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Genes determining yeast replicative life span in a long-lived genetic background

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Abstract

Here we describe the replicative life spans of more than 50 congenic *Saccharomyces cerevisiae* strains, each carrying a mutation previously implicated in yeast aging. This analysis provides a direct comparison, in a single, long-lived strain background, of a majority of reported yeast aging genes. Of the eleven deletion mutations previously reported to increase yeast life span, we find that deletion of *FOB1*, deletion of *SCH9*, and deletion of *GPA2*, *GPR1*, or *HXK2* (three genetic models of calorie restriction) significantly enhanced longevity. In addition, overexpression of *SIR2* or growth on low glucose increased life span. These results define a limited number of genes likely to regulate replicative life span in a strain-independent manner, and create a basis for future epistasis analysis to determine genetic pathways of aging.

Keywords: Yeast aging; Caloric restriction; Genetic pathways

1. Introduction

The budding yeast *Saccharomyces cerevisiae* has served as a useful model for aging research, leading to the identification of new longevity genes and pathways whose counterparts can be examined in higher eukaryotes (Kaeberlein et al., 2001). One measure of aging in yeast is the finite replicative life span (RLS) of mother cells, defined as the number of mitotic cycles completed prior to senescence (Mortimer and Johnston, 1959). An alternative measure of yeast aging, termed chronological aging, is defined by the ability of cells to maintain viability in a non-dividing, metabolically active state (MacLean et al., 2001; Fabrizio and Longo, 2003).

Several processes have been implicated in the determination of yeast RLS, including the accumulation of extrachromosomal rDNA circles (ERCs), transcriptional silencing at the rDNA mediated by the Sir2 and Rpd3 histone deacetylases, genomic instability, mitochondrial signaling to the nucleus, and oxidative stress (Sinclair et al., 1998; Bitterman et al., 2003). Numerous studies have examined the role these processes play in yeast aging; however, little effort has been made to determine which regulate aging in a general manner and which are strain-specific.

The degree to which strain-specific features determine RLS in yeast remains an open question. At one extreme, it is possible that the regulatory events controlling yeast aging are invariant in all strains. This seems unlikely, given that different wild-type strains have been reported to have highly variable mean and maximum RLSs (Table 1), and polymorphisms in genes such as *MPT5* and *SSD1* are known to have a significant effect on RLS (Kennedy et al., 1997; Kaeberlein et al., 2004a). At the other extreme, it is possible that each yeast strain might have highly divergent aging properties, and therefore most genetic interventions reported to affect RLS would act in a strain-specific fashion. This is also not the case, as some interventions, such as calorie restriction (CR) or decreased ERC levels, have been reported to increase life span in multiple genetic backgrounds. Thus, it seems clear that some proteins act as general regulators of replicative aging and others act in a strain-specific manner.

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Table 1
Comparison of replicative life span for strains commonly used in yeast aging research

Strain	% Difference	Mean MRLS	Reference
BY4742	26.5	–	This study
PSY316AT	22.4	18	Bitterman et al. (2002)
SP1-1	22.4	18	Kirchman et al. (1999)
A364A	21.4	24	Lin et al. (2000)
SGY	21.4	24	Lin et al. (2000)
W303R	20.8	27	Kaeberlein et al. (1999)
YPK9	20.4	30	Kirchman et al. (1999)
YPK4	19	39	Kirchman et al. (1999)
BWG1-7A	16	66	Austriaco (1996)
BKy4-14c	15.6	70	Kennedy et al. (1995)
X2180-1A	13	104	Egilmez and Jazwinski (1989)

Mean replicative life span (RLS) and the relative percent difference in RLS for each strain compared to BY4742 is shown for selected strains used in yeast aging research. Together, these strains comprise the vast majority of published yeast replicative life span data.

According to the Science of Aging Knowledge Environment's Aging Genes/Interventions Database (Kaeberlein et al., 2002b), more than 50 genes have been reported to alter RLS when mutated or over-expressed. To date, no attempt has been made to determine which of these genes act to determine longevity in a general manner across multiple strain backgrounds. In addition, for a majority of these "aging genes", little effort has been made to place them into genetic or molecular pathways with respect to their longevity phenotype.

Two approaches should be used to assess the relative importance of the genes reported to affect yeast replicative aging. First, the RLS of each mutation should be determined in a variety of strain backgrounds. Mutations that alter RLS, in particular those that increase life span, in a similar manner in multiple strain backgrounds are likely to correspond to genes that act as general determinants of longevity. This type of information has been determined for only a few mutations, thus far. Among those, mutations that are likely to behave in a general manner with respect to longevity include those impacting ERC levels, such as deletion of *SIR2* and deletion of *FOB1* (Defossez et al., 1999; Kaeberlein et al., 1999, 2002a, 2004a, 2004b; Lin et al., 2003; McMurray and Gottschling, 2003; Takeuchi et al., 2003; Borghouts et al., 2004). One example of a mutation known to impact longevity in a strain-specific manner is loss of mitochondrial DNA (rho^0), which increases RLS in one background, decreases RLS in two other backgrounds, and has no effect on longevity in a fourth (Kirchman et al., 1999).

As a second approach to assess the importance of putative "aging genes", a majority of the mutations reported to affect RLS should be analyzed in a single, long-lived strain. Thus, the relative effect of each mutation on mortality can be directly compared in a standardized genetic background. Spontaneous mutations that have occurred since divergence from a common ancestor have led to much strain specific variability between different yeast strains. Since the vast majority of these mutations are likely to be neutral or detrimental to life span, performing this analysis in a long-lived strain will reduce the likelihood that strain-specific suppressors are isolated as longevity-enhancing mutations. Additionally, this approach will allow epistasis analyses through the creation of double (and more complex) mutants in order to characterize genetic pathways regulating longevity.

As a first step toward the goal of a comprehensive understanding of yeast aging genes, we have determined the effect of more than 50 mutations and interventions on RLS in the long-lived strain, BY4742. The S288C strain background, from which BY4742 is derived, has not been used extensively in yeast aging analysis. It was chosen: (1) because nearly all non-essential yeast gene deletions have already been created in this strain (Winzeler et al., 1999); and (2) because we reasoned that a long-lived strain background would be less likely to contain deleterious mutations or polymorphisms that reduce overall fitness. This analysis has allowed us to compare directly the relative effects of different mutations and, thus, to determine which genes are general regulators of aging, and which are not. While many of the mutations reported to shorten RLS have the expected phenotype in this strain background, only five single-gene deletions resulted in a statistically significant increase in mean RLS: *fob1Δ*, *gpa2Δ*, *gpr1Δ*, *hxk2Δ*, and *sch9Δ*. In addition, over-expression of *SIR2*, or CR by growth on low glucose, also increased RLS. Intriguingly, with the exception of *FOB1*, all of these longevity-enhancing interventions have counterparts that influence pathways linked to life span extension in higher eukaryotes.

2. Materials and methods

2.1. Strains and plasmids

All yeast strains used in this study are derived from BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), or BY4743 (*MAT*a/*MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0*). Unless otherwise stated, deletion strains were obtained from the *MATα* ORF deletion collection, with the KanMX marker replacing the deleted ORF (Winzeler et al., 1999). The *MATα* haploid ORF deletion collection, the *MAT*a haploid ORF deletion collection, and the parental wild-type strains BY4742, BY4741 and BY4743 were obtained from Research Genetics. Gene disruptions were carried out by transforming yeast with PCR-amplified deletion constructs containing 45 nucleotides of homology to regions flanking the ORF to be deleted and either *HIS3*, *LEU2*, or *URA3* amplified from pRS403, pRS405, or pRS406 (Sikorski and Hieter, 1989), respectively. In each case, the entire open reading frame of the deleted gene was removed. All gene disruptions were verified by PCR. The rho^0 strain used for life span analysis

was generated by treatment with ethidium bromide. The *MAT*a/*MAT*a diploid strain was created by transforming BY4743 with a plasmid containing the *HO* gene expressed under the control of a galactose-inducible promoter. Transformants were grown in the presence of galactose then transferred to glucose media and allowed to form colonies that were subsequently tested for the ability to mate with haploid tester strains. The strain carrying the phosphorylation defective *SIR3S275* allele was constructed by integrating the plasmid pRS303-SIR3S275A into the *URA3* locus of the *sir3Δ* strain from the *MATα* ORF deletion collection. As a control, a wild-type copy of *SIR3* was integrated into the *sir3Δ* strain using the plasmid pRS303-SIR3. Proper integration was confirmed in both cases by complementation of the mating defect caused by deletion of *SIR3*. Plasmids pRS303-SIR3S275A and pRS303-SIR3 are described elsewhere (Ray et al., 2003), and were generously provided by K. Runge.

