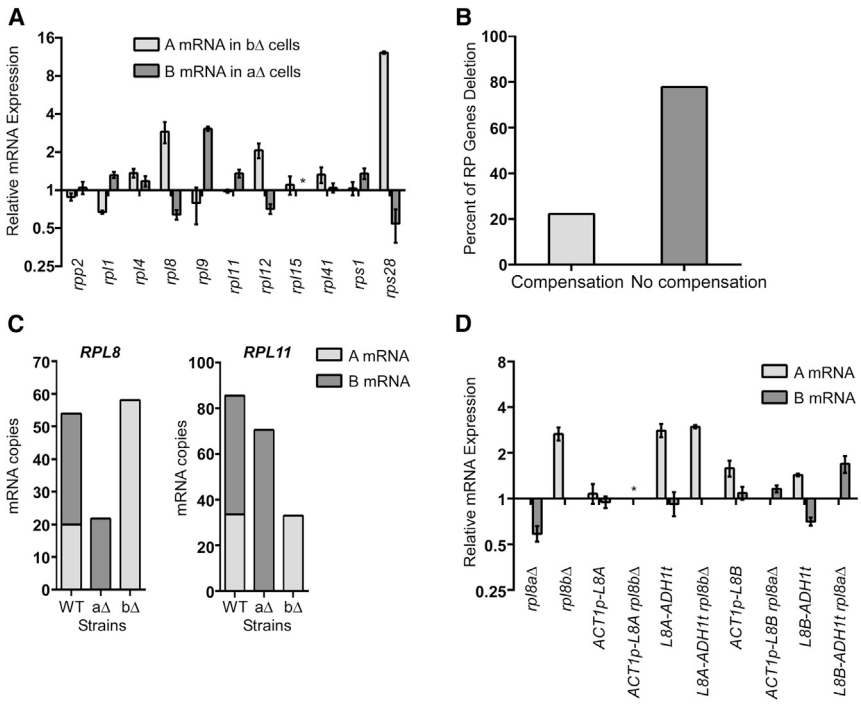

Figure 3. Expression of niRPGs Is Regulated by an Ohnolog-Specific Negative Feedback Loop

(A) One copy of each duplicated gene set was deleted and the effect on the expression level of the other remaining copy was examined using qRT-PCR. mRNA expression is presented relative to the expression detected in WT cells in the form of bar graph. The asterisk indicates lethal ohnolog deletion.

(B) A bar graph summarizing the response of RPGs to ohnolog deletions. Compensation (light gray) means that sum of the RPG pairs (A and B) mRNA in WT cells equals that detected in strain lacking one of the two ohnologs. No compensation (dark gray) means that the sum of A and B differ by 20% or more upon ohnolog deletion.

(C) The relative expression levels of *RPL8* and *RPL11* ohnologs were determined using qRT-PCR and converted into mRNA copy numbers using pre-established expression values (http://transcriptome.ens.fr/ymgv). The number of A copies is shown in light gray and those of the B copies in dark gray.

(D) The expression levels of *RPL8 genes* were determined using qRT-PCR in cells lacking one or the other ohnolog (aΔ and bΔ), or in cells expressing copies of *RPL8* fused to either heterologous promoter (*ACT1p-L8A* and *ACT1p-L8B*) or termination signals (*L8A-ADH1t* and *L8B-ADH1t*), and are presented relative to the value of WT cells. All experiments were performed in at least three biological and two technical replicates and the SDs are indicated by error bars (see also Figure S4).

