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The *SIR2/3/4* complex and *SIR2* alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms

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The *SIR* genes are determinants of life span in yeast mother cells. Here we show that life span regulation by the Sir proteins is independent of their role in nonhomologous end joining. The short life span of a *sir3* or *sir4* mutant is due to the simultaneous expression of a and α mating-type information, which indirectly causes an increase in rDNA recombination and likely increases the production of extrachromosomal rDNA circles. The short life span of a *sir2* mutant also reveals a direct failure to repress recombination generated by the Fob1p-mediated replication block in the rDNA. Sir2p is a limiting component in promoting yeast longevity, and increasing the gene dosage extends the life span in wild-type cells. A possible role of the conserved SIR2 in mammalian aging is discussed.

In the budding yeast *Saccharomyces cerevisiae*, the *SIR* (silent information regulator) genes serve several functions. *SIR1*, *SIR2*, *SIR3*, and *SIR4* were first identified as necessary components for the transcriptional repression of the silent mating type loci, *HML* and *HMR* (Ivy et al. 1986; Rine and Herskowitz 1987). Loss of silencing at these loci results in coexpression of **a** and α mating type genes and sterility in haploid strains. An additional function of *SIR2*, *SIR3*, and *SIR4* was demonstrated by showing that reporter genes positioned at telomere-proximal sequences exhibit positional effect variegation (PEV) of gene expression (Gottschling et al. 1990). Sir3p and Sir4p can be visualized at telomeric locations microscopically, and *sir* mutations result in a loss of PEV, telomere shortening, and the constitutive expression of telomeric reporter genes (Aparicio et al. 1991; Palladino et al. 1993).

A growing body of evidence suggests that the Sir proteins are also involved in nonhomologous end joining (NHEJ), which is used to repair breaks in DNA by ligation of the free ends (for review, see Critchlow and Jackson 1998). In mammalian cells, DNA protein kinase and KU70/85 carry out NHEJ, which can occur in response to double strand breaks. In yeast, *HDF1* and *HDF2* encode Ku70p and Ku80p and, together with other genes including *DNL4*, *XRS2*, *MRE11*, and *RAD50*, are required for NHEJ (Boulton and Jackson 1996a,b; Moore and Haber 1996; Teo and Jackson 1997). Sir4p interacts with Hdf1p in a two-hybrid assay, and efficient NHEJ

requires *SIR2*, *SIR3*, and *SIR4* (Tsukamoto et al. 1997; Boulton and Jackson 1998). Furthermore, genetic and biochemical experiments imply a direct role for the Sir proteins and Hdf1p in NHEJ (Martin et al. 1999; Mills et al. 1999). Immunofluorescence and chromatin immunoprecipitation have shown that Sir3p, as well as Hdf1p, relocalizes from the telomeres to sites of double-strand breaks created by the *Eco*RI or HO endonucleases (Martin et al. 1999; Mills et al. 1999).

In addition to its roles in *HM* silencing, telomeric silencing, and NHEJ, Sir2p also functions at the ribosomal DNA (rDNA) locus. In yeast, the rDNA consists of a 9.1 kb unit that is tandemly repeated 100–200 times on chromosome XII (Petes and Botstein 1977; Philippsen et al. 1978; Rustshenko and Sherman 1994). Each unit contains genes encoding the 35S rRNA and the 5S rRNA, separated by a nontranscribed spacer (NTS). Transcription of these genes and ribosome assembly takes place in a subnuclear compartment called the nucleolus (Scheer and Benavente 1990, Melese and Xue 1995; Shaw and Jordan 1995). *SIR2* was initially shown to play a role in the suppression of mitotic recombination in the rDNA (Gottlieb and Esposito 1989). More recently, *SIR2* was shown to be required for transcriptional silencing of reporter genes integrated at the rDNA (Bryk et al. 1997; Smith and Boeke 1997). The majority of cellular Sir2p, as assayed by immunofluoresence, is found in the nucleolus (Gotta et al. 1997), and the accessibility of rDNA chromatin is responsive to Sir2p dosage (Fritze et al. 1997).

Recently, it was demonstrated that a cause of aging in yeast is the accumulation of circular species of rDNA

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(Sinclair and Guarente 1997). These extrachromosomal rDNA circles (ERCs) are able to replicate via an ARS sequence contained within the rDNA repeat, and are preferentially segregated to mother cells during division. Deletion of *RAD52*, a gene required for homologous recombination, results in the absence of ERCs, thereby implicating a recombinational repair process in their formation (Park et al. 1999).

Replication within the rDNA repeats is blocked in one direction at a site in the NTS termed the replication fork barrier or RFB (Brewer and Fangman 1988). Thus, rDNA replication is unidirectional, with the replication fork progressing in the same direction as transcription of the 35S rRNA (Brewer et al. 1992; Kobayashi et al. 1992). The replication block at the RFB requires the *FOB1* gene (Kobayashi and Horiuchi 1996), and Fob1p has been shown to localize to the nucleolus (Defossez et al. 1999). Strains with *fob1* mutations have a reduced rate of rDNA recombination (Kobayashi et al. 1998) and ERC formation, and have an extended life span compared with wild-type cells. (Defossez et al. 1999). The *FOB1*-dependent replication block may cause a DNA double-strand break within the rDNA. This break can be repaired by homologous recombination, which results in the formation of an ERC (Gangloff et al. 1996; Defossez et al. 1999; Johnson et al. 1999).

Interestingly, Sir3p and Sir4p both relocalize from telomeres to the nucleolus in aging yeast cells (Kennedy et al. 1997). In addition, the *sir4-42* allele results in both a 30% increase in life span (Kennedy et al. 1995) and a constitutive redistribution of Sir3p and Sir4p to the nucleolus in young cells (Kennedy et al. 1997). These observations led to a model proposing that Sir3p and Sir4p move to the nucleolus to delay an event that leads to the accumulation of ERCs and ultimately, cell death. Taken together with the role of the *SIR* genes in DNA damage repair, the question that emerges is: Do the Sir proteins move to the nucleolus via an active mechanism to forestall or repair DNA damage and thereby slow the aging process?

In this paper, we investigate the roles of *SIR2*, *SIR3*, and *SIR4* in yeast aging. Our results indicate that the *sir3Δ* and *sir4Δ* mutations cause a moderate shortening of haploid life span, which is due to derepression of the silent mating type loci and the simultaneous expression of both **a** and α mating-type information. In contrast, the *sir2Δ* mutation has a much more severe effect on life span, which is largely due to an inability to suppress the generation of ERCs. Genetic analysis suggests that the primary role of Sir2p is to prevent homologous recombination at the blocked replication fork in the rDNA. Over-expression of *SIR2* increases life span beyond that of wild type, showing that Sir2p is a limiting component of the aging process in yeast.