In three cases (*cpr7Δ*, *rpd3Δ*, and *sgs1Δ*), we observed that strains obtained from the *MATα* deletion collection yielded low spore viability when crossed to BY4741 and sporulated. This may suggest that these particular mutants are aneuploid or contain some other genomic irregularity. Therefore, we generated new deletion alleles for these genes in BY4742 and determined RLS. In only one case (*sgs1Δ*), was RLS significantly different in the newly generated strain compared to the deletion collection strain. Upon analysis of the *SGS1* locus by PCR using primers flanking the *SGS1* gene, aneuploidy was observed based on the presence of PCR products corresponding to both a wild-type *SGS1* allele and a *sgs1::kanMX* deletion allele. Aneuploidy at the deleted locus was not observed in either *cpr7Δ* or *rpd3Δ*.

2.2. Identification of reported yeast aging genes

The Science of Aging Knowledge Environment's Aging Genes/Interventions Database (http://sageke.sciencemag.org/cgi/genesdb) was used to identify yeast genes previously studied with respect to replicative aging. An advanced search of the database was performed to generate a list of single-gene deletion mutations for which RLS had been determined (see supplementa1 table). A subset of these genes were selected for RLS analysis in BY4742 based on the availability of the particular mutant in the ORF deletion set and the reported effect on RLS, with preference given to mutations reported to increase RLS.

2.3. Replicative life span analysis

Yeast strains for replicative life span (RLS) analysis were removed from frozen stock (25% glycerol, −80 °C) and streaked onto YPD. After 2 days growth, single colonies were selected and patched to YPD. The next evening, cells were lightly patched to the plates used for life span analysis. After overnight growth, cells were arrayed on the YPD plate using a micromanipulator and allowed to undergo 1–2 divisions. Virgin cells were then selected and subjected to life span analysis. Plates were sealed with Parafilm, except during dissection. Cells were grown at 30 °C during the day and stored at 4 °C at night. Daughter cells were removed by gentle agitation with a dissecting needle and tabulated every 2–4 h. All life span experiments were carried out on standard YPD plates (2% glucose, unless otherwise noted). In order to prevent possible bias, strains were coded such that the researcher performing the life span experiment had no knowledge of the strain genotype for any particular strain. For each experiment, each strain was randomly assigned a numerical identifier at the time of removal from frozen stock. One individual was responsible for assigning strain designations (K.T.K.) while a different individual (M.K. or B.K.K.) performed the RLS analysis.

2.4. Data format and analysis

For statistical analysis, life span datasets were compared by a two-tailed Wilcoxon rank-sum test. Wilcoxon *P*-values were calculated using the MATLAB 'rank-sum' function. Data shown in Table 2 and used to calculate *P*-values were derived from pair-matched, pooled experiments where each mutant was compared to wild-type cells examined within the same experiment(s). Strains are stated to have a significant difference in life span for $P < 0.01$. The wild-type data contained in each figure is the union of the sets of pair-matched data for each mutant.

3. Results

The SAGE KE Aging Genes/Interventions Database lists fewer than 70 single-gene deletion mutations for which RLS has been determined (see supplementa1 table). The vast majority of these reported life span phenotypes, however, were determined in short-lived strains, and few mutations have been examined in combination or in more than one genetic background. In order to develop a more complete picture of the pathways that determine longevity in a long-lived genetic background, we performed a large-scale analysis of genes reported to affect RLS. Congenic single-ORF deletion strains from the *MATα* yeast ORF deletion collection (Winzeler et al., 1999), corresponding to 43 genes previously implicated in yeast aging, were subjected to RLS analysis (Fig. 1; Table 2). In addition, the life span phenotypes associated with CR by growth on low glucose; with over-expression of *SIR2*; with a phosphorylation defective allele of *SIR3* (S275A); with loss of mitochondrial DNA; and with two double mutant strains were determined. In general, mutations reported to decrease RLS displayed the expected phenotype; however, of the 17 strains examined that are reported to have increased life span (Table 3), only seven exhibited a statistically significant increase in RLS ($P < 0.01$) in our study: *fob1Δ*, *gpa2Δ*, *gpr1Δ*, *hxk2Δ*,

Table 2
Replicative life span analysis of genes previously linked to yeast aging

Strain	Mean RLS (*n*)	Experiment-matched BY4742 mean RLS (*n*)	% Change RLS	*P*-value
BY4742	26.5 (470)	26.5 (470)	0	0.50
BY4741	27.3 (60)	26.1 (120)	5	0.20
BY4743	37.5 (110)	26.8 (80)	40	2.5×10^{-10}
BY4743 *MAT*a/*MAT*a	34.3 (20)	27.4 (20)	25	8.9×10^{-3}
cdc73Δ	22.4 (40)	26.1 (40)	−14	0.053
cpr7Δ::kanMX[a]	30.3 (80)	26.1 (80)	16	0.090
cpr7Δ::URA3[b]	25.7 (99)	26.8 (80)	−4	0.22
cyt1Δ	18.2 (40)	28.6 (30)	−36	2.3×10^{-5}
fob1Δ	36.5 (190)	25.8 (220)	42	$<10^{-15}$
fob1Δ sir2Δ	29.8 (200)	26.2 (250)	14	1.8×10^{-3}
gpa2Δ	34.9 (40)	26.7 (60)	31	7.4×10^{-4}
gpr1Δ	34.3 (40)	26.7 (60)	28	3.6×10^{-3}
hog1Δ	24.2 (40)	27.6 (40)	−12	0.059
hpr1Δ	9.8 (40)	26.1 (40)	−63	1.5×10^{-11}
hpr5Δ	16.1 (40)	27.6 (40)	−42	3.9×10^{-8}
hsc82Δ	25.1 (20)	30.4 (20)	−17	0.047
hsp104Δ	32.4 (20)	30.4 (20)	6	0.33
hxk2Δ	34.7 (180)	25.9 (220)	34	5.6×10^{-10}
lag1Δ	27.8 (40)	26.1 (40)	6	0.22
lag2Δ	28.8 (40)	26.1 (40)	10	0.18
mpt5Δ	17.8 (40)	28 (60)	−36	9.6×10^{-9}
mpt5Δ ssd1Δ	14.4 (20)	24.6 (20)	−41	3.7×10^{-4}
pde2Δ	18.6 (40)	26.1 (40)	−29	2.1×10^{-4}
phb1Δ	13.8 (40)	26.1 (40)	−47	3.9×10^{-10}
phb2Δ	14.3 (40)	26.1 (40)	−45	9.3×10^{-9}
pnc1Δ	27 (60)	25.7 (60)	5	0.16
rad16Δ	24 (40)	26.1 (40)	−8	0.16
rad52Δ	9.9 (40)	26.1 (40)	−62	1.2×10^{-12}
ras1Δ	28.4 (40)	26.1 (40)	9	0.097
ras2Δ	18.3 (60)	26.1 (80)	−30	2.2×10^{-7}
rho^{0}[b]	27.1 (40)	27.5 (60)	−2	0.28
rpd3Δ::kanMX[a]	28.2 (40)	26.8 (80)	5	0.23
rpd3Δ::URA3[b]	25.3 (40)	26.8 (80)	−6	0.16
rtg2Δ	20.1 (40)	26.1 (10)	−23	0.014
rtg3Δ	24.8 (40)	26.7 (60)	−7	0.14
sch9Δ::URA3[b]	37.1 (60)	26.9 (90)	38	4.9×10^{-6}
scp1Δ	26.4 (40)	23.4 (40)	13	0.043
sgs1Δ::kanMX[a]	32.5 (40)	24.8 (20)	31	0.013
sgs1Δ::URA3[b]	10.9 (40)	27.5 (60)	−60	4.2×10^{-14}
sin3Δ	18.9 (60)	26.8 (80)	−30	1.6×10^{-5}
sip2Δ	28.5 (60)	26.8 (80)	6	0.18
sir2Δ	13.9 (110)	26.1 (150)	−47	$<10^{-15}$
SIR2-ox[b]	34.6 (60)	27.8 (60)	25	7.0×10^{-4}
sir3Δ	23.8 (40)	26.1 (40)	−9	0.18
sir3Δ SIR3S275A[b]	24 (40)	27.6 (40)	−13	0.079
sir3Δ SIR3-WT[c]	24.2 (20)	27.6 (40)	−13	0.086
sir4Δ	24.3 (40)	26.1 (40)	−7	0.21
slt2Δ	24.3 (40)	27.6 (40)	−12	0.030
snf4Δ	21.7 (50)	26.9 (30)	−19	9.2×10^{-3}
sod1Δ	2.8 (20)	24.8 (20)	−89	3.1×10^{-8}
sod2Δ	28.6 (40)	26.1 (40)	9	0.13
soh1Δ	20.2 (40)	26.1 (40)	−22	3.9×10^{-3}
ssd1Δ	24.8 (60)	25.6 (60)	−3	0.30
uth1Δ	29.4 (40)	27.5 (60)	7	0.16
zds1Δ	27.7 (40)	26.1 (40)	6	0.23
zds2Δ	29.5 (40)	26.1 (40)	13	0.033
0.05% glu	31.8 (60)	26.3 (70)	21	2.2×10^{-3}

Replicative life span (RLS) was determined for each strain. Mean RLS for each strain (number of cells examined) compared to experiment-matched BY4742 mother cells (number of cells examined) is shown. *P*-values were calculated using a two-tailed Wilcoxon rank-sum test.

