Regulation of Longevity and Stress Resistance by Sch9 in Yeast

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The protein kinase Akt/protein kinase B (PKB) is implicated in insulin signaling in mammals and functions in a pathway that regulates longevity and stress resistance in *Caenorhabditis elegans*. We screened for long-lived mutants in nondividing yeast *Saccharomyces cerevisiae* and identified mutations in adenylate cyclase and *SCH9*, which is homologous to Akt/PKB, that increase resistance to oxidants and extend life-span by up to threefold. Stress-resistance transcription factors Msn2/Msn4 and protein kinase Rim15 were required for this life-span extension. These results indicate that longevity is associated with increased investment in maintenance and show that highly conserved genes play similar roles in life-span regulation in *S. cerevisiae* and higher eukaryotes.

Mutations that extend life-span in *C. elegans*, *Drosophila melanogaster*, and mice are associated with increased resistance to oxidative stress (*1, 2*). However, the mechanisms that regulate aging in these multicellular organisms are poorly understood. As in higher eukaryotes, the unicellular yeast *Saccharomyces cerevisiae* undergoes an age-dependent increase in cell dysfunction and mortality rates (*3, 4*). Aging in yeast is associated with an enlargement of the cell and a slowing in the budding rate, and is commonly measured by counting the number of buds generated by a single mother cell (replicative life-span) (*5, 6*). The replicative life-span of yeast is regulated by the Sir2 protein, which mediates chromatin silencing in a nicotinamide adenine dinucleotide–dependent manner (*6, 7*). However, yeast can also age chronologically as a population of nondividing cells (*2, 4, 6*). *Saccharomyces cerevisiae* grown in complete glucose medium [synthetic complete (SC) medium] stop dividing after 24 to 48 hours and survive for 5 to 7 days while maintaining high metabolic rates (*2, 8, 9*), a situation more akin to their experience in nature where they are likely to survive as nondividing populations exposed to scarce nutrients. For these reasons, and to avoid extended growth and entry into the hypometabolic stationary phase induced by incubation in the nutrient-richer yeast extract/peptone/dextrose (YPD) medium (*10*), our studies were performed exclusively in SC medium. The survival of nondividing yeast is shortened by null mutations in either or both superoxide dismutases (SODs) (*2, 11, 12*) and is modestly extended by overexpressing the antiapoptotic protein Bcl-2 (*8*).

To understand the molecular mechanism that regulates yeast longevity, we transposon-mutagenized yeast cells and isolated long-lived mutants (*13*). Because of the association between stress resistance and longevity in higher eukaryotes, we screened for mutants that survived both a 1-hour heat stress at 52°C and a 9-day treatment with the superoxide-generating agent paraquat (1 mM). From 2 billion cells screened, we isolated 4000 thermotolerant colonies and 40 paraquat-resistant colonies carrying transposons. From the 4040 stress-resistant mutants, we isolated nine that were able to survive to day 9, when most of the wild-type cells are dead. The only two long-lived mutants isolated independently in both the paraquat and heat shock selections, designated Tn3-5 and Tn3-24, were also the longest lived (Fig. 1A), suggesting that resistance to multiple stresses is associated with increased longevity. Allele rescue of the mutants revealed that transposons had integrated in the promoter region of the Sch9 protein kinase gene (*sch9::mTn*) (Tn3-5) (33 base pairs upstream of the start codon) and in the NH_2-terminal regulatory region of adenylate cyclase (*cyr1::mTn*) (Tn3-24) (between codon 208 and 209). The mean life-spans of *sch9::mTn* and *cyr1::mTn* were extended by 30 and 90%, respectively. Transformation of Tn3-5 cells with wild-type *SCH9*, and of Tn3-24 cells with *CYR1*, abolished the survival extension, strongly suggesting that the decreased expression or activity of Sch9 and Cyr1 extends survival (not shown).

To investigate further the role of *SCH9* in chronological survival, we deleted the *SCH9* gene (*14*). The *sch9*Δ mutants grew slowly, but survived three times longer than wild-type cells (Fig. 1B). To determine whether the protein kinase activity of Sch9 accelerates mortality in nondividing yeast, we transformed mutants with either wild-type *SCH9* or with forms of *SCH9* bearing kinase-inactivating mutations: $sch9_{K441A}$ and $sch9_{D556R}$ (*15*). Transformation of *sch9*Δ with wild-type *SCH9* reversed the life-span extension, whereas transformation