Mass spectrometry analysis of the protein amount produced by the different substitutions indicated that most changes in *RPL8A* mRNA result in corresponding changes in protein levels (Figure S4A; Table S2). In contrast, variations in the levels of *RPL8B* mRNA did not result in corresponding changes in protein levels. The most striking example of this discrepancy was observed upon the deletion of *RPL8A* (*rpl8aΔ*), where the decrease in Rpl8b mRNA was associated with an increase in the level of L8B protein (Figure S4A; Table S2). Opposite effects were also observed after the substitution of the promoter (*ACT1p-L8B*) or the terminator regions in *rpl8aΔ* cells (*L8B-ADH1t rpl8aΔ*). In this case, an increase (or no difference) in RNA levels resulted in reduced protein amounts (Table S2). Overall, the most significant *RPL8A*-dependent changes in ohnologs ratio were induced by the modification of *RPL8A* terminator, while the most *RPL8B*-dependent changes in ohnolog ratio were induced by the modification of *RPL8B* promoter (Figure S4B). Together, these data suggested that the expression of *RPL8B* might be regulated at the level of translation. Examination of the polysome-associated Rpl8a and b mRNAs indicated that the discrepancy between the RNA and protein levels are indeed the result of differences in translation levels (Figure S4C; Table S2). For example, translation of the *L8B-ADH1t rpl8aΔ* construct was reduced explaining why it produced less protein despite marked increase in mRNA amounts. Similarly changes in the promoter region of *RPL8B* (*ACT1p-L8B*) also reduced translation explaining the decrease in protein amounts generated by this construct (Figure S4C; Table S2). We conclude that the ohnologs of *RPL8* use distinct regulatory pathways that permit copy-specific interdependent regulation of gene expression.

Duplication of niRPGs Improves Cell Fitness

To evaluate the level of functional redundancy between the duplicated genes, we deleted one or the other copy of each gene pair and monitored the impact on cell growth and fitness. In most cases, ohnolog deletions produced different growth defects leading to different levels of growth inhibitions (Figure 4A). In five cases (*RPP2*, *RPL1*, *RPL11*, *RPL12*, and *RPS1*), the deletions reduced growth by more than 15%, and in one case (*RPL15A*) the deletion was lethal. Only one gene pair (*RPL41*) did not exhibit any (<1%) ohnolog-dependent reduction in growth under normal condition (Figure 4A). The role of the ohnologs in cell fitness was evaluated by growing the deletion strains in competition with a WT strain for 50 generations. As indicated in Figure 4B, all but five of the mutated strains tested had greatly reduced cell fitness. In general, all cells missing one ohnolog, except *RPL41* deletions, were outcompeted by WT cells, providing a potential explanation of the evolutionary preservation of RPG duplications in yeast. Indeed, other growth conditions may reveal the need for preserving the ohnologs of *RPL41*.

The effect of an RPG deletion on cell fitness is not directly related to the capacity of its ohnolog to compensate for gene expression (Figures 3A and 4B). For example, deletion of *RPL8A* and *B* decreased fitness despite the increase in the expression levels of *RPL8A* in cells lacking *RPL8B*. Consistently, *RPL41A* and *B* deletions did not affect fitness despite the lack of gene compensation. This could be due to differences in the total protein produced or differences in the capacity of the different protein isoforms to sustain growth independently. To determine the gene features influencing the ohnologs' capacity to sustain growth as a single copy, we measured the impact on growth of

