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Chronological aging-independent replicative life span regulation by Msn2/Msn4 and Sod2 in *Saccharomyces cerevisiae*

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Abstract Mutations in *RAS2*, *CYR1*, and *SCH9* extend the chronological life span in *Saccharomyces cerevisiae* by activating stress-resistance transcription factors and mitochondrial superoxide dismutase (Sod2). Here we show that mutations in *CYR1* and *SCH9* also extend the replicative life span of individual yeast mother cells. However, the triple deletion of stress-resistance genes *MSN2/MSN4* and *RIM15*, which causes a major decrease in chronological life span, extends replicative life span. Similarly, the overexpression of superoxide dismutases, which extends chronological survival, shortens the replicative life span and prevents budding in 30–40% of virgin mother cells. These results suggest that stress-resistance transcription factors Msn2/Msn4 negatively regulate budding and the replicative life span in part by increasing *SOD2* expression. The role of superoxide dismutases and of other stress-resistance proteins in extending the chronological life span of yeast, worms, and flies indicates that the negative effect of Sod2, Msn2/Msn4/Rim15 on the replicative life span of *S. cerevisiae* is independent of aging.
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Key words: Stress resistance; Longevity; Superoxide; Budding

1. Introduction

In organisms ranging from *Caenorhabditis elegans* to mammals aging is measured by monitoring chronological survival and the age-specific increase in mortality rates [1]. Certain cells that function within these organisms, such as fibroblasts and lymphocytes, can also undergo a form of aging termed 'replicative senescence', which is measured by counting the number of times a cell population can divide and double in size [2]. *Saccharomyces cerevisiae* ages chronologically and undergoes an age-dependent increase in mortality rates [3,4], but can also undergo replicative senescence, measured by counting the maximum number of buds generated by an individual mother cell [5,6]. Thus, aging yeast share similarities with aging organisms and with specific cells, within these organisms, that undergo replicative senescence.

Chronological aging in non-dividing yeast is associated with the inactivation of the superoxide-sensitive mitochondrial aconitase, and is extended by mutations that decrease the activity of Ras/Cyr1/PKA and Sch9 pathways [4,7,8]. Life span extension in *ras2*, *cyr1*, and *sch9* mutants is mediated by transcription factors Msn2/Msn4 and by mitochondrial Sod2 [4,9]. By contrast, replicative senescence in yeast is associated with the accumulation of rDNA circles in the nucleolus and is decreased by a mutation in the transcriptional silencing gene *SIR4* [10], and by increasing the gene dosage of *SIR2*, another gene that mediates genomic silencing [11]. Mutations in the Ras/cAMP/PKA pathway and calorie restriction also extend replicative life span by a mechanism that depends on Sir2, but not on stress-resistance proteins [12]. Replicative life span extension by calorie restriction requires an increase in mitochondrial respiration [13]. Furthermore, the replicative life span of mother cells that are maintained in stationary phase decreases progressively with the age of the mother cell, suggesting that the chronological and replicative aging paradigms may be regulated by some overlapping mechanisms [14].

Here, we study the role of stress-resistance proteins that mediate chronological life span extension in the replicative life span to explore the relationship between the two yeast longevity paradigms.

2. Materials and methods

2.1. Yeast strains

All *S. cerevisiae* strains used in this study were derived from DBY746 [15] and are listed in Table 1. The *RIM15* disruption in strain PF112 was produced using the *Xho*I–*Sac*II fragment of plasmid pSV117 [16]. The *MSN2* overexpressor was generated by transforming wild-type DBY746 with multicopy plasmid YEp24 containing the *MSN2* gene [17].

Strains overexpressing *SOD1-SOD2* and control strain YEp351-YEp352 were originated in the DBY746 background and have been described previously [9]. Yeast transformations were carried out by a modification of the lithium acetate method [18] and gene disruption was tested by PCR analysis.

2.2. Chronological life span

All experiments were performed in liquid synthetic dextrose complete medium (SDC) with 2% glucose, supplemented with amino acids, adenine, as well as a four-fold excess of tryptophan, leucine, histidine, uracil. The latter supplements were required because of the auxotrophies of the wild-type strain used and were added in excess to prevent any growth limitation due to their depletion. In a standard experiment overnight cultures were grown in SDC and inoculated into flasks with a flask volume/medium volume ratio of 5:1 and grown at 30°C with shaking at 220 rpm. The maximum size of the viable population was approximately 100 million cells/ml. To determine the number of viable yeast, two 10 μl aliquots were removed from each flask and serially diluted. Each aliquot was then plated twice onto

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YPD plates for a total of 4 platings/population/day. Serial dilutions were performed in order to plate approximately 100 viable organisms per plate. Viability is defined as the ability of a single organism to reproduce and form a colony within 48 h (colony forming units or CFU).

