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Yeast Life Span Extension by Depletion of 60S Ribosomal Subunits Is Mediated by Gcn4

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SUMMARY

In nearly every organism studied, reduced caloric intake extends life span. In yeast, span extension from dietary restriction is thought to be mediated by the highly conserved, nutrient-responsive target of rapamycin (TOR), protein kinase A (PKA), and Sch9 kinases. These kinases coordinately regulate various cellular processes including stress responses, protein turnover, cell growth, and ribosome biogenesis. Here we show that a specific reduction of 60S ribosomal subunit levels slows aging in yeast. Deletion of genes encoding 60S subunit proteins or processing factors or treatment with a small molecule, which all inhibit 60S subunit biogenesis, are each sufficient to significantly increase replicative life span. One mechanism by which reduced 60S subunit levels leads to life span extension is through induction of Gcn4, a nutrient-responsive transcription factor. Genetic epistasis analyses suggest that dietary restriction, reduced 60S subunit abundance, and Gcn4 activation extend yeast life span by similar mechanisms.

INTRODUCTION

Invertebrate model organisms serve as valuable tools for aging research, largely because of their short lifespans and ease of genetic manipulation. The most commonly used model organisms include fruit flies, nematodes, and yeast. In the budding yeast *Saccharomyces cerevisiae*, two models of cellular aging have been developed: replicative and chronological (Kaeberlein, 2006). Replicative lifespan (RLS) is defined as the number of mitotic cycles completed by a mother cell before senescence and may model the aging of mitotically active cells in multicellular organisms (Mortimer and Johnston, 1959). Chronological lifespan refers to the length of time that a nondividing yeast cell retains viability during stationary phase and may model the aging of postmitotic cells (Fabrizio and Longo, 2003).

Dietary restriction (DR) increases lifespan and delays the onset of age-associated diseases in a variety of evolutionarily divergent organisms, including mammals (Masoro, 2005; Weindruch et al., 1988). In yeast, DR by a reduction in either glucose or amino acid concentration in the media results in lifespan extension in both chronological and replicative aging models (Fabrizio and Longo, 2003; Jiang et al., 2000; Lin et al., 2000; Powers et al., 2006). Genetic epistasis experiments support the hypothesis that lifespan extension from DR in yeast is mediated by the coordinated activity of three nutrient-responsive kinases: TOR (target of rapamycin), Sch9, and protein kinase A (PKA) (Fabrizio et al., 2001, 2004; Kaeberlein et al., 2005b; Lin et al., 2000; Powers et al., 2006). Decreased activity of these kinases extends both replicative and chronological lifespan (Fabrizio et al., 2001, 2004; Kaeberlein et al., 2005b; Lin et al., 2000; Powers et al., 2006). In response to nutrients, these three kinases regulate multiple important cellular processes, including ribosome biogenesis (Carey, 2003; Jorgensen et al., 2004; Martin et al., 2004; Powers and Walter, 1999), stress response (Beck and Hall, 1999), autophagy (Noda and Ohsumi, 1998), and mitochondrial retrograde metabolism (Dilova et al., 2002). Lifespan extension by deletion of *TOR1* or *SCH9* is not additive with DR and is independent of Sir2 protein deacetylase (Kaeberlein et al., 2005b).

TOR was first identified as a regulator of yeast lifespan through a random screen of 564 yeast strains, each lacking a single nonessential gene (Kaeberlein et al., 2005b). Along with *TOR1* and *SCH9*, deletion of several other genes in the TOR signaling pathway were identified to be long lived, including *RPL31A* and *RPL6B* (Kaeberlein et al., 2005b). The observation that *rpl31a*Δ or *rpl6b*Δ cells are long lived suggests that one mechanism by which DR might slow replicative aging is by decreasing ribosomal protein (RP) production through downregulation of TOR and Sch9 activity. Consistently, several reports have since linked a reduction in RP levels to increased lifespan in both yeast and *C. elegans*. Deletion of *RPL10*, *RPS6B,* or *RPS18A,B* also increases yeast RLS (Chiocchetti et al., 2007). In *C. elegans*, individual knockdown of nine different 40S subunit RP genes and

seven different 60S subunit RP genes has been reported to extend lifespan (Chen et al., 2007; Curran and Ruvkun, 2007; Hansen et al., 2007). In addition, inhibition of several translation initiation factors including the eIF2β/γ, eIF3A/B/F, eIF4A/E/G, or eIF5A homologs has been shown to increase *C. elegans* lifespan (Chen et al., 2007; Curran and Ruvkun, 2007; Hamilton et al., 2005; Hansen et al., 2007; Henderson et al., 2006; Pan et al., 2007; Syntichaki et al., 2007). Inhibition of the ribosomal protein S6 kinase has also been linked to lifespan extension in both worms (Chen et al., 2007; Hansen et al., 2007; Pan et al., 2007) and flies (Kapahi et al., 2004), and recent data suggests that Sch9 is the functional ortholog of S6 kinase in yeast (Powers, 2007; Urban et al., 2007).

To better understand the relationship between ribosomal proteins and aging, we measured the RLS for each of 107 RP gene deletion strains present in the yeast deletion collection and determined that multiple different 60S RP gene deletions significantly extend RLS. Consistently, we found that decreasing the abundance of 60S ribosomal subunits by deletion of 60S-specific ribosomal processing factors or by treatment with the small molecule diazaborine also leads to increased RLS. Epistasis analyses allowed us to conclude that depletion of 60S subunits extends lifespan by a mechanism similar to DR and independent of Sir2. Finally, we show that the transcription factor Gcn4 is required for full RLS extension in mutants with depleted 60S subunits, demonstrating a novel longevity-promoting function of Gcn4.

RESULTS

Longevity Analysis of RP Gene Deletion Strains

The yeast ribosome consists of two subunits, the 40S (small) and the 60S (large), which together contain four discrete rRNA species and 78 ribosomal proteins (RPs). In yeast, about 85% of RP genes are present in duplicate copies, allowing for the viable deletion of either paralog, but generally not both paralogs simultaneously. Of the 137 genes encoding RPs, 107 are present as quality-control verified (see Experimental Procedures) deletions in the *MATα* ORF deletion collection (Winzeler et al., 1999). We measured the RLS for each of these 107 RP single-gene deletion strains, corresponding to 46 RP paralog pairs (e.g., *RPL31A* and *RPL31B*) and 15 unpaired RP genes, 3 of which are nonessential single-copy genes and 12 of which have paralogs not represented in the deletion set.