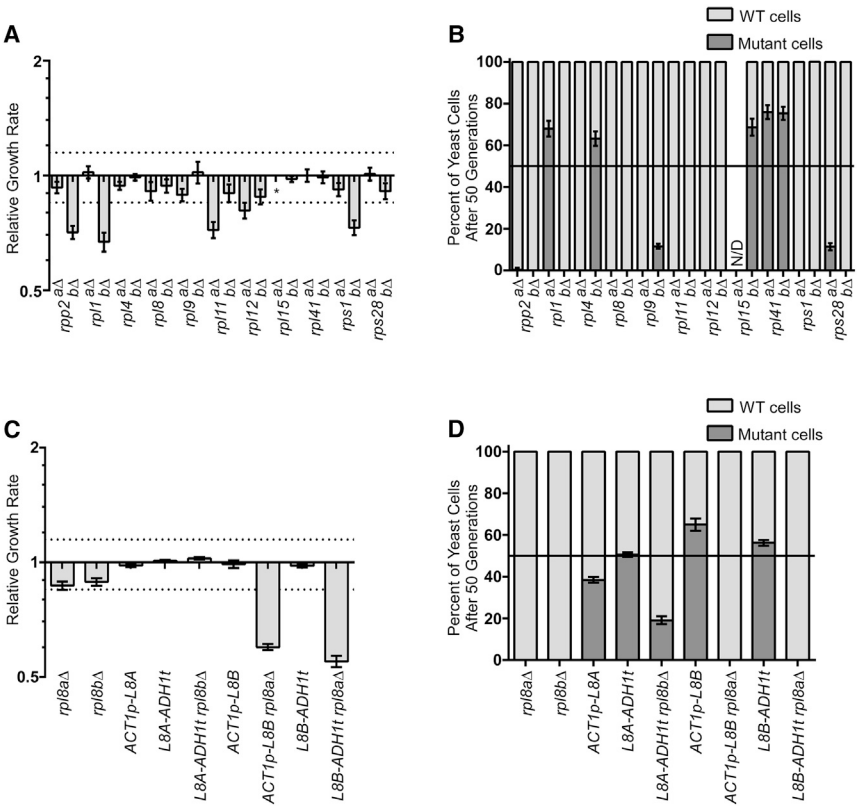

Figure 4. Duplication of niRPGs Promotes Growth and Improves Cell Fitness

(A) The doubling time of cells carrying deletions in one or the other copy of the duplicated niRPGs was determined in rich media and is presented relative to the values of WT cells in the form of a bar graph. Changes in doubling time by more than 15% (dotted line) were considered significant to exclude the natural variation in growth observed with WT cells. The asterisk indicates unavailable data due to lethality caused by the deletion of *RPL15A*. Experiments were performed in triplicate and the SDs are indicated by error bars.

(B) Cells carrying niRPG deletions (dark gray) were grown in competition with WT cells (light gray), and the ratio of the competing strains was determined using a colony color assay after 50 generations. Experiments were performed in duplicate and the SDs are indicated by error bars. No data (N/D) are available for the lethal *RPL15A* deletion.

(C) The growth rate of cells lacking *RPL8A* (*rpl8aΔ*) or *RPL8B* (*rpl8bΔ*) and cells expressing *RPL8* ohnologs fused to heterologous promoter (*ACT1p-L8A* and *ACT1p-L8B*) and termination signals (*L8A-ADH1t* and *L8B-ADH1t*) was determined and plotted relative to that of WT cells. Experiments were performed using three different spores, and the SDs are indicated by error bars. Variations in growth were considered significant when greater than 15% (dotted line).

(D) The fitness of cells lacking *RPL8A* (*rpl8aΔ*) or *RPL8B* (*rpl8bΔ*) and cells expressing the *RPL8* ohnologs fused to heterologous promoter (*ACT1p-L8A* and *ACT1p-L8B*) and termination signals (*L8A-ADH1t* and *L8B-ADH1t*) was determined as described in (B).

replacing the promoter and termination region of the *RPL8* gene pairs with constitutive promoter and terminator of the *ACT1* and *ADH1* genes (Figures 4C and 4D). Changing the promoter or termination regions in cells expressing both ohnologs did not affect growth or fitness except in the case of the *RPL8A* promoter substitution (*ACT1p-L8A*), which slightly reduced cell fitness (Figures 4C and 4D). Substitution of the *RPL8B* promoter or terminator (*ACT1p-L8B rpl8aΔ and L8B-ADH1t rpl8aΔ*) repressed the gene's ability to sustain growth in the absence of *RPL8A*, while the substitution of the *RPL8A* terminator (*L8A-ADH1t rpl8bΔ*) restored the fitness defect produced by the deletion of *RPL8B* (*rpl8bΔ*) (Figure 4D). The capacity of the different substitutions to support growth correlated with the amount of proteins produced by these mutations (Figure S4A). This suggests that the nature of the promoter and transcription termination sequences influence the RPGs' capacity to compensate for ohnolog deletions by simply controlling gene expression.

Changes in the Expression Levels of RPGs Confer Ohnolog-Specific Stress Response

We previously showed that introns modulate the response of duplicated RPs to growth under stress (Parenteau et al., 2011). Therefore, we measured the impact of drugs on the expression of niRPG ohnologs and evaluated their requirement for growth under stress (Figure 5A). We used a pre-established battery of 14 growth conditions to cover the main categories of cell function (Parenteau et al., 2008). As indicated in Figure 5A, the majority of the tested conditions affected cell growth in an ohnolog-specific manner. Most of the growth defects were observed after exposures to the protein kinase C inhibitor Staurosporine (Yoshida and Anraku, 2000) and the peptide chain elongation inhibitor Hygromycin B (González et al., 1978). Some dRPG deletions exhibited growth defects only under one growth condition with little effect on cell fitness (e.g., *RPL41A*), illustrating the need of examining large number of condition to uncover the advantages of maintaining two copies of RPGs. Surprisingly, in several cases, the deletion of the under-expressed ohnolog (i.e., the ohnolog that produces the least amount of mRNA) was more sensitive to stress than the deletion of its abundantly expressed counterpart (Figure 5B). This indicates, that decreased resistance to stress is not directly linked to the amount of RP produced in the cell.