[a] Represents strain from the *MATα* deletion set that is likely to be aneuploid based on low spore viability as a heterozygous diploid (see Materials and Methods).

[b] Represents a newly constructed allele in the BY4742 parental strain.

[c] *sir3Δ SIR3-WT* refers to the strain constructed as a control for the *sir3Δ SIR3S275A* allele. Both the *sir3Δ SIR3-WT* and *sir3Δ SIR3S275A* strain were constructed by integrating a plasmid carrying either the wild-type allele of *SIR3* or the *SIR3S275A* allele into the *sir3Δ::kanMX* strain from the Research Genetics *MATα* haploid deletion set.

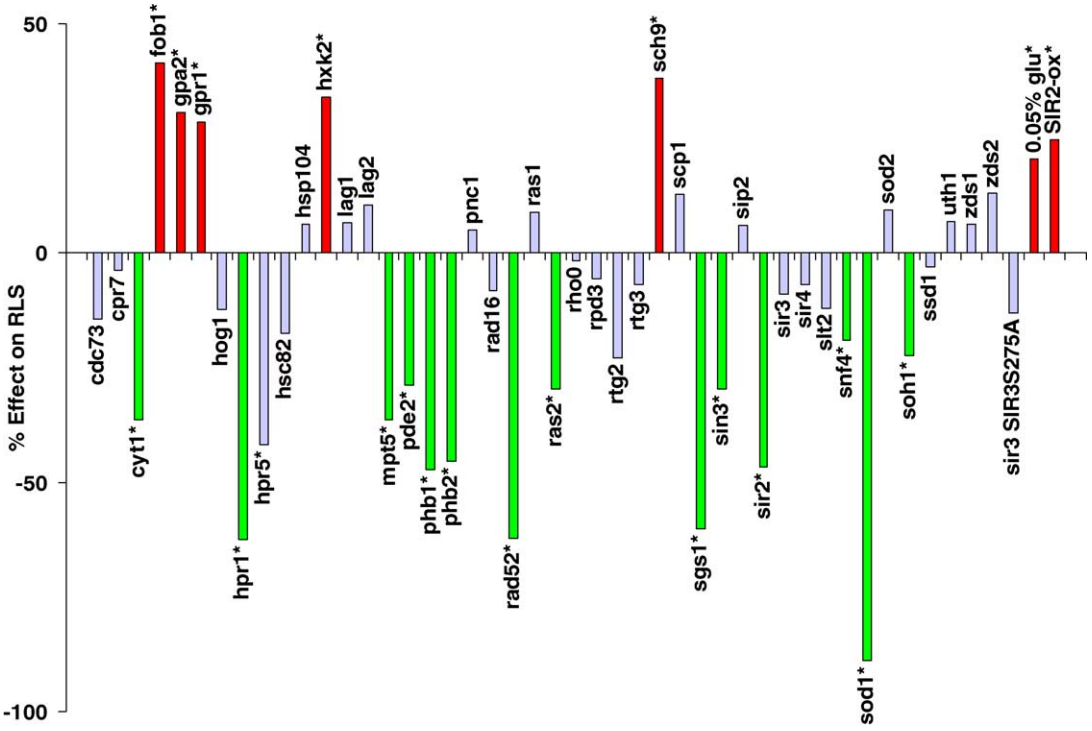

Fig. 1. Large-scale analysis of mutations reported to affect replicative life span. Percent effect on mean replicative life span relative to experiment-matched BY4742 mother cells is shown. All strains are *MATα* haploids derived from BY4742. *Significant difference in life span relative to experiment-matched wild-type cells ($P < 0.01$).

sch9Δ, *SIR2* over-expression, and growth on low glucose (discussed below).

3.1. Strains with increased replicative life span

In general, mutations that increase life span are likely to be more informative about the underlying aging process than mutations that decrease life span. A mutation can shorten life span either by accelerating the aging process or by increasing mortality in a manner unrelated to normal aging. Often, it is difficult to differentiate between these two possibilities. In contrast, a substantial increase in life span can only be accomplished by altering the normal cause(s) of mortality, thus providing insight into the genetic and molecular mechanisms of aging.

3.1.1. ERCs determine longevity in a long-lived strain

Life span extension by deletion of *FOB1* has been reported in multiple strain backgrounds (Defossez et al., 1999; Kaeberlein et al., 1999, 2004b; Lin et al., 2003; McMurray and Gottschling, 2003; Takeuchi et al., 2003; Borghouts et al., 2004) and is thought to be the result of decreased rDNA recombination and ERC formation (Defossez et al., 1999). Deletion of *FOB1* had a robust effect on life span in BY4742 (Fig. 2A), suggesting that ERC accumulation is one factor limiting the longevity of this strain. The Sir2 protein is also thought to regulate longevity by modulating rDNA recombination and ERC formation, although in a manner antagonistic to *FOB1*. Consistent with data previously reported for W303R (Kaeberlein et al., 1999), we observed that deletion of *SIR2* shortened life span by approximately 60% (Fig. 2A), while over-expression increased life span by 30% (Fig. 2B). The *sir2Δ fob1Δ* double mutant had a life span comparable to wild-type (Fig. 2A), as previously observed for W303R and PSY316 (Kaeberlein et al., 1999; Lin et al., 2000). Thus, *SIR2* and ERCs are determinants of longevity in a long-lived strain.

3.1.2. Calorie restriction increases replicative life span in BY4742

Life span extension by calorie restriction can be accomplished in yeast by reducing the glucose concentration of the media from 2 to 0.5% (Lin et al., 2000), or lower (Kaeberlein et al., 2002a, 2002b). In addition, several genetic models of calorie restriction have been described. These include deletion of the gene coding for hexokinase, *HXK2*, and several mutations that decrease cAMP-dependent protein kinase (PKA) activity, such as *gpa2Δ*, *gpr1Δ*, *cdc25-10*, *cdc35-1*, and *tpk1Δ tpk2-63 tpk3Δ*. Three of these genetic models (*hxk2Δ*, *gpa2Δ*, and *gpr1Δ*) were included in our analysis, all of which showed a comparable 30–40% increase in life span (Kaeberlein et al., 2004b) (Fig. 2C). In addition, we found that growth on low glucose resulted in a significant life span increase, with maximum extension observed at 0.05%. Thus, CR is effective at slowing aging in a long-lived strain background.

Table 3
Strains previously reported have increased life span

Mutation or intervention	Reference	Strain previously reported	Reported effect on mean RLS (%)	BY4742 effect on mean RLS (%)
fob1Δ	Defossez et al. (1999)	W303-1A	35	41*
gpa2Δ	Lin et al. (2000)	PSY316	35	31*
gpr1Δ	Lin et al. (2000)	PSY316	35	28*
hxk2Δ	Lin et al. (2000)	PSY316	35	34*
lag1Δ	D'Mello et al. (1994)	YPHDF-1A	50	6
ras1Δ	Sun et al. (1994)	SP1	20	9
rpd3Δ	Kim et al. (1999)	YPK9	40	−6
rtg3Δ	Jiang et al. (2000)	YPK9	55	−7
sch9Δ	Fabrizio et al. (2004)	DBY746	20	38*
scp1Δ	Gourlay et al. (2004)	KAY159	65	13
snf4Δ	Ashrafi et al. (2000)	S288C	20	−22
uth1Δ	Austriaco (1996)	BKy4-14c	20	7
zds1Δ	Roy and Runge (2000)	W303-1A	35	6
rho^0	Kirchman et al. (1999)	YPK9	30	−2
Low glucose	Lin et al. (2000)	PSY316	35	21*
SIR2-ox	Kaeberlein et al. (1999)	W303R	35	25*
sir3Δ Sir3S275A	Ray et al. (2003)	W303-1A	35	−13

Replicative life span was determined in the BY4742 background for twelve single-gene deletion mutations, a phosphorylation defective allele of Sir3 (Sir3S275A), over-expression of *SIR2*, and CR by growth on low glucose, all previously reported to increase life span in different strain backgrounds.
* Significant increase in life span relative to experiment-matched wild-type cells ($P < 0.01$).

