

Sir2 Blocks Extreme Life-Span Extension

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Summary

Sir2 is a conserved deacetylase that modulates life span in yeast, worms, and flies and stress response in mammals. In yeast, Sir2 is required for maintaining replicative life span, and increasing Sir2 dosage can delay replicative aging. We address the role of Sir2 in regulating chronological life span in yeast. Lack of Sir2 along with calorie restriction and/or mutations in the yeast AKT homolog, Sch9, or Ras pathways causes a dramatic chronological life-span extension. Inactivation of Sir2 causes uptake and catabolism of ethanol and upregulation of many stress-resistance and sporulation genes. These changes while sufficient to extend chronological life span in wild-type yeast require severe calorie restriction or additional mutations to extend life span of *sir2Δ* mutants. Our results demonstrate that effects of *SIR2* on chronological life span are opposite to replicative life span and suggest that the relevant activities of Sir2-like deacetylases may also be complex in higher eukaryotes.

Introduction

Recent findings suggest that the molecular pathways that regulate longevity may have been partially conserved throughout evolution (Kenyon, 2001; Longo and Finch, 2003). In organisms ranging from yeast to mammals, longevity is extended by inactivation of either nutrient- or insulin/insulin growth factor 1 (IGF-I)-like signaling pathways and the consequent activation of stress-resistance transcription factors (Msn2/4 and the FOXO transcription factors) (Longo and Finch, 2003). In the unicellular *Saccharomyces cerevisiae*, the inactivation of Ras2, Cyr1 or Sch9, homologs of the mammalian G protein Ras, adenylate cyclase, and the serine/threonine protein-kinase Akt, respectively, increases stress resistance and extends the chronological life span of nondividing populations (Fabrizio and Longo, 2003) up to 3-fold (Fabrizio et al., 2001, 2003).

In yeast, aging is also studied by counting the maximum number of daughter cells produced by individual

mother cells (replicative life span) (Jazwinski, 2004; Mortimer, 1959). A key regulator of the replicative life span is the silent information regulator protein Sir2. An extra copy of Sir2 extends yeast replicative longevity by 40% by reducing both rDNA recombination and the accumulation of extrachromosomal DNA circles (ERCs) (Kaeberlein et al., 1999). Conversely, the deletion of *SIR2* dramatically decreases replicative life span (Kaeberlein et al., 1999). Sir2 is a highly conserved NAD-dependent histone-deacetylase that plays a crucial role in the formation of silent chromatin and is involved in several other cellular functions including the maintenance of genome stability via the repair of double-stranded DNA breaks by nonhomologous end-joining (NHEJ) (Guerente, 1999; Tanny et al., 1999; Tanny and Moazed, 2001; Tsukamoto et al., 1997). Recently, Sir2 has been shown to promote the asymmetric inheritance of oxidatively damaged proteins during cytokinesis. Sir2 activity is required for yeast mother cells to retain carbonylated proteins during cell division (Aguilaniu et al., 2003). The overexpression of Sir2 homologs can also extend life span in *C. elegans* (Tissenbaum and Guarente, 2001) and *Drosophila* (Rogina and Helfand, 2004) although the effect of lowering Sir2 activity on the life span of *Drosophila* is not clear (Astrom et al., 2003; Newman et al., 2002).

Calorie restriction (CR), an intervention known to extend the life span of many organisms, causes a substantial increase of both the yeast replicative and chronological life span (Fabrizio et al., 2004a; Fabrizio and Longo, 2003; Lin et al., 2000; Longo et al., 1997). A role for Sir2 in mediating replicative life-span extension under CR has been proposed by Lin et al. (Lin et al., 2000, 2002), but it has recently been challenged (Kaeberlein et al., 2004).

Here, we study *S. cerevisiae* to investigate further the role of Sir2 in stress resistance and chronological life-span regulation.

Results

Sir2 Blocks Extreme Chronological Life-Span Extension in Long-Lived *sch9Δ* and *cyr1* Mutants

To study the effect of Sir2 on the chronological life span, we studied cells lacking the *SIR2* gene (*sir2Δ*) in the BY4741 genetic background. In agreement with the role of Sir2 in the reduction of the replicative life span, *sir2Δ* mutations caused a minor reduction in the chronological life span in the BY4741 background (Figure 1A). However, no significant difference in chronological life span was observed between control and *sir2Δ* mutants in the DBY746 or W303 genetic backgrounds (Figures 1B–1D). Notably, the high number of experiments performed (>15) in the DBY746 background revealed that in some experiments the *sir2Δ* mutation could extend life span (Figure 1D).

To investigate further the role of Sir2 on chronological life span, we deleted *SIR2* in two long-lived mutant strains that we had previously identified (Fabrizio et al.,

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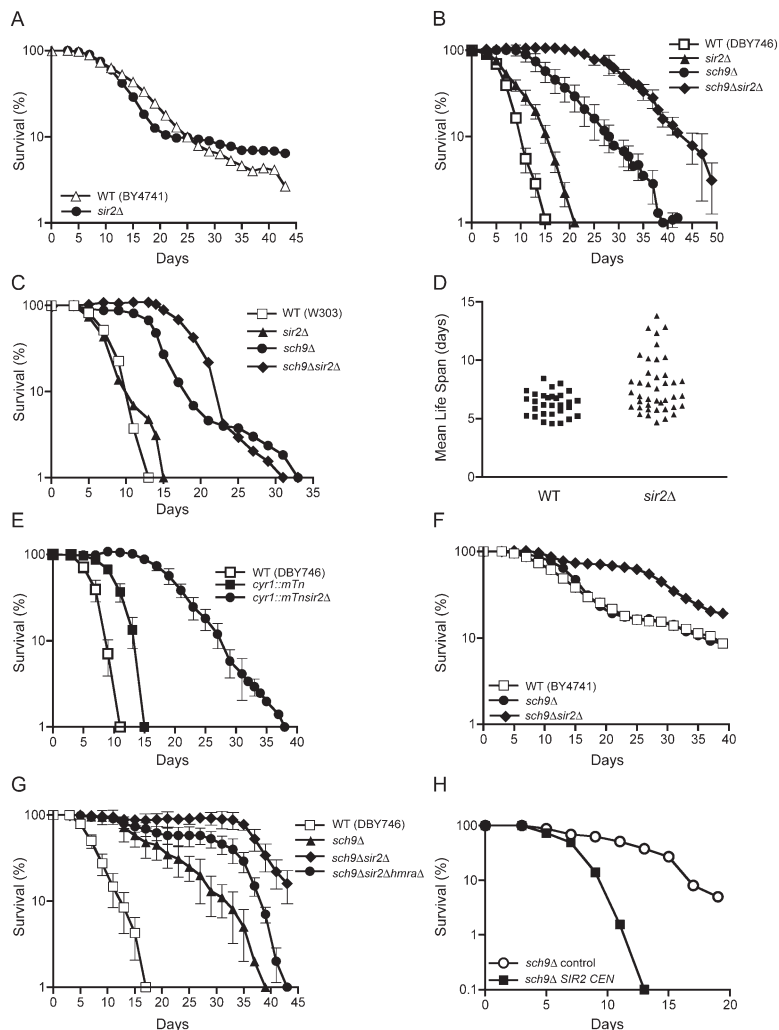


Figure 1. Chronological Life Span of Yeast *sir2* Mutants in SDC/Ethanol Medium

Chronological survival in minimal medium (SDC) of (A) wild-type (BY4741) and mutants lacking Sir2; (B) wild-type (DBY746) and mutants lacking Sir2, Sch9, or both; (C) wild-type (W303AR) and mutants lacking Sir2, Sch9, or both; (D) distribution of the mean chronological life span of wild-type (DBY746) and *sir2Δ* mutants; (E) wild-type (DBY746), mutants with a transposon insertion in the *CYR1* gene (*cyr1::mTn*), and *cyr1::mTnsir2Δ* double mutants; (F) wild-type BY4741 and mutants lacking Sch9 or both Sch9 and Sir2; (G) wild-type (DBY746), *sch9Δ*, *sch9Δsir2Δ*, and *sch9Δsir2ΔhmraΔ* mutants; (H) mutants lacking Sch9 transformed with a centromeric plasmid carrying the *SIR2* gene and relative control strain transformed with the empty vector. When error bars (SEM) are shown, the plots represent the average of 3–10 experiments. When no bars are shown, the plots show a representative experiment repeated 2–3 times with similar results.

