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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 α 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.

[Key Words: Aging; yeast; SIR; rDNA; recombination; nonhomologous end-joining]

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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 α 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 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, relocates from the telomeres to sites of double-strand breaks created by the EcoRI or HO endonucleases [Martin et al. 1999; Mills et al. 1999].

In addition to its roles in HML 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 [Sheer 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 immunofluorescence, 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...
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 35S rRNA barrier or RFB [Brewer and Fangman 1988]. Thus, rDNA direction at a site in the NTS termed the replication fork barrier or RFB [Brewer and Fangman 1988].

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To examine roles of the Sir proteins in yeast aging, null alleles of either sir2, sir3, or sir4 were introduced into a W303 MATa 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 mutations 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 a 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 a 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 a 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 a. The HMRα 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
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 MATa integrated at the URA3 locus (W303Ra/α). The mean life span of the W303Ra/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, and not a 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.
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 HMLα 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

Figure 2. The Sir2/3/4 complex extends life span by silencing the HM loci and preventing a/a coexpression. (A) The life span defect of a W303R MATα 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 MATα 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 MATα 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 20.4 (n = 48), sir2Δ 15.2 (n = 47), sir2Δ hmlΔ 15.4 (n = 49), and W303R URA3/MATα 15.2 (n = 48). (●) 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/MATα. 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/MATα 15.2 (n = 48). (●) W303R; (■) sir4; (▲) sir3; (×) W303a/a.
We were not able to observe a clear increase in the levels of ERCS in \textit{sir3} or \textit{sir4} mutants relative to wild type (not shown). This does not rule out the possibility that \textit{sir3} or \textit{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 \textit{sir} mutants relative to wild type. As a more accurate measure of recombination rates in the rDNA, we measured the loss rate of the \textit{ADE2} marker integrated into a single rDNA repeat. This marker was lost at a two- to threefold higher frequency in a \textit{sir3} or \textit{sir4} mutant strain compared with wild type (Table 1). Furthermore, this elevated rate of rDNA recombination was suppressed by deleting \textit{HML\alpha} to restore the \(\alpha\) mating type. These data suggest that the short life span in the \textit{sir3} or \textit{sir4} nonmating haploids is caused by a small increase in recombination at the rDNA due to the expression of both \(\alpha\) and \(\alpha\) mating-type genes.

Also consistent with the notion that \textit{sir3} and \textit{sir4} mutants have an elevated rate of ERC formation, the release of an ERC into wild-type or \textit{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 3. SIR2, but not SIR4, is required for longevity in diploid cells.](image)

A) Diploid life span is not regulated by SIR4. Life spans were determined for W303R/W303R, the isogenic derivative, \textit{sir4Δ/sir4Δ}, W303R MAT\(\alpha\), and W303R MAT\(\alpha\). Mean life spans and the number of mothers examined were as follows: W303R/W303R \(18.2 \ (n = 49)\), \textit{sir4Δ/sir4Δ} \(18.1 \ (n = 50)\), W303R MAT\(\alpha\) \(21.1 \ (n = 50)\), and W303R MAT\(\alpha\) \(20.5 \ (n = 50)\). (\(\blacklozenge\)) W303R/W303R; (\(\blacksquare\)) \textit{sir4Δ/sir4Δ}; (\(\blacktriangle\)) W303R MAT\(\alpha\); (\(\blacklozenge\)) W303R MAT\(\alpha\). (B) Homozygous mutation of \textit{sir2} causes a significant reduction in diploid life span. The \textit{sir2Δ/SIR2} heterozygote has a life span that is intermediate between the wild-type diploid and the \textit{sir2Δ/sir2Δ} homozygote. Life spans were determined for W303R/W303R, and isogenic derivatives, \textit{sir2Δ/SIR2}, and \textit{sir2Δ/sir2Δ}. Mean life spans and the number of mothers examined were as follows: W303R/W303R \(18.8 \ (n = 50)\), \textit{sir2Δ/SIR2} \(14.0 \ (n = 47)\), and \textit{sir2Δ/sir2Δ} \(12.1 \ (n = 48)\). (\(\blacklozenge\)) W303R/W303R; (\(\blacksquare\)) \textit{sir2Δ/SIR2}; (\(\blacktriangle\)) \textit{sir2Δ/sir2Δ}.

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

![Figure 4. FOB1-dependent accumulation of ERCS occurs more rapidly in a \textit{sir2} mutant than in wild-type mother cells.](image)

We were not able to observe a clear increase in the levels of ERCS in \textit{sir3} or \textit{sir4} mutants relative to wild type (not shown). This does not rule out the possibility that \textit{sir3} or \textit{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 \textit{sir} mutants relative to wild type. As a more accurate measure of recombination rates in the rDNA, we measured the loss rate of the \textit{ADE2} marker integrated into a single rDNA repeat. This marker was lost at a two- to threefold higher frequency in a \textit{sir3} or \textit{sir4} mutant strain compared with wild type (Table 1). Furthermore, this elevated rate of rDNA recombination was suppressed by deleting \textit{HML\alpha} to restore the \(\alpha\) mating type. These data suggest that the short life span in the \textit{sir3} or \textit{sir4} nonmating haploids is caused by a small increase in recombination at the rDNA due to the expression of both \(\alpha\) and \(\alpha\) mating-type genes.

Also consistent with the notion that \textit{sir3} and \textit{sir4} mutants have an elevated rate of ERC formation, the release of an ERC into wild-type or \textit{sir4} mother cells caused an identical shortening of life span. For this experiment, we used the method of Sinclair and Guarente (1997) to re-
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 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

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**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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of rDNA marker loss ( (\times 10^3) )</th>
</tr>
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<tbody>
<tr>
<td>W303R</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>sir4</td>
<td>2.3 (0.1)</td>
</tr>
<tr>
<td>sir4 hml</td>
<td>0.9 (0.3)</td>
</tr>
<tr>
<td>sir2</td>
<td>18.4 (4.6)</td>
</tr>
<tr>
<td>sir2 hml</td>
<td>9.0 (0.6)</td>
</tr>
<tr>
<td>sir2 fob1 hml</td>
<td>0.8 (0.1)</td>
</tr>
</tbody>
</table>

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 at least 20,000 colonies from three independent isolates were examined for each strain.
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-type 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-
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 hdf2 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; Schidl 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 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 (Gottlieb 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 strain. Disruption was accomplished by transform-
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-sectorred 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 determined 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.

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**References**