2.3. Replicative life span

The replicative life span was measured by counting the number of buds produced by 40–169 virgin cells. The buds were removed as they were formed by micromanipulation using a Singer MSM series 200 micromanipulator apparatus [19].

Lifetime reproductive output was analyzed primarily using analysis of variance (ANOVA) [20]. For the ANOVA we tested differences in total reproductive output among lines, which are treated as fixed effects in the analysis. When comparing differences in reproductive output across many lines, multiple comparisons were carried out using Dunnet's methods of comparing treatment lines to the control. Lines were considered different in their total reproductive output if the Dunnet's test revealed a $P < 0.05$. Inspection of the distribution of the measurements suggested the possibility that a log transformation was required to normalize the data. Analyses were carried out on both the natural and the log scale, with nearly identical results.

2.4. Budding assay

Closely synchronized log phase cells were obtained as described previously [10]. For each line, 100 cells were distributed on a 10×10 grid across a plate of YPD medium. Grid points were examined every 24 h for 8 days, and each grid point was given a score according to the following scale: 0 = No budding; 1 = < 5 cells; 2 = 5–10 cells; 3 = small colony (< half the size of the visual field) 4 = large colony (> half the field, but < entire field) 5 = covered the entire visual field. The grid point was considered as having a successful budding cell when the score was ≥ 2.

2.5. Stress resistance

Heat-shock resistance was tested by spotting serial dilutions of cells removed from day 1/day 3 SDC postdiauxic phase cultures onto YPD plates and incubating at 55°C (heat-shocked) or at 30°C (control) for 1 h. Pictures were taken after 3–4 days of incubation at 30°C.

Oxidative stress resistance was studied by exposing cells to superoxide/hydrogen peroxide-generating agent menadione. Yeast removed from day 3 postdiauxic phase cultures were diluted to an OD_{600} of 0.1 in potassium phosphate buffer and exposed to 20 μM of menadione for 1 h.

3. Results

3.1. Transposon insertional mutations in CYR1 and SCH9 increase the replicative life span

Our selection for transposon-mutagenized yeast that are resistant to heat and oxidative stress resulted in the isolation of two strains with mutations in the serine/threonine kinase Sch9 (*sch9::mTn*, PF108) and adenylate cyclase (*cyr1::mTn*, PF101) that also survived chronologically between 50 and 100% longer than wild-type controls [4]. Complete inactivation of Sch9 by the deletion of the *SCH9* gene results in a three-fold extension of chronological life span [4]. To investigate the relationship between the chronological and the replicative life span of *S. cerevisiae* we measured the budding potential of individual *cyr1::mTn*, *sch9::mTn* and *sch9Δ* mother cells. The *cyr1::mTn* mutation causes a 21% increase in the mean replicative life span compared to wild-type DBY746 controls (Fig. 1A) ($P < 0.05$). Similarly, the mean replicative life span of *sch9::mTn* mutants is extended by 18% (Fig. 1A) ($P < 0.05$). The mean number of buds generated increases from 18.7 in wild-type to 22.6 and 22 in *cyr1::mTn* and *sch9::mTn* mutants, respectively (Table 2). Surprisingly, the deletion of *sch9* (*sch9Δ*, PF102), which extends survival in non-dividing yeast by three-fold, causes only a small (not significant) increase in the budding life span (Table 2).

3.2. The deletion of stress-resistance genes increases further the replicative life span of cyr1::mTn mutants

We tested whether the extension of the replicative life span in *cyr1::mTn* mutants is mediated by the stress-resistance proteins Msn2/Msn4 and Rim15. Surprisingly, the deletion of *MSN2* and *MSN4* and *RIM15* has opposite effects on the replicative and chronological life spans (Fig. 1B,C). The mean replicative life span of *cyr1::mTn msn2/4 Δrim15Δ* (PF112) mutants is 52% longer than that of wild-type ($P < 0.05$) and is 26% longer than that of *cyr1::mTn* mutants ($P < 0.05$) (Fig. 1B, Table 2).

By contrast the triple deletion of *MSN2*, *MSN4*, and *RIM15* abolishes the chronological life span extension caused by *cyr1::mTn* mutations (Fig. 1C). In fact, the deletion of *RIM15* alone, is sufficient to cause a major reduction in chronological life span compared to wild-type cells [4].