2001). Yeast lacking the serine/threonine protein kinase Sch9, a functional homolog of mammalian Akt (Geykens et al., 2000), live three times longer than wild-type cells (Fabrizio et al., 2001). Analogously, the mean chronological life span of mutants with a decreased activity of Ras2 or adenylate cyclase (*Cyr1*) is extended by approximately 2-fold (Fabrizio et al., 2001, 2003). Our results show that the deletion of *SIR2* extends further the chronological life span of long-lived mutants ($p < 0.001$ for *sir2Δ sch9Δ* versus *sch9Δ*) (Figure 1B). The mean chronological life span of *sch9Δsir2Δ* was five times longer and that of *cyr1::mTnsir2Δ* three times longer compared to those of wild-type cells (Figures 1B and 1E). The synergistic effect of the deletion of *SCH9* and *SIR2* in the regulation of the chronological life span was confirmed using two additional genetic backgrounds commonly used in replicative life span studies (W303 and BY4741) (Aguilaniu et al., 2003; Kaerberlein et al., 1999), although the effect of the *sir2Δ* mutation in these genetic backgrounds was smaller (Figures 1C and 1F). Taken together, these results suggest that Sir2 serves as a “blocker” of extreme longevity extension in cells with low Sch9 or Ras/Cyr/PKA activity. Notably,

the conditions under which chronological life span is studied model those encountered by *S. cerevisiae* in the wild (Fabrizio et al., 2004a), indicating that the inhibition of extreme longevity phases may represent an important function for Sir2.

Given that lack of Sir2 causes sterility due to the expression of both silent mating-type loci, we deleted the *HMRA* locus in our *sir2* double and single mutants (mating type α). This deletion restored fertility but caused no significant difference in mean chronological life span compared to the corresponding nonsterile strains (*sch9Δsir2ΔhmraΔ* versus *sch9Δsir2Δ*, no significant difference; *sch9Δsir2ΔhmraΔ* versus *sch9Δ*, $p < 0.05$) (Figure 1G and data not shown). Although the chronological life span of *sch9Δsir2ΔhmraΔ* is not significantly different than that of *sch9Δsir2Δ* mutants, the deletion of *HMRA* shortened the survival of *sch9Δsir2Δ* mutants in several experiments as apparent from the average survival shown in Figure 1G. This decrease of life span may be caused by the effect of the single *hmraΔ* mutation on the expression of many genes (data not shown).

To determine whether *SIR2* overexpression, shown to extend replicative life span, could also play the oppo-

site role in the regulation of chronological life span, we overexpressed *SIR2* in both wild-type and *sch9Δ* mutants. *SIR2* low-copy overexpression reduced the chronological life-span extension caused by the *sch9Δ* mutation but did not affect the life span of wild-type cells (Figure 1H and data not shown). Considering the role of stress resistance in the extension of chronological life span in *sch9Δ* mutants (Fabrizio et al., 2001), it is possible that increased dosage of Sir2 may abolish the effect of *sch9Δ* on chronological life span by reducing the expression of stress-resistance genes (Table 1).

Sir2 Blocks Extreme Chronological Longevity Extension by CR

During the fermentative growth phase, yeast release ethanol, which is utilized as a carbon source by surviving cells. To model extreme starvation conditions encountered in the wild, we switched the cells from the ethanol-containing medium to water at day 3 (Fabrizio and Longo, 2003). This severe form of CR causes entry into a low metabolic phase and a major chronological life-span extension (Fabrizio and Longo, 2003; Longo et al., 1997). Since the Sch9 and Ras/PKA pathways are downregulated during starvation and their inactivation may partially mimic CR, we tested whether CR could substitute the mutations in *SCH9* and *CYR1* and extend longevity in cells lacking *SIR2*. Whereas *sir2Δ* mutations did not extend the life span in ethanol-containing medium (Figure 1), the chronological life span of *sir2Δ* mutants (DBY746 background) incubated in water was extended by 60% compared to that of wild-type yeast under the same conditions ($p < 0.05$) and 4-fold compared to that of wild-type cells grown and maintained in glucose/ethanol medium (Figure 2A). Analogously, the mean chronological life span of CR *sch9Δsir2Δ* mutants was approximately 20% longer than that of CR *sch9Δ* mutants ($p < 0.05$) and 3-fold longer than that of CR wild-type cells (Figure 2A). Similar results were obtained using strains generated in the BY4741 background although under CR conditions, *sch9Δsir2Δ* mutants did not survive longer than *sch9Δ* mutants (Figure 2B). Thus, Sir2, which may be required for replicative life-span extension by CR (Anderson et al., 2003; Lin et al., 2000), reduces the effect of CR on chronological life span. Notably, *sch9Δ* mutants also live longer than wild-type under CR, suggesting that downregulation of Sch9 may only mediate a portion of the effects of CR.

Lack of Sir2 Deacetylase Activity Induces Stress Resistance and Reduces Age-Dependent DNA Mutations

Since chronological longevity extension in *ras2* and *cyr1* mutants is associated with stress resistance and requires stress-resistance transcription factors Msn2/Msn4 as well as Mn-superoxide dismutase (Sod2) (Fabrizio et al., 2003), we hypothesized that lack of Sir2 may increase longevity by further activation of proteins involved in protection, repair, and replacement. To test this hypothesis, we exposed *sir2Δ* mutants and wild-type (DBY746) to superoxide-generating agent menadione (20–100 μ M, 30 min–1 hr) or heat (55°C, 1 hr) (Figure 3A). Stress resistance was measured in both exponentially growing cells and in chronologically aging

cells at day 3. Lack of Sir2 did not modify stress resistance in exponentially growing cells (Figure 1A, top panel) as previously shown (Lin et al., 2002) but increased resistance to heat and oxidative damage in 3-day-old wild-type yeast (Figure 3A, middle and bottom panels). Analogous results were obtained when the stress resistance of the fertile *sir2Δhmr1Δ* mutants was monitored, suggesting that sterility does not affect the ability of yeast to respond to stress (Figure 3B). To test whether the deacetylase activity of Sir2 was responsible for the reduction in stress resistance in chronologically aging yeast, we monitored both heat (55°C, 2 hr) and oxidative stress resistance (100 mM H_2O_2 , 30 min) of cells expressing either mutated alleles *sir2-H364Y* and *sir2-G262A*, encoding enzymatically inactive Sir2 proteins (Hoppe et al., 2002; Tanny et al., 1999), or wild-type *SIR2*. The stress-resistant phenotype of cells expressing the enzymatically inactive Sir2 suggests that the deacetylase activity is required to downregulate stress-resistance systems in aging cells at days 1–3 (Figure 3C and data not shown).

Sir2 was reported to be implicated in the repair of DNA double-strand breaks by NHEJ, which is particularly efficient in the G1 phase of the cell cycle and in G0-arrested cells (Karathanasis and Wilson, 2002). Since chronologically aging cells are cell cycle arrested, lack of Sir2 might be associated with higher sensitivity to DNA damage. To test this hypothesis, we measured the age-dependent frequency of mutations in *sir2Δ* strains. Our results show an age-dependent mutation-frequency increase in the wild-type (DBY746) (Figure 3D). The mutation frequency of the *sir2Δ* mutants was 2–4 times higher than that of wild-type cells at days 1–3 but did not show any age-dependent increase between days 1 and 7 (Figure 3D). These results are consistent with the previously reported role for Sir2 in maintaining genomic stability and increasing bud-ding potential during the growth phase (Karathanasis and Wilson, 2002; Kaeberlein et al., 1999) but suggest that Sir2 may play a role in promoting a chronological age-dependent increase in DNA mutations.

To further elucidate the role played by Sir2 in the aging process, we obtained the gene expression profile of 3-day-old *sir2Δ* mutants and compared it with that of wild-type cells (Table 1 and Supplemental Data available with this article online). This comparison by DNA microarray analysis showed an increase of many genes in the DNA repair, stress response, and sporulation functional groups (Table 1). Consistent with our hypothesis that Sir2 may regulate a set of genes that block entry into a spore-like state, the deletion of *SIR2* appears to cause upregulation of many more sporulation and DNA repair-related genes compared to those downregulated (Table 1). Surprisingly, in the stress-resistance category, a larger number of genes appear to be downregulated compared to those upregulated. However, many genes listed in the “downregulated” *sir2Δ* stress-resistance group are implicated in the negative regulation of stress resistance and/or chronological life span. Thus, a lower expression of these genes is expected to increase stress resistance and life span. For example, the downregulation of *HSP82* (Table 1, stress resistance) reduces Hsp90 activity, which has been shown to negatively regulate stress resistance

Table 1. Differentially Expressed Genes in the *sir2Δ* and *sir2ΔhmrΔ* Mutants at Day 3 and Variation in Their Expression in the *sir2Δ* Mutant during Exponential Growth (Fold Increase/Decrease)