To confirm the ohnolog-specific nature of the various niRPGs' contribution to drug resistance, we directly tested the impact of drug exposure on their expression. The RNA was extracted from cells at a drug concentration that reduces growth of WT by 50%, and the expression of the different RPGs was compared to that of housekeeping genes using qRT-PCR (Figure 5C). For the most part, drugs inhibited the expression of both ohnologs, presumably due to the drug-associated inhibition of cell growth, which

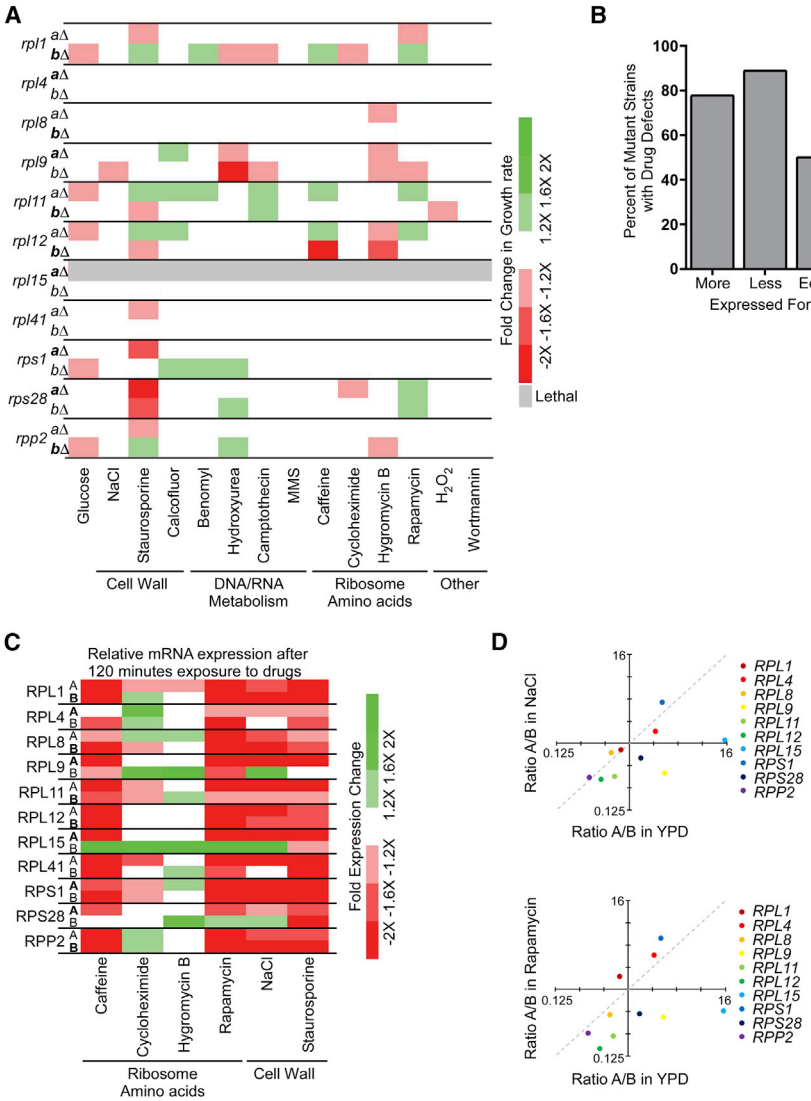

Figure 5. Cell Exposure to Stress Induces Ohnolog-Specific Modulation of Gene Expression

(A) The relative growth rate of niRPG deletion strains was determined under different stress conditions and presented in the form of a heatmap. The drugs were selected to cover different metabolic pathways (Parenteau et al., 2008) indicated at the bottom. The values represent the average of three independent experiments and differences less than 20% were not considered.

(B) The percentage of genes affected by the exposure to drugs are plotted as a function of the RPGs expression levels. "More" and "Less" indicate genes that are more and less expressed than their ohnologs. Ohnologs that are equally expressed are indicated as "Equal."