3.3. MSN2/MSN4 and RIM15 regulate stress resistance in cyr1::mTn mutants

To study the relationship between replicative life span and stress resistance we tested resistance to heat and oxidative stress of *cyr1::mTn* mutants lacking *MSN2/4* and *RIM15*. The deletion of *MSN2/4* decreases the resistance of *cyr1::mTn* mutants to heat stress at days 1–3. The triple deletion of *MSN2/4* and *RIM15* abolishes the increased thermotolerance (Fig. 2A), suggesting that Rim15 is a central regulator of heat-resistance, whereas transcription factors Msn2/4 play a less important role.

The downregulation of the Ras2/Cyr1/PKA pathway in exponentially growing cells increases *SOD2* expression by activation of transcription factors Msn2/Msn4, Gis1 (regulated by Rim15) and Hap2/3/4/5, which bind, respectively, to the STRE, PDS, and HAPB elements in the *SOD2* promoter [21,22]. To test whether the disruption of *MSN2/4* or of *MSN2/4ΔRIM15Δ* abolishes the increased resistance of *cyr1::mTn* mutants to superoxide we tested the resistance to

Table 1
Yeast strains used in this study

Strain	Genotype	Source
DBY746	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$	Gralla [15]
PF101	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *cyr1::mTn*	Fabrizio [4]
PF102	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *sch9::URA3*	Fabrizio [4]
PF103	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *msn2::HIS3 msn4::LEU2*	Fabrizio [4]
PF108	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *sch9::mTn*	Fabrizio [4]
PF109	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *rim15::TRP1*	Fabrizio [4]
PF110	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *cyr1::mTn msn2::HIS3*	Fabrizio [4]
PF111	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *cyr1::mTn msn2::HIS3 msn4::LEU2*	Fabrizio [4]
PF112	*MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^+$ *cyr1::mTn msn2::HIS3 msn4::LEU2 rim15::TRP1*	This study