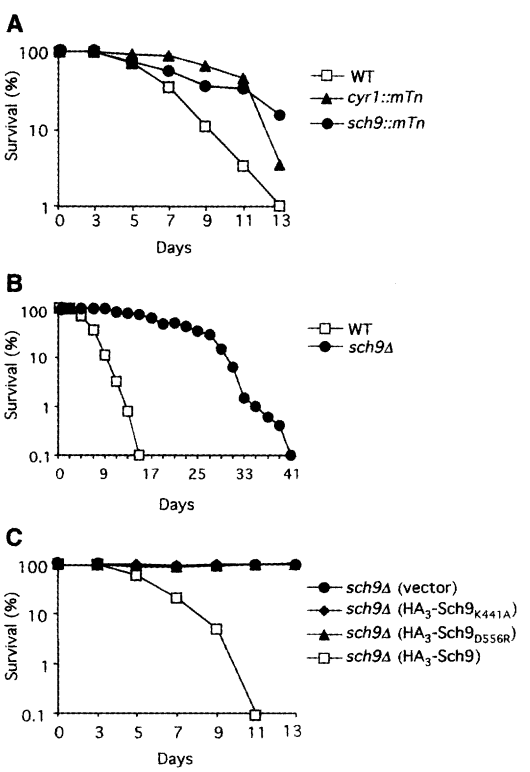

Fig. 1. Mutations in *CYR1* and in *SCH9* increase chronological life-span of *S. cerevisiae*. Survival of (**A**) the wild type (DBY746), and transposon-mutagenized *cyr1::mTn* (Tn3-24) and *sch9::mTn* (Tn3-5); (**B**) the wild type and *sch9*Δ; (**C**) *sch9*Δ transformed with vector alone wild-type *SCH9* or with a mutated *sch9* encoding for a catalytically inactive proteins ($Sch9_{K441A}$, $Sch9_{D556R}$). Cell viability was measured every 2 days starting at day 3 (*14*). Experiments were repeated between three and seven times with two or more samples per experiment with similar results. The average of all experiments is shown. The mean life-span increase in *cyr1::mTn* (90%), *sch9::mTn* (30%), and *sch9*Δ (300%) is significant [$P < 0.05$, analysis of variance (ANOVA)].

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with the genes encoding for the inactive $Sch9_{K441A}$ or $Sch9_{D556R}$ kinases did not (Fig. 1C).

Both Sch9 and Cyr1 function in pathways that mediate glucose-dependent signaling, stimulate growth and glycolysis, and decrease stress resistance, glycogen accumulation, and gluconeogenesis (*16*). The COOH-terminal region of Sch9 is highly homologous to the AGC family of serine/threonine kinases, which includes Akt/PKB, whereas the NH_2-terminal region contains a C2 phospholipid and calcium-binding motif. The 327–amino acid serine/threonine kinase domain of yeast Sch9 is, respectively, 47 and 45% identical to that of *C. elegans* AKT-2 and AKT-1, which function downstream of the insulin-receptor homolog DAF-2 in a longevity/diapause regulatory pathway (*14, 17, 18*). In this domain conserved from yeast to mammals, Sch9 is also 49% identical to human AKT-1/AKT-2/PKB, which are implicated in biological functions including insulin signaling, the translocation of glucose transporter, apoptosis, and cellular proliferation (*19*).

The *CYR1* gene encodes for adenylate cyclase, which stimulates cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKA) activity required for cell cycle progression and growth. The catalytic subunits of PKA are also 35 to 42% identical to *C. elegans* and human AKT-1/AKT-2, although PKA belongs to a different family of serine/threonine kinase. The inactivation of the Ras/cAMP/PKA pathway in *S. cerevisiae* increases resistance to thermal stress, in part, by activating transcription factors Msn2 and Msn4, which induce the expression of genes encoding for several heat shock proteins, catalase (*CTT1*), and the DNA damage inducible gene *DDR2* (*14, 16*). MnSOD also appears to be regulated in a similar manner (*20*). To determine whether *MSN2/MSN4* mediate survival extension, we deleted both genes in the *cyr1::mTn* mutants. The absence of both transcription factors abolished the life-span extension conferred by *cyr1::mTn*, but did not affect the survival of wild-type cells (Fig. 2A). By contrast, the deletion of *MSN2/MSN4* did not reverse the survival extension in *sch9*Δ cells (Fig. 2B).

The protein kinase Rim15 regulates genes containing a PDS (postdiauxic shift) element T(T/A)AG_3AT involved in the induction of thermotolerance and starvation resistance by a Msn2/Msn4-independent mechanism (*21*). To test the role of Rim15 in survival, we generated *sch9*Δ *rim15*Δ mutants. The life-span of the double mutant was decreased compared to *sch9*Δ (Fig. 2B). The deletion of *RIM15* also abolished the life-span extension in *cyr1::mTn* cells (Fig. 2A). However, it is difficult to establish whether Rim15 mediates the survival extension in these mutants, because *rim15* single mutants are short-lived (Fig. 2A).

