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Elimination of Replication Block Protein Fob1 Extends the Life Span of Yeast Mother Cells

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Summary

A cause of aging in yeast is the accumulation of circular species of ribosomal DNA (rDNA) arising from the 100–200 tandemly repeated copies in the genome. We show here that mutation of the *FOB1* gene slows the generation of these circles and thus extends life span. Fob1p is known to create a unidirectional block to replication forks in the rDNA. We show that Fob1p is a nucleolar protein, suggesting a direct involvement in the replication fork block. We propose that this block can trigger aging by causing chromosomal breaks, the repair of which results in the generation of rDNA circles. These findings may provide a novel link between metabolic rate and aging in yeast and, perhaps, higher organisms.

Introduction

Does a specific molecular event trigger aging? In mammals, one paradigm shown to slow aging is a calorically restricted diet. Mice, rodents, and primates fed calorically restricted diets live up to twice as long as controls and exhibit robust health for most of their lives (reviewed in Weindruch and Sohal, 1997). Along parallel lines, genetic studies of aging in *C. elegans* have identified a gene *clk-1*, which appears to slow the metabolic rate of animals and extend their life span (Lakowski and Hekimi, 1996; Ewbank et al., 1997). Additional studies in *C. elegans* point to the importance of a genetic pathway dedicated to channeling worms to a dormant state early in development termed Dauer. The activation of this pathway in adults extends their life span significantly, presumably by activating some of the mechanisms normally used for longevity in the Dauer (Kenyon et al., 1993). In both *C. elegans* (Morris et al., 1996; Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997) and *Drosophila* (Lin et al., 1998), the identity of genes that can mutate to extend life span indicates that extracellular signaling molecules and receptor-mediated signal transduction pathways are of central importance.

In the yeast *S. cerevisiae*, a specific molecular pathway has been identified that causes aging in mother cell lineages (Sinclair and Guarente, 1997). Mothers can be distinguished from daughters in budding yeasts because they are larger and contain structures that become older each cell division. Mothers can divide about 20 times and exhibit characteristic phenotypic changes as they age, such as sterility (Muller, 1985) and cell enlargement (Mortimer and Johnston, 1959). Aging in mothers is caused, at least in part, by the fragmentation and enlargement of the nucleolus (described below; Sinclair et al., 1997).

The nucleolus is a nuclear structure that is anchored by 100–200 copies of ribosomal DNA (rDNA) arrayed in tandem on chromosome XII. Each repeat of rDNA contains sequences that encode 35S rRNA and 5S RNA. In addition, the spacer between adjacent 35S coding sequences encodes the initiator and enhancer for transcription of 35S by RNA pol I and an *ARS* sequence for the initiation of DNA replication (Figure 1). rDNA replication is halted in one direction at a replication fork block (RFB) preventing replication from proceeding into a 35S rDNA transcription unit (Brewer and Fangman, 1988; Brewer et al., 1992). Thus, rDNA is unique in the yeast genome in that replication is unidirectional, that is, in the same direction as transcription of 35S rRNA. The site of the RFB is just 3′ to the termination of 35S rRNA and overlaps the pol I enhancer (Kobayashi et al., 1992). The product of the *FOB1* gene is required to elicit the replication block; in a *fob1* mutant rDNA replication is bidirectional (Kobayashi and Horiuchi, 1996).

The rDNA also contains an activity termed *HOT1*, which is embodied in an rDNA spacer fragment and enhances recombination when placed near markers outside of the rDNA (Keil and Roeder, 1984). *HOT1* activity requires the RNA pol I transcriptional enhancer and initiator, as well as sequences at the replication fork block (Voelkel-Meiman et al., 1987). Mutations in *FOB1* reduce the rates of recombination promoted by *HOT1* in an assay involving markers outside of the rDNA (Kobayashi and Horiuchi, 1996). In addition, *fob1* mutants show reduced recombination in the rDNA as measured by loss of a marker gene inserted into the rDNA (Kobayashi and Horiuchi, 1996). It has been suggested that a complex among proteins that bind to the pol I promoter and enhancer assembles (Brewer et al., 1992) to set up a strong replication block, which, in turn, causes high rates of recombination in DNA sequences in the vicinity.