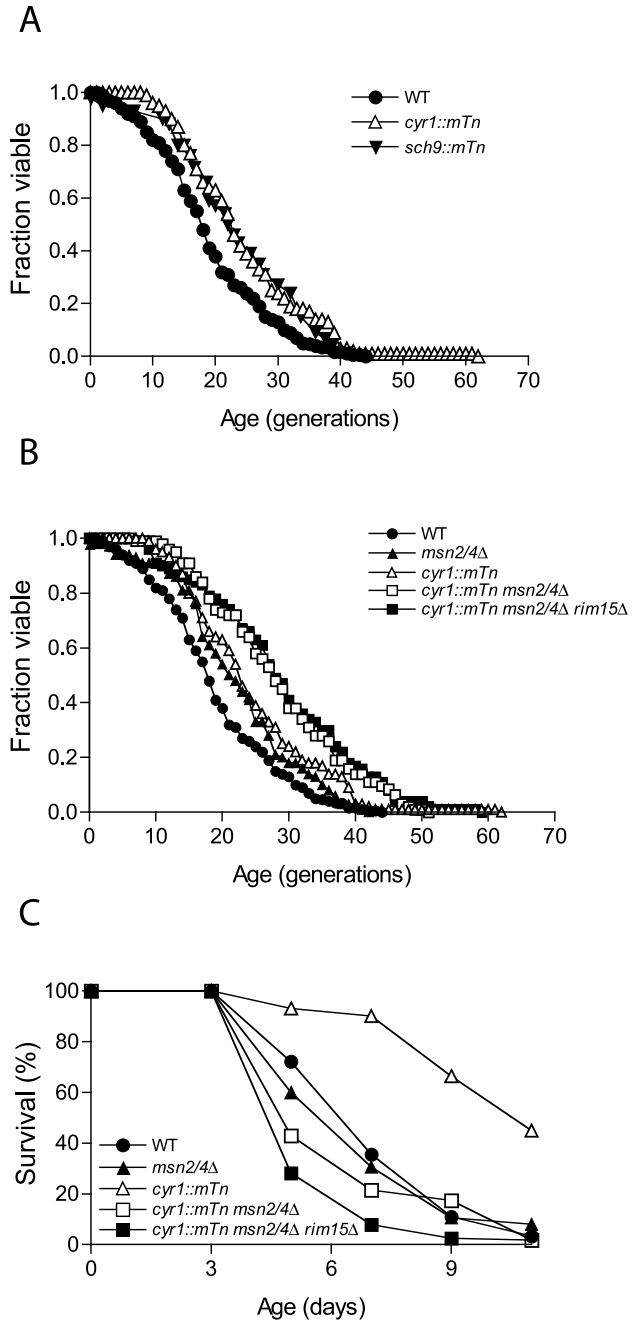

Fig. 1. A: The replicative life span is increased in *cyr1::mTn* (PF101) and *sch9::mTn* mutants (PF108) ($P<0.05$, see Section 2). The replicative life span was determined for 40–169 cells (Table 2). B: The deletion of stress-resistance genes *MSN2/MSN4* and *RIM15* increases further the replicative life span in the *cyr::mTn* mutant (52% vs. WT, 26% vs. *cyr::mTn* $P<0.05$). C: Chronological life span extension in *cyr1::mTn* mutants requires transcription factors Msn2/Msn4 and the protein kinase Rim15. Cell viability was measured every 2 days starting at day 3. Experiments were repeated between three and seven times with similar results. The average of all experiments is shown.

menadione, which generates superoxide/hydrogen peroxide. The deletion of *MSN2/MSN4* or of *MSN2/MSN4* and *RIM15*, which results in a 52% replicative life span extension compared to wild-type cells, decreases resistance to menadione to a level similar to that of wild-type cells (Fig. 2B).

3.4. SOD1SOD2 *overexpression decreases the replicative life span*

To test further the relationship between resistance to superoxide/hydrogen peroxide and the replicative life span we measured the budding potential of cells overexpressing cytosolic (*SOD1*) and mitochondrial (*SOD2*) superoxide dismutases, which survive 30% longer than wild-type under non-dividing conditions (Fig. 3B) [9]. The increase in activity was over three-fold for both Sod1 and Sod2 [9]. The double overexpression of *SOD1* and *SOD2* decreased the mean replicative life span from 18.7 to 14.5 (Fig. 3A) ($P<0.05$). Furthermore, the overexpression of *MSN2*, which is sufficient to induce STRE-regulated stress-resistance genes [23], decreased the mean replicative life span from 18.7 to 16.8 (Table 2). Thus, the extension of the replicative life span is not mediated by either Msn2/Msn4/Rim15 or by Sod1/Sod2, as shown for the chronological life span. Instead, stress-resistance proteins and specifically Sods shorten the replicative life span.

The overexpression of *SOD1* extends longevity in *Drosophila* [24–26] but can either decrease or increase oxidative damage in mammalian cells [27,28]. To test whether the double overexpression of *SOD1* and *SOD2* caused a pro-oxidant effect that may decrease replicative life span by a mechanism independent of its superoxide dismutase activity, we treated *SOD1*ox*SOD2*ox cells with the superoxide/hydrogen peroxide-generating agent menadione (20 μM). Treatment of oxidatively stressed cells with superoxide/hydrogen peroxide should increase cell death. Instead yeast overexpressing both Sods were more resistant to a 1 h treatment with menadione at different stages of the life span, suggesting that the overexpression of both Sods in strain DBY746 decreases superoxide levels and does not cause a pro-oxidant effect (Fig. 3C).

3.5. *Increased stress resistance is associated with decreased budding*

SOD1SOD2 double overexpressors are more resistant to superoxide toxicity and survive 30% longer than controls, but have a reduced replicative life span compared to wild-type cells (Fig. 3). To test whether this may be the result of a negative regulation of budding by superoxide dismutases, we monitored the ability of individual mother cells overexpressing *SOD1*, *SOD2*, or both *SOD1* and *SOD2* to divide and form a colony. For this purpose we plated 100 individual virgin overexpressor cells and the same number of virgin wild-type mother cells. Whereas less than 3% of wild-type and *SOD1* overexpressor mother cells failed to form a dense colony by day 3, 27% of *SOD1SOD2* (data not shown) and 40% of *SOD2* overexpressors never formed a colony and the cells that budded grew at a slower rate compared to wild-type cells (Fig. 4A,B). The role of stress-resistance transcription factors Msn2/Msn4 in negatively regulating growth is consistent with our results [29,30]. This effect of *SOD1SOD2* overexpression on the growth of approximately 30% of the cells does not affect the chronological life span (Fig. 3B) considering that 'chronological cultures' are derived from four to five colonies that have formed on selective medium. Therefore, the chronological cultures are obtained from *SOD1SOD2* overexpressors that do not have growth defects.