Results

The SIR2/3/4 complex regulates life span

To examine roles of the Sir proteins in yeast aging, null alleles of either *sir2*, *sir3*, or *sir4* were introduced into a

W303 MAT**a** RAD5 strain (*W303R*). Disruption of either *sir3* or *sir4* resulted in a 20% decrease in mean life span (Fig. 1A). In contrast, the *sir2* mutant strain demonstrated a more severe reduction in mean life span of ~50% (Fig. 1A). To ascertain whether the short life span of a *sir* mutant could be the result of a defect in NHEJ, *hdf1* and *hdf2* were disrupted in *W303R*, and the life spans of the mutants were determined. Both *hdf* mutants had life spans that were significantly shorter than wild type (Fig. 1B). Thus, these observations initially raised the possibility that the short life span of *sir* mutants was due to a defect in NHEJ.

Because *hdf1* and *hdf2* mutants are also defective for telomere maintenance and are temperature sensitive (Feldmann et al. 1996; Boulton and Jackson 1996a,b; Barnes and Rio 1997; Gravel et al. 1998), we wished to measure the life span of a mutant specifically defective for NHEJ, without the pleiotropic effects associated with *hdf* mutations. The yeast ligase IV gene, *DNL4*, is required for NHEJ (Schar et al. 1997; Teo and Jackson 1997; Wilson et al. 1997), but is not required for growth at 37°C in the *W303R* strain background (not shown). The life span of the *dnl4* mutant was indistinguishable from wild type (Fig. 1C), demonstrating that NHEJ does not affect aging, and suggesting that the short life span of a *sir* mutant is not caused by a defect in NHEJ.

The short life span of sir3 and sir4 mutant haploid strains is due to the simultaneous expression of **a** and α mating-type genes

In addition to their role in NHEJ, the *SIR* genes are also required for transcriptional repression. Mutation in any of the *sir* genes results in a loss of silencing at the *HM* loci, coexpression of **a** and α mating-type genes, and a corresponding sterility phenotype in haploid *S. cerevisiae*. This raised the possibility that the short life span of *sir* mutants was due to the inappropriate expression of loci that are normally silenced in a *SIR+* strain (e.g., see Astrom et al. 1999). Therefore, we wished to test whether the simultaneous expression of **a** and α mating-type genes affected life span.

HMLα was deleted from each of the *sir* mutant strains above. As expected, deletion of *HMLα* resulted in a restoration of the **a** mating phenotype, as demonstrated by the ability of the *sir hml* strains to mate with a *MATα* strain (not shown). Strikingly, haploid *sir4 hml* and *sir3 hml* strains showed a significant extension of mean life span relative to the *sir4* and *sir3* strains (Fig. 2A,B), and were indistinguishable from *W303R*. In contrast, the *sir2 hml* strain had a life span that was indistinguishable from the *sir2* strain (Fig. 2C). Deleting *HML* from the wild-type parent did not alter the life span (not shown). Similarly, the *sir4* gene was disrupted in an isogenic strain of mating type α. The *HMR***a** locus was then deleted from this strain, restoring the ability of these cells to mate. Once again, a 20% shortening of life span was observed in the *sir4* mutant, and this life span defect was suppressed by deletion of *hmr* (not shown). Thus, it appears that the life span defect caused by a *sir4* or *sir3*

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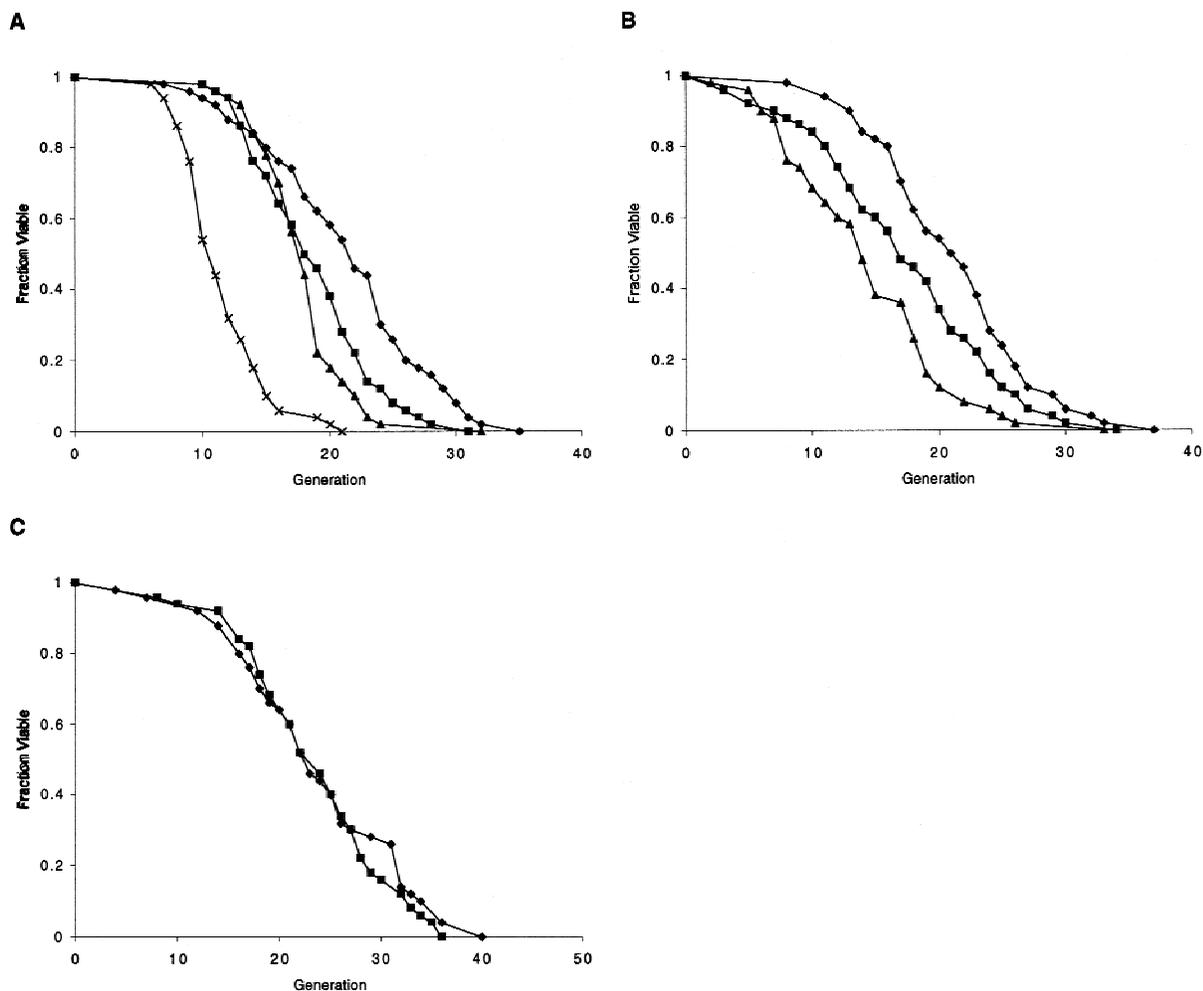
Figure 1. *SIR2*, *SIR3*, and *SIR4*, but not NHEJ, are required for longevity of haploid mother cells. (*A*) Mutation of either *sir3* or *sir4* results in a 20% reduction in mean life span. Mutation of *sir2* reduces mean life span by ~50%. Life spans were determined for *W303R*, and isogenic *sir4Δ*, *sir3Δ*, and *sir2Δ* mutants. Mean life spans and the number of mothers examined were as follows: *W303R* 21.4 (*n* = 50), *sir4Δ* 18.8 (*n* = 50), *sir3Δ* 18.0 (*n* = 50), and *sir2Δ* 11.6 (*n* = 50), (♦) *W303R*; (■) *sir4*; (▲) *sir3*; (x) *sir2*. (*B*) Mutation of *hdf1* or *hdf2* shortens life span. Life spans were determined for haploid *W303R*, *hdf1Δ*, and *hdf2Δ* strains. Mean life spans and the number of mothers examined were as follows: *W303R* 21.2 (*n* = 50), *hdf1Δ* 17.3 (*n* = 50), and *hdf2Δ* 14.3 (*n* = 50), (♦) *W303R*; (■) *hdf1*; (▲) *hdf2*. (*C*) Mutation of *dnl4* does not affect life span. Life spans were determined for haploid *W303R* and *dnl4Δ* strains. Mean life spans and the number of mothers examined were as follows: *W303R* 23.7 (*n* = 50) and *dnl4Δ* 23.4 (*n* = 50), (♦) *W303R*; (■) *dnl4*.