Of the 107 RP gene deletion strains analyzed, 28 were found to be significantly long lived relative to experiment-matched wild-type cells ($p < 0.05$) in the *MATα* deletion set. To verify these results, we then measured the RLS of the 28 corresponding deletion strains derived from the *MAT*a ORF deletion collection (Figures 1A–1C; Table S1). In total, 14 RP gene deletion strains were verified to be significantly long lived in both the *MATα* and *MAT*a ORF deletion collections. Based on prior studies (Kaeberlein et al., 2005b), we would expect a similar analysis of 107 randomly chosen deletion strains to yield 2–3 (2.5) strains with significantly increased RLS; therefore, the percent of verified long-lived RP gene deletions is enriched approximately 5-fold relative to the entire set of nonessential ORFs. Strikingly, all 14 of the verified long-lived RP gene deletion strains lack protein components of the 60S subunit, whereas none of the deletion strains lacking RP genes encoding 40S subunit proteins

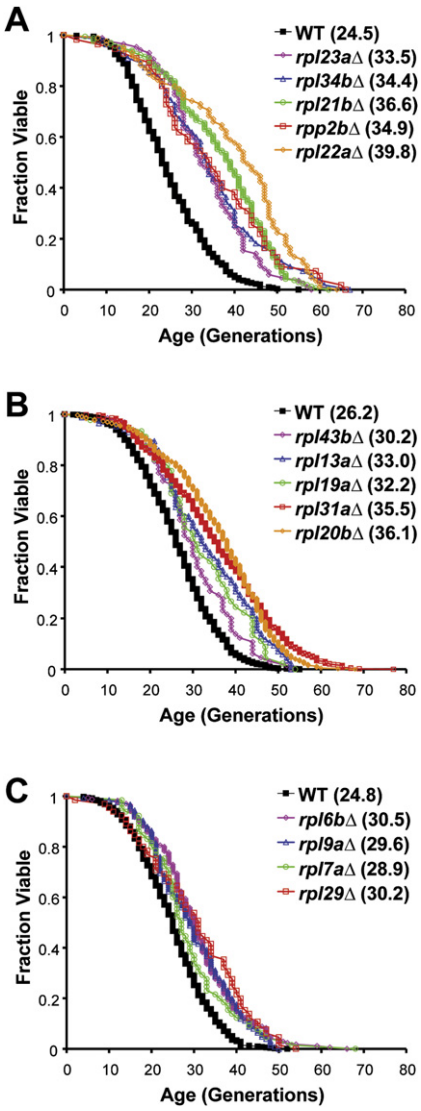


Figure 1. Genome-Wide Screen of RP Gene Deletion Strains Verifies 14 Significantly Long-Lived Strains, Each Lacking an RPL Gene

(A–C) Survival curves for *RP* deletion strains that are significantly ($p < 0.05$) long lived in both the *MATα* and *MAT*a ORF deletion collections. Data from *MAT*a and *MATα* deletion strains are pooled, and experiment-matched wild-type cells are shown. Mean lifespans are shown in parentheses. (See also Table S1.)

were verified to be long lived. Some long-lived *rplΔ* mutations, such as *rpl22aΔ* and *rpp2bΔ*, resulted in lifespan extension exceeding 50% (Figures 1A–1C), with longevity comparable to the longest-lived single-gene deletion mutants reported in yeast (Kaeberlein et al., 2005a, 2005b). Not all *rplΔ* strains were long lived however, and some were short lived (Table S1), for example *rpl20aΔ* (Figure 2B). These findings indicate that ribosomal proteins of the large subunit (RPLs) are important determinants of longevity in yeast.

Differential Longevity of RPL Paralog Deletion Strains

Although multiple RPL gene deletions were long lived, many were not. To characterize this apparent specificity, we further

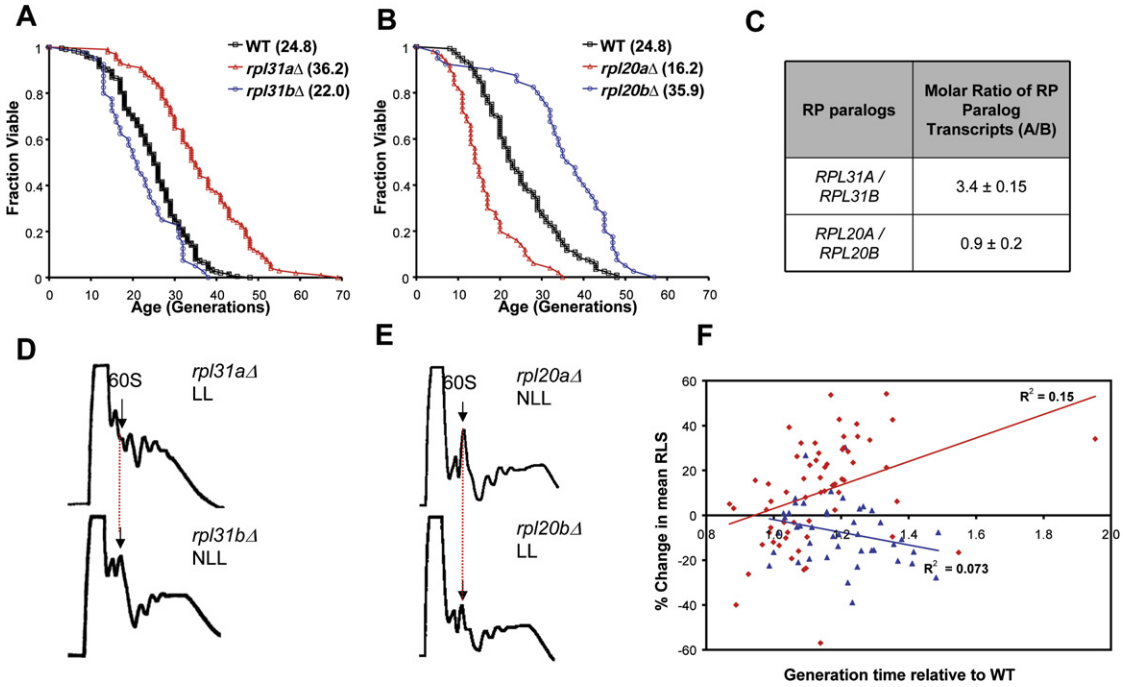


Figure 2. Abundance of 60S Ribosomal Subunits Correlates with RLS

(A and B) Survival curves for RP paralog gene deletions and experiment-matched wild-type cells. Mean lifespans are shown in parentheses.

(C) Molar ratios of RP paralog transcripts (A/B). (See also Table S2).