4. Discussion

The downregulation of signal transduction pathways acti-

Table 2
Replicative life span

Strain	*N*	Mean (Max)	S.E.M.
DBY746 (WT)	169	18.7 (46)	0.979
PF101 (*cyr1::mTn*)	87	22.6* (62)	1.27
PF108 (*sch9::mTn*)	74	22.0* (48)	1.33
PF102 (*sch9Δ*)	72	21.2 (37)	1.47
PF103 (*msn2/4Δ*)	70	21.0 (55)	1.64
PF109 (*rim15Δ*)	62	18.8 (46)	1.67
PF110 (*cyr1::mTn msn2Δ*)	55	25.4* (46)	1.52
PF111 (*cyr1::mTn msn2/4Δ*)	94	28.0*~ (55)	1.4
PF112 (*cyr1::mTnmsn2/4Δ rim15Δ*)	82	28.5*~ (60)	1.47
YEp351-YEp352 (WT)	56	18.7 (39)	1.56
*SOD1*ox *SOD2*ox	40	14.5* (34)	1.72
*MSN2*ox	73	16.8 (38)	1.46

*$P < 0.05$ vs. WT.
~ $P < 0.05$ vs. *cyr1::mTn*.
The mean and maximum replicative life spans, standard error of the mean, and total number of cells analyzed are shown for *sch9* mutants, *cyr1::mTn*, *cyr1::mTn* with deletions in *MSN2*, *MSN4*, and *RIM15* and wild-type cells overexpressing *SOD1*, *SOD2* and *MSN2*. YEP351-YEP352 is the wild-type plasmid control for the overexpressor strains. The replicative life span of *SOD1*ox *SOD2*ox, *cyr1::mTn*, *cyr1::mTn msn2Δ*, *cyr1::mTn msn2/4Δ*, *cyr1::mTn msn2/4Δ rim15Δ*, and *sch9::mTn* lines is significantly different from that of controls ($P < 0.05$) as determined by using both ANOVA and the Dunnet's method for comparing treatment lines to controls.

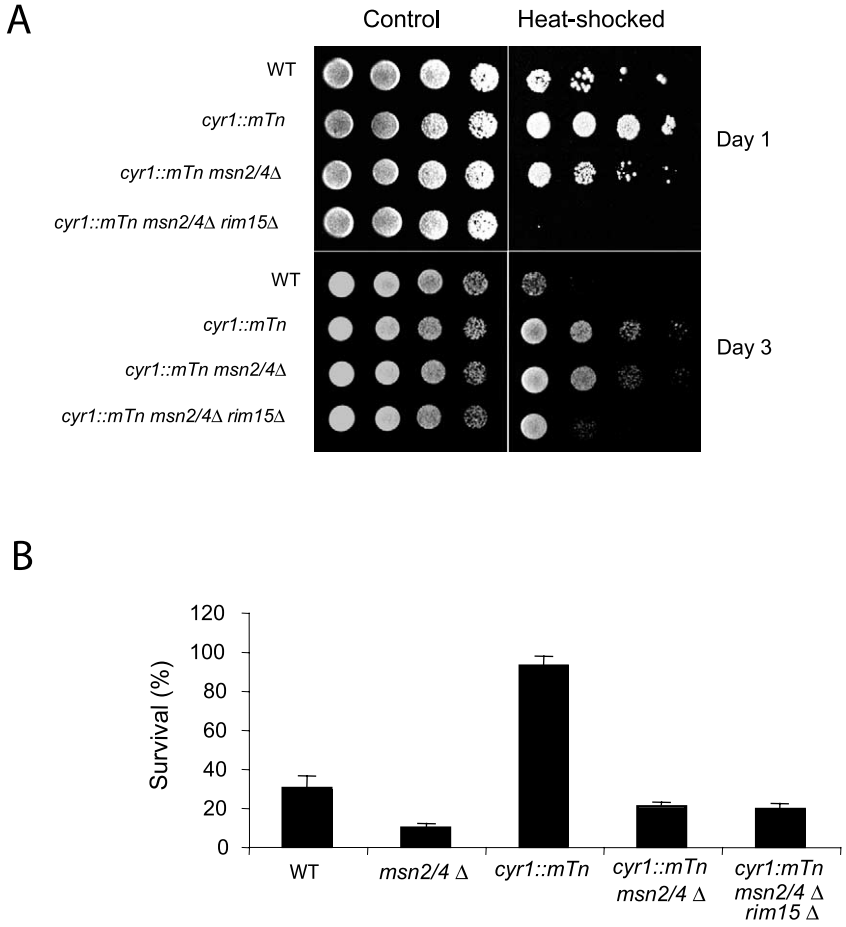

Fig. 2. *cyr1::mTn*-dependent increase in stress resistance is mediated by Msn2/Msn4 and Rim15. A: Serial dilutions of cells removed from day 1/day 3 postdiauxic phase cultures were spotted onto YPD plates and incubated at 55°C (heat-shocked) or at 30°C (control) for 1 h. Pictures were taken after 3–4 days of incubation at 30°C. The experiment was performed twice with similar results. B: Cells removed from day 3 postdiauxic phase culture were diluted to an OD_{600} of 0.1 in potassium phosphate buffer and treated with 20 μM of the superoxide-generating agent menadione for 60 min. The experiment was performed twice with similar results. The data shown are the average of the two experiments. Error bars show S.E.M.