Name	Function	Day 3 <i>sir2Δ</i> versus wt	<i>sir2ΔhmrΔ</i> versus wt	Log Phase <i>sir2Δ</i> versus wt
Stress-Response Genes (Upregulated)				
<i>SIP18</i>	Salt-induced protein	16.56	15.35	1.39
<i>GRE1</i>	Hydrophilin	13.37	16.28	1.22
<i>COS8</i>	Nuclear membrane protein	5.32	7.98	2.88
<i>SSA3</i>	Heat-shock protein of HSP70 family	3.31	5.55	1.40
<i>PHO5</i>	Acid phosphatase	3.04	3.01	1.21
<i>ALO1</i>	D-arabinono-1,4-lactone oxidase	2.87	2.69	-1.03
<i>PER1</i>	Protein processing in the ER	2.67	1.64	1.12
<i>KCS1</i>	Inositol polyphosphate kinase	2.45	3.01	1.10
<i>PAU4</i>	Part of seripauperin multigene family	2.39	2.10	1.20
<i>DAK2</i>	Dihydroxyacetone kinase	2.26	0.30	1.27
<i>MCR1</i>	NADH-cytochrome b5 reductase	2.21	2.26	1.36
<i>PTC2</i>	Protein phosphatase type 2C	2.19	1.42	1.03
<i>TEL1</i>	Protein kinase	2.12	3.01	1.17
<i>TPS3</i>	Trehalose-6-phosphate synthase subunit	2.11	1.61	1.08
<i>ATX1</i>	Copper chaperone	2.04	2.29	1.27
Stress-Response Genes (Downregulated)				
<i>MRK1</i>	MDS1-related protein kinase	-21.17	-12.90	1.34
<i>MSN4</i>	Zinc-finger protein	-6.75	-10.57	1.01
<i>SSA4</i>	HSP70 family	-5.85	-3.41	1.29
<i>HSP78</i>	Heat-shock protein 78	-4.39	-1.79	1.07
<i>HCH1</i>	Heat-shock protein regulator	-4.35	-4.28	1.13
<i>HSP42</i>	Stress-induced chaperone	-4.08	-2.49	1.63
<i>HSP104</i>	Heat-shock protein 104	-4.04	-2.30	1.48
<i>PUP2</i>	Proteasome subunit	-4.04	-2.26	1.13
<i>NTH2</i>	Neutral trehalase	-3.99	-5.23	-1.07
<i>UGA2</i>	Succinate semialdehyde dehydrogenase	-3.96	-6.16	1.04
<i>HSP82</i>	Heat-shock protein 90	-3.86	-1.78	1.46
<i>GRX3</i>	Glutaredoxin	-3.70	-2.22	1.02
<i>PHO4</i>	Myc-family transcription factor	-3.28	-3.41	1.22
<i>OCA1</i>	Putative protein tyrosine phosphatase	-3.08	-1.41	1.05
<i>CKB2</i>	Protein kinase CK2, beta subunit	-3.06	-1.88	1.11
<i>CCP1</i>	Cytochrome c peroxidase	-3.04	-2.95	1.23
<i>UBC4</i>	Ubiquitin-conjugating enzyme e2	-3.02	-2.43	1.19
<i>GPX1</i>	Phospholipid hydroperoxide glutathione peroxidase	-2.61	-3.32	1.12
<i>AHA1</i>	Hsp90 system cochaperone	-2.57	-0.84	1.17
<i>DEF1</i>	Rad26-interacting protein	-2.41	-1.36	1.10
<i>MXR1</i>	Peptide methionine sulfoxide reductase	-2.34	-1.24	1.26
<i>GRE3</i>	Aldose reductase	-2.32	-1.86	1.00
<i>ATH1</i>	Acid trehalase	-2.13	-1.63	1.12
Sporulation-Related Genes (Upregulated)				
<i>SPS100</i>	Sporulation-specific cell wall maturation protein	12.39	1.14	1.37
<i>SPO75</i>	Required for spore wall formation during sporulation	3.93	-1.47	1.29
<i>SPO77</i>	Required for spore wall formation during sporulation	3.61	1.01	1.38
<i>SPO71</i>	Required for spore wall formation during sporulation	3.30	2.58	1.46
<i>SPR3</i>	Septin	2.96	1.51	1.27
<i>DIT2</i>	N-formyltyrosine oxidase	2.67	1.32	1.24
<i>SPO21</i>	Component of the meiotic outer plaque of the spindle pole	2.62	-1.05	1.14
<i>DIT1</i>	Converting L-tyrosine to N-formyl-L-tyrosine	2.61	1.83	1.23
<i>SPO74</i>	Component of the meiotic outer plaque of the spindle pole	2.56	2.05	1.22
<i>IRA1</i>	GTPase-activating protein (GAP)	2.54	2.27	1.10
<i>ATG18</i>	Phosphatidylinositol 3,5-bisphosphate-binding protein	2.52	2.20	1.20
<i>IRR1</i>	Cohesin complex subunit	2.50	3.25	1.03
<i>SPO73</i>	Required for spore wall formation during sporulation	2.39	4.05	1.07
<i>SMC3</i>	SMC chromosomal ATPase family member	2.38	3.39	1.09
<i>CDA2</i>	Chitin deacetylase	2.35	1.39	1.17
<i>SSP2</i>	Sporulation Specific	2.29	1.63	1.38
<i>SPR1</i>	Exo-1,3-beta-glucanase, sporulation-specific	2.14	1.14	1.34
<i>SPS2</i>	Middle-late gene of meiosis	2.11	-1.32	1.44
<i>SMK1</i>	MAP kinase	2.03	-1.06	1.38

(continued)

Table 1. Continued

Name	Function	Day 3 <i>sir2Δ</i> versus wt	<i>sir2ΔhmrΔ</i> versus wt	Log Phase <i>sir2Δ</i> versus wt
Sporulation-Related Genes (Downregulated)				
<i>PUP2</i>	Proteasome subunit	-4.04	-2.26	1.12
<i>UBC4</i>	Ubiquitin-conjugating enzyme e2	-3.02	-2.43	1.19
<i>SHP1</i>	Putative regulator of Glc7p	-2.48	-1.09	1.10
<i>SPS19</i>	2,4-dienoyl-CoA reductase	-2.34	-3.05	1.23
<i>RAS2</i>	Small GTP binding protein	-2.22	1.57	-1.01
DNA Repair-Related Genes (Upregulated)				
<i>CAC2</i>	Chromatin assembly factor-I (CAF-I) p60 subunit	3.30	2.59	1.18
<i>REV3</i>	DNA polymerase zeta subunit	2.89	3.00	1.19
<i>MMS4</i>	Endonuclease	2.71	2.33	1.16
<i>MRE11</i>	Subunit of a complex with Rad50p and Xrs2p	2.34	2.79	1.18
<i>PAN3</i>	Pab1p-dependent poly(A) ribonuclease (PAN) subunit	2.32	1.76	1.29
<i>MUS81</i>	Mms and UV sensitive	2.27	2.01	1.26
<i>SMC5</i>	Structural maintenance of chromosomes protein	2.23	2.62	1.20
<i>RAD53</i>	Protein kinase	2.23	1.93	1.00
<i>TDP1</i>	Tyrosine-DNA phosphodiesterase	2.20	1.26	1.11
<i>MMS1</i>	Sensitive to MMS, diepoxybutane, and mitomycin C	2.20	1.02	1.33
<i>HPR5</i>	DNA helicase	2.18	-1.30	1.32
<i>REV7</i>	DNA polymerase zeta (pol-zeta) subunit	2.14	1.44	1.46
<i>RAD18</i>	ATPase (putative) zinc-finger protein	2.03	2.65	1.09
DNA Repair-Related Genes (Downregulated)				
<i>RAD6</i>	Ubiquitin-conjugating enzyme	-5.04	-2.72	-1.05
<i>SIT4</i>	Similar to catalytic subunit of bovine PP2A	-2.81	-1.88	1.04
<i>LRP1</i>	Homolog of mammalian C1D	-2.78	-1.10	-1.07
<i>MPH1</i>	Member of the DEAH family of helicases	-2.55	-1.00	-1.44
<i>DDR48</i>	Flocculent specific protein	-2.54	-1.69	-1.15
<i>HPR5</i>	DNA helicase	-2.34	-1.63	-1.33

and chronological life span in *S. cerevisiae* (Harris et al., 2001; Lotz et al., 2003). *ATH1* and *NTH2*, also downregulated in *sir2Δ* mutants (Table 1, stress resistance), encode for proteins involved in either the utilization or breakdown of trehalose (Nwaka and Holzer, 1998), which is important for chronological survival (Werner-Washburne et al., 1996). Analogously, *GRE3* (Table 1, stress resistance) encodes for a glycerol dehydrogenase whose downregulation increases the levels of glycerol, a cryoprotectant similar to trehalose, also important for stress resistance and chronological survival (Izawa et al., 2004) (Werner-Washburne et al., 1996). Considering our stress-resistance results (Figure 3) and the role of the genes listed above, it is likely that the downregulation of many of the stress-resistance-associated genes in *sir2Δ* mutants protects, rather than sensitizes, cells against age-dependent damage.