(D and E) Polysome profiles of *rpl*Δ paralogs. Long-lived deletion strains *rpl31a*Δ or *rpl20b*Δ show a reduced level of 60S ribosomal subunits relative to its paralog deletion strain (not long lived).

(F) Generation time of *RPL* (red diamonds) and *RPS* (blue triangles) gene deletion strains relative to wild-type plotted versus the percent change in mean RLS relative to experiment-matched wild-type cells. Linear regressions for *RPL* (red) and *RPS* (blue) gene deletions are shown separately.

examined paralog pairs for which deletion of one gene significantly increased RLS while deletion of the other did not (Table S1). For example, *rpl31a*Δ and *rpl20b*Δ increased RLS, but their corresponding paralog gene deletions did not (Figures 2A and 2B). The protein products encoded by the majority of *RPL* paralog pairs (26 of 33) are greater than 98% identical, and in the case of Rpl31a and Rpl31b, only a single conservative amino acid change differentiates the paralogs. Thus, it seems unlikely that one of the two paralogous RP proteins has evolved a specialized longevity-modulating function in the cases where *rpl*Δ paralog pairs have divergent RLS phenotypes. Furthermore, we found no correlation between RLS and any other functional role reported for ribosomal proteins (Komili et al., 2007).

One possible explanation for divergent RLS phenotypes among paralog pair deletion strains is that one paralog is transcribed at a higher level than the other and thus accounts for a disproportionate amount of the total protein produced from both paralogous genes. To test this possibility, we examined expression of two RPL paralog pairs by quantitative RT-PCR analysis of RNA isolated from three independent cultures of the wild-type strain, using paralog-specific primers (Figure 2C; Table S2). In the case of *RPL31A*, its mRNA transcript is more abundant than that of *RPL31B*, which is consistent with the longevity of *rpl31a*Δ cells when compared to *rpl31b*Δ cells. This correlation does not extend however to the *RPL20A/B* paralogs, which had nearly equimolar steady-state mRNA levels. Therefore, transcriptional bias among paralog pairs cannot account for divergent RLS phenotypes in every case.

In addition to transcriptional control, yeast cells can use a variety of mechanisms to regulate the level of RPs, including mRNA splicing, translation initiation, and turnover of excess protein (Tsay et al., 1988; Warner et al., 1985). To more directly assay whether deletion of one paralog more robustly affects the total amount of protein produced, we analyzed overall polysome profiles because cells limited for a particular RP should display a reduced abundance of the corresponding subunit. Polysome profiles were generated for several *rpl*Δ paralog pairs using high-salt conditions (to disrupt nontranslating 80S monosomes) (Figures 2D, 2E, and S1). In all examples studied, 60S subunit levels and overall polysome profile were more profoundly decreased in the *rpl*Δ paralog with significantly increased RLS.

Depressed polysome profiles (Figures 2D and 2E) indicate that translation is reduced in these long-lived mutants. In yeast, a sufficient reduction in translation will slow growth rate. In order to determine whether growth rate among RP deletion strains is a predictor of longevity, we measured the doubling time for each of the 107 *rp*Δ strains (Table S3) and compared growth rate to the percent change in mean RLS relative to wild-type (Figure 2F). The set of RPL gene deletion strains differed markedly from the set of RPS gene deletions strains with respect to the relationship between growth rate and RLS. For the set of *rpl*Δ strains, growth rate inversely correlated with RLS, while

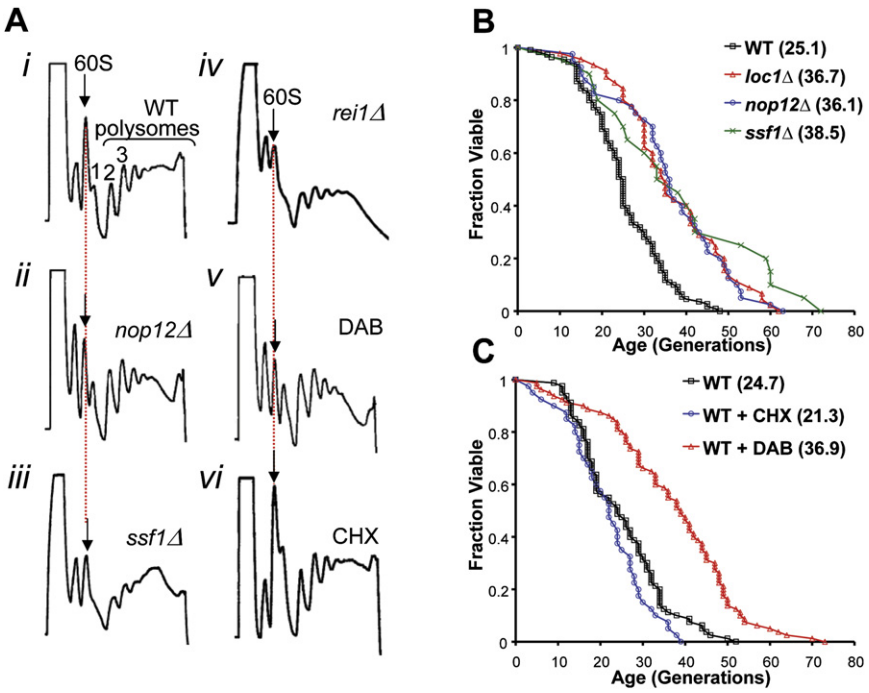


Figure 3. Interventions that Decrease 60S Ribosomal Subunits Extend RLS

(A) Relative to (i) wild-type, polysome profiles for (ii) *nop12*Δ, (iii) *ssf1*Δ, (iv) *rei1*Δ, and (v) diazaborine-treated (15 μg/ml) cells show significant reduction of 60S subunit levels while (vi) cycloheximide-treated (25 ng/ml) cells do not.

(B) Deletion of 60S-specific processing factor genes *NOP12*, *SSF1*, or *LOC1* increases lifespan relative to experiment-matched wild-type cells.

(C) Treatment of wild-type cells with 15 μg/ml diazaborine extends lifespan relative to experiment-matched wild-type cells while treatment with 25 ng/ml cycloheximide does not. Mean lifespans are shown in parentheses.

the opposite trend was observed for *rps*Δ strains. The growth rate analysis of *rp*Δ strains is complicated, however, by the strong selection for suppressors of slow growth among RP gene deletion strains in the ORF deletion set. We have observed three different cases where growth rate suppressors are present as spontaneously arising mutations in *rpl*Δ strains from the deletion collection (see Supplemental Data for details). We suspect that the inverse correlation between growth rate and RLS among *rpl*Δ strains would be more highly significant if it were possible to prevent spontaneous mutations suppressing growth rate defects. Regardless, these data suggest that differential lifespan potential among RPL gene deletion strains is related to the abundance of functional 60S subunits as indicated by polysome profile and perhaps overall translation rate.