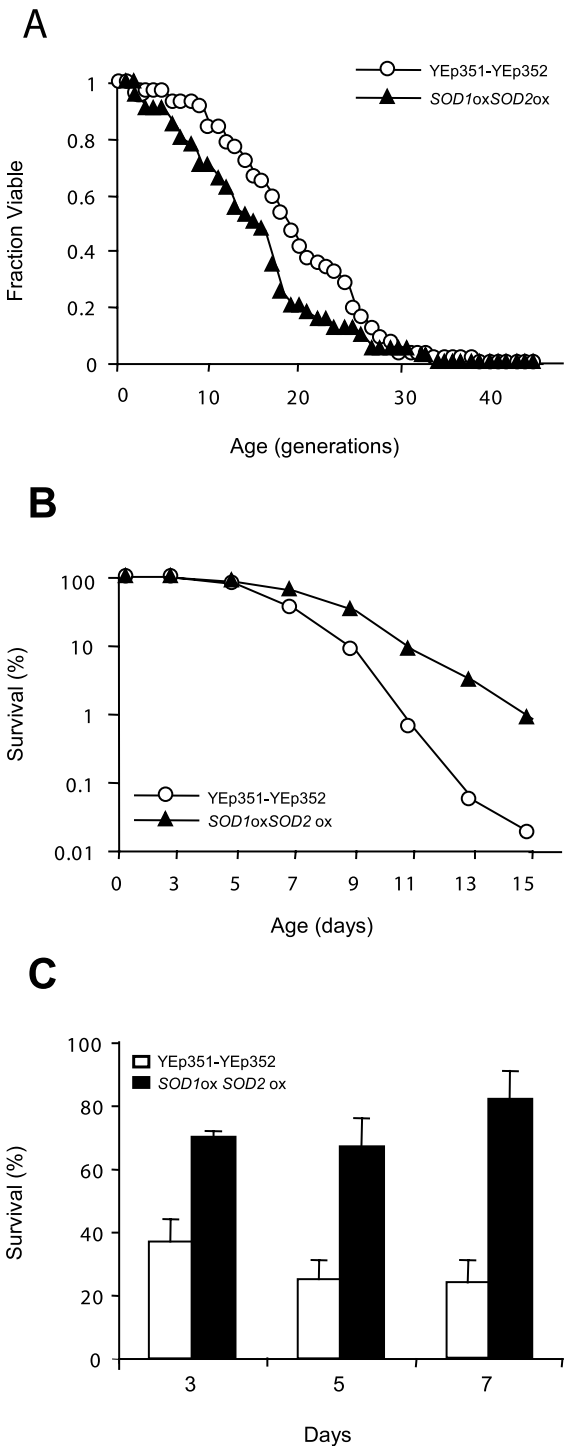

Fig. 3. Effects of the double overexpression of *SOD1* and *SOD2* on the replicative and chronological life span. Replicative (A) and chronological (B) life span of non-dividing DBY746 plasmid control cells (YEp351-YEp352) and overexpressors of cytosolic and mitochondrial superoxide dismutases (*SOD1*ox*SOD2*ox). Both the replicative and chronological life span of *SOD1-SOD2* overexpressors, were different from those of vector controls as determined by ANOVA ($P < 0.05$). C: Survival of DBY746 plasmid control cells and *SOD1*ox*SOD2*ox, removed from day 3–7 cultures, after a 1 h treatment with 20 μM of the superoxide/hydrogen peroxide-generating agent menadione. The experiment was performed twice in duplicate. Error bars show S.E.M.