To determine whether the differential gene expression observed in the *sir2Δ* mutants was caused by sterility, we compared the gene-expression profiles of 3-day-old *sir2ΔhmrΔ* and *sir2Δ* mutants and found a very similar differential gene-expression pattern compared to wild-type cells (Table 1 and Supplemental Data). Thus, we show that the deletion of *SIR2* causes changes in gene expression, which may be relevant to chronological life-span regulation, and which are independent of loss of fertility. To complete the gene-expression analysis of yeast lacking Sir2, we compared the gene-expression profiles of exponentially growing wild-type yeast and *sir2Δ* mutants and found that the majority of the genes that are differentially expressed in the *sir2Δ* mutants at day 3 are expressed at levels

comparable to those of wild-type cells during exponential growth (Table 1 and Supplemental Data). These results suggest that studies of the effect of mutations on chronological and replicative survival can yield very different results in part because mutations may have very different effects in growing and nondividing cells.

To further explore the role of Sir2 in stress resistance and DNA damage, we monitored heat-shock (55°C, 1.5–2 hr) and oxidative stress resistance (200 μM menadione, 1 hr) of *sch9Δsir2Δ* and *cyr1::mTnsir2Δ* double mutants. The deletion of *SIR2* increased further the resistance of *sch9Δ* mutants to heat shock and menadione (Figure 3E, top panels), but it did not further improve stress resistance of the already highly resistant *cyr1* mutants (Figure 3E, bottom panels), suggesting that “extreme life-span extension” does not necessarily require an additional increase in stress resistance. Consistent with the results obtained with the *sir2Δ* single mutant, the deletion of the *HMRa* locus did not affect the stress resistance of *sch9Δsir2Δ* and *cyr1::mTnsir2Δ* mutants (Figure 3E).

In the long-lived *sch9Δ* mutants, the frequency of *can^R* mutations was significantly lower than that of wild-type but increased at day 13 and continued increasing until day 17 (Figure 3F). By contrast, in the *sch9Δsir2Δ* mutants, it remained low until day 17 (Figure 3F). This effect appears to be *HMRa* independent since it was not abolished by its deletion (Figure 3F). Thus, Sir2 appears to play an important role in protecting against genomic instability during the growth phase (Figure 3D) but contributes to chronological age-dependent DNA mutations in *S. cerevisiae* (Figures 3D and 3F). These

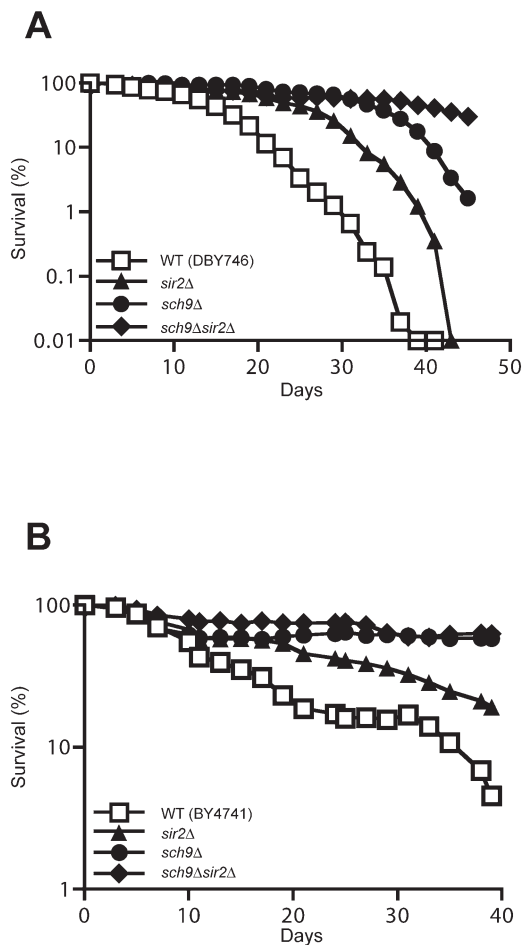


Figure 2. Chronological Life Span of *sir2Δ* Mutants under Calorie Restriction

Survival of calorie-restricted (CR) (A) DBY746 and mutants lacking *SIR2*, *SCH9*, or both and (B) BY4741 and mutants lacking *SIR2*, *SCH9*, or both. The experiments shown are representative and were repeated twice with similar results.

results are also consistent with a role for Sir2 in blocking entry into an extreme longevity phase characterized by increased stress resistance and improved DNA protection and/or repair. The reduced genomic instability caused by the deletion of *SIR2* is likely to play a role in extending further the chronological life span of both CR yeast and *sch9Δ* mutants.

Sir2 Blocks the Depletion of Extracellular Ethanol in Part by Inactivating Adh2

Our recent study suggests that yeast chronological aging can be programmed and altruistic (Fabrizio et al., 2004a). This aging and death program, which requires the acidification of the extracellular medium, can be postponed or prevented by severe CR (incubation in water) (Fabrizio et al., 2004a) (Figures 2A and 2B). In an attempt to understand further the mechanisms of CR's effects on life span, we measured the chronological age-dependent ethanol concentration in the expired media of wild-type (DBY746), *sir2Δ*, *cyr1::mTn*, and *sch9Δ* mutants. Interestingly, while the ethanol content

remained high in wild-type cultures until the majority of cells had died (days 9–11), it was depleted from the *sir2Δ* cultures by day 3 and from the *sch9Δ/cyr1* cultures by days 5–7 (Figures 4A and 4B and data not shown). The effect of *sir2Δ* was partially dependent on the glucose-repressible alcohol dehydrogenase isoenzyme 2 (Adh2) as indicated by the higher levels of ethanol at days 3 and 5 in *sir2Δadh2Δ* double mutants (Figure 4A).

We performed additional studies on the role of Sir2 and Adh2 in the depletion of extracellular ethanol. Adh2 is normally activated when yeast start breaking down ethanol and switch from fermentative to respiratory metabolism. Since *ADH2* expression can be activated by the deletion of the genes encoding for deacetylases Hda1 and Rpd3, whose activities reduce the acetylation of the chromatin at the promoter region of the *ADH2* gene (Verdone et al., 2002), we hypothesized that Sir2 might also regulate the acetylation state of the *ADH2* promoter and consequently the expression level of *ADH2* during chronological aging. To test this hypothesis we performed both RT-PCR to study the expression level of *ADH2* and chromatin immunoprecipitation (ChIP) to monitor the acetylation state of the *ADH2* promoter. Our results do not indicate an increase in the *ADH2* gene expression level in *sir2Δ* mutants at days 1–2 (data not shown). Consistently, our ChIP studies did not reveal any difference in the acetylation state of histones H3 and H4 at the *ADH2* promoter between wild-type and *sir2Δ* cells at days 1–2 (data not shown). Taken together, these results indicate that Sir2 histone deacetylase activity does not regulate *ADH2* gene expression.

We also monitored the alcohol dehydrogenase activity of the *sir2Δ* and wild-type strains. We observed Adh2 activity starting from days 1–2 in the *sir2Δ* mutants but not in wild-type cells (lower band, Figure 4C). The deletion of *ADH2* in the *sir2Δ* mutants caused the disappearance of the lower band in the activity gels, confirming that the band corresponds to Adh2 (Figure 4C). The deletion of *HMRa* in the *sir2Δ* mutants did not abolish the induction of Adh2 activity, suggesting that sterility is not responsible for Adh2 activation (Figure 4D). Consistent with the results above, *hmraΔ* mutations did not abolish the effect of *sir2Δ* on the depletion of ethanol in the medium obtained from day 3 cultures (data not shown).