mutation, but not a *sir2* mutation, is due to derepression of the *HM* loci.

To determine whether mating-type heterozygosity in an otherwise wild-type background (*SIR+*) is sufficient to reduce haploid longevity, we directly compared the life spans of the *sir4*, *sir3*, and *W303R* strains with an isogenic strain with *MATα* integrated at the *URA3* locus (*W303R***a**/α). The mean life span of the *W303R***a**/α strain was significantly shorter than the wild-type strain and indistinguishable from the *sir3* and *sir4* strains (Fig. 2D). Thus, simultaneous expression of **a** and α mating-type information causes a short life span even in the presence of a functional Sir2/3/4 complex.

To further test the hypothesis that mating-type heterozygosity shortens life span, we constructed homozygous *sir4/sir4* and *sir2/sir2* mutant diploids. The life

span of the homozygous *sir4/sir4* diploid was indistinguishable from the wild-type diploid, and ~20% shorter than isogenic **a** or α haploid strains (Fig. 3A). We have found previously in other strain backgrounds that the life spans of **a**/α diploids and isogenic haploids were not significantly different (Kennedy et al. 1995; Park et al. 1999). The effect of **a**/α heterozygosity in diploid cells therefore appears to be strain specific, for reasons that are presently unclear.

In contrast, the homozygous *sir2/sir2* diploid displayed a life span significantly shorter than the wild-type or *sir4/sir4* homozygous strains (Fig. 3B), consistent with the conclusion that the short life span of the *sir2* mutant is not related to cell mating type. Interestingly, the *sir2/SIR2* heterozygous diploid had a life span that was much closer to the *sir2/sir2* homozygote than to wild type,
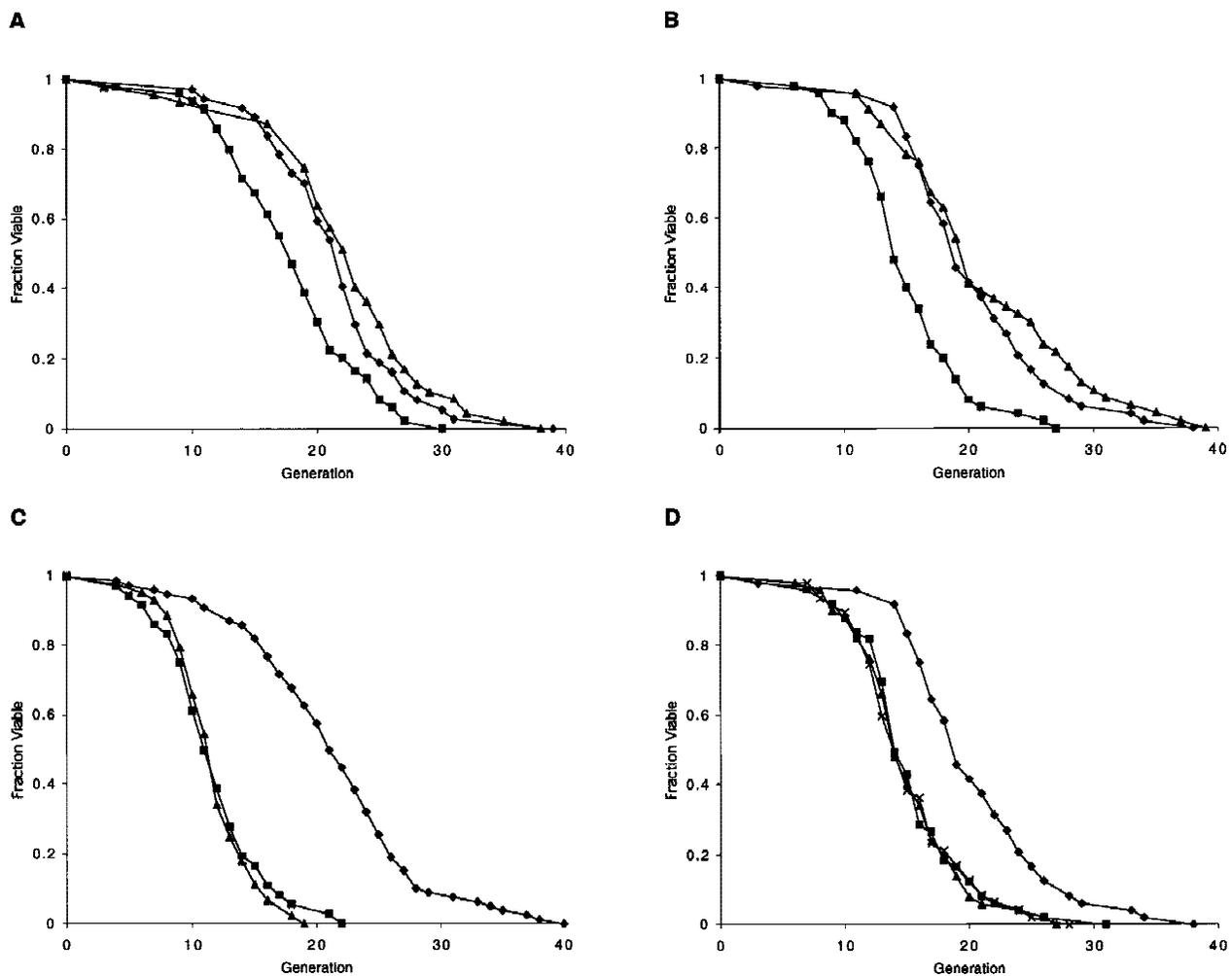
Figure 2. The Sir2/3/4 complex extends life span by silencing the *HM* loci and preventing **a**/α coexpression. (*A*) The life span defect of a *W303R MAT***a** *sir4Δ* mutant is suppressed by deletion of *hmlα*. Life spans were determined for wild-type *W303R*, and isogenic derivatives, *sir4Δ* and *sir4Δ hmlΔ*. Mean life spans and the number of mothers examined were as follows: *W303R* 21.6 (*n* = 37), *sir4Δ* 18.2 (*n* = 49), and *sir4Δ hmlΔ* 22.4 (*n* = 48). (◆) *W303R*; (■) *sir4*; (▲) *sir4 hml*. (*B*) The life span defect of a *W303R MAT***a** *sir3Δ* mutant is suppressed by deletion of *hmlα*. Life spans were determined for *W303R*, and isogenic derivatives, *sir3Δ* and *sir3Δ hmlΔ*. Mean life spans and the number of mothers examined were as follows: *W303R* 20.3 (*n* = 48), *sir3Δ* 15.2 (*n* = 47), and *sir3Δ hmlΔ* 21.3 (*n* = 46). (◆) *W303R*; (■) *sir3*; (▲) *sir3 hml*. (*C*) The short life span of a *W303R MAT***a** *sir2Δ* mutant is not suppressed by deletion of *hmlα*. Life spans were determined for wild-type *W303R*, and isogenic derivatives, *sir2Δ*, and *sir2Δ hmlΔ*. Mean life spans and the number of mothers examined were as follows: *W303R* 21.4 (*n* = 58), *sir2Δ* 11.8 (*n* = 48), and *sir2Δ hmlΔ* 11.9 (*n* = 43). (◆) *W303R*; (■) *sir2*; (▲) *sir2 hml*. (*D*) Mating-type heterozygosity is sufficient to shorten haploid life span to the level of a *sir3Δ* or *sir4Δ* mutant. Life spans were determined for wild-type *W303R*, and isogenic derivatives, *sir3Δ*, *sir4Δ*, and *W303R URA3/MAT*α. Mean life spans and the number of mothers examined were as follows: *W303R* 20.4 (*n* = 48), *sir3Δ* 15.2 (*n* = 47), *sir4Δ* 15.4 (*n* = 49), and *W303R URA3/MAT*α 15.2 (*n* = 48). (◆) *W303R*; (■) *sir4*; (▲) *sir3*; (×) *W303***a**/α.