To test whether the long-lived strains were stress-resistant, we exposed the mutants to hydrogen peroxide, menadione, or heat. All mutants were resistant to a 1-hour heat shock treatment at 55°C (Fig. 3A). Similarly, 3- to 5-day-old mutants were resistant to a 30-min treatment with 100 mM hydrogen peroxide (Fig. 3B) or with the superoxide/H_2O_2-generating agent menadione (20 μM) (Fig. 3C).

In yeast *sod2*Δ mutants, superoxide specifically inactivates aconitase and other [4Fe-4S] cluster enzymes and causes the loss of mitochondrial function and cell death (*11, 12*). To investigate further the role of superoxide toxicity in aging, we monitored the activity and reactivation of mitochondrial aconitase, which can also serve as an indirect measure of superoxide concentration (*22*). In agreement with the pattern of resistance to superoxide toxicity (Fig. 3C), aconitase specific activity decreased by 50% in wild-type cells, and by 30% in *cyr1::mTn* mutants, but did not decrease in *sch9::mTn* and *sch9*Δ mutants at day 7 compared to day 3 (*14*). The percent reactivation of aconitase was lowest in the long-lived *sch9*Δ mutants and highest in wild-type cells (Fig. 4A) and correlated with death rates (Fig. 4B), suggesting that *cyr1* and *sch9* mutants increase survival, in part, by preventing superoxide toxicity. However, the overexpression of both *SOD1* and *SOD2* only increases survival by 30% (*9*), indicating that additional systems, regulated by Msn2, Msn4, and Rim15, are responsible for the major portion of chronological life-span extension in *cyr1::mTn* and *sch9*Δ mutants.

There are many phenotypic similarities between long-lived mutants in *S. cerevisiae*, *C. elegans*, *Drosophila*, and mice (*1, 2*). *Cae-*

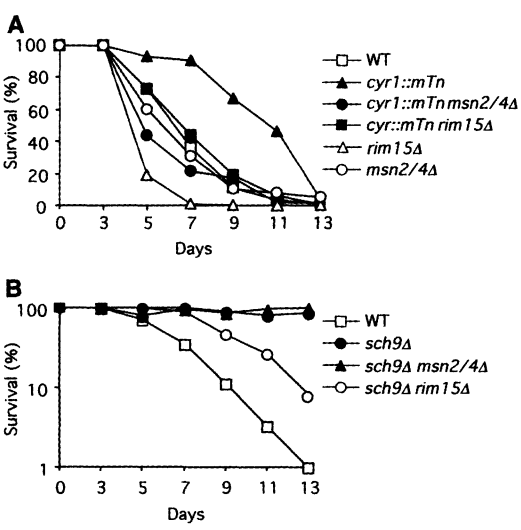

Fig. 2. Transcription factors Msn2, Msn4, and protein kinase Rim15 are required for the chronological life-span extension of *cyr1::mTn* and *sch9*Δ mutants. (**A**) Survival of the wild type and *cyr1::mTn* mutants lacking either the stress-resistance genes *MSN2/MSN4* or *RIM15*. (**B**) Survival of the wild type and *sch9*Δ mutants lacking either *MSN2/MSN4* or *RIM15*. Experiments were repeated between three and seven times with two or more samples per experiment with similar results. The average of all experiments is shown.

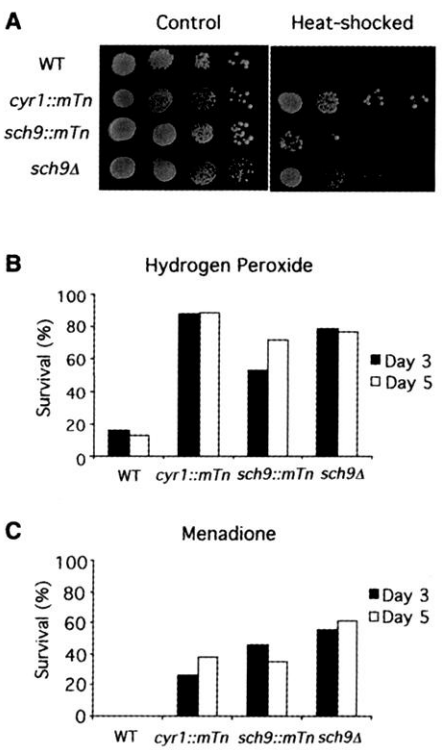

Fig. 3. Heat-shock and oxidative stress resistance are increased in long-lived mutants. (**A**) Serial dilutions (1:1 to 1:1000, left to right) of cells removed from day 1 postdiauxic phase cultures were spotted onto YPD plates and incubated at 30°C (control) or 55°C (heat-shocked) for 1 hour. Pictures were taken after a 4-day incubation at 30°C. The experiment was performed twice with two or more samples per experiment with similar results. Cells removed from days 3 or 5 in the postdiauxic phase were (**B**) diluted to an OD_{600} (optical density at 600 nm) of 1 in expired medium and incubated with hydrogen peroxide (100 mM) for 30 min or (**C**) diluted to an OD_{600} of 0.1 in potassium phosphate buffer and treated with 20 μM of the superoxide/H_2O_2-generating agent menadione for 60 min. Viability was measured by plating cells onto YPD plates after the treatment. The experiments were performed twice with similar results. The average of the two experiments is shown.