To test whether Sir2 controls the enzymatic activity of Adh2 by regulating the protein level of Adh2, we detected the presence of Adh2 by Western blot analysis in wild-type yeast, *sir2Δ*, and *sir2ΔhmraΔ* mutants (Figure 4E). Our results show a low but similar level of Adh2 protein in the *sir2Δ* and *sir2ΔhmraΔ* mutants. By contrast, no band corresponding to Adh2 was observed in the wild-type cells, suggesting that Sir2 affects either the translation or the stability of the Adh2 protein during chronological aging. Consistent with the results relative to the activity of Adh2, the deletion of *HMRa* does not affect the protein level of Adh2 observed in the *sir2Δ* mutants (Figure 4E). To establish whether the effect of Sir2 on Adh2 activity was deacetylase dependent, we used strains expressing a deacetylase-defective Sir2 (see above). The increase in Adh2 activity in *sir2Δ* mutants was reversed by transformation with wild-type *SIR2* but not with mutated alleles of *SIR2* coding for

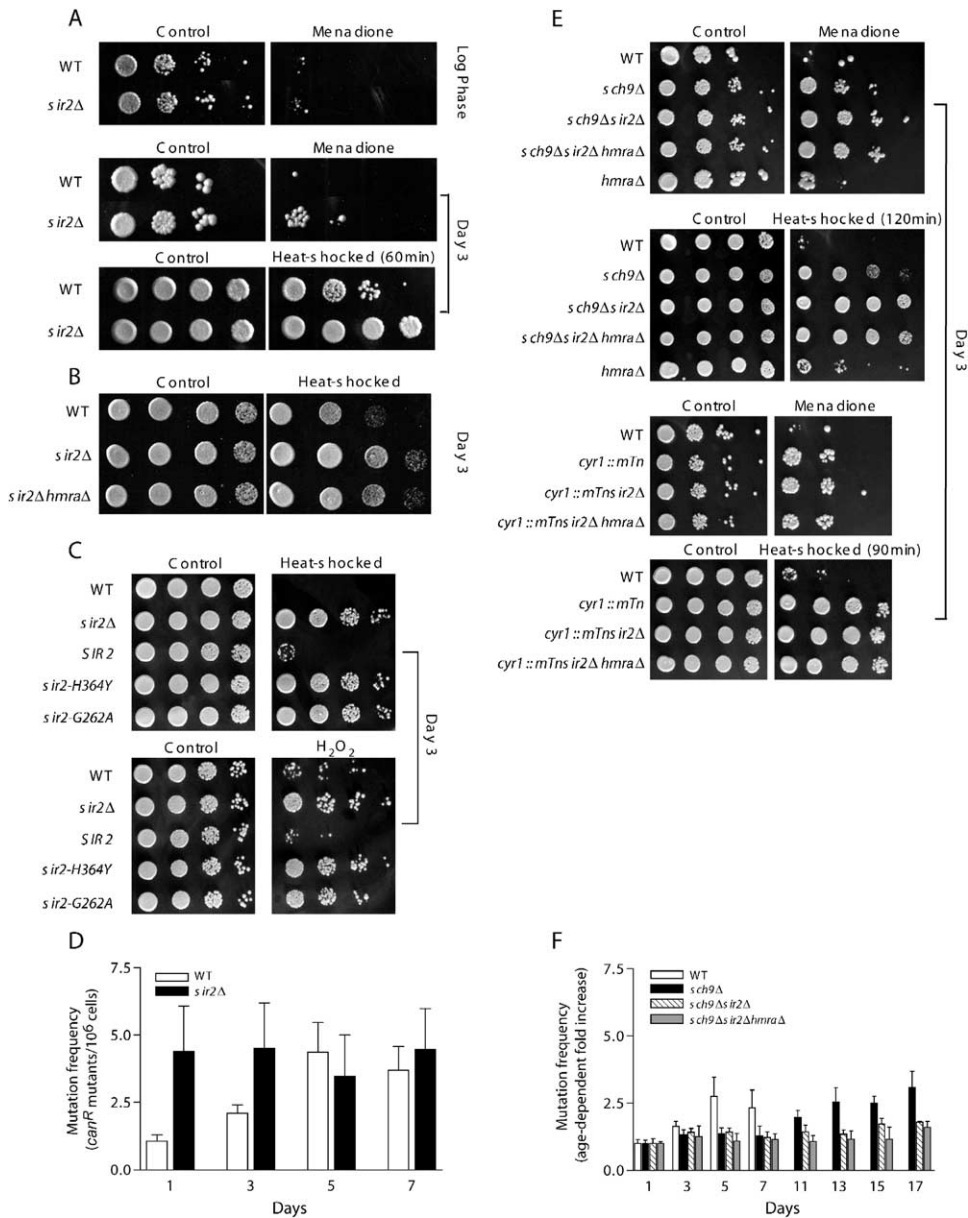


Figure 3. Sir2 Reduces Stress Resistance and Increases Mutation Frequency in Yeast Aging Chronologically

(A) Oxidative stress resistance of wild-type (DBY746) and *sir2Δ* mutants. Exponentially growing and day 3 cells were treated with 20 μ M of menadione for 30 min and 100 μ M for 1 hr, respectively. After treatment, cells were serially diluted, spotted onto YPD plates, and incubated at 30°C for 3–4 days (top and middle panels). The bottom panel shows heat-shock resistance of wild-type (DBY746) and mutants lacking Sir2. Serial dilution of day 3 cultures were spotted onto YPD plates and incubated at 55°C for 60 min. Pictures were taken after a 3 day incubation at 30°C.

(B) Heat-shock resistance of day 3 wild-type (DBY746), *sir2Δ*, and *sir2ΔhmraΔ* mutants.

(C) Heat-shock and oxidative stress resistance of day 3 wild-type (DBY746), *sir2Δ* mutant, and *sir2Δ* mutant transformed with centromeric plasmid pSIR2, which carries the *SIR2* wild-type gene, and plasmids pSIR2-H364Y and pSIR2-G262A, which carry *sir2* alleles coding for deacetylase-defective enzymes. Yeast cells were either heat-shocked at 55°C for 2 hr as described above or treated with 100 mM H₂O₂ for 30 min, serially diluted, and spotted onto YPD plates.

(D) Mutation frequency over time measured as *can^R* mutants/10⁶ cells of wild-type (DBY746) and *sir2Δ* mutants. The average of six experiments is shown. Error bars show SEM.

(E) Heat and oxidative stress resistance of wild-type (DBY746), *sch9Δ* and *cyr1::mTn* mutants, *sch9Δsir2Δ* and *cyr1::mTnsir2Δ* double mutants, *sch9Δsir2ΔhmraΔ* and *cyr1::mTnsir2ΔhmraΔ* triple mutants. Cells were either heat-shocked at 55°C for 90–120 min or treated with menadione as described above (A).

(F) Mutation frequency over time measured as fold-increase relative to frequency of *can^R* mutants measured at day 1 of wild-type (DBY746), *sch9Δ*, *sch9Δsir2Δ*, and *sch9Δsir2ΔhmraΔ* mutants. The plot represents the average of four experiments; error bars show SEM. The average mutation frequencies at day 1 were 1.05/10⁶ cells (wt), 1.08/10⁶ cells (*sch9Δ*), 0.71/10⁶ cells (*sch9Δsir2Δ*), and 1.53/10⁶ cells (*sch9Δsir2ΔhmraΔ*).