suggesting that the levels of Sir2p are limiting for life span, at least in diploid strains.

The short life span of sir2, sir3, *or* sir4 *haploids is likely due to an increase in the rate of ERC formation*

As deleting *HML*α extends the life span of a *sir3* or a *sir4* mutant, but fails to suppress the life span defect of a *sir2* mutant, Sir2p must function to forestall senescence by a mechanism(s) that does not involve silencing at *HM* loci. Sir2p is known to localize to the rDNA independently of

Sir3p and Sir4p, in which it functions to repress homologous recombination (Gottlieb and Esposito 1989). Because homologous recombination is absolutely required for ERC formation (Park et al. 1999), the short life span of a *sir2* mutant is likely to be caused, at least in part, by an increased rate of ERC formation. As a direct test of this hypothesis, we quantitated ERC levels in age-matched *W303R* and the isogenic *sir2 hml* mutant cells. In both young (unsorted) and old (sorted) cells, the *sir2 hml* strain accumulated a greater number of high molecular weight ERCs than *W303R* (Fig. 4). The fact that we can

A

B
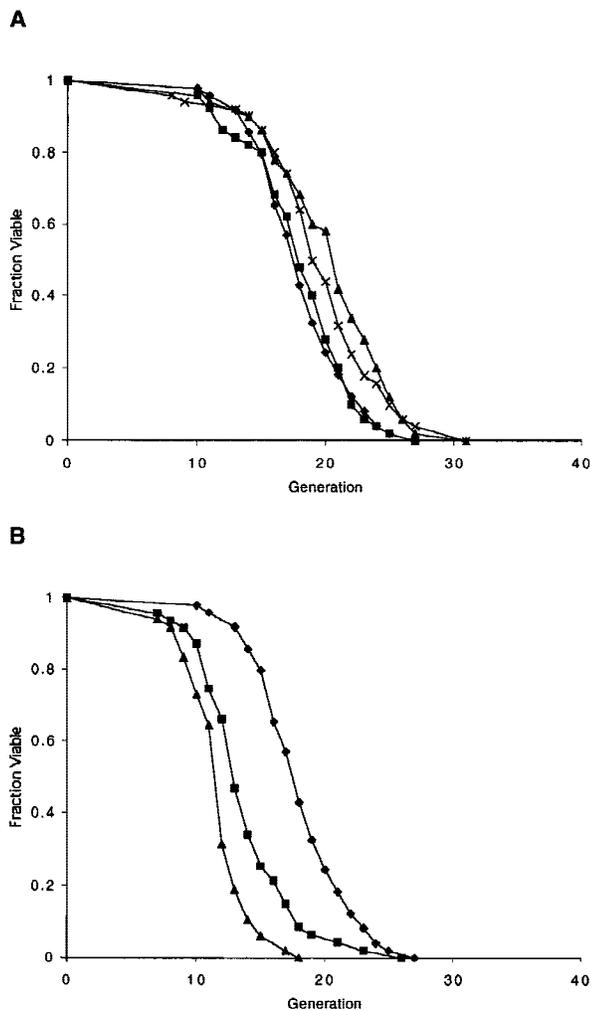
Figure 3. *SIR2*, but not *SIR4*, is required for longevity in diploid cells. (*A*) Diploid life span is not regulated by *SIR4*. Life spans were determined for *W303R/W303R*, the isogenic derivative, *sir4Δ/sir4Δ*, *W303R MATα*, and *W303R MAT***a**. Mean life spans and the number of mothers examined were as follows: *W303R/W303R* 18.2 (*n* = 49), *sir4Δ/sir4Δ* 18.1 (*n* = 50), *W303R MATα* 21.1 (*n* = 50), and *W303R MAT***a** 20.5 (*n* = 50). (◆) *W303R/W303R*; (■) *sir4/sir4*; (▲) *W303R MATα*; (×) *W303R MAT***a**. (*B*) Homozygous mutation of *sir2* causes a significant reduction in diploid life span. The *sir2Δ/SIR2* heterozygote has a life span that is intermediate between the wild-type diploid and the *sir2Δ/sir2Δ* homozygote. Life spans were determined for *W303R/W303R*, and isogenic derivatives, *sir2Δ/SIR2*, and *sir2Δ/sir2Δ*. Mean life spans and the number of mothers examined were as follows: *W303R/W303R* 18.8 (*n* = 50), *sir2Δ/SIR2* 14.0 (*n* = 47), and *sir2Δ/sir2Δ* 12.1 (*n* = 48). (◆) *W303R/W303R*; (■) *sir2/SIR2*; (▲) *sir2/sir2*.

detect more ERCs in an unsorted population of *sir2 hml* cells, relative to wild-type, indicates that a significant fraction of the *sir2 hml* cells form an ERC in an early mother cell division. This suggests that increased homologous recombination at the rDNA in a *sir2* mutant directly results in the formation of ERCs, which shortens the life span.