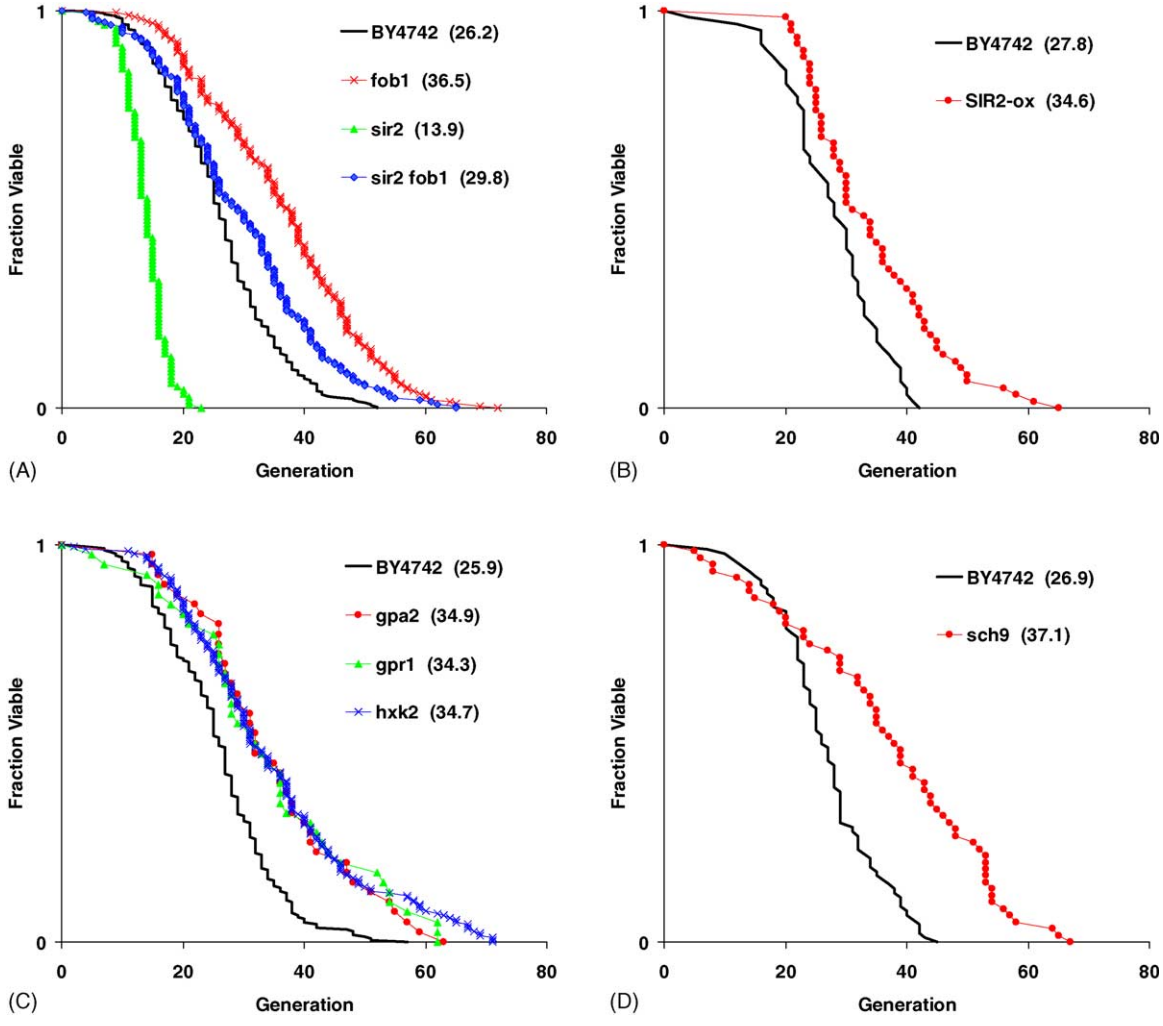

Fig. 2. CR, ERCs, and SCH9 determine longevity in BY4742. *SIR2* and *FOB1* act in opposite ways to determine life span. (A) Deletion of *FOB1* increases life span and suppresses the short life span caused by deletion of *SIR2*, while (B) over-expression of *SIR2* increases life span in BY4742. (C) Three genetic models of CR increase life span in BY4742 as does (D) deletion of *SCH9*.

3.1.3. Life span extension by SCH9 deletion

The Sch9 protein kinase acts in a signaling pathway responsive to environmental nutrients (Longo, 2003). Deletion of *SCH9* is reported to increase chronological life span as well as resistance to heat and oxidative stress (Fabrizio et al., 2001), and was also found to increase RLS (Fabrizio et al., 2004). The *sch9Δ* strain is not present in the ORF deletion collection, therefore we constructed a *sch9::URA3* allele in BY4742. Consistent with the prior report, we find that *sch9Δ* mother cells live approximately 40% longer than wild-type cells in BY4742 (Fig. 2D).

Recently, we found that calorie restriction increases RLS in a Sir2-independent manner (Kaeberlein et al., 2004b), leading to a model whereby at least two pathways influence aging. One pathway culminates in ERC formation and is regulated by Sir2 and Fob1. The other pathway is influenced by calorie restriction. Since even *fob1Δ hxk2Δ* double mutants, which are extremely long-lived due to life span extension through both known pathways, display Gompertz-like mortality, it is reasonable to speculate on the existence of additional aging pathways that remain to be discovered.

3.2. Mutations that influence replicative life span in a strain-specific manner

From our comprehensive analysis of yeast aging genes, we identified several mutations previously reported to increase life span in other strains that failed to significantly increase life span in BY4742: *lag1Δ*, *ras1Δ*, *rho*^{0}, *rpd3Δ*, *rtg3Δ*, *scp1Δ*, *SIR3S275A*, *snf4Δ*, *uth1Δ*, and *zds1Δ*. Of these, a few modestly increased RLS relative to pair-matched wild-type controls, but not to a statistically significant extent at a *P*-value cutoff of 0.01. It is possible that with analysis of additional cells, statistical significance would be achieved for some of these mutations; however, in each case, the magnitude of the observed extension was much less than that previously reported. For example, deletion of *SCP1*, which is reported to increase life span by 67% in the KAY446 strain background (Gourlay et al., 2004), resulted in only a modest 15% ($P = 0.04$) life span extension in BY4742. Likewise, deletion of *LAG1* is reported to increase RLS by 50% in the YPHDF-1A background (D'Mello et al., 1994), but resulted in only a 6% increase in RLS in BY4742. Thus, it is highly unlikely that analysis of additional cells would result in a life span phenotype comparable to that previously reported for these mutations.

3.2.1. RPD3 and SIN3

Sin3 and Rpd3 are two members of a chromatin remodeling complex that regulates the transcriptional activity of many genes (Struhl, 1998) and promotes transcriptional silencing of reporter genes inserted into rDNA repeats (Smith et al., 1999). Rpd3 functions as a histone deacetylase (Rundlett et al., 1996; Taunton et al., 1996), while Sin3 interacts with a number of gene-specific transcriptional repressors, assisting in Rpd3 recruitment (Kadosh and Struhl, 1997). Given that deletion of *RPD3* has been reported to enhance longevity in flies (Rogina et al., 2002) as well as in a short-lived yeast strain (Kim et al., 1999), we were surprised to find that the *rpd3Δ* allele failed to increase life span in the BY4742 background (Fig. 3A). We therefore generated a new *rpd3Δ::URA3* allele in the parental strain, which also failed to increase RLS (Fig. 3A). In addition, the *rpd3Δ* strain from the *MAT*a deletion collection was found to have a RLS indistinguishable from wild-type (not shown).