(C) The RNA was extracted from WT cells growing under normal growth conditions or after exposure to different drugs, and the relative levels of the ohnologs' mRNAs were determined by qRT-PCR and shown in the form of a heatmap.

(D) The expression levels of the niRPGs were determined as described in (C), before and after exposure to NaCl or Rapamycin, and converted into copy-per-cell as described in Figure 3C. The ratio of each gene pair was calculated and presented as a dot plot.

is expected to inhibit ribosome production. However, in many cases exposure to drug repressed RPG expression in an ohnolog-specific manner, and in a few cases the drug inhibited one copy and induced the other (e.g., *RPL1*, *RPL8*, *RPL9*, *RPL15*, and *RPS28*). Indeed, the ratio of many ohnologs was modified in a drug-dependent manner, and this modification of ratio was observed regardless of the initial mRNA ratio detected under normal growth conditions (Figure 5D). Together the data suggest that cells modify the ratio of the dRPGs favoring the expression of certain ohnologs over others in response to stress, which explain why the deletion of one copy of dRPGs affect growth under stress while the deletion of the other does not.

Ohnolog Functional Specificities Are Generated by Differences in Expression Patterns and Protein Sequence

The purpose of functional specificity of the RPGs ohnologs remains unclear. Differences in ohnolog functions may stem from variations in protein functions or differences in the modality of gene expression (Komili et al., 2007; Parenteau et al., 2011). To differentiate between these two possibilities, we studied the effects on growth of *RPL8* regulatory and coding sequences under stress. The *RPL8A* and *RPL8B* coding sequences differ by 5%, while the 5′ and 3′ regulatory sequences exhibit only 46% and 43% identity, respectively (Table S1). Deletion of the minor ohnolog *RPL8A* increased sensitivity to Hygromycin B, while the deletion of the predominant copy (i.e., *RPL8B*) did not alter drug resistance (Figure 5A). Consistently, Hygromycin B specifically increased the expression of *RPL8A* (Figures 5C and S4D). This suggests that *RPL8A* acts as a dedicated stress-response gene that is mostly repressed under normal conditions but required for growth in the presence of Hygromycin B. To directly evaluate this hypothesis, we created yeast strains that express two copies of *RPL8B* and measured the impact of this ohnolog homogenization on hygromycin resistance (Figures 6A and S5). As expected, transformation of *rpl8aΔ* cell with a plasmid carrying a copy of *RPL8A* gene (*aΔ/pL8A*) completely rescued the hygromycin sensitivity phenotype. A plasmid carrying *RPL8B* only partially rescued the phenotype of *rpl8aΔ* cells (*aΔ/pL8B*) indicating that the expression of two copies of *RPL8B* is not sufficient for optimal resistance to drugs. Plasmids expressing L8B under the control of *RPL8A* promoter and termination sequence (*pL8ABA*) or L8A under the control of *RPL8B* regulatory sequence (*pL8BAB*) fully rescued the

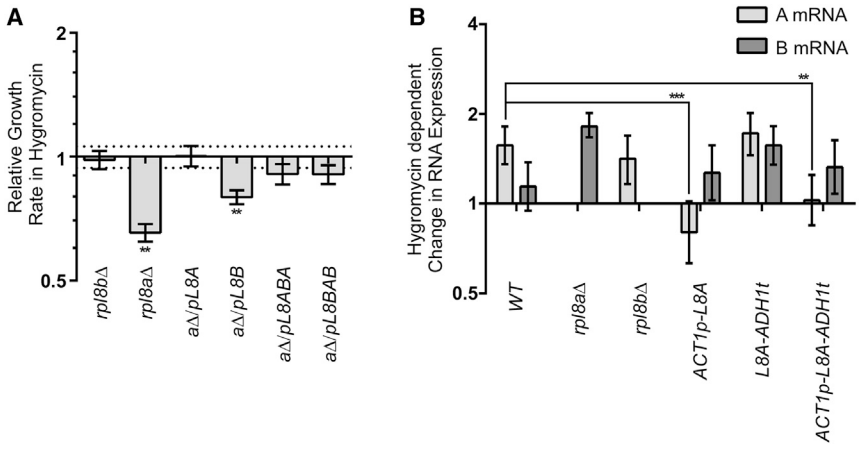

Figure 6. The Functional Specialization of the *RPL8* Ohnologs Depends on Both Coding and Non-coding Sequences