We were not able to observe a clear increase in the levels of ERCs in *sir3* or *sir4* mutants relative to wild type (not shown). This does not rule out the possibility that *sir3* or *sir4* mutations increase ERC formation, because an undetectable difference in levels of ERCs might be able to cause the small difference in the life spans of these *sir* mutants relative to wild type. As a more accurate measure of recombination rates in the rDNA, we measured the loss rate of the *ADE2* marker integrated into a single rDNA repeat. This marker was lost at a two- to threefold higher frequency in a *sir3* or *sir4* mutant strain compared with wild type (Table 1). Furthermore, this elevated rate of rDNA recombination was suppressed by deleting *HMLα* to restore the **a** mating type. These data suggest that the short life span in the *sir3* or *sir4* nonmating haploids is caused by a small increase in recombination at the rDNA due to the expression of both **a** and α mating-type genes.

Also consistent with the notion that *sir3* and *sir4* mutants have an elevated rate of ERC formation, the release of an ERC into wild-type or *sir4* mother cells caused an identical shortening of life span. For this experiment, we used the method of Sinclair and Guarente (1997) to re-
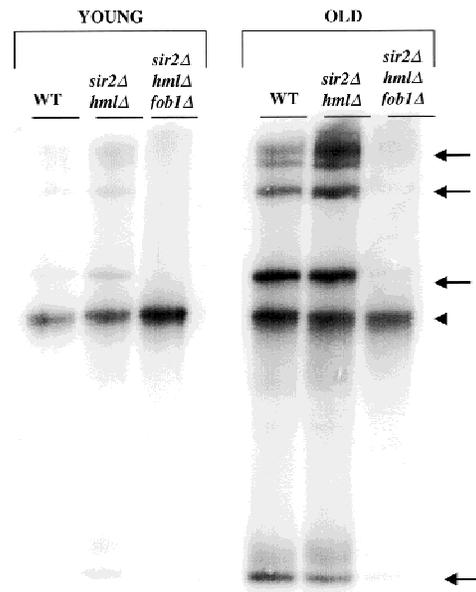
Figure 4. *FOB1*-dependent accumulation of ERCs occurs more rapidly in a *sir2* mutant than in wild-type mother cells. DNA from young (unsorted) and old (mean age = 7 generations) mother cells was isolated and electrophoresed as described (Defossez et al. 1999). The gel was transferred and probed with rDNA. Chromosomal DNA is denoted by an arrowhead and ERC species by arrows. Both sorted and unsorted *W303R sir2Δ* mother cells show a greater accumulation of higher oligomeric species of ERCs than wild type. Mutation of *fob1* in *sir2Δ hmlΔ* mother cells decreases ERC accumulation below levels observed in wild-type cells. Intensity of bands was quantitated on a PhosophorImager. The ratio of ERC DNA to genomic rDNA in young wild-type and *sir2Δ hmlΔ* cells is 0.2 and 0.4, respectively. In old wild-type and *sir2Δ hmlΔ* cells, this ratio is 2.1 and 3.1, respectively.

Table 1. *Mating-type heterozygosity increases rDNA marker loss of the* W303R *haploid—deletion of* fob1 *suppresses the rDNA recombination defect of a* sir2 hml *mutant*

Strain	Rate of rDNA marker loss ($\times 10^3$)
W303R	1.1 (0.1)
sir4	2.3 (0.1)
sir4 hml	0.9 (0.3)
sir2	18.4 (4.6)
sir2 hml	9.0 (0.6)
sir2 fob1 hml	0.8 (0.1)

Strain *W303R* carries a copy of *ADE2* integrated into the rDNA (see Materials and Methods for a discussion of the half sector assay). Mean values for the rate of marker loss are given with standard deviation in parentheses. A total of least 20,000 colonies from three independent isolates were examined for each strain.

lease an ERC into the nucleus of wild-type and *sir4* virgin cells. On release of an ERC, *W303R+ERC* cells had a mean life span that was shortened by ~25% and did not differ significantly from the *sir4+ERC* strain (Fig. 5). This finding indicates that the *sir4* (and presumably *sir3*) mutation does not affect the rate of accumulation of ERCs and strengthens the idea that it increases the rate of formation of ERCs.

Deletion of FOB1 *suppresses the rDNA hyperrecombination phenotype and the life span defect of a* sir2 *mutant*

A key gene that regulates rDNA recombination and life
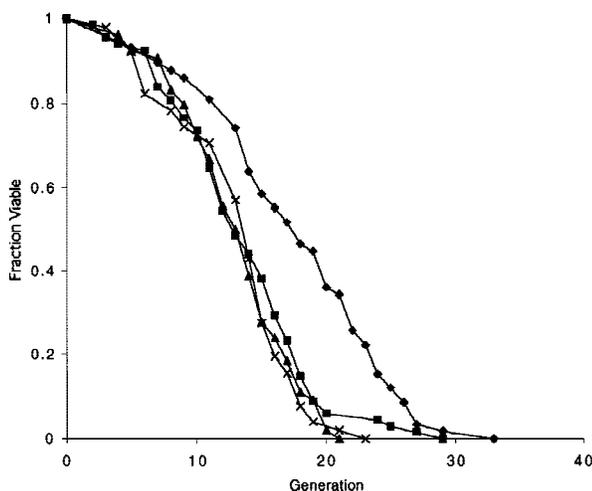
Figure 5. Sir4p does not function to extend life span after release of an ERC. ERCs were released in virgin cells as described (Sinclair and Guarente 1997). Life spans were determined for *W303R*, *W303R* induced to release an ERC (*W303R + ERC*), the isogenic derivative, *sir4*, and *sir4* induced to release an ERC (*sir4 + ERC*). Mean life spans and the number of mothers examined were as follows: *W303R* 17.6 (*n* = 58), *W303R + ERC* 13.5 (*n* = 68), *sir4* 13.5 (*n* = 55), and *sir4 + ERC* 13.0 (*n* = 51). (♦) *W303R*; (■) *W303R + ERC*; (▲) *sir4*; (×) *sir4 + ERC*.

span is *FOB1*. Deletion of *fob1* gives rise to bidirectional rDNA replication (Kobayashi and Horiuchi 1996), decreases the rate of rDNA recombination (Kobayashi et al. 1998), dramatically reduces ERC formation, and extends life span by 30%–60% (Defossez et al. 1999). We therefore wished to examine the relationship between *sir2* and *fob1* with respect to life span, rDNA recombination, and ERC formation.

To eliminate any effects of the *sir2* mutation on *HM* expression, the *sir2 hml* strain was used. When *fob1* was deleted in this strain, the short life span of the *sir2* mutant was largely suppressed, giving rise to a life span extended by ~50% (Fig. 6). The mean and maximum life span of the *sir2 fob1 hml* strain was comparable with *W303R*, but slightly shorter than the *fob1* single mutant. This suppression, along with the experiment described below (Fig. 7B), suggests that *SIR2* and *FOB1* act in the same pathway to determine yeast life span (see Discussion).

Consistent with the life span data, deletion of *fob1* also suppressed the rDNA hyperrecombination phenotype of the *sir2 hml* strain (Table 1). An *ADE2* marker inserted into the rDNA array was lost at an ~10-fold higher rate in the *sir2 hml* strain than in *W303R*. Deletion of *fob1* in this background reduced this high rate of marker loss to approximately that of the *SIR2 FOB1* strain.