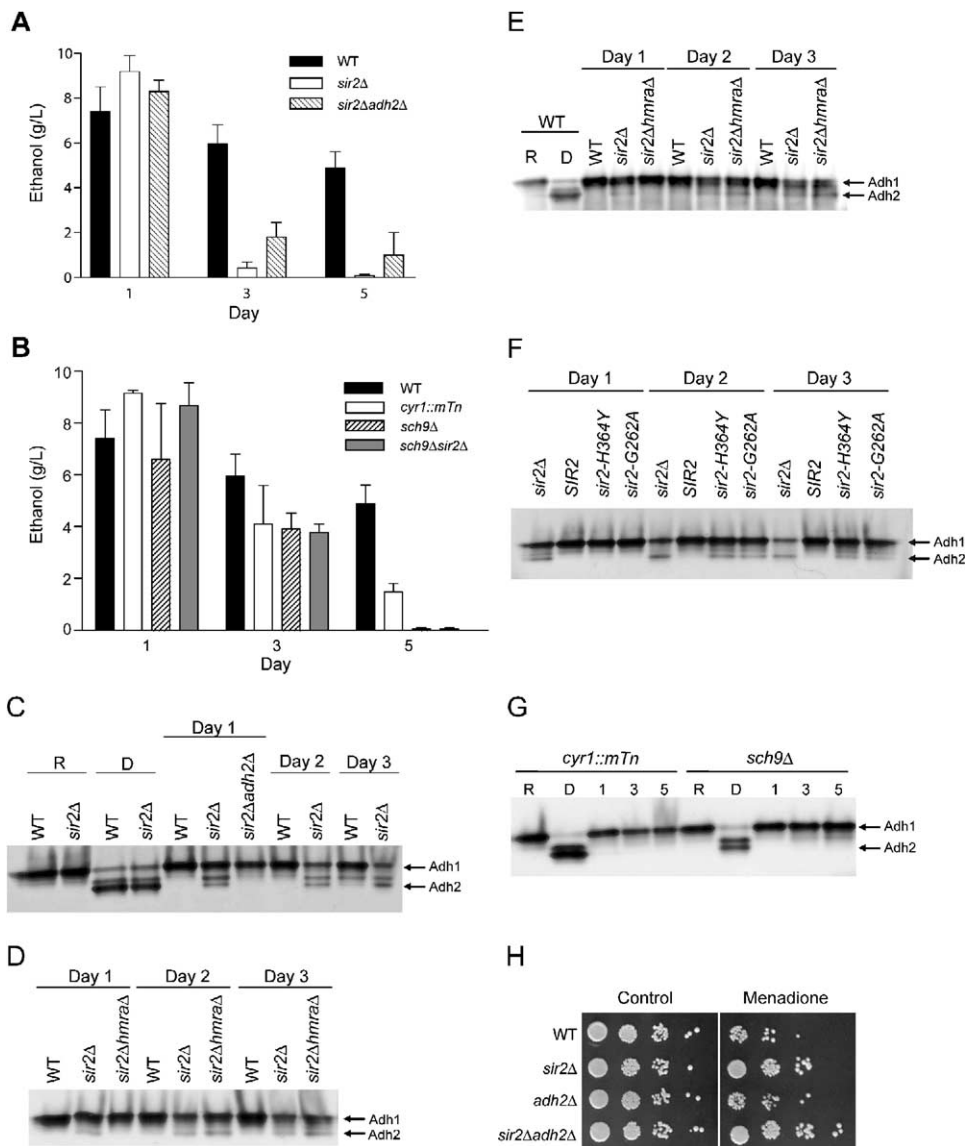


Figure 4. Sir2 Affects Ethanol Catabolism in Part by Inhibiting Adh2 Activation

(A) Ethanol concentration in expired media obtained from day 1–5 cultures of wild-type (DBY746) and mutants lacking either Sir2 or both Sir2 and Adh2.

(B) Ethanol concentration in expired media obtained from day 1–5 cultures of wild-type (DBY746), *sch9Δ*, *sch9Δsir2Δ*, and *cyr1::mTn* mutants. Plots represent the average of 5–8 experiments. Error bars show SEM.

(C) Adh activity in whole-cell extracts obtained from wild-type and *sir2Δ* mutants grown in medium containing glucose (repressed) or ethanol (derepressed) or from day 1–3 postdiauxic wild-type and *sir2Δ* cultures. The panel shows the relevant portion of the native gel after staining for Adh activity. The slower-migrating band corresponds to the Adh1 isozyme (tetramer), the faster-migrating band corresponds to the Adh2 isozyme (tetramer), and the intermediate band corresponds to a hybrid protein (2 Adh1 subunits and 2 Adh2 subunits).

(D) Adh activity in wild-type, *sir2Δ*, and *sir2ΔhmraΔ* mutants at days 1–3.

(E) Western blot analysis of Adh2 in wild-type under repression and derepression conditions and of wild-type, *sir2Δ*, and *sir2ΔhmraΔ* mutants at days 1–3. Proteins were separated on a native gel and Adh2 was detected using an anti-alcohol dehydrogenase polyclonal antibody.

(F) Adh activity in native whole-cell extracts obtained from day 1–3 postdiauxic *sir2Δ* mutants and *sir2Δ* mutants transformed with pSIR2, pSIR2-H364Y, and pSIR2-G262A.

(G) Adh activity in *sch9Δ* and *cyr1::mTn* mutants grown under repressing and derepressing conditions and at days 1–3–5.

(H) Oxidative stress resistance of day 3 wild-type (DBY746) and wild-type cells lacking Sir2, Adh2, or both. Cells were treated with 200 μ M of menadione for 1 hr. A representative experiment is shown.

deacetylase-deficient proteins (Figure 4F), indicating that the Sir2-dependent deacetylation is required to prevent increased Adh2 activity, although this effect may be indirect. Interestingly, the long-lived *sch9Δ* and *cyr1* mutants, which depleted extracellular ethanol by

day 5 instead of day 3, did not show any Adh2 activity between days 1 and 5 (Figure 4G), suggesting that other enzymes break down ethanol during the survival phase. The role of other enzymes in age-dependent ethanol depletion is also supported by the inability of

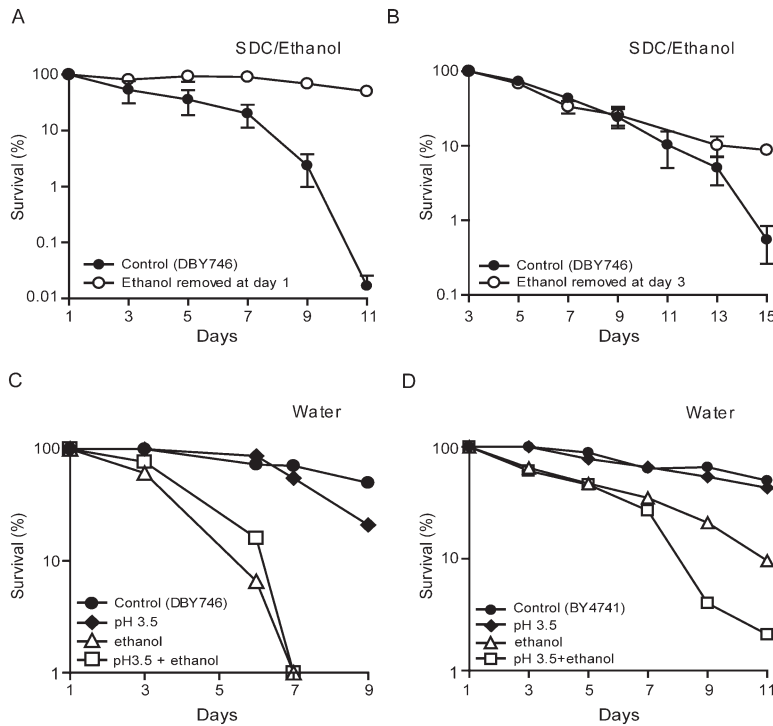


Figure 5. Ethanol Is a Mediator of Yeast Chronological Aging

(A and B) Chronological life span of wild-type (DBY746) cells incubated in either regular or ethanol-depleted SDC medium. Yeast were switched to ethanol-depleted medium at day 1 (A) or day 3 (B). Ethanol was removed by evaporation in a vacuum centrifuge. Both experiments were performed twice with similar results.

(C and D) Chronological survival of wild-type DBY746 and BY4741 yeast incubated in water (CR), water adjusted to pH 3.5, water and ethanol in concentration equal to that commonly found in day 1 expired media (~8 g/l), water adjusted to pH 3.5 and ethanol (8 g/l). Yeast cultures were switched from regular SDC to water at day 1 and washed with water every 2 days to remove nutrients released from dead cells. Ethanol content was monitored and adjusted to 8 g/l every 2 days.

the deletion of *ADH2* to cause a complete reversal of the effect of *sir2Δ* mutations on ethanol catabolism (Figure 4A).

To test whether the removal of ethanol from the incubation medium was required to induce the stress resistance observed in the *sir2Δ* mutants, we monitored both oxidative and heat resistance of the *sir2Δadh2Δ* mutants, which deplete ethanol at a lower rate compared to the *sir2Δ* mutants (Figure 4H). Our results showed no reversion of the stress-resistance phenotype in the *sir2Δadh2Δ* mutants, suggesting that the two effects caused by deleting the *SIR2* gene, namely the activation of Adh2 and the increased stress resistance, are independent of each other.

Uptake of Extracellular Ethanol Mediates Chronological Life-Span Extension

To understand the role of ethanol on yeast aging and death, we monitored the chronological life span of a DBY746 culture in a medium obtained from a different day 1 DBY746 culture in which ethanol was removed by evaporation. Incubation in ethanol-free medium caused an approximately 2-fold increase of mean chronological life span compared to incubation in regular medium, suggesting that uptake of this nonfermentable carbon source plays a central role in life-span extension by CR (Figure 5A). By contrast, the removal of ethanol at days 3 and 5 caused chronological life-span extension only in a minor portion of the population (Figure 5B and data not shown), indicating that the ethanol depletion dependent changes must occur early to cause a significant mean life-span extension.

To test whether ethanol could reverse the effect of CR on chronological life span, we monitored the survival

of wild-type strains incubated in water containing an amount of ethanol comparable to that found in wild-type cultures grown in glucose medium at day 1 (~8 g/l). Our results show that whereas the addition of ethanol is sufficient to reverse the effects of CR, the acidification is not (Figures 5C and 5D). In fact, ethanol reversed the effect of CR in two genetic backgrounds (DBY746 and BY4741) (Figures 5C and 5D). In summary, our results indicate that ethanol triggers the yeast aging and death program. In fact, the depletion of extracellular ethanol also occurs in long-lived *sch9Δ* and *cyr1::mTn* mutants, albeit at a later stage.