(A) Cells carrying deletions in *RPL8A* were transformed with plasmids expressing *RPL8A* (*aΔ/pL8A*), *RPL8B* (*aΔ/pL8B*), L8B protein under the control of *RPL8A* promoter, and terminator sequence (*aΔ/pL8ABA*) or a plasmid expressing the L8A protein under the control of *RPL8B* promoter and terminator sequence (*aΔ/pL8BAB*). Growth rates relative to the WT cells were determined before and after exposure to hygromycin. The relative growth rates of *rpl8aΔ* and *rpl8bΔ* strains were also included for comparison. The experiments were performed at least five times, and the SDs are indicated by error bars. The dotted lines represented the SDs of the WT strain. The asterisks indicate statistically significant differences in the growth rate detected in WT and mutant strains (p value (t test) <0.01 indicated by two asterisks; see also Figure S5).

(B) The levels of Rpl8A (light gray) and Rpl8B (dark gray) mRNAs in *rpl8aΔ* and *rpl8bΔ* cells or in cells expressing the *RPL8* genes under the control of a heterologous promoter (*ACT1p-L8A* and *ACT1p-L8B*) or termination signals (*L8A-ADH1t* and *L8B-ADH1t*) were determined using qRT-PCR before and after exposure to hygromycin. The data were plotted relative to the values obtained in the absence of hygromycin for each strain. The asterisks indicate statistically significant differences in the mRNA levels detected in WT and mutant strains (p value [t test] <0.001 indicated by three asterisks or 0.01 indicated by two asterisks).

rpl8aΔ phenotype suggesting that both the regulatory and coding sequences contribute to the ohnolog-specific drug response. Consistently, substitution of the *RPL8A* promoter with a heterologous promoter (e.g., *ACT1*) completely abolished the drug-dependent induction of *RPL8A* (Figure 6B). On the other hand, the substitution of the terminator sequence of *RPL8A* (*L8A-ADH1t*) resulted in constitutive induction of *RPL8A* (Figure 6B). The expression levels obtained after the replacement of both terminator and promoter sequence appeared to be the average of that produced by each mutation separately, regardless of the drug concentrations. Together the data suggest that *RPL8A* response is achieved by a combination of terminator-based repression under normal conditions and promoter-dependent induction when exposed to stress. We conclude that the specificity of RPG ohnologs may evolve from differences in the modality of gene expression and small differences in coding sequence.

DISCUSSION

We have shown that functional specialization of dRPGs is generated through variations in ohnolog expression patterns. Differences in ohnolog expression are driven by variations in the flanking regulatory sequences that determine the site of transcription termination and the RNA stability of each ohnolog (Figures 1 and 2). Almost all niRPG pairs were unequally expressed, and their deletions led to partial non-reciprocal dose compensation (Figures 2 and 3) consistent with the proposed non-redundant functions of the dRPGs. Indeed, yeast cells responded to most stresses, which mimic the resource limitations encountered in nature, in an ohnolog-specific manner and exposure to stress altered the ohnolog protein ratio (Figures 5 and S4D). Strikingly, we found that inclusion of two copies of the highly expressed gene (e.g., *RPL8B*) required for growth under normal condition do not fully restore growth of cells lacking the stress response copy (e.g., *RPL8A*) (Figures 6A and S5). This clearly eliminates the overall expression levels of RPGs as the sole reason for drug sensitivity and points at differences in ohnolog expression pattern and/or function. Homogenization of different parts of the duplicated gene pair indicated that both the coding and regulatory sequence contribute to growth under stress (Figures 6 and S5). Together, the data support a model where gene duplication permits cells to modulate its composition of RPs in response to changes in growth conditions. Indeed, mass spectrometry analysis indicated that growth under stress, while reducing the overall amount of RP, modify the ratio of the protein isoforms to favor those required for growth under stress (Figure S4D). Together the data suggest that differences in the ohnolog expression pattern provide advantages for growth under different growth conditions.