Given that BY4742 is longer-lived strain than YPK9 (Table 1), the strain in which *rpd3Δ* is reported to increase longevity (Kim et al., 1999), we wished to test whether deletion of *RPD3* would RLS in a second short-lived strain, W303R. Taking advantage of the *ADE2* marker present at the rDNA in this strain, we were able to confirm that, as reported (Smith et al., 1999; Imai et al., 2000; Armstrong et al., 2001 #89), the *rpd3Δ::URA3* mutant demonstrated increased rDNA silencing that was partially Sir2-independent (data not shown). As was the case for BY4742, however, deletion of *RPD3* in W303R had no effect on life span (Fig. 3B).

Rpd3 is targeted to gene-specific promoters through its interaction with the transcriptional co-repressor Sin3 (Struhl, 1998). Unlike *rpd3Δ*, deletion of *SIN3* significantly shortened RLS (Fig. 3C). This result suggests that Sin3 has a function independent of Rpd3 that is required for normal longevity in yeast.

3.2.2. The effect of Sir3 phosphorylation on replicative aging

The Sir complex (Sir2/Sir3/Sir4) was first implicated in yeast aging with the identification of a semi-dominant allele of *SIR4* (*SIR4-42*) that could suppress the temperature sensitivity and life span defect caused by mutation of *MPT5* (*UTH4*) (Kennedy et al., 1995, 1997). Consistent with prior reports (Kennedy et al., 1995; Kaeberlein et al., 1999), we found that deletion of either Sir3 or Sir4 modestly shortened life span (Table 3), although not significantly. Deletion of *SIR2*, on the other hand, shortened mean life span by nearly 60% and over-expression increases mean life span by approximately 30% (Fig. 2).

Sir3, which helps direct Sir2 to subtelomeric regions and silent mating type loci, is a phosphoprotein (Stone and Pillus, 1996); with Sir3 in the phosphorylated state, transcriptional silencing near telomeres and HM loci, but not rDNA, is enhanced (Ray et al., 2003). Several proteins have been implicated in Sir3 phosphorylation, including the Slt2 kinase, and two paralogs, Zds1 and Zds2 (Roy and Runge, 2000; Ray et al., 2003). Deletion of *ZDS1* results in reduced Sir3 phosphorylation and is reported to increase RLS, while deletion of *ZDS2* is reported to have opposite effects (Roy and Runge, 2000).

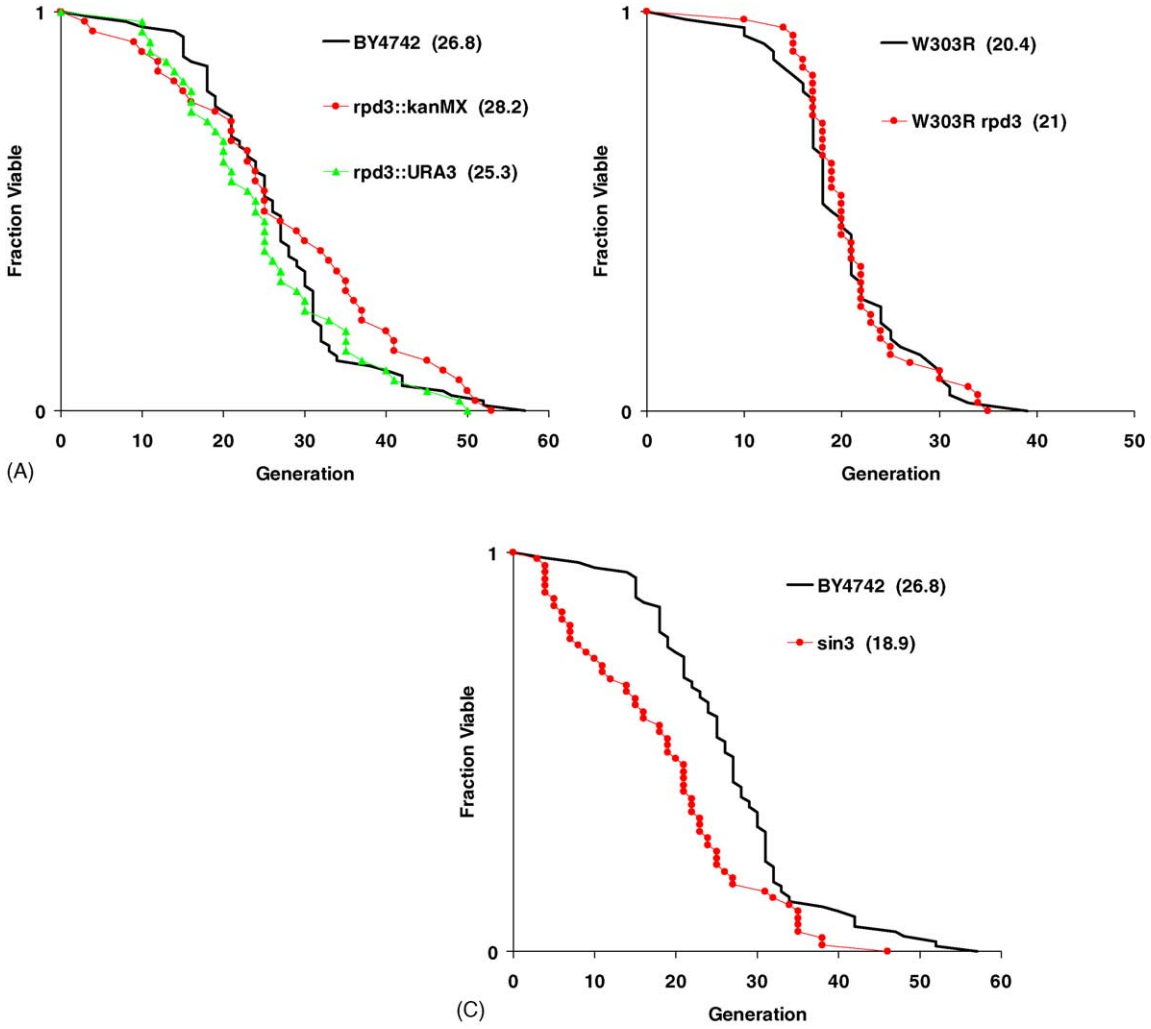

Fig. 3. Differential roles for *RPD3* and *SIN3* in life span determination. Deletion of *RPD3* has no effect on life span in (A) BY4742 or (B) W303R. The *rpd3::kanMX* strain was obtained from the *MATα* deletion collection and the *rpd3::URA3* strain was constructed in BY4742. (C) Deletion of *SIN3* shortens life span, suggesting a role for *SIN3* that is independent of *RPD3* but necessary for wild-type longevity.

We examined RLS in the BY4742 background for *slt2Δ*, *zds1Δ*, *zds2Δ* (Fig. 4A), and a phosphorylation site mutation in Sir3, S275A (Fig. 4B). None of these genetic modifications altered RLS to a large extent, suggesting that Sir3 phosphorylation may not play a major role in longevity determination in the BY4742 background. It seems likely that the mechanism by which Sir3 phosphorylation alters life span in W303 is by altering Sir2 dosage at the rDNA. Since Sir2 dosage also determines life span in BY4742 (Fig. 2), it is surprising that Sir3 phosphorylation appeared to have little effect. Perhaps, localization of the Sir complex is less dependent on Sir3 phosphorylation state in this strain. In this regard, it may be noteworthy that deletion of Sir3 has a less pronounced shortening effect on life span in BY4742 (Fig. 4B) than in W303 (Kaeberlein et al., 1999).

3.2.3. Mitochondrial function and retrograde response

The importance of mitochondrial function as a determinant of RLS appears to be highly dependent on strain background. One study (Kirchman et al., 1999) found that spontaneous loss of respiratory capacity (rho$^-$) shortened life span in two strain backgrounds (SP1-1 and A364A), had no effect on life span in a third strain (W303-1A) and increased life span in a fourth (YPK9). In order to determine the role of mitochondrial DNA and respiratory capacity in the aging of a long-lived strain, we created a BY4742 variant lacking mitochondrial DNA (rho^0) and determined its life span. Rho^0 cells are defective for respiration and are unable to grow on a non-fermentable carbon source, but the structure of the mitochondria remains intact. The BY4742 rho^0 cells had a life span indistinguishable from wild-type cells (Fig. 5A), suggesting that respiratory capacity has neither a beneficial nor deleterious affect on aging in BY4742.