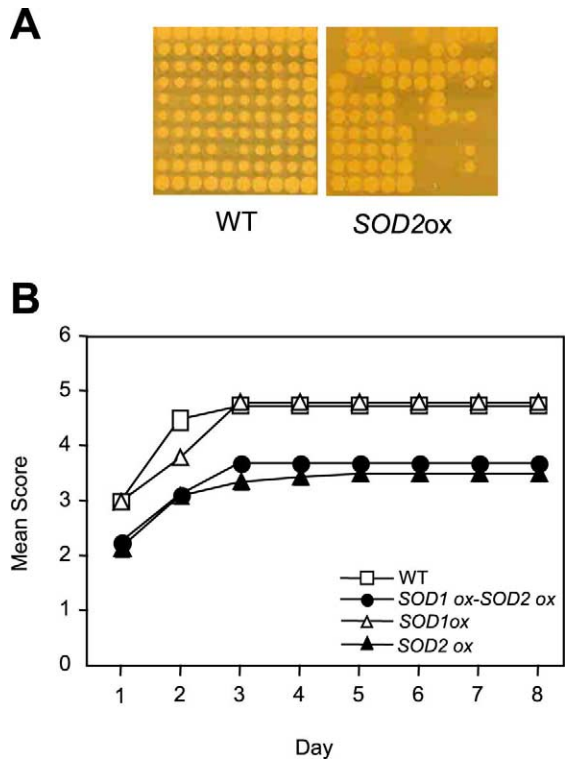

Fig. 4. A: Single mother cells of wild-type and *SOD1-SOD2* overexpressor strains were tested for their ability to produce buds. One hundred virgin cells of each strain were micromanipulated onto YPD plates and set in a series of columns to monitor their budding ability and the size of the colonies produced. B: A score ranging from 0 to 5 was assigned to each of the 100 colonies depending on its density starting after 24 h (day 1) (0 = no colony, 5 = maximum size colony) after the micromanipulation of an individual mother cell onto the plate. The average score of all the colonies tested for each strain (WT, *SOD1*ox*SOD2*ox, *SOD1*ox, or *SOD2*ox) is shown for up to 8 days and it includes cells that did not form a colony of any size.

vated in response to glucose or insulin-like growth factors extends longevity in organisms ranging from *S. cerevisiae* to *C. elegans* to mammals [8,31,32]. The present study shows that transposon insertional mutations that extend chronological life span can also extend the replicative life span. However, the replicative life span is decreased by the overexpression of *SOD1*/*SOD2* and is extended by the deletion of Msn2/Msn4/Rim15 suggesting that these stress-resistance proteins limit budding potential independently of aging (Fig. 5).

Whereas the life span of most eukaryotes is determined exclusively by measuring chronological survival, the longevity of the unicellular *S. cerevisiae* is also measured by counting the number of buds generated by an individual mother cell [5,6]. The assumption is that if a mother cell lives longer chronologically, it will bud more. In fact, the *cyr1::mTn* and *sch9::mTn* mutants cells have both an extended chronological and replicative life span [4]. The role of a reduced adenylate cyclase activity (*cyr1::mTn*) in survival extension (Fig. 1) is consistent with recent results demonstrating that attenuation of cAMP-PKA signaling by mutations in *CDC25*, *CDC35*, and *TPKs* increases the replicative life span of various yeast strains [12]. However, the present study suggests that the stress-resistance proteins regulated by the Ras2/Cyr1/PKA pathway have opposite effects on replicative and chronological longevity (Fig. 5).