Translation May Provide a Secondary Level of Ohnolog-Specific Gene Regulation

Modulation of translation initiation is often associated with variations in the size and internal structure of the 5′ UTR, which are not observed in dRPGs (Paul et al., 2015). Most RPGs possess short 5′ UTRs that vary in size between 24 and 150 nucleotides and the majority of the ohnologs exhibit 5′ UTRs with similar sizes. However, comparison between the mRNA and protein levels of RPGs indicates that, at least in some cases, translation may influence ohnolog expression patterns. For example, changing the 5′ and 3′ end sequence of *RPL8A* with constitutive promoter and terminator sequences of unrelated housekeeping genes increased its association with the ribosome and increased the amount of proteins produced in the same direction as the changes in mRNA levels (e.g., Figures 3 and S4). In contrast, similar substitutions of *RPL8B* promoter and terminator sequences resulted in a marked decrease in translation and protein amounts regardless of the effect of these mutations on RNA levels (Figures 3 and S4). Indeed, in most cases we found a better correlation between the RNA and protein level of *RPL8A* than

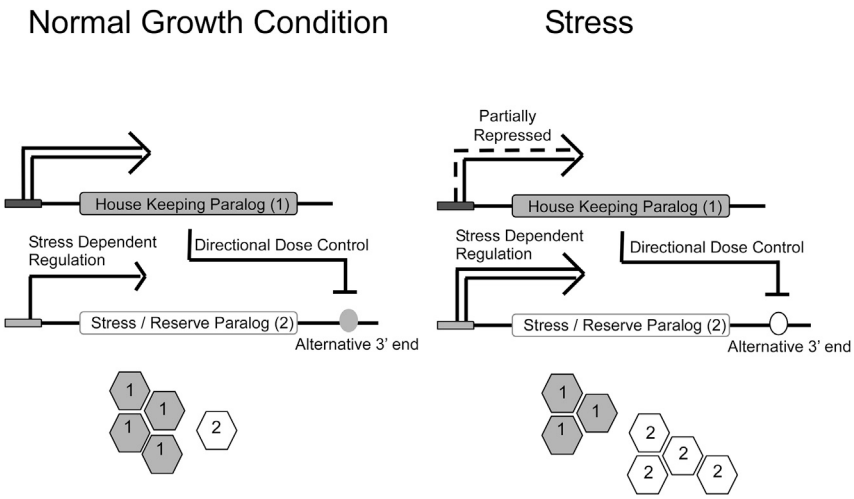

Figure 7. Schematic Representation of Intron-Independent Regulation of Duplicated RPGs

The state of the niRPG ohnologs is illustrated before (left) and after (right) exposure to stress. The response of the promoter and terminator regions to changes in growth conditions or changes in the levels of RPs may vary between gene pairs. Genes, transcription start sites, and termination sites are indicated by boxes, thick lines, and circles, respectively. The arrows and flat arrowheads indicate induction and termination of transcription.

RPL8B (Table S2). Together the data suggest that dRPGs are subjected to different levels of gene regulation permitting independent copy-specific modulation of gene expression. The divergence of ohnolog regulatory mechanisms permits discrete responses to different stimuli and increases the number of potential regulatory and sensory circuits that could be linked to ribosome production.

Origin of Ohnolog Functional Specificity

Several studies now support the functional specialization of yeast RPG ohnologs, mostly based on phenotypic differences between the ohnolog deletions or the deletion of their associated introns (Komili et al., 2007; Parenteau et al., 2011). However, prior to these studies the origin of these phenotypic differences were not clear due to difficulty in differentiating between the importance of the threshold of protein dose or the functional changes due to variations of protein sequence (Abovich and Rosbash, 1984; Komili et al., 2007; Lucioli et al., 1988). The argument for dose effects is supported by the very small differences in ohnolog amino acid sequences, evidence for evolutionary pressure for coding-region gene conversion, and the absence of amino acid differences in certain ohnologs (Evangelisti and Conant, 2010; Langkjaer et al., 2003). On the other hand, protein-encoded functional differences are supported by the fact that ohnologs with similar expression levels may have different effects on cellular phenotype, have different localization pattern, and affect the expression of genes implicated in different metabolic pathway (Komili et al., 2007). The data in this study reconcile these two seemingly opposing explanations for the origin of functional specialization of ohnologs by revealing a level of redundancy between the ohnologs' regulatory and coding sequence in promoting cell response to stress (Figures 6 and S5). This cooperativity is evident from the failure of extra copies of the same gene to restore the phenotype of ohnolog deletion and the restoration of the phenotype by expression of either the coding sequence or regulatory sequence of the two ohnologs separately (Figures 6 and S5). Most importantly, these findings may also explain why deletions of genes with identical amino acid sequence may result in different phenotypes by underlining the potential of differences in the regulatory sequences as means for ohnologs specificity. Deletions of genes with identical coding sequence or similarly regulated coding sequence may affect cell function by decreasing the overall dose of RPs. However, in this case the deletion of either copy of the dRPG would have been expected to result in similar effects, which is not the case for the majority of dRPGs (Table S1).