To test whether the *fob1* deletion reduced the rate of ERC accumulation in the *sir2* mutant, we determined ERC levels in young and old *sir2 hml fob1* cells. Deletion of *fob1* reduced ERC levels in both young and old cells, even below those seen in *W303R* (Fig. 4). A similarly dramatic reduction in ERCs is observed when *fob1* is mutated in a wild-type background (Defossez et al. 1999). The fact that the *sir2 hml fob1* strain does not live
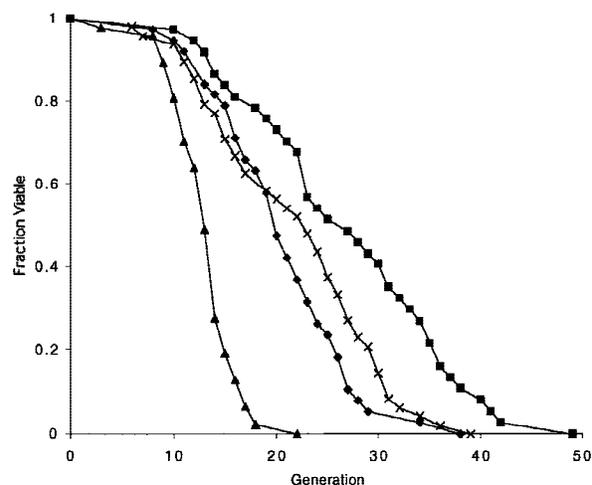
Figure 6. Mutation of *fob1* suppresses the life span defect of a *sir2* mutant. Life spans were determined for *W303R*, and the isogenic derivatives, *sir2Δ hmlΔ*, and *sir2Δ hmlΔ fob1Δ*. Mean life spans and the number of mothers examined were as follows: *W303R* 20.7 (*n* = 38), *fob1Δ* 27.0 (*n* = 37), *sir2Δ hmlΔ* 13.0 (*n* = 47) and *sir2Δ hmlΔ fob1Δ* 21.9 (*n* = 48). (♦) *W303R*; (■) *fob1*; (▲) *sir2 hml*; (×) *sir2 hml fob1*.

significantly longer than *W303R*, even though the levels of ERCs are lower than wild type, suggests that Sir2p may play an additional role as a regulator of longevity.

Increasing the dosage of SIR2 *extends life span*

The above analysis of *SIR2* suggested that this gene played a central role in yeast aging. The short life span of the *SIR2/sir2* heterozygous diploid further suggested that the levels of this protein may be limiting. If this were so, then increasing the levels of Sir2p might extend the life span of wild-type cells.

Thus, a second copy of *SIR2* was integrated into the genome of *W303R* and *W303R sir2* (see Materials and Methods), and the life spans of the resulting transformants were determined. The integrated *SIR2* restored the wild-type life span (Fig. 7A) and mating ability (not shown) to the *sir2* mutant, indicating that the cloned *SIR2* was functional. Strikingly, integration of a second copy of *SIR2* into the wild-type strain gave rise to an ~30% extension of life span (Fig. 7A,B). This finding shows that the levels of Sir2p are limiting for the life span of wild-type mother cells.

The above finding afforded another possibility to test whether *FOB1* and *SIR2* function in the same pathway. If the role of *SIR2* were to prevent recombination at blocked replication forks in the rDNA, then a second copy of *SIR2* should not extend further the elongated life span in a *fob1Δ* strain. Thus, a second *SIR2* copy was integrated into the *fob1Δ* strain and life spans were determined (Fig. 7B). The increased levels of Sir2p did not extend further the long life span in the *fob1Δ* strain,

providing additional evidence that the role of *SIR2* in extending life span involves preventing recombination at the Fob1p-arrested replication fork.

Discussion

The results presented here define two distinct roles of the *SIR* genes as negative regulators of aging in *S. cerevisiae*. First, the *SIR2/3/4* encoded complex acts indirectly to extend haploid life span by repressing transcription at *HML* and *HMR*. In *sir3* and *sir4* mutants, coexpression of the **a** and α mating-ype genes results in an elevated rate of rDNA recombination, likely increasing the rate of formation of ERCs. The short life span of a *sir3* or *sir4* mutant is suppressed by deleting the relevant *HM* locus. Second, *SIR2* acts directly to suppress ERC formation by inhibiting homologous recombination at a blocked replication fork in the rDNA (see below). Thus, the short life span of a *sir2* mutant is not significantly altered by deleting the *HM* loci. Sir2p appears to be a limiting component in yeast aging, because increasing the dosage of this gene extends the life span well beyond that of wild type.

The role of cell type in aging

Several previous experiments led us to propose that the role of the Sir complex in yeast longevity might be to repair damage in the rDNA. First, the wild-type Sir complex redistributes from telomeres to the nucleolus in aging mother cells (Kennedy et al. 1997). Second, the *sir4-*
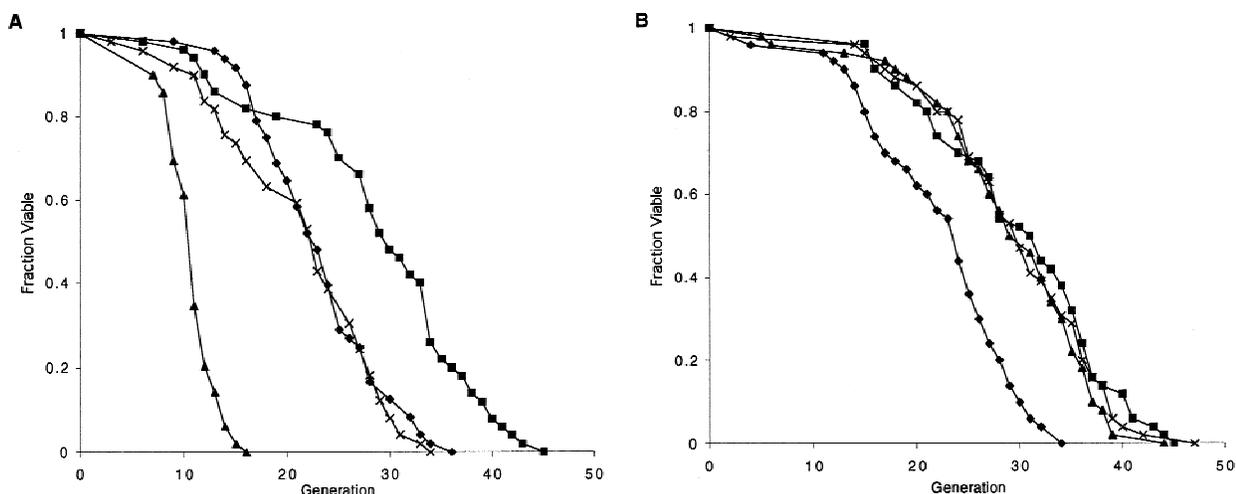
Figure 7. Sir2p is a limiting component of yeast longevity. (*A*) Overexpression of *SIR2* extends the life span of *W303R* by ~30%. A second copy of *SIR2* was integrated at the *URA3* locus (denoted *SIR2/URA3*) of strains *W303R* and *sir2Δ*. Life spans were determined for *W303R*, and the isogenic derivatives *sir2Δ*, *W303R SIR2/URA3*, and *sir2Δ SIR2/URA3*. Mean life spans and the number of mothers examined were as follows: *W303R* 23.0 (*n* = 48), *W303R SIR2/URA3* 28.8 (*n* = 50), *sir2Δ* 11.0 (49), and *sir2Δ SIR2/URA3* 21.3 (49). (◆) *W303R*; (■) *W303R SIR2/URA3*; (▲) *sir2*; (×) *sir2 SIR2/URA3*. (*B*) Overexpression of *SIR2* does not affect the life span of a *fob1* mutant. A second copy of *SIR2* was integrated at the *URA3* locus of *fob1Δ*. Life spans were determined for *W303R*, and the isogenic derivatives *W303R SIR2/URA3*, *fob1Δ*, and *fob1Δ SIR2/URA3*. Mean life spans and the number of mothers examined were as follows: *W303R* 22.2 (*n* = 50), *W303R SIR2/URA3* 29.9 (*n* = 50), *fob1Δ* 29.0 (*n* = 50), and *fob1ΔSIR2/URA3* 29.4 (*n* = 49). (◆) *W303R*; (■) *W303R SIR2/URA3*; (▲) *fob1*; (×) *fob1 SIR2/URA3*.