Loss of Nonessential 60S Processing Factors Increases Life Span

If RLS extension in long-lived *rpl*Δ strains is a result of reduced 60S subunit levels, then mutations in nonribosomal proteins important for 60S maturation might also extend life span. To test this hypothesis, three single-gene deletion strains—*nop12*Δ, *loc1*Δ, and *ssf1*Δ, each of which lacks a factor specifically involved in a different stage of pre-60S subunit maturation—were characterized. Deletion of *LOC1* has been previously shown to cause decreased abundance of 60S subunits (Harnpicharnchai et al., 2001), and a similar depletion of 60S subunits was observed in polysome profiles of *ssf1*Δ or *nop12*Δ cells relative to wild-type (Figure 3A). Consistent with our prediction, each of these deletion mutants had an RLS significantly longer than wild-type cells (Figure 3B).

In our previously reported screen of 564 random deletion mutants, deletion of either *REI1* or *YBR266C* extended RLS (Kaeberlein et al., 2005b). These two ORFs are encoded on opposite strands and overlap. It has since been determined that the slow-growth phenotype of both of these mutants is due to loss of Rei1 function and is unrelated to Ybr266c (Figure S3), which is designated dubious and unlikely to encode a functional protein. Rei1 has recently been implicated in late-stage pre-60S processing (Hung and Johnson, 2006; Lebreton et al., 2006). Like *nop12*Δ, *ssf1*Δ, and *loc1*Δ cells, *rei1*Δ cells have reduced 60S subunits (Figure 3A). Thus, we conclude that nonribosomal mutations which impair 60S maturation can increase RLS in a manner similar to deletion of large subunit RP genes.

Pharmacological Inhibition of 60S Maturation Increases Life Span

We next determined whether RLS extension could also be achieved by a pharmacological intervention that depletes 60S subunit levels. Diazaborine is a synthetic antibiotic effective against Gram-negative bacteria (Baldock et al., 1998) that has been shown to reduce levels of 60S ribosomal subunits in yeast by a mechanism that likely involves pre-rRNA processing (Pertschy et al., 2004). Consistent with the above results, sublethal concentrations of diazaborine (15 μg/ml) reduced 60S subunit abundance (Figure 3A) and significantly increased RLS (Figure 3C).

In parallel, we determined the effect of adding sublethal concentrations of the general translation inhibitor cycloheximide (10–100 ng/ml) to the media. The lowest concentration of cycloheximide tested resulted in only a modest reduction in growth rate, whereas the highest concentrations substantially slowed growth. In contrast to diazaborine, cycloheximide neither increased RLS (Figures 3C and S4) nor led to reduced levels of 60S subunits (Figure 3A). Thus, pharmacological depletion of 60S subunits with diazaborine, but not general inhibition of translation with cycloheximide, is sufficient to increase yeast RLS.

60S Subunit Deficiency Increases Life Span Independently of Sir2

Enhanced Sir2 activity increases yeast RLS, an effect thought to be mediated by repression of extrachromosomal rDNA circle (ERC) formation (Kaeberlein et al., 1999). Similarly, deletion of *FOB1*, encoding the rDNA replication fork barrier protein,

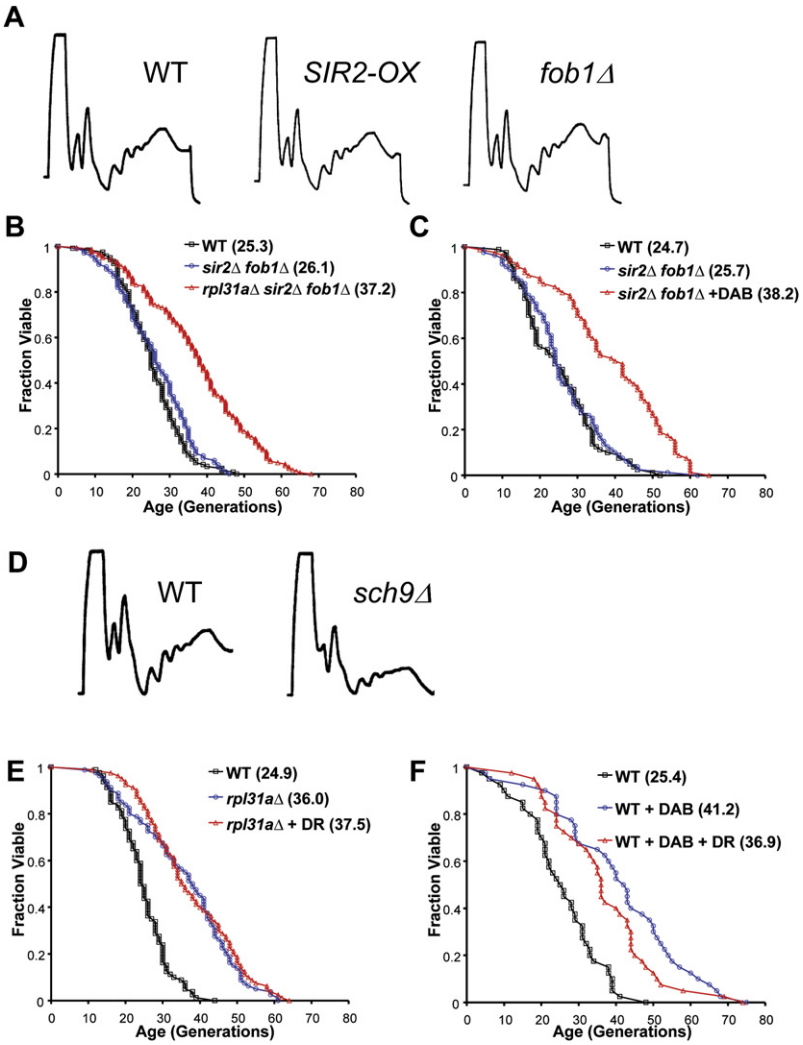


Figure 4. Depletion of 60S Subunits Extends RLS by a Mechanism Independent of Sir2 and Similar to DR

(A) *SIR2* overexpression and *fob1*Δ cells show polysome profiles similar to that of wild-type.