Discussion

Increasing the dosage of Sir2 extends the replicative life span of yeast and the chronological life span of *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001) and *Drosophila melanogaster* (Rogina and Helfand, 2004; Wood et al., 2004). However, mice lacking Sirt1 activity show growth defects but also many similarities with long-lived IGF-I-deficient mice (McBurney et al., 2003), raising the possibility that low Sir2-like deacetylase activity may also play an anti-aging function. Our studies in *S. cerevisiae* show that Sir2 blocks entry into an extreme life-span extension phase induced by CR and/or mutations in the Sch9 or Ras/Cyr1/PKA pathways and characterized by depletion of extracellular ethanol, increased resistance to heat shock and oxidative stress, and decreased age-dependent DNA mutations. Whereas the overexpression of Sir2/Sirt1 can cause relatively minor increases in the replicative life span of yeast and in the chronological life span of worms and flies, the deletion of *S. cerevisiae* *SIR2*, in

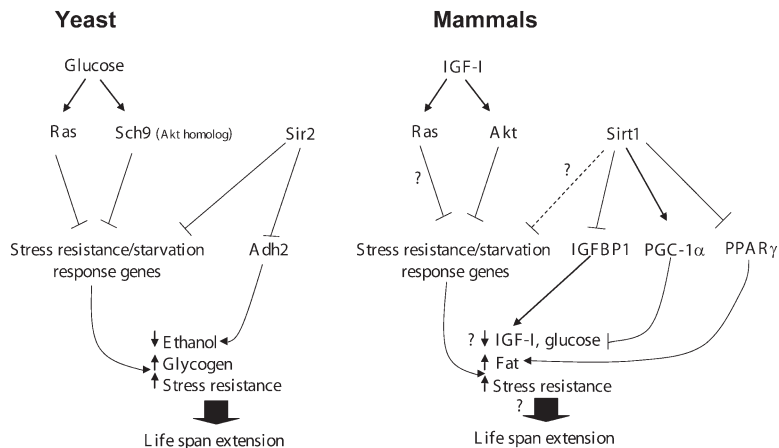


Figure 6. A Model for Sir2- and Sirt1-Dependent Regulation of Stress Resistance and Chronological Life Span

In yeast and mammals, glucose/IGF-I signaling downregulates stress-resistance genes, inhibits the accumulation of glycogen/fat, and promotes aging and death. CR or mutations that decrease the activity of the glucose/IGF-I signaling pathways promote longevity extension. In yeast, Sir2 activity downregulates several stress-resistance, sporulation, and DNA-repair genes whose activity may be fundamental to promote a “hibernation-like” phase and extreme longevity extension. Moreover, Sir2 inhibits the activity of alcohol dehydrogenase 2 (Adh2), a key enzyme for ethanol catabolism, and causes the accumulation of ethanol in the incubation medium, which contributes to reducing survival. In mammalian cells, Sirt1 reduces fat accumulation while promoting the increase of blood glucose levels and may reduce IGF-I signaling via the downregulation of IGFBP-1.

combination with CR and mutations in glucose signaling pathways, causes one of longest chronological life-span extensions reported for any organism. This effect was confirmed in different genetic backgrounds.

We show that cells lacking the Sir2 deacetylase activate the Adh2 enzyme between days 1 and 2 and deplete extracellular ethanol by day three (Figure 4). In a previous study, we showed that aging yeast undergo an altruistic aging program triggered by an unidentified “molecule or factor” in the medium. Our present results suggest that ethanol is a central mediator of the aging and death program. In fact, we show that the addition of ethanol causes a dramatic reduction of life span in two different genetic backgrounds (DBY746 and BY4741) that were severely calorie restricted (Figure 5). Notably, the age-dependent level of ethanol was measured only in *sir2* and long-lived mutants in the DBY746 background. Thus, it will be important to determine whether this mechanism may contribute to aging in different yeast genetic backgrounds as we have demonstrated for the effect of Sir2 and Sch9 on life span (Figures 1 and 2). Considering that ethanol is the major extracellular carbon source utilized by postdiuaxic *S. cerevisiae*, it is possible that ethanol uptake may represent a form of self-induced CR aimed at preparing for severe starvation phases and possibly at preventing the uptake of nutrients by competing organisms. Thus, if the effect of Sir2-like deacetylases in blocking life-span extension under severe CR is conserved in higher eukaryotes, we speculate that it may not involve the regulation of mammalian ADH2 homologs but may instead involve the regulation of enzymes that regulate glucose or fat metabolism (Figure 6).

The age-dependent depletion of extracellular ethanol, which occurs at days 2–3 in *sir2Δ* mutants and at days 5–7 in mutants with inactive Sch9 or Ras2/Cyr1/PKA pathways, is not sufficient to explain the effect of *sir2Δ* mutations on chronological life span. In fact, the removal of ethanol from the medium at day 1 does not extend chronological life span to the level caused by mutations in *SCH9* or CR. We propose that the removal

of ethanol must be accompanied by the induction of many stress-resistance, DNA-repair, and sporulation genes to cause the up to 6-fold chronological life-span extension that we observed in calorie-restricted *sch9Δsir2Δ* double mutants. Consistent with this hypothesis, we have previously shown that stress-resistance proteins Msn2/Msn4, Rim15, and Sod2 play essential roles in chronological life-span extension (Fabrizio et al., 2003; Fabrizio et al., 2001). The further reduction in age-dependent mutations and increase in stress resistance in the *sch9Δsir2Δ* double mutants compared to *sch9Δ* single mutants may depend on the increased expression of many DNA-repair and stress-resistance genes (Table 1). Since the effect of Sir2 on stress resistance appears to be independent of Adh2 activity and ethanol uptake, we conclude that Sir2 may function as a general blocker of many changes required to enter very long-lived phases under starvation conditions.

Increased dosage of Sir2 was shown to extend the yeast replicative life span (Kaeberlein et al., 1999). Sir2 was also shown to mediate the effect of CR on the extension of replicative life span (Lin et al., 2000), although it was later shown that CR could extend replicative life span independently of Sir2 (Kaeberlein et al., 2004). We attribute the discrepancy between the role of Sir2 in extending the yeast replicative and chronological life span to the differential Sir2 deacetylase-dependent regulation of stress-resistance genes and Adh2 activity in the growth and survival phases. In fact, stress resistance and Adh2 activity in *sir2Δ* mutants are elevated beginning at day 1 but are comparable to those of wild-type cells earlier during the growth phase (Figures 3A and 4C). Similarly the lack of *SIR2* increases spontaneous DNA mutation rate during the growth phase but does not affect age-dependent mutation frequency, suggesting that the *sir2Δ* mutation may have deleterious effects during the growth phase but may upregulate systems that under certain conditions can cause a major survival extension (Figure 3F; Table 1). Thus, assuming that similar mechanisms regulated rep-

licative and chronological life span in *S. cerevisiae*, *sir2Δ* mutations would not be expected to extend replicative life span since growing *sir2Δ* mutants have increased genomic instability and wild-type-like levels of stress resistance and Adh2 activity. Furthermore, increased resistance to damage does not appear to delay replicative aging since deletion of stress-resistance factors Msn2/Msn4 does not reverse the replicative life-span extension caused by mutations that mimic CR (Fabrizio et al., 2004b; Lin et al., 2000).

Although, a pro-aging role for Sirt1 has not been demonstrated in higher eukaryotes, several studies are consistent with a potential role for Sirt1 in blocking longevity extension in mammals. Both IGFBP-1-overexpressing (Gay et al., 1997) and *sirt1*^{-/-} deletion mice (McBurney et al., 2003) show many phenotypes observed in the long-lived IGF-I-deficient dwarf mice, including decreased IGF-I levels and decreased body weight and reproductive function. Recent results showing that *sirt1*^{-/-} deletion mice have increased IGFBP-1 expression (Lemieux et al., 2005) raise the possibility that the previously reported phenotypes of *sirt1*^{-/-} mice may be caused by IGFBP-1-dependent inhibition of IGF-I signaling. In fact, Sirt1 inhibits FOXO1 activity through the activation of FHL2 (Yang et al., 2005). Notably, FOXO1 phosphorylation in response to insulin causes inhibition of basal IGFBP-1 transcription (Tomizawa et al., 2000). Analogously to the effect of Sir2 in the depletion of extracellular ethanol in yeast, Sirt1 may regulate the level and/or activity of IGF-I in mammals (Figure 6). Other studies are consistent with a pro-aging role for Sirt1: (1) Motta et al. showed that mammalian Sirt1 represses the DAF-16 homolog FOXO3 and point out that "this regulation appears to be in the opposite direction from the genetic interaction of Sir2 with forkhead in *C. elegans*" (Motta et al., 2004); (2) Picard et al. showed that Sirt1 promotes fat mobilization in mammalian adipocytes (Picard et al., 2004). Although this effect may have a role in preventing certain diseases of aging, the storage and not the mobilization of fat has been consistently associated with longevity extension in worms, flies, and mice (Longo and Finch, 2003); (3) Sirt1 upregulates hepatic glucose release (Rodgers et al., 2005). These characteristics of Sirt1-deficient cells and mice, with the obvious exception of fat storage, are also generally consistent with those of calorie-restricted mice (Longo and Finch, 2003). In summary, the increased fat storage, reduced insulin and glucose levels, and other similarities between the long-lived IGF-I-deficient dwarf mice (Longo and Finch, 2003) and mammalian cells or mice with reduced Sirt1 activity suggest that Sirt1 may play both pro- and anti-aging roles (Figure 6). Notably the potential for low Sir2/Sirt1 deacetylase activity to increase life span is far from straightforward since the deletion of Sir2 in yeast causes severe defects including decreased replicative life span (Kaeberlein et al., 1999), increased growth-dependent mutations (Figure 3D), and asymmetric inheritance of oxidatively damaged proteins (Aguilaniu et al., 2003), and *sirt1*^{-/-} mice die prematurely and are sterile (McBurney et al., 2003). Thus, it is important to test whether, as observed in *S. cerevisiae*, either CR or mutations in the IGF-I/Ras or IGF-I/Akt pathways in combination with the *sirt1*^{-/-} mu-

tation may prevent the growth, sterility, and survival defects and cause longevity extension in mice.