42 mutation, identified previously as an allele-specific suppressor for the short life span of a strain carrying a carboxy-terminal deletion in *uth4* (Kennedy et al. 1995), results in the constitutive localization of the Sir complex to the nucleolus. One specific possibility was that the Sir complex might function by repairing DSBs in the rDNA via NHEJ. Although one study indicated that the role of the Sir complex in NHEJ was an indirect consequence of the **a**/α cell type (Astrom et al. 1999), more recent studies showed that the Sir complex redistributes directly from telomeres to induced DSBs and likely functions directly in promoting their repair (Martin et al. 1999; Mills et al. 1999).

Mutations in *hdf1* and *hdf2* shorten life span significantly. However, the short life spans of these mutants do not result from a defect in NHEJ. This follows from the fact that the *dnl4* mutant is also defective in NHEJ, but has a wild-type life span. *Hdf* mutants show an aberrant organization of telomeres (Gravel et al. 1998) and are temperature sensitive for growth (Feldmann et al. 1996; Boulton and Jackson 1996a,b; Barnes and Rio 1997). Thus, the short life spans of *hdf1* and *hdf2* strains are caused by defects in processes other than NHEJ, perhaps related to telomere maintenance or function.

Rather, it is the expression of both **a** and α mating-type genes in *sir3* or *sir4* mutants that appears to cause the short life span in these strains. Deletions of the *HM* loci that preserve haploid mating reverse the short life span of *sir3* or *sir4* mutants. Further, homozygous deletion of *sir4* in an **a**/α diploid does not shorten life span.

How might **a**/α expression in haploids shorten life span? It has been suggested that *RAD52*-mediated recombination is increased in cells expressing both **a** and α mating-type genes (Heude and Faber 1993; Schild 1995; Fasullo et al. 1999), thus accounting for the increased resistance of diploid cells to certain DNA damaging agents. Consistent with these findings, we demonstrate an increase in the rate of marker loss from the rDNA due to a mutation in *sir4*, and this increase is suppressed by deleting *HMLα* (thereby preventing **a**/α coexpression). In addition, *RAD52* is absolutely required for the formation of ERCs (Park et al. 1999). Thus, we propose that ERCs are generated at a higher rate in *sir* mutants, and that this increase in ERC formation results in a shortened life span (Fig. 8).

How then can we understand the *sir4-42* mutation and the redistribution of the wild-type Sir complex in old cells? We speculate that the constitutive localization of the *sir4-42* mutant complex to the rDNA might alter the recombinational properties in that locus to forestall the generation of ERCs. In this way, the mutation is really a neomorph and does not correspond to any normal activity of the Sir complex. In addition, accumulation of ERCs in old cells results in an amplification of rDNA sequence, which may titrate the Sir2/3/4p complex to the nucleolus.

A direct role of Sir2p in forestalling aging

The extremely short life span of a *sir2* mutant is not a
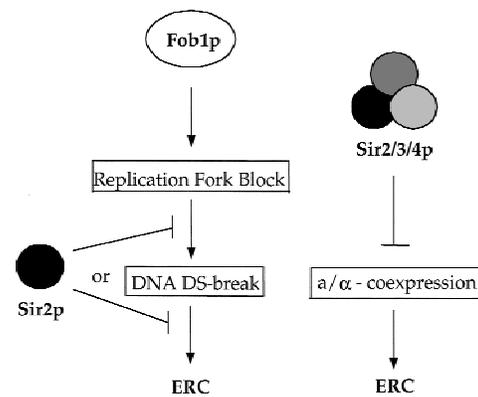
Figure 8. The Sir proteins regulate longevity by reducing the rate of ERC formation by two mechanisms. The Sir2/3/4p complex acts indirectly to inhibit ERC formation by preventing the simultaneous expression of **a** and α mating-type genes. When expressed, the **a**/α cell type increases recombination in the rDNA and, presumably, the formation of ERCs. Sir2p acts by a second mechanism in the rDNA to inhibit Fob1p-mediated formation of ERCs, presumably at blocked replication forks.

consequence of the **a**/α cell type. Rather, elevated levels of rDNA recombination (Gottlieb and Esposito 1989) result in an increase in the rate of formation of ERCs and thus accelerated aging. Strikingly, deletion of the replication fork block protein gene, *FOB1*, largely suppresses the short life span of a *sir2* mutant. This epistasis of *FOB1* to *SIR2* implies that these genes function in the same pathway. The inability of a second *SIR2* copy to extend life span in a *fob1* strain strengthens this view, although we cannot exclude the possibility that *fob1* mutant cells senesce for reasons unrelated to *SIR2* function.

We propose that Sir2p stabilizes blocked replication forks in the rDNA to prevent DNA breaks, recombination, and the generation of ERCs (Fig. 8). In the absence of Fob1p, the replication fork is not blocked in the rDNA, and Sir2p is not required to prevent the formation of ERCs. This suppression of the short life span of *sir2* mutants by mutation in *fob1* contrasts with the short life span in mutants lacking the Sgs1p DNA helicase, which is not suppressed by *fob1* (Defossez et al. 1999). Therefore, unlike the case in a *sir2* mutant, the short life span of an *sgs1* mutant is not caused by a Fob1p-blocked replication fork.

Further support for this model comes from observations that *rad52* or *fob1* mutations suppress the rDNA hyperrecombination phenotype of a *sir2* mutant (Gottleib and Esposito 1989; Table 1). This demonstrates that in the absence of Sir2p, the predominant form of repair at the rDNA is through the *RAD52* pathway of homologous recombination, most likely at the replication fork block. Correspondingly, ERCs are dramatically reduced in *fob1* and *sir2 fob1 hml* mutant strains (Defossez et al. 1999; Fig. 4) and eliminated in a *rad52* strain (Park et al. 1999).