(B and C) Deletion of *RPL31A* or diazaborine treatment (15 μg/ml) increases the RLS of *sir2*Δ *fob1*Δ cells.

(D) A genetic model of DR (*sch9*Δ) results in cells with reduced levels of both 40S and 60S ribosomal subunits and polysomes relative to wild-type.

(E and F) DR does not further extend the RLS of *rpl31a*Δ cells or cells treated with diazaborine (15 μg/ml). Mean lifespans are shown in parentheses.

extends yeast RLS by limiting accumulation of ERCs (Defossez et al., 1999). Because Sir2 and Fob1 regulate rDNA recombination, they might also influence 60S subunit levels by modulating the rate of rDNA transcription. Contrary to this idea, however, neither overexpression of Sir2 nor deletion of *FOB1* had a detectable effect on 60S subunit levels or on overall polysome profile relative to wild-type cells (Figure 4A).

As long as ERC levels are kept low by deletion of *FOB1*, RLS extension by DR (via growth on reduced glucose media or genetic models of DR) is independent of Sir2 (Kaeberlein et al., 2004; Kaeberlein et al., 2006). Similarly, deletion of *RPL31A* or treatment with diazaborine significantly increased the RLS of *sir2*Δ *fob1*Δ cells (Figures 4B and 4C). These data indicate that, similar to *tor1*Δ or *sch9*Δ (Kaeberlein et al., 2005b), depletion of 60S subunits extends life span independently of Sir2.

60S Subunit Deficiency Increases Life Span by a Mechanism Similar to DR

During DR, the activity of TOR and Sch9 are reduced, resulting in decreased RP transcription (Jorgensen et al., 2004). Therefore, it is possible that TOR and Sch9 mediate lifespan extension in response to DR by depleting 60S subunits. Inhibition of TOR by treatment with rapamycin results in a moderately depressed polysome profile (Powers and Walter, 1999), although unlike diazaborine treatment, inhibition of TOR does not specifically affect 60S subunit abundance. Strains lacking *SCH9* display a polysome profile in which both free 40S and 60S subunit levels as well as polysomes are reduced (Figure 4D). The polysome profiles of these genetic models do not show a specific reduction in the abundance of 60S subunits, possibly indicating that the simultaneous decrease of both 40S and 60S subunits is compatible with long life span and that the decrease in 60S abundance is dominant. Another possibility is that the young cells used for polysome analysis (log-phase cultures) cannot accurately model aging cells, in which important changes in polysome profile may occur over time.

DR does not further increase the RLS of long-lived *tor1*Δ or *sch9*Δ cells (Kaeberlein et al., 2005b), which is consistent with these genes acting in a genetic pathway with DR. DR also failed to significantly increase the long RLS of *rpl31a*Δ cells (Figure 4E) or of cells grown on media containing diazaborine (Figure 4F). Together these data support a model in which the depletion of 60S subunits promotes longevity by a mechanism independent of Sir2 and similar to *tor1*Δ, *sch9*Δ, and DR.

Gcn4 Is Required for Full Life Span Extension by Depletion of 60S Subunits

Gcn4 is a nutritionally regulated transcriptional activator important for activating transcription of amino acid biosynthetic genes in response to amino acid starvation (reviewed in Hinnebusch, 2005) as well as regulating diverse cellular processes including purine biosynthesis, autophagy, biosynthesis of organelles, ER stress response, and induction of mitochondrial transport carrier proteins (Jia et al., 2000; Natarajan et al., 2001; Patil et al., 2004). Gcn4 protein levels are primarily determined by translation and protein degradation rather than by transcription. Translation of *GCN4* mRNA is regulated by four small upstream open reading frames (uORFs1–4) in the 5′ leader region of the *GCN4* mRNA, and both amino acid starvation (reviewed in Hinnebusch, 2005)

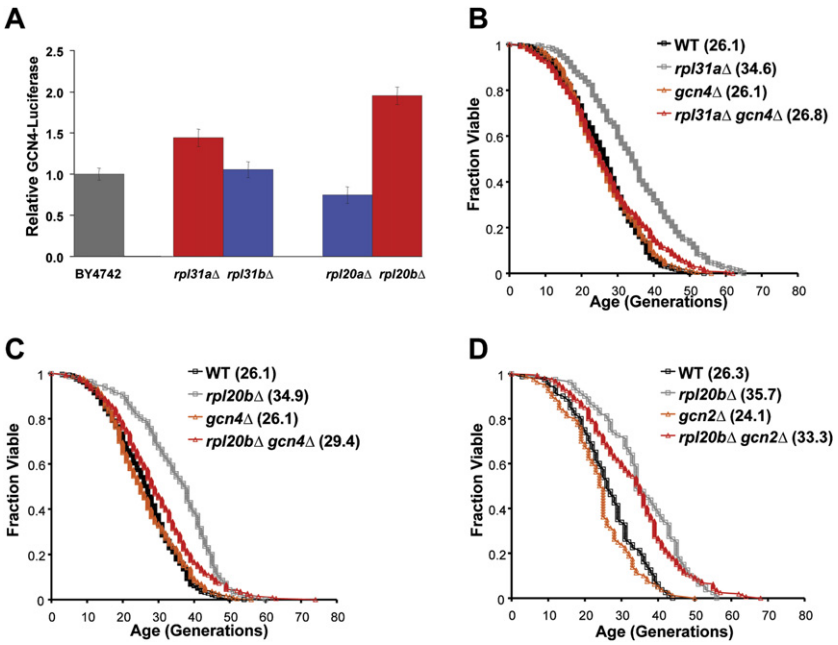


Figure 5. Cells Lacking RPL Genes Require *GCN4*, but Not *GCN2*, for Increased Longevity

(A) Gcn4-luciferase levels for *rpl31a*Δ, *rpl31b*Δ, *rpl20a*Δ, and *rpl20b*Δ relative to wild-type cells show that translation of *GCN4*-luciferase RNA correlates with long life span. Red bars represent long-lived strains, and blue bars represent strains that are not long lived.

(B and C) Long-lived strains *rpl20b*Δ and *rpl31a*Δ require *GCN4* for full life span extension.

(D) *GCN2* is not required for lifespan extension by deletion of *RPL20B*. Mean life spans are shown in parentheses.

and glucose limitation (similar to DR) (Yang et al., 2000) are known to induce Gcn4 activity in a Gcn2-dependent manner. *RPL* mutations have also been shown to induce expression of Gcn4 reporters (Foiani et al., 1991; Martín-Marcos et al., 2007), as has inhibition of TOR signaling (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003; Valenzuela et al., 2001).