Gene Reserve, a New Mechanism for Stress Response

In prior work, the majority of the ohnolog-specific defects were observed in intron deletion strains grown under stress (Parenteau et al., 2011), suggesting that gene duplications enhance the response to unexpected changes in the growth environment. Consistently, in most cases deletion of only one of the two RPG copies affects cell growth in rich media while the other only affects cell growth under stress (Figures 4A and 5A). To explain these observations, we propose a new model (Figure 7), where the duplication of an RPG provides the cells with one copy for growth under normal condition and another for response to stress. This model is consistent with subfunctionalization as mechanism for the retention of duplicated genes (Force et al., 1999) and is supported by the fact that most duplicated genes include one primary ohnolog expressed at high levels and one secondary ohnolog expressed at low levels under normal growth conditions (Table S1) (Parenteau et al., 2011). The secondary ohnolog is often less required for growth under normal conditions but needed for growth under stress (Table S1) suggesting that, at least in some cases, one RPG may function as gene supplement needed under certain growth conditions. This gene reserve could support growth by increasing the pool of RPs, by increasing the level of a protein with amino acid sequence optimized for growth under stress or by providing a combination of dose and amino acid optimization. The increase in the expression of the normally suppressed gene may not alter the overall stoichiometry of the ribosome components since it is often coupled to reduction, albeit limited, in the expression of its ohnolog (Figure S4D; data not shown). Based on this model, gene duplication represents a means to accommodate extra-ribosomal functions of RPs or to modify ribosome composition in response to changes in growth conditions. However, while currently the most obvious function of dRPGs is their capacity to support growth under limiting growth conditions, it is possible

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that they were retained originally due to small advantages in growth normal conditions. Slight changes in the expression or function of the ancestral gene may not have great impact under optimal conditions in vitro but generate enough differences to alter cell survival during evolution. This possibility also explains why both RPG copies are expressed at least at low levels under both normal and stress condition and why the deletion of most ohnologs affects cell fitness.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

Plasmid manipulation and bacterial culture were carried out as previously described (Sambrook et al., 1989). Yeast cells were transformed and grown in standard yeast media (Gietz and Woods, 2006; Rose et al., 1990). Primers used for cloning are available in Table S3. Gene replacements and deletions were carried out essentially as described (Parenteau et al., 2011). Details of gene deletion, replacement, and plasmid construction, as well as the sequence of primers used, are described in Supplemental Experimental Procedures.

Sequence Comparison

Ohnolog sequences were extracted from the *Saccharomyces* Genome Database (SGD) genome v.R64-1-1 and aligned using Needleman-Wunsch global alignment tools from the European Molecular Biology Open Software Suite (EMBOSS 6.1.0). RPG UTR lengths were obtained from deep sequencing data (Nagalakshmi et al., 2008).

qRT-PCR Analysis

RNA extraction, primer validation, and qPCR analysis were performed as previously described (Parenteau et al., 2011). The sequences of the primers used for PCR are shown in Table S4.

Northern Blot

Total RNA from exponentially growing cells was isolated and blotted as described earlier (Abou Elela and Ares, 1998; Elela et al., 1996). Details are in Supplemental Experimental Procedures. Primers used to create probes are listed in Table S5.

Growth Assays and Fitness Test

Growth assays were performed as described (Parenteau et al., 2008; Toussaint et al., 2006). Details can be found in the Supplemental Experimental Procedures section.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.033.

AUTHOR CONTRIBUTIONS

J.P. designed and performed experiments, analyzed data, produced figures, and participated in the writing of the paper, M.L., M.C., and M.M.-G. performed experiments, J.G. performed statistical analysis and phylogenetic comparison, and S.A. designed experiments, analyzed data, and participated in the writing of the paper.

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