However, because the *sir2 fob1 hml* strain has a shorter life span than the *fob1* mutant, Sir2p must func-

tion to extend life span by an, as yet undefined, additional mechanism. Thus, *FOB1* is only partially epistatic to *SIR2* for life span, but is completely epistatic to *SIR2* for ERC formation. Therefore, we propose that *SIR2* promotes longevity by at least three distinct mechanisms; first, Sir2p functions, along with Sir3p and Sir4p, to silence *HM* loci; second, Sir2p directly inhibits ERC formation by antagonizing homologous recombination at the blocked replication fork in the rDNA; finally, *SIR2* apparently helps extend life span by a third mechanism that is independent of ERCs or cell type.

Sir2p as the limiting component in yeast aging

It is of interest that introduction of a second copy of *SIR2* into the yeast genome extends the life span 30% beyond wild type. Sir2p is, therefore, limiting in wild-type cells, and rDNA recombination at the replication fork block is not fully suppressed. In the *fob1* mutant, in which the replication fork block is absent and life span is extended, a second copy of *SIR2* does not extend life span further. The short life span of a *sir2/SIR2* heterozygous diploid reveals a haplo insufficiency, also indicating that levels of Sir2p are limiting for life span. This is consistent with the observation that rDNA silencing is extremely sensitive to Sir2p dosage (Smith et al. 1998), and suggests that rDNA silencing, rDNA recombination, and life span are all tightly coupled to nucleolar levels of Sir2p.

Of all of the *SIR* genes, *SIR2* is evidently the only one conserved in higher eukaryotes. Whereas the function of *SIR2*-related genes in mammals is not presently known, it is interesting to speculate that an alteration in Sir2p levels or activity may alter the pace of aging in these organisms, as well. If so, a key molecular link in the aging process of many organisms would be highlighted in the form of the function of this gene.

Materials and methods

Strains, plasmids, and media

The yeast strains used in this work are listed in Table 2. Strain *W303R* is described in Mills et al. (1999), in which it is referred to as W303AR5. The *sir3Δ::URA3* disruption was generated as described in Sinclair and Guarente (1997). The *sir4Δ::HIS3*, *sir2Δ::TRP1*, and *hmlΔ::LEU2* disruptions were generated as described in Mills et al. (1999).

All other gene disruptions were generated by targeted PCR disruption. Marker genes were amplified from the appropriate pRS integrating vector (Sikorski and Heiter 1989) with primers designed to incorporate 40 nucleotides of homology to genomic DNA at each end of the disruption cassette. In each case, the entire coding region of the disrupted gene and up to 100 nucleotides of flanking sequence were removed. All disruptants were verified by PCR prior to subsequent analysis. Additionally, *hdf1* and *hdf2* mutations were verified by the inability of these strains to grow at 37°C. Lack of NHEJ in the *dnl4* and *hdf1* mutants was verified by a plasmid end-joining assay as described (Mills et al. 1999).

Integration of *SIR2* at *URA3* was accomplished by transforming cells with plasmid p306SIR2 digested with *Xcm*I. In addition

Table 2. *Yeast strains used in this study*

Strain	Genotype
W303R	*W303-1A MATaRDN1::ADE2 RAD5*
DSY1036	*W303R sir4Δ::HIS3*
DSY880	*W303R sir3Δ::URA3*
DSY1034	*W303R sir2Δ::TRP1*
MMY98	*W303R hdf2Δ::LEU2*
MMY134	*W303R dn14Δ::URA3*
MKY576	*W303R hdf1Δ::URA3*
MKY545	*W303R sir4ΔHIS3 hmlΔ::LEU2*
MKY543	*W303R sir3ΔURA3 hmlΔ::LEU2*
MKY541	*W303R sir2ΔTRP1 hmlΔ::LEU2*
MKY549	*W303R URA3/MATα*
MKY1033	*W303-1A MATα RDN1::ADE2 RAD5 sir4Δ::HIS3*
MKY1046	*MKY1033 hmrΔ::URA3*
MKY547	*MKY545 fob1Δ::URA3*
MKY501	*W303R fob1Δ::URA3*
MKD104	*W303R/W303R MATa/MATα sir4Δ::HIS3/sir4Δ::HIS3 hmlΔ::LEU2/HML hmrΔ::URA3/HMR*
MKD105	*W303R/W303R MATa/MATα hmlΔ::LEU2/HML hmrΔ::URA3/HMR*
MKD106	*MKD105 sir2Δ::TRP1/SIR2*
MKD107	*MKD105 sir2Δ::TRP1/sir2Δ::TRP 1*
MKY546	*MKY541 fob1Δ::URA3*
MMY094	*W303R URA3/SIR2*
MMY095	*W303R sir2Δ::TRP1 URA3/SIR2*
MMY112	*W303R fob1Δ::URA3*
MMY113	*W303R fob1Δ::URA3 URA3/SIR2*

Strain *W303R* is described in Mills et al. (1999). All other strains are isogenic derivatives of *W303R*. The notation *URA3/SIR2* indicates that a single copy of the *SIR2* gene has been integrated at the *URA3* locus.

to the entire coding region of *SIR2*, ~1000 nucleotides of upstream sequence and 200 nucleotides of downstream sequence are present. Plasmid p306SIR2 was constructed by ligating the PCR-amplified *SIR2* gene into the *Xba*I and *Xho*I sites of pRS306.

Unless otherwise stated, all strains were cultured at 30°C and grown on YEPD containing 2% glucose.

Life span analysis

Micromanipulation and life span analysis were performed as described previously (Kennedy et al. 1994), with the following modifications. Prior to analysis, strains were patched onto solid YPD for at least two consecutive days. Cells were then transferred at low density to fresh YPD and allowed to grow for ~3 hr. Virgin daughter cells were then isolated as buds from mother cells and subjected to life span analysis. Each experiment consisted of 37–50 mother cells and was carried out at least twice, independently. Statistical significance was determined by a Wilcoxon rank sum test. Average life span is stated to be different for $P < 0.05$. Each figure represents data derived from a single experiment.

Release of ERCs into virgin daughter cells

The method of Sinclair and Guarente (1997) was used to release an ERC into virgin cells, with the following modifications. Life spans were performed on plates lacking adenine and containing 1% galactose and 2% raffinose. Mother cells whose daughters

formed large colonies on this medium failed to induce the Cre recombinase and were therefore not included in the data set.

Determination of rDNA recombination rate

Strains carrying an *ADE2* marker integrated into the rDNA array were grown overnight and then plated onto solid YPD. Colonies were allowed to grow for 48 hr at 30°C then placed at 4°C for 48 hr. The number of half-red/half-white colonies was determined; each was assumed to represent a marker loss event during the first cell division after plating. The number of half-sectored colonies divided by the total number of colonies (not including entirely red colonies) was reported as the rate of marker loss.

Purification of old cell DNA and ERC analysis

Old cells were obtained by the biotin-labeling procedure (Smeal et al. 1996) and age was determined by counting bud scars (Sinclair et al. 1997). ERC levels were detemined as described (Defossez et al. 1999), with the following modifications. ERC species were separated on a 0.6% agarose gel (without ethidium bromide) run at 1 Volt/cm for 44 hr.

Acknowledgments

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