We speculate that in cells limited for 60S subunits, ternary complexes containing initiation factors and a 40S subunit will more frequently scan through the inhibitory uORFs present in the *GCN4* 5′ leader region before binding a 60S subunit and translating the *GCN4* ORF. The resulting increased expression of Gcn4 protein may be related to the increased RLS of these cells. Consistent with this hypothesis, two different long-lived strains, *rpl20b*Δ and *rpl31a*Δ, displayed elevated expression of Gcn4-luciferase relative to wild-type in a dual-luciferase reporter assay (Figure 5A), while the deletion strains corresponding to their paralogs, which are not long lived, did not induce Gcn4-luciferase (Figure 5A).

We next tested whether the long life span of strains lacking RPL genes is dependent upon the presence of Gcn4. In each of the 11 cases examined, the percent increase in mean RLS observed by deletion of an RPL gene was diminished when *GCN4* was simultaneously deleted ($p < 0.001$) (Figures 5B, 5C, 6A, and S5). In addition, the life span extension observed by deletion of *RPL20B* was independent of the eIF2 α kinase Gcn2 (Figure 5D), consistent with a model in which translation of Gcn4 occurs more frequently due to a lack of 60S subunits available to initiate translation at upstream inhibitory uORFs. Together, these data support a model in which cells deficient for 60S subunits induce expression of Gcn4 to achieve maximum life span extension and for the first time identify Gcn4 as a potential longevity factor.

Full Life Span Extension by DR Is Dependent on Gcn4

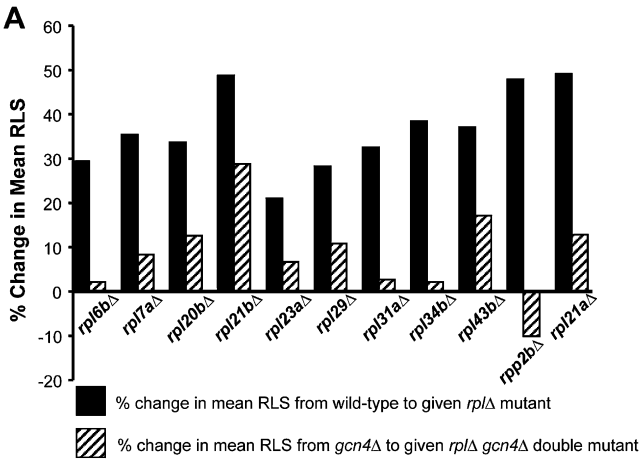
Since DR or TOR inhibition is known to reduce RP levels and increase *Gcn4* translation, we considered the possibility that DR might promote longevity in part via induction of *GCN4*. If so, then deletion of *GCN4* should attenuate the RLS extension afforded by DR or TOR inhibition. Consistent with this hypothesis, the life span extension from *TOR1* deletion is significantly reduced in *gcn4*Δ cells, relative to wild-type cells ($p = 0.028$) (Figures 6B and S6). A similar trend was observed in *sch9*Δ cells or in response to DR by growth on 0.05% glucose media; however, statistical significance was not attained in triplicate replicates. Thus, we conclude that Gcn4 is required for full lifespan extension in response to depletion of 60S subunits or reduced TOR signaling, and may also play a role in the response to DR (Figure 7).

DISCUSSION

The 60S Ribosomal Subunit Modulates Longevity in Yeast

Accumulating evidence suggests that regulation of mRNA translation is an evolutionarily conserved mechanism for modulating longevity (Kaeberlein and Kennedy, 2007). From a comprehensive analysis of 107 different RP gene deletions in yeast, we have determined that at least 14 different RPL gene deletions confer long RLS. In addition, deletion of any one of four different 60S-specific processing factors or treatment of cells with the 60S inhibitor diazaborine is also sufficient to increase RLS. Interestingly, we find no evidence that reduction of 40S subunits has a similar effect on life span, even when translation and polysomes are decreased to an extent similar to that of long-lived *rpl*Δ mutants (Figure S7). Thus, we conclude that the RLS extension reported here does not result solely from reduced translation but by a specific reduction in 60S ribosomal subunit levels.

The relatively greater importance of 60S subunits over 40S subunits for longevity in yeast is interesting given that no such specificity has been observed in *C. elegans*, where RNAi knockdown of multiple large and small subunit RPs increases adult longevity (Chen et al., 2007; Curran and Ruvkun, 2007; Hansen et al., 2007). In yeast, two 40S proteins have been reported to influence RLS (Chiocchetti et al., 2007). It is possible that a number of RPS gene deletions are long lived in the set we analyzed, and the change in RLS is not statistically significant at the current level of analysis. Anomalies in the yeast ORF deletion collection could also account for differential results in RLS experiments



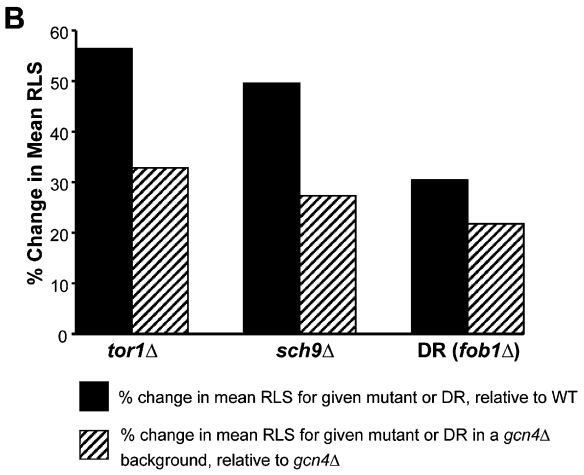


Figure 6. *GCN4* Is Required for Full Life Span Extension by Depletion of 60S Subunits or by DR

(A) Mean lifespan extension by deletion of any of 11 different RPL genes is largely dependent on *GCN4* ($p = < 0.001$). Solid bars represent the percent change in mean RLS for each *rpl*Δ strain, relative to experiment-matched wild-type cells; hashed bars represent the percent change in mean RLS for each corresponding *rpl*Δ *gcn4*Δ double mutant, relative to experiment-matched *gcn4*Δ cells.

(B) Full life span extension by *tor1*Δ ($p = .03$), *sch9*Δ ($p = .21$), or DR ($p = .44$) is dependent on *GCN4*. Solid bars represent the percent change in mean RLS from *tor1*Δ, *sch9*Δ or DR, relative to experiment-matched wild-type cells; hashed bars represent the percent change in mean RLS from *tor1*Δ, *sch9*Δ, or DR in a *gcn4*Δ background, relative to experiment-matched *gcn4*Δ cells.