Experimental Procedures

Yeast Strains and Growth Conditions

The majority of the experiments were performed in DBY746 (*MAT α* , *leu2-3, 112*, *his3Δ1*, *trp1-289*, *ura3-52*, *GAL⁺*). Strains BY4741 (*MAT α* , *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and W303AR (*MAT α* , *leu2-3, 112*, *trp1-1*, *ura3-52*, *his3-11*) were used to confirm the results obtained with DBY746. *SIR2* gene disruption was produced by one-step gene replacement using plasmid pJH103.1 (provided by D. Moazed, Harvard University). The *sch9Δ* and *cyr1::mTn* mutants have been described previously (Fabrizio et al., 2001). The *adh2Δ* and *hmraΔ* strains were originated in the DBY746 background by one-step gene replacement according to Brachmann et al. (Brachmann et al., 1998). Strains expressing Sir2 proteins lacking deacetylase activity were originated transforming the *sir2Δ* mutant (DBY746 background) with centromeric plasmids p-sir2-H364Y-LEU2 and p-sir2-G262A-LEU2 carrying mutated forms of *SIR2* coding for deacetylase-deficient enzymes. The control strain was obtained transforming the *sir2Δ* mutant with a centromeric plasmid containing wild-type *SIR2* (p-SIR2-LEU2). All the centromeric *SIR2* plasmids were provided by D. Moazed, Harvard University. Overexpression of *SIR2* in the *sch9Δ* strain was obtained by transformation with plasmid p-SIR2-LEU2.

Chronological life span of cells incubated in either minimal medium containing glucose (SDC) or water was measured as described previously (Fabrizio and Longo, 2003). Briefly, yeast were grown in SDC containing 2% glucose and supplemented with amino acids, adenine, and uracil as described (Kaiser et al., 1994) as well as a 4-fold excess of the supplements tryptophan, leucine, uracil, and histidine. Chronological life span was monitored in expired SDC medium by measuring colony-forming units (CFUs) every 48 hr. The number of CFUs at day 3 was considered to be the initial survival (100%) and was used to determine the age-dependent mortality. For life-span experiments in water (CR), yeast were grown in SDC for 1–3 days, washed with sterile distilled water, and resuspended in water. Yeast cells were washed with water every 48 hr to avoid the accumulation of nutrients released from dead cells.

Survival in Ethanol-Free Medium

The ethanol accumulated in the media obtained from day 1–3 DBY746 cultures was removed by evaporation in a vacuum centrifuge. These ethanol-free expired media were filter-sterilized and used to resuspend day 1–3 DBY746 yeast from different cultures. Viability of the ethanol-free cultures and their relative controls was measured as described above (survival in SDC). The presence of ethanol was monitored in 200 μ l aliquots of expired medium as described below (ethanol measurement).

Survival in Water Containing Ethanol

Experiments were performed as described for the CR survival (see above). Cells were switched from medium to water at day 1, and ethanol was added to the culture at a concentration equal to that found in expired SDC at day 1. The same amount of ethanol was added to the cultures after washing the cells with water every 48 hr. To study the effect of pH on chronological survival, cells were also resuspended in water whose pH had been adjusted to 3.5.

For the Adh activity experiments, repressing growth medium contained 5% glucose (YP5%D) and derepressing growth medium contained 2% ethanol (YPE). Cells were diluted in both media to an initial OD₆₀₀ of 0.1 and harvested when they reached OD₆₀₀ of 0.8 (repression) and 2 (derepression).

Longevity curves were analyzed by either Student's *t* test (*p* < 0.01) or Mann-Whitney (*p* < 0.05) test on the survival rates at each day for each pair of strains.

Stress-Resistance Assays and Mutation-Frequency Measurement

Heat-shock resistance was measured by spotting serial dilutions of cells removed from day 3 postdiauxic phase cultures onto YPD plates and incubating at 55°C (heat-shocked) and at 30°C (control) for 60–120 min. After the heat shock, plates were transferred at 30°C and incubated for 2–3 days.

For oxidative stress-resistance assays, either exponentially growing ($OD_{600} = 1$) or day 3 postdiauxic cells were diluted to an OD_{600} of 0.1 in K-phosphate buffer, pH 7.4, and treated with 20–250 μ M of menadione for 30–60 min. Serial dilutions of untreated and menadione-treated cells were spotted onto YPD plates and incubated at 30°C for 2–3 days. Alternatively, cells were diluted to an OD_{600} of 1 in K-phosphate buffer, pH 6, and treated with 100–200 mM of hydrogen peroxide for 30 min.

Spontaneous mutation frequency was evaluated by measuring the frequency of mutations of the *CAN1* gene. Briefly, overnight inoculations were diluted in liquid SDC medium and incubated at 30°C. Cells' viability was measured every 2 days starting at day 3 by plating appropriate dilutions onto YPD plates. To identify the canavanine-resistant mutants (*can^R*) in the liquid culture, an appropriate number of cells was harvested by centrifugation, washed once with sterile water, and plated on selective medium (SD-ARG, 60 mg/l L-canavanine sulfate). Colonies were counted after 3–4 days.

DNA Microarray Analysis

Log phase or day 3 wild-type, *sir2 Δ* and *sir2 Δ hmr Δ* cultures were used to extract total RNA according to the acid phenol method.

Total RNA from independent cultures of each strain was used as a template to synthesize complementary RNA (cRNA). cRNA was hybridized to GeneChip Yeast Genome S98 array (Affymetrix). Three to six arrays were generated, each obtained using RNA from an independent population of wild-type, *sir2 Δ* , *sir2 Δ hmr Δ* . After excluding the arrays lacking high correlation with other arrays for the same mutant in the probe level (less than 0.88), at least two arrays per strain were analyzed. Background correction was performed using the rma method. Invariant set normalization and "pmonly" PM correction (using perfect match probe as signal) were also performed. Differentially expressed genes were identified (Table 1) using pair wise comparison. 2×2 comparison results in four ratios, which are averaged to get the mean fold change (FC). Bioconductor affy package software was used for the analysis (<http://www.bioconductor.org/>).

The change in expression level between a baseline and an experimental array was included in Table 1, when there was at least a fold change of two in the *sir2 Δ* versus wt comparison at day 3 (He et al., 2005).

Ethanol Measurement and Alcohol Dehydrogenase Activity

Two hundred microliters of yeast cultures were centrifuged and supernatants frozen at -20°C . Ethanol concentration was analyzed enzymatically using the Boehringer Mannheim kit in media collected from day 1–7 yeast cultures.

For the alcohol dehydrogenase activity assays, native all cell extracts were prepared by vortexing the cells in ADH buffer (30 mM Tris-Cl, pH 7.5, 85 mM KCl, 3 mM magnesium acetate, 25% glycerol, 0.07% 2-mercaptoethanol) with glass beads three times for 3 min. Extracts were then centrifuged and supernatants collected. Protein content was determined in supernatants and 50 μ g of proteins were loaded on native Tris-Acetate acrylamide gels. Electrophoresis and in situ detection of enzymes by chromogenic staining for Adh activity were performed as previously described (Williamson et al., 1980).

Western Blotting

For detection of Adh2, whole-cell extracts were obtained as described in the previous section and proteins were separated on a native Tris-Acetate acrylamide gel. Proteins were transferred to a PVDF membrane (Millipore) overnight at 4°C and blocked in 3% nonfat dried milk. The resulting membrane was incubated at 4°C overnight in 1:1000 dilution of anti-alcohol dehydrogenase (Plysciences Inc.).

Supplemental Data

Supplemental Data include two tables and can be found with this article online at <http://www.cell.com/cgi/content/full/123/4/655/DC1/>.

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