In contrast to rho^0, deletion of either mitochondrial prohibitin, *PHB1* or *PHB2*, shortened RLS by approximately 50% (Fig. 5B). This result is consistent with the previously reported life span defect of these mutants (Coates

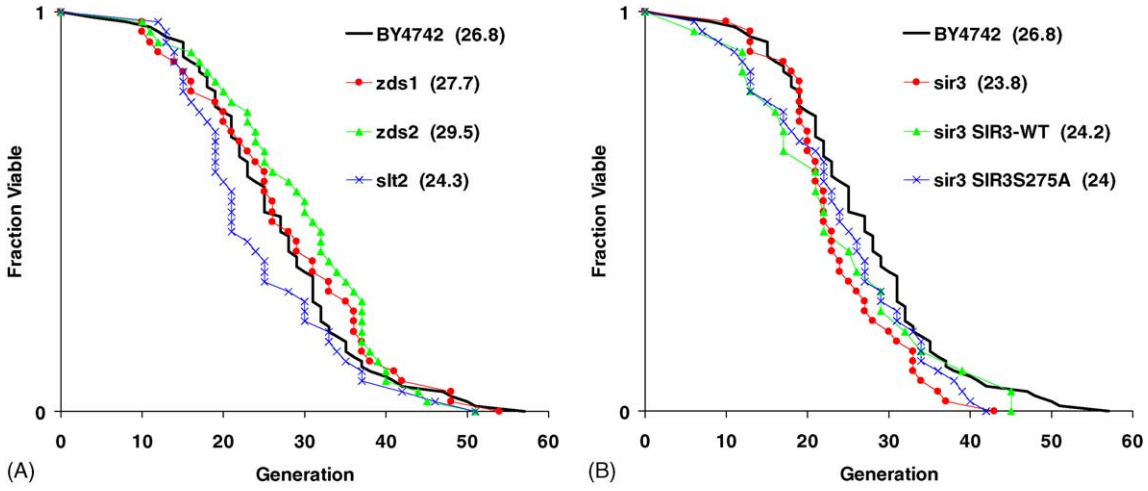

Fig. 4. Sir3 phosphorylation state appears to be unimportant for life span in BY4742. (A) Zds1, Zds2, and Slt2 regulate Sir3 phosphorylation state but have no significant impact on life span. (B) A phosphorylation-site mutant allele of *SIR3*, *SIR3S275A*, also fails to alter life span.

et al., 1997; Piper and Bringloe, 2002), and suggests that, while respiratory function per se is not critical, either (1) certain mitochondrial activities are necessary for wild-type life span; or (2) interfering with mitochondrial function(s) in specific ways can cause toxicity resulting in an abnormally short life span. The distinction between these two possibilities is an important one and should be examined in future studies.

Another intriguing link between mitochondrial function and aging has been suggested by reports that retrograde response can impact yeast aging (Kirchman et al., 1999; Jiang et al., 2000; Borghouts et al., 2004). The retrograde response is initiated by reduced mitochondrial function and transduces signals to the nucleus, resulting in changes in expression of a variety of nuclear genes (Liao and Butow, 1993). Cells lacking *RTG2* and *RTG3*, two genes required for the retrograde response are reported to have different effects on RLS. Deletion of *RTG3* is reported to increase RLS in one strain background (Jiang et al., 2000) and have no effect on life span in another (Borghouts et al., 2004), whereas deletion of *RTG2* shortens life span in both of these strains. In BY4742, we found that deletion of *RTG2* resulted in a decreased RLS and deletion of *RTG3* had no effect on RLS (Fig. 5C). Given the link between retrograde response and mitochondrial function, it seems likely that the stain-specific roles of *RTG2* and *RTG3* in life span determination are related to the observation that loss of respiratory capacity (rho^0 and rho^-) has variable longevity effects in different genetic backgrounds.

Based on our findings and those of others, it is reasonable to conclude that altered mitochondrial function or respiratory capacity can affect RLS in certain cases, but has no consistent effect across strain backgrounds. Interestingly, respiration is reported to be required for life span extension by CR in the short-lived PSY316 strain background (Lin et al., 2002). Whether respiratory capacity is required for life span extension by CR in other yeast strains remains to be determined.

3.2.4. *Oxidative stress and replicative aging*

A role of oxidative stress and reactive oxygen species (ROS) as a cause of eukaryotic aging has been widely theorized (Droge, 2003). To date, there is little evidence suggesting that ROS limit yeast RLS. It is certainly the case that elevated production of ROS can artificially shorten RLS; however, no reports of increased RLS under standard growth conditions have been provided that can be solely attributed to increased antioxidant capacity or reduced ROS production. Increased expression of the ROS detoxification enzyme Sod1 has no effect on RLS (Kirchman et al., 1999), and over-expression of Sod2 is reported to shorten RLS (Fabrizio et al., 2004). Further, long-lived mutants in the CR pathway are no more resistant to oxidative stress than wild-type cells (Lin et al., 2002), suggesting that enhanced antioxidant capacity is not a prerequisite for enhanced longevity in yeast.

Recently, a potential link between ROS and replicative aging was suggested by a report that deletion of the actin bundling protein Scp1 dramatically increases RLS (Gourlay et al., 2004). Cells with elevated Scp1 levels are short-lived and generate increased ROS, suggesting that Scp1 might affect longevity by regulating mitochondrial turnover and ROS production. In BY4742, deletion of Scp1 resulted in only a modest increase in RLS (Fig. 6A), suggesting that the relative importance of this gene as a determinant of longevity is strain-specific. Whether the effect of Scp1 on RLS is caused by, or simply correlates with, a reduction in ROS remains to be determined.

Yeast, like other eukaryotes, have a cytosolic copper, zinc superoxide dismutase (*SOD1*) and a mitochondrial manganese superoxide dismutase (*SOD2*) (Fridovich, 1995). Deletion of either of these genes has been reported to

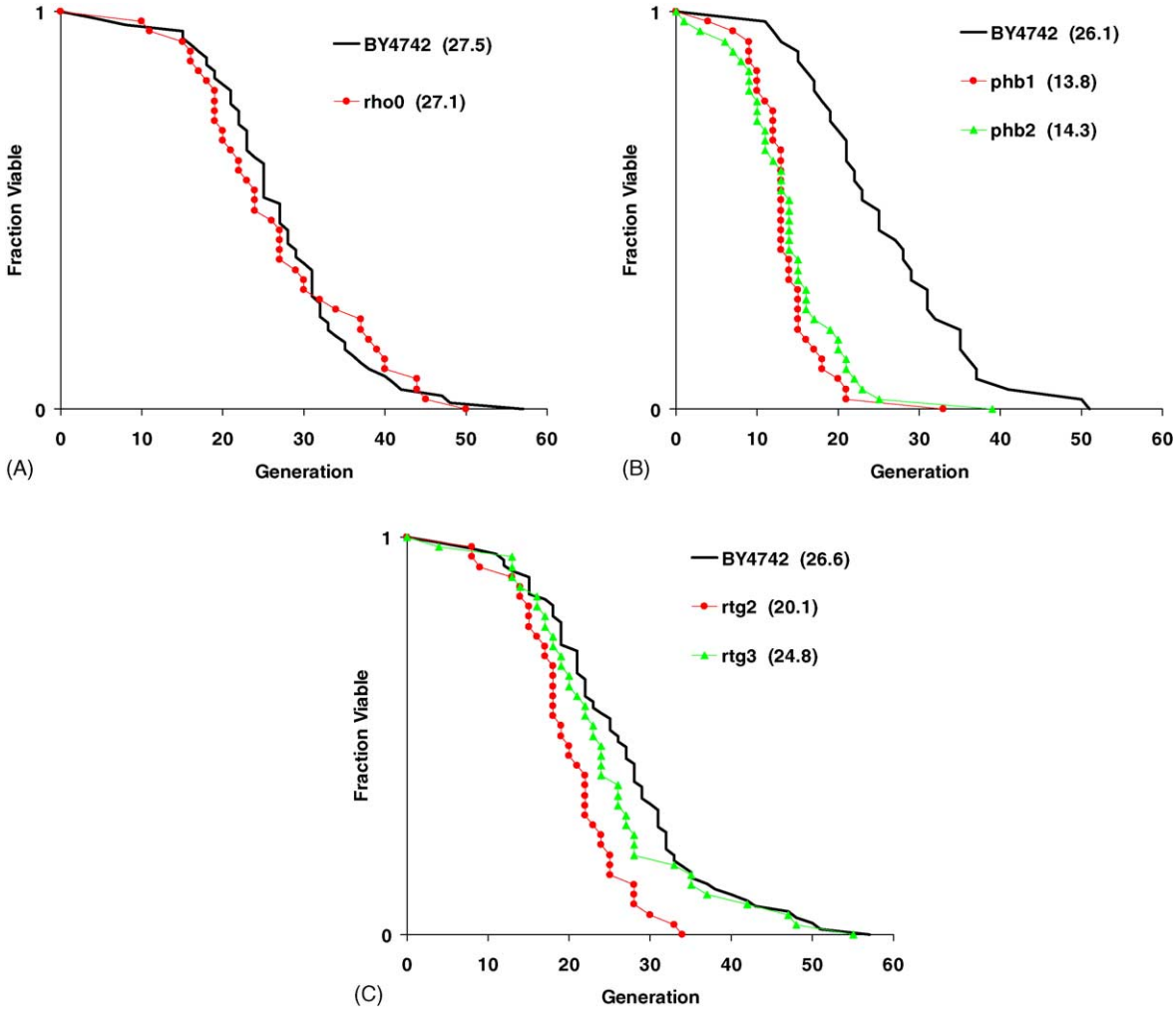

Fig. 5. Mutations affecting mitochondrial function have differential effects on life span. (A) Complete lack of mitochondrial DNA has no effect on life span, even though cells are unable to respire. (B) Loss of either mitochondrial prohibitin, Phb1 or Phb2, on the other hand significantly shortens life span. (C) The role of the retrograde response in life span determination is ambiguous, as deletion of *RTG2* shortens, but deletion of *RTG3* has no effect on longevity.