(see Supplemental Data). One possible explanation for these observations is that a subset of 40S RP mutations can alter the translational machinery in a manner that may induce *GCN4*. Altered *GCN4* translational regulation due to RPS mutations is consistent with previously published data (Mueller et al., 1998). Alternatively, there may be a life span benefit derived directly from reduced translation, such as improved protein homeostasis with age (Kaeberlein and Kennedy, 2007). Our data suggest that, at least in yeast, the primary contribution to extended RLS is from specific depletion of 60S subunits and enhanced Gcn4 translation.

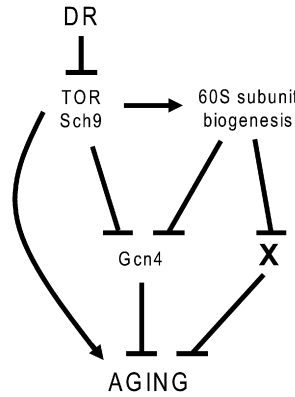


Figure 7. A Genetic Model for Life Span Extension by DR

Lifespan extension by depletion of 60S subunits, *tor1*Δ, *sch9*Δ, or DR, is mediated in part by Gcn4; however, a portion of the lifespan extension in each case can occur via at least one Gcn4-independent mechanism.

Gcn4 Modulates Longevity

We have provided evidence that Gcn4 is required for full life span extension by depletion of 60S subunits, DR, or genetic mimics of DR, *tor1*Δ, and *sch9*Δ. Gcn4 induces the transcription of over 500 target genes, many of which are implicated in processes linked to life span regulation (Natarajan et al., 2001). For example, both Gcn4 and TOR are involved in regulating autophagy (Jia et al., 2000; Natarajan et al., 2001), and in *C. elegans*, lifespan extension by *daf-2* requires beclin, the ortholog of yeast *ATG6* (Melendez et al., 2003). In future studies, it will be important to determine which of Gcn4's many target genes are most important for life span regulation. The mammalian Gcn4 ortholog (ATF4) is regulated via a similar Gcn2-dependent translational mechanism (Lu et al., 2004), raising the possibility that this pathway could play a similar role in multicellular organisms.

Our data provide evidence that Gcn4 is required for full lifespan extension by deletion of *RPL* genes in yeast. Although this dependence is significant for all the *RPL* mutants examined ($p = < 0.001$), the degree to which different *RPL* mutants depend on Gcn4 for life span extension varied (Figures 5B, 5C, 6A, and S5). Perhaps this reflects that deletion of single *RPL* genes can result in varied levels of *GCN4* induction. The large number of Gcn4 target genes and the stringent nature of its regulation lead us to suspect that genetic approaches to attain an "optimal level" of Gcn4 for life span may be difficult.

A Model for Translational Control of Replicative Life Span

Epistasis analyses place reduction of 60S subunits together with DR, *tor1*Δ, and *sch9*Δ, characterized by a lack of response to DR and an ability to extend RLS independently of Sir2. However, a single linear path from DR to increased Gcn4 synthesis is not entirely supported by the data presented here, because the loss of *GCN4* does not completely prevent the life span extension observed by either DR (environmental or genetic) or of strains lacking RPL genes (Figures 5B, 5C, 6A, 6B, S5, and S6). This indicates that one or more additional Gcn4-independent pathways exist for life span regulation in response to nutrients (Figure 7).

What might a Gcn4-independent pathway be? A number of other yeast genes encode mRNAs containing 5′ uORFs (Vilela and McCarthy, 2003; Zhang and Dietrich, 2005), including *HAP4* and *CLN3*, both of which are also important for appropriate response to nutrients, and increased expression of Hap4 increases yeast RLS (Lin et al., 2002). The translational regulation of these messages is largely unstudied, leaving open the possibility that reduced 60S subunit levels may be influencing expression of these genes similarly to *GCN4*. It is also possible that changes in 60S subunit abundance or structural composition (if ribosomes lacking a protein are being composed) affect translation of particular messages independently of uORFs.

Another possible means by which cells depleted for 60S subunits could increase RLS is by modulating the cellular response to ER stress (Miyoshi et al., 2002; Zhao et al., 2003). Yeast carrying the *sly1-1* mutation or cells treated with tunicamycin, both of which induce an ER stress response, activate the PKC pathway leading to a dramatic decrease in RP gene transcription. For unknown reasons, this signal is abrogated in cells lacking *RPL* but not *RPS* genes (Miyoshi et al., 2002; Zhao et al., 2003). Interestingly, this signal is still abrogated in double mutants lacking both an *RPL* and an *RPS* gene (Zhao et al., 2003), perhaps supporting the idea that cells like *tor1*Δ and *sch9*Δ, which display polysome profiles with both decreased 40S and 60S subunits, should act similarly to cells in which only 60S subunits are limited. As cells age, they may experience ER stress, which results in inhibition of RP gene transcription to a point at which translation can no longer be supported. Reduction of 60S subunits may specifically block this signal, allowing the cells to maintain a level of protein translation sufficient to support additional replicative cycles. Gcn4 is required for activating a majority of unfolded protein response target genes in response to ER stress (Patil et al., 2004), and ER stress has been proposed to play a role in *C. elegans* lifespan regulation (Viswanathan et al., 2005).

Conclusions

Our findings demonstrate that the abundance of 60S ribosomal subunits is a key determinant of yeast RLS. Genetic evidence places DR, TOR inhibition, *SCH9* deletion, and depletion of 60S subunits in a longevity pathway that is partially dependent on the Gcn4 transcription factor. Gcn4 activity is enhanced in long-lived mutants and is required for full life span extension by reduction of 60S subunits, *tor1*Δ, *sch9*Δ, or DR. Evidence from multicellular eukaryotes is consistent with DR being mediated in part by an altered translational program. Decreased activity of TOR and Sch9 orthologs results in increased life span in worms and flies (Hertweck et al., 2004; Jia et al., 2004; Kapahi et al., 2004; Vellai et al., 2003), and inhibition of factors important for translation initiation has been shown to increase the lifespan of *C. elegans* (Chen et al., 2007; Curran and Ruvkun, 2007; Hamilton et al., 2005; Hansen et al., 2007; Henderson et al., 2006; Pan et al., 2007; Syntichaki et al., 2007). Thus, we propose the existence of an evolutionarily conserved pathway linking DR, protein translation, and longevity. The high level of conservation among Gcn4 orthologs in multicellular eukaryotes merits the future investigation of their potential roles in life span regulation.