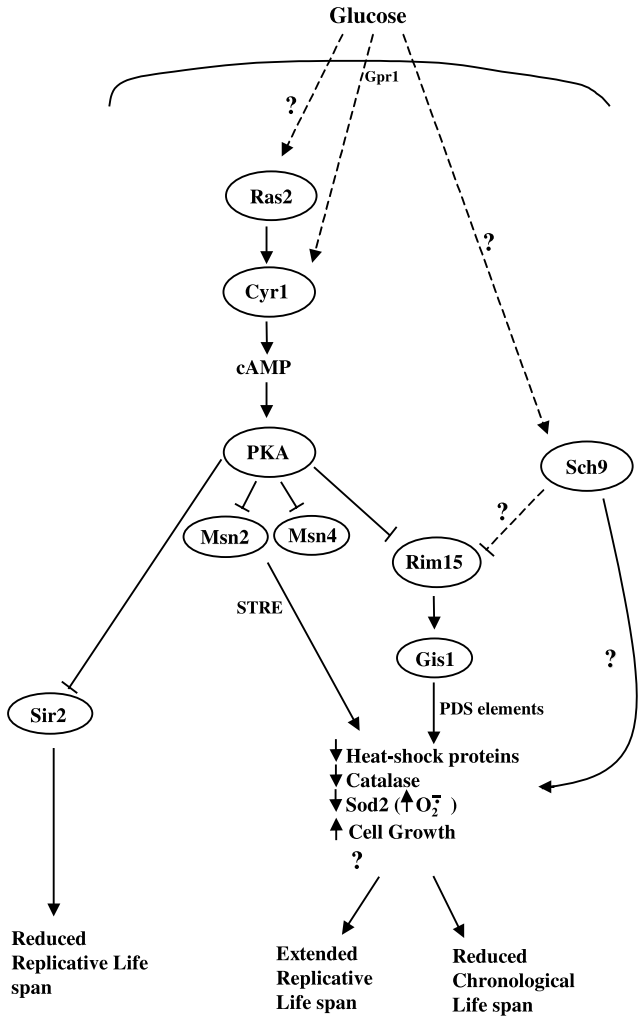

Fig. 5. Model for the regulation of replicative and chronological life span in yeast. Glucose activates the Ras2/Cyr1/PKA and Sch9 pathways. The activation of these pathways decreases both replicative and chronological life span. Msn2/Msn4 and Rim15, which are inactivated by PKA, are required for extension of chronological life span but limit the Cyr1/PKA-dependent extension of replicative life span. Transcription factors Msn2/Msn4 and protein kinase Rim15 control the expression of several stress-resistance proteins including mitochondrial Sod2 (required for chronological life span extension in *ras2Δ*, *cyr1::mTn*, and *sch9Δ* mutants). The overexpression of *SOD1/SOD2* extends the mean chronological life span by 30% but decreases the replicative life span as well as budding in virgin mother cells.

Stress-resistance genes that extend chronological survival may decrease the replicative life span by affecting the budding process. Several experiments support this possibility: (a) the overexpression of *SOD1SOD2* decreases the replicative life span but also decreases budding rates and prevents budding in 27–40% of mother cells. A similar result has been recently reported for *SOD2* overexpressors in strain FY1679-28c[33], (b) the deletion of *SCH9*, which triples chronological life span, causes a major decrease in budding rates but only causes a small increase in budding life span, (c) overactivation of the heat-shock response extends chronological but not replicative life span [34], (d) The deletion of stress-resistance transcription factors *MSN2*, *MSN4*, and serine-threonine kinase *RIM15* in *cyr1::mTn* mutants shortens chronological survival by over 50% but extends the replicative life span by 50% (Fig. 1). Notably, Msn2/Msn4 and Rim15 negatively regulate budding [29,35]. These results are consistent with the optimization of replicative life span by the induction of stress-resistance genes to levels that slow down aging without negatively affecting budding. In fact, the deletion of *sch9* promotes high stress resistance and slow growth [4] but does not extend replicative life span. By contrast, the *sch9::mTn* mutation, which promotes lower levels of stress resistance compared to the *sch9Δ* mutant [4], does not affect growth and extends both the chronological and replicative life span. An alternative explanation is that increased resistance to stress is not required to slow down aging and the effect of Sods, Msn2/4, Rim15, and Sch9 on chronological longevity is independent of the aging process. However, this possibility is unlikely considering the role of these stress-resistance proteins in protecting cells against damage and their effect in extending chronological longevity in yeast and higher eukaryotes [8,31,36].

Although a role for superoxide in increasing budding and the replicative life span may appear to be counterintuitive, superoxide has been shown to promote growth in mammalian cells [37,38]. Thus, a high concentration of superoxide is toxic and decreases both the chronological and replicative life span [39,40]. By contrast, physiological levels of superoxide play a major role in chronological aging and death but may extend the replicative life span. However, we cannot exclude that the effect of Sod overexpression may be independent of a role for superoxide in cell division. In fact, a recent study suggests that *SOD2* overexpression decreases the budding in old mother cells by causing a defect in mitochondria segregation from mother to daughter [33].

In summary, the inactivation of the Ras/cAMP/PKA pathway can increase both the chronological and replicative life span, but the induction of stress-resistance genes plays opposite roles in these survival paradigms. The effect of increased stress resistance in reducing the replicative life span may be explained in part by a role for mitochondrial Sod2 in preventing budding in mother cells. The signal transduction pathways that regulate chronological longevity in yeast and worms share several homologous proteins including superoxide dismutases, catalase, heat-shock proteins and the serine-threonine kinases Sch9 (yeast) and AKT-1/AKT-2 (worm) [41]. Furthermore, similar pathways appear to regulate longevity in flies and mice [41]. By contrast, the major link between the yeast replicative life span and aging in higher eukaryotes is the role of *SIR2* overexpression in the extension of yeast replicative life span and worm chronological life span [11,42]. Thus, the identification of mutations that extend the yeast chronological life span can serve as a strategy to understand the fundamental mechanisms of aging in higher eukaryotes. By contrast, the replicative life span may be linked with chronological aging but may also reflect the inability of a mother cell to divide at a point when it is neither senescent nor dead.

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