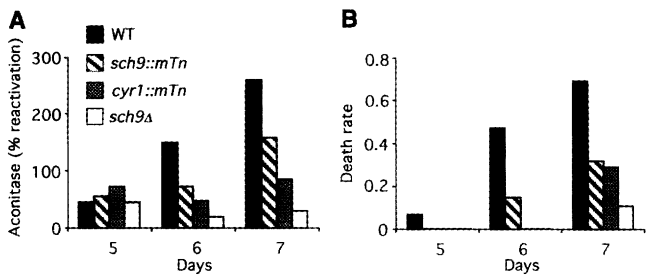

Fig. 4. Mutations in *cyr1* and *sch9* delay the reversible inactivation of the superoxide-sensitive enzyme aconitase in the mitochondria. (**A**) Mitochondrial aconitase percent reactivation after treatment of whole-cell extracts of yeast removed from cultures at day 5 through 7 with agents (iron and Na_2S) able to reactivate superoxide inactivated [4Fe-4S] clusters. (**B**) Death rate reported as the fraction of cells that lose viability in the 24-hour period following the indicated day.

norhabditis elegans age-1 and *daf-2* mutations extend the life-span in adult organisms by 65 to 100%, by decreasing AKT-1/AKT2 signaling and activating transcription factor DAF-16 (*14, 18, 23*). These changes are associated with the induction of superoxide dismutase (MnSOD), catalase, and the heat shock proteins HSP70 and HSP90 (*14, 17*). A role for oxidants in the aging of *C. elegans* was confirmed by the extended survival of wild-type worms treated with small synthetic SOD/catalase mimetics (*24*). Thus, the yeast Gpr1/Cyr1/PKA/Msn2/4-Sch9/Rim15 and the *C. elegans* DAF-2/AGE-1/AKT/DAF16 pathways play similar roles in regulating longevity and stress resistance (*14*). Analogously, a *Drosophila* line with a mutation in the heterotrimeric guanosine triphosphate–binding protein (G protein)–coupled receptor homolog *MTH* gene displays a 35% increase in life-span and is resistant to starvation and paraquat toxicity (*25*). Furthermore, in flies, aconitase undergoes age-dependent oxidation and inactivation (*26*), and the overexpression of *SOD1* increases survival by up to 40% (*27, 28*). A mutation in a signal-transduction gene also increases resistance to stress and lengthens survival in mammals. A knockout mutation in the signal transduction $p66^{SHC}$ gene increases resistance to paraquat and hydrogen peroxide and extends survival by 30% in mice (*29*).

We propose that yeast Sch9 and PKA and worm AKT-1/AKT-2 evolved from common ancestors that regulated metabolism, stress resistance, and longevity in order to overcome periods of starvation. Analogous mechanisms triggered by low nutrients may be responsible for the extended longevity of dietary restricted rodents (*3*). The phenotypic similarities of long-lived mutants ranging from yeast to mice (*1, 2*), and the role of the conserved yeast Sch9 and PKA and mammalian Akt/PKB in glucose metabolism, raise the possibility that the fundamental mechanism of aging may be conserved from yeast to humans.

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Functional Specialization in Rhesus Monkey Auditory Cortex

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Neurons in the lateral belt areas of rhesus monkey auditory cortex prefer complex sounds to pure tones, but functional specializations of these multiple maps in the superior temporal region have not been determined. We tested the specificity of neurons in the lateral belt with species-specific communication calls presented at different azimuth positions. We found that neurons in the anterior belt are more selective for the type of call, whereas neurons in the caudal belt consistently show the greatest spatial selectivity. These results suggest that cortical processing of auditory spatial and pattern information is performed in specialized streams rather than one homogeneously distributed system.

Hearing plays a dual role in the identification of sounds and in their localization. Although it is undisputed that auditory cortex participates in the analysis of spectro-temporal patterns for the identification of complex sound objects, including speech and music, the neural basis of auditory spatial perception remains a matter of controversy. Brainstem structures play a significant role in the processing of binaural cues, which contain important information for sound localization (*1*). However, lesions of auditory cortex also impair auditory spatial analysis (*2, 3*). With the recent discovery of multiple cochleotopic maps in nonprimary auditory cortex of the rhesus monkey (*4, 5*), the question arises whether neurons in some of these areas show greater specificity for sound source location than in others. This could indicate the existence of a specialized cortical stream for the processing of auditory space, similar to what

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