EXPERIMENTAL PROCEDURES

Strains and Media

All yeast strains were derived from the parent strains of the haploid yeast ORF deletion collections (Winzeler et al., 1999), BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0)*, and BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The *MATα* haploid ORF deletion collection and the *MAT*a haploid ORF deletion collection, along with the parental strains, were obtained from Research Genetics. Of the RP gene deletion strains from the *MATα* deletion collection, *rps12*Δ failed to pass quality control during construction and was therefore excluded from our analysis. Figure 1 contains pooled data from both *MAT*a and the corresponding *MATα* deletion collection strains (and remade strains in the cases of *rpl31a*Δ and *rpl20b*Δ; see Supplemental Data). Data represented in all other figures were generated from strains in the *MATα* deletion collection. The multiple gene deletion strains represented in Figures 4–6 were constructed by standard PCR-based gene disruption as described (Kaeberlein et al., 2004). The *SIR2* overexpression strain (Figure 4A) was constructed by genomic integration of an extra copy of *SIR2* at the *LEU2* locus, as described (Kaeberlein et al., 1999).

Cells were grown in standard YPD containing 1% yeast extract, 2% peptone, and 2% glucose, with the exception of the life span assays, which were done using YPD containing 0.05% glucose where noted. Diazaborine was a generous gift from Gregor Hogenaur (Graz, Austria).

Replicative Life Span Analysis

Lifespan assays were carried out as described previously (Kaeberlein et al., 2005b). All lifespan experiments were carried out on standard YPD plates (2% glucose, unless otherwise noted). DR experiments were carried out on 0.05% glucose; we have previously shown that 0.05% glucose is an optimal concentration for DR in RLS studies using BY4742 (Kaeberlein et al., 2004). For life span studies with diazaborine or cycloheximide, the drug was added from frozen stock to melted and cooled YPD at the appropriate concentration. Statistical significance for RP mutants were determined using a Wilcoxon Rank-Sum test (MATLAB "ranksum" function) using a $p = 0.05$ cutoff. For each of the 107 *rp*Δ strains analyzed in this study, mean life span and p values can be found in Table S1. For Figure 6A, a t test was used to determine the statistical significance for the dependence of *RPL* mutants on *GCN4* for long lifespan. Independent t tests were used to determine the statistical significance for the percent change in mean RLS for DR, *tor1*Δ, and *sch9*Δ with or without *GCN4* (Figure 6B).

Polysome Analysis

Polysome analysis was carried out as described previously (MacKay et al., 2004). Briefly, log-phase yeast cultures were quick-chilled with crushed frozen YPD containing 100 μg/ml cycloheximide. Cells were harvested by centrifugation, washed with 10 ml lysis buffer (25 mM Tris-HCl, pH 7.5, 40 mM KCl, 7.5 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml heparin, 100 μg/ml cycloheximide) and resuspended in 1 ml lysis buffer. Cells were lysed by vortexing with glass beads. Triton X-100 and sodium deoxycholate were added (1% final concentration each) with vortexing and the samples stood on ice for 5 min before the supernatant was clarified by centrifugation. All reagents were ice-cold and all steps were done in a 4°C cold room. For separation on gradients, 1 ml containing 20 (or 25, Figure 2E) A260 units of lysate were loaded onto 11–ml linear 7%–47% sucrose gradients in 50 mM Tris-HCl, pH 7.5, 0.8 M KCl, 15 mM $MgCl_2$, 0.5 mg/ml heparin, 100 μg/ml cycloheximide and sedimented at 39,000 rpm at 4°C in an SW40 Ti swinging bucket rotor (Beckman) for 2 hr (or 1.5 hr, Figure 2E). Gradients were collected from the top and profiles were monitored at 254 nm.

Quantitative Real-Time PCR Analysis of RPL RNAs

Lysates of strain BY4742 were prepared as described above and previously (MacKay et al., 2004), total RNA was purified using QIAGEN RNeasy mini columns, and 2 μg RNA were converted to cDNA with Invitrogen Ss III reverse transcriptase and an oligo$(dT)_{25}$ primer with a G/C/A 3′ anchor. Lysates, RNA purification, and reverse transcription reactions were performed on different days for three independently grown cultures. Specific cDNAs were quantitated with an iCycler (from Bio-Rad in Hercules, CA) and SYBRGreen

detection of products (according to manufacturer's specifications). See Supplemental Data for primer sequences and details.

Gcn4-Luciferase Assays

Gcn4 expression was assayed using a dual-luciferase reporter plasmid pVW31, modified from the *URA3*-2μ plasmid pDB688 (Keeling et al., 2004; Salas-Marco and Bedwell, 2005) (kindly provided by David Bedwell). Plasmid pVW31 contains (1) a *GCN4*-firefly luciferase cDNA fusion under transcriptional control of a 772 bp *GCN4* 5′ fragment containing all of the promoter, upstream open reading frames (uORFs), and other 5′ regulatory elements and the *CYC1* transcription terminator and (2) an independent transcriptional unit with a Renilla luciferase cDNA transcribed from the constitutive *S. cerevisiae PGK1* promoter and terminated with a 3′ fragment from *GCY1*. Strains transformed with pVW31 were grown in synthetic glucose minimal medium lacking uracil and containing required amino acids as well as isoleucine and valine (Lucchini et al., 1984). A dual-luciferase reporter assay system (Promega) and a Perkin Elmer Victor Light Model 1420 luminometer were used to measure luciferase activities. Firefly luciferase activity was normalized to the Renilla luciferase activity.

Growth Rate Analysis

Growth curves for the RP gene deletion strains were generated using a Bioscreen C machine (Growth Curves USA). Overnight cultures of the strains were grown in 250 μl YPD in 96-well plates (inoculated from single colonies). The next day, 8 μl of overnight culture were added to 250 μl fresh YPD medium in Bioscreen C Honeycomb microplates and cultures were grown in the Bioscreen C at 30°C for 24 hr. Optical density was measured every 30 min, the plates were shaken every 10 min for 20 s. The doubling time was calculated between every 30 min interval. The generation time was defined as the average of the four lowest doubling times (steepest part of the growth curve), after dropping the lowest single number. Independent growth curves were generated for each strain on three different days; the average generation time ± standard deviation for at least three independent assays is given in Table S3. The raw data collected for each of the independent experiments are provided in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures (including primer nucleotide sequences), four tables, and seven figures, and can be found online at http://www.cell.com/cgi/content/full/133/2/292/DC1/.

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