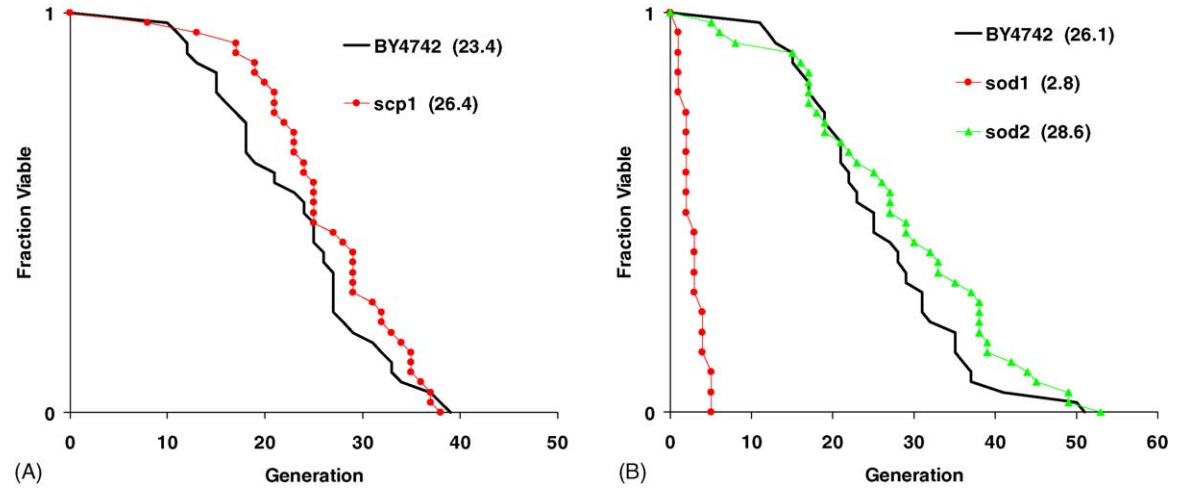

Fig. 6. Mutations that impact oxidative stress have variable effects on life span. (A) Deletion of the gene coding for the acting bundling protein *SCP1* is reported to increase resistance to oxidative stress but fails to significantly increase life span. (B) Deletion of the gene coding for cytosolic superoxide dismutase, *SOD1*, dramatically shortens life span, whereas deletion of the gene coding for mitochondrial superoxide dismutase, *SOD2*, has no effect on life span.

dramatically reduce longevity (Wawryn et al., 1999, 2002). In BY4742, we found that deletion of these genes had differential effects. Cells lacking *SOD1* had a mean RLS of 2.8 generations, nearly a 90% reduction relative to pair-matched wild-type cells (Fig. 6B). In contrast, *sod2Δ* cells had a mean RLS of 28.6, not significantly different from wild-type (Fig. 6B). Both mutations, however, resulted in enhanced sensitivity to oxidative stress and dramatically shortened chronological life span (not shown), as expected. These findings suggest that oxidative stress and ROS play, at best, a modest role in the normal replicative aging process of a long-lived strain.

3.2.5. Polymorphic loci

At least two polymorphic loci have been demonstrated to affect replicative aging in yeast: *MPT5* and *SSD1*. In one short-lived strain background, BKy4-14c, a naturally occurring C-terminal truncation allele of *MPT5* has been identified (Kennedy et al., 1995, 1997). Addition of full-length *MPT5* increases life span in BY4-14c, indicating that the C-terminal truncation has a negative effect on longevity. Deletion of *MPT5* has been found to decrease RLS in several other strains, while over-expression increases RLS (Kennedy et al., 1997; Kaeberlein and Guarente, 2002; Kaeberlein et al., 2004a), suggesting that *MPT5* is a general regulator of longevity, perhaps by acting to regulate the distribution of Sir2 within the cell (Kennedy et al., 1997; Kaeberlein et al., 2004a). Two types of *SSD1* alleles have also been isolated from laboratory and naturally occurring yeast strains: a fully function *SSD1-V* allele and mutant *ssd1-d* alleles (Kaeberlein et al., 2004a). It appears likely that the *SSD1* polymorphism confers selective advantage in different environmental conditions, as *ssd1-d* is associated with increased virulence of clinical isolates (Wheeler et al., 2003). Interestingly, *SSD1* interacts genetically with *MPT5*. In three *ssd1-d* strains, BKy4-14c, PSY316 and W303R, addition of a single *SSD1-V* allele suppresses the short life span, as well as several cell integrity defects, caused by deletion of *MPT5* (Kaeberlein and Guarente, 2002; Kaeberlein et al., 2004a). In PSY316, addition of *SSD1-V* to *MPT5* wild-type cells results in a further 60–70% increase in life span (Kaeberlein et al., 2004a).

Unlike most of the strains commonly used in aging research, BY4742 carries the functional *SSD1-V* allele. We therefore wished to determine whether deletion of *MPT5* or *SSD1* would shorten life span in this background. Consistent with prior reports, we found that deletion of *MPT5* shortened life span by approximately 35% in BY4742 (Fig. 7A), whereas *ssd1Δ* cells had a life span only slightly shorter than wild-type cells, suggesting that *SSD1* is less important than *MPT5* for wild-type life span in this strain. Deletion of both *SSD1* and *MPT5*, however, resulted in an additional shortening of life span beyond that of either single mutant (Fig. 7A), consistent with the idea that *MPT5* and *SSD1* function in parallel pathways to promote longevity in BY4742, as in other strains.

3.2.6. Effects of mating type and ploidy on life span

Budding yeast can grow vegetatively either as haploid *MAT*a or *MATα* cells, or as diploid a/α cells. The effect of mating type and ploidy on replicative life span has been examined in several strain backgrounds (Muller, 1971; Kennedy et al., 1997, Kaeberlein et al., 1999). In every case reported, a and α haploid cells have indistinguishable life spans, whereas diploid cells have been reported to either have the same life span as haploid cells (Muller, 1971; Kennedy et al., 1997) or a shortened life span (Kaeberlein et al., 1999). We found that *MAT*a haploid cells (BY4741) have a life span indistinguishable from *MATα* cells in the BY4742 background (Fig. 7B). Surprisingly, diploid cells in this background (BY4743) were significantly longer lived than cells of either haploid mating type (Fig. 7B). The increased life span of diploid cells is apparently not due to coexpression of both mating type genes, as a *MAT*a/*MAT*a diploid was longer lived than a congenic *MAT*a haploid (Fig. 7C). Thus, while mating type appears to consistently have no effect on haploid life span, further studies will be necessary to determine the molecular basis for the strain-specific longevity effects of ploidy.

4. Discussion

We used the long-lived BY4742 strain background to carry out a large-scale analysis of single-gene deletion mutations previously reported to alter yeast RLS. This analysis of greater than 3500 individual mother cell life spans (~100,000 daughter cells) will provide the foundation for determining which genes represent the best candidates for general determinants of longevity and will provide a reference genetic background for future studies. BY4742 is closely related to S288C, the genetic background for which the complete genome sequence has been determined (Cherry et al., 1997). In addition, this is the strain from which the various yeast ORF deletion sets are derived (Winzeler et al., 1999), making it a useful genetic background for comparative analysis of longevity across multiple mutations and interventions. Fortuitously, BY4742 is long-lived relative to other genetic backgrounds, with a mean RLS 20–100% longer than strains previously utilized in yeast aging research (Table 1).

The disparity in RLS among different strains is likely explained by the presence of genetic polymorphisms. Mutations that shorten life span have been reported to accumulate during laboratory propagation in other model systems, and can have significant confounding effects on aging studies performed in short-lived genetic backgrounds (Spencer and Promislow, 2002). We speculate that many common laboratory yeast strains contain mutations that result in a shorter RLS, making it possible that life span extending mutations isolated in these backgrounds are merely suppressors of these deleterious mutations and not general aging factors. Indeed, preliminary data derived from

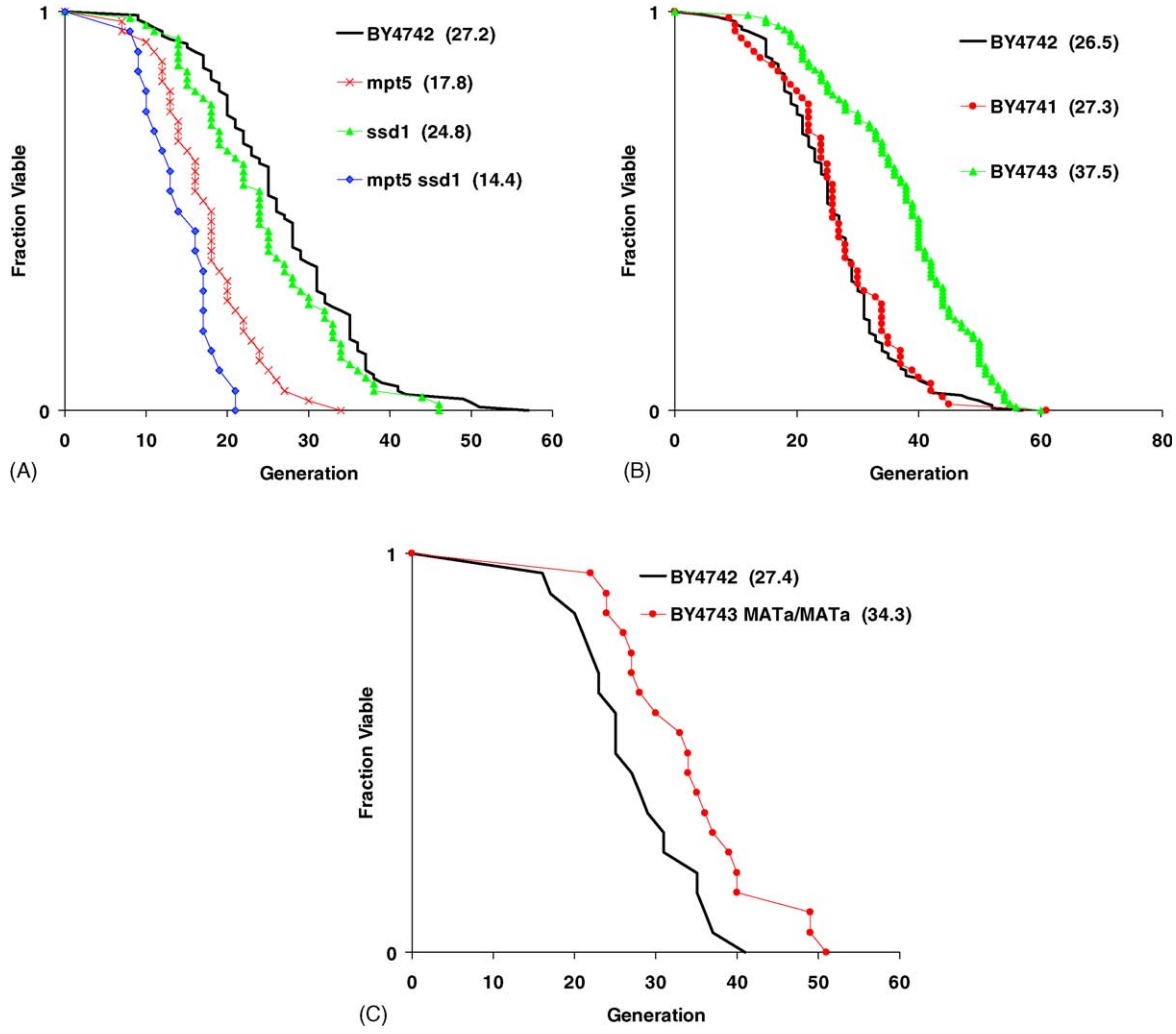

Fig. 7. Effects of polymorphic loci and ploidy on life span. (A) *MPT5* is a more important determinant of life span in BY4742 than *SSD1*. (B) Mating type has no effect on life span, but diploid cells are longer-lived than haploid cells. (C) The increased life span of diploid cells is not solely due to a/α-coexpression.

an ongoing genome-wide analysis of yeast RLS indicate that at least 20% of non-essential single-gene deletions result in a statistically significant reduction in RLS (M.K., K.T.K., S.F., B.K.K., unpublished). This finding suggests that a broad spectrum of spontaneous mutations is capable of decreasing longevity. Further supporting this argument, a yeast strain recently derived from the wild was observed to have a RLS longer than that of common laboratory strains (M. McMurray and D. Gottschling, personal communication), and polymorphic loci have been identified that significantly alter the RLS of some short-lived strains used in aging research (Kennedy et al., 1997; Kaeberlein et al., 2004a, 2004b).

From this analysis, we conclude that a number of genetic interventions previously reported to affect RLS act in a strain-specific manner. This does not necessarily make these genes uninformative with respect to aging in yeast (and potentially in higher eukaryotes), but it does make interpretation difficult without knowledge of the strain-specific genetic differences that underlie the specificity.

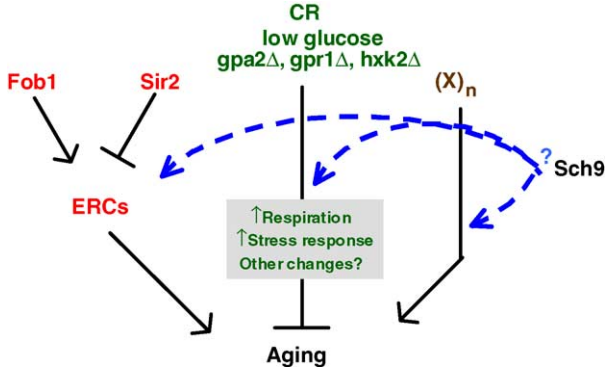

Fig. 8. Pathways determining longevity in a long-lived strain. Extrachromosomal rDNA circles (levels of which are regulated by Sir2 and Fob1) and calorie restriction define two genetic pathways that regulate replicative life span, and it is likely that additional longevity pathways, $(X)_n$, remain to be identified. *SCH9* may represent one of these unidentified pathways or may act in either the ERC pathway or the CR pathway.

Because our study was carried out in an unusually long-lived yeast strain, mutations observed to increase RLS in BY4742 are more likely to be general regulators of aging compared to those that increase life span in a short-lived background. Most of the mutations found to enhance longevity in BY4742 either regulate ERC levels or are genetic models of CR. Of the mutations found to increase life span at the $P < 0.01$ significance level, only deletion of *SCH9* is not currently implicated in one of these pathways. Interestingly, however, *SCH9* has been implicated in nutrient sensing as well as oxidative stress response, two recurrent themes in aging amongst diverse eukaryotic species. Further analysis will be needed to determine whether life span extension by deletion of *SCH9* occurs through the ERC pathway, the CR pathway, or another, as yet unknown, mechanism (Fig. 8).

The recent discovery that CR and Sir2 represent genetically distinct pathways in yeast (Kaeberlein et al., 2004b) is consistent with similar observations in the nematode *Caenorhabditis elegans* (Kaeberlein and Kennedy, 2005). Over-expression of the Sir2 ortholog, Sir-2.1, increases life span in *C. elegans* through a pathway that is genetically separable from life span extension by CR (Lakowski and Hekimi, 1998; Tissenbaum and Guarente, 2001; Houthoofd et al., 2003). Interestingly, deletion of SGK-1, a putative *SCH9* ortholog, also increases life span in *C. elegans* (Hertweck et al., 2004). Thus, CR, Sir2, and Sch9 represent three unique regulators of longevity that act similarly to determine replicative life span in yeast and post-mitotic life span in worms. Further dissection of the genetic and molecular basis by which these interventions increase life span in simple eukaryotes may provide important insight into evolutionarily conserved aspects of aging.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.mad.2004.10.007.

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