In aging lineages of yeast mother cells, DNA circles containing a variable number of rDNA repeats arise and replicate in each subsequent cell division, which give the nucleolus a fragmented morphology. Because these extrachromosomal rDNA circles (ERCs) segregate in a highly biased fashion to mother cells at cell division, they accumulate in direct proportion to the number of

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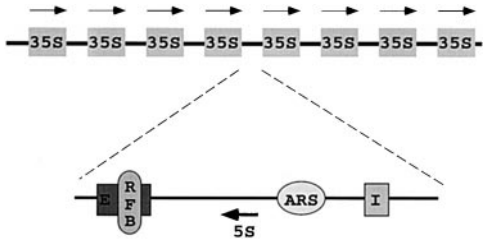

Figure 1. The rDNA Locus in *S. cerevisiae*

The rDNA repeats, present in 100–200 copies on yeast chromosome XII, are indicated. Each repeat contains the coding sequence for 35S rRNA, transcribed in the direction of the arrows, and the spacer, which is blown up. The spacer contains the enhancer (E) and initiator (I) for 35S transcription, the *ARS* for initiation of DNA replication, the 5S RNA-coding sequence, and the replication fork block site (RFB).

cell divisions occurring in the mother cell lineage subsequent to their formation (Sinclair and Guarente, 1997). The segregation bias also assures that daughter cells are born ERC-free and enjoy a full life span. If ERCs are created artificially early in the life span of mothers by use of a site-specific recombinase, the life span of mothers is shortened, suggesting that the ERCs are a cause of aging (Sinclair and Guarente, 1997). Very old wild-type cells contain about 1000 ERCs, which may interfere with cell growth by titrating essential replication and transcription factors. No other DNA species accumulates to a significant degree in old mother cells (Sinclair and Guarente, 1997).

What causes the formation of ERCs? We recently found that ERCs do not form in cells in which double-strand break repair has been eliminated by mutation in genes involved in homologous recombination, such as *RAD52* (P. U. Park, P.-A. D., and L. G., unpublished data). These mutant cells, however, do not live longer and appear to die early because of unrepaired DNA breaks that occur throughout the genome. These findings are consistent with the model that ERCs are generated in a recombination process initiated by double-strand breaks (DSBs) in the rDNA (discussed in Defossez et al., 1998).

Is the rDNA vulnerable to ERC formation simply because it is highly repeated, or is there some underlying molecular Achilles heel in this region of the genome? In this paper, we study a class of mutations termed *hrm* that uniquely affect recombination in the rDNA and not other genomic sequences (Lin and Keil, 1991). *hrm1* mutants enjoy a much longer life span than the wild-type parent. The cloning of *HRM1* reveals that it is identical to *FOB1*, thus suggesting that a blocked replication fork in the rDNA may be a unique molecular structure that renders cells vulnerable to DNA breaks, ERC formation, and aging.

Results

hrm1 Mutations Extend Life Span

Mutations in *HRM1–4* have been shown to reduce *HOT1*-promoted recombination to levels that approach those observed in the absence of the *HOT1* site (Lin and Keil, 1991). The *hrm* mutations also reduce the rates of recombination in the rDNA as measured by loss of a *URA3* marker inserted into that locus. Recombination in substrates not involving *HOT1* are not affected by these mutations (Lin and Keil, 1991). Thus, the *HRM* genes selectively promote recombination in the rDNA and are therefore unlikely to encode general recombination functions used throughout the genome. As such, these mutations might selectively interfere with production of ERCs, without compromising the repair of DSBs generally in the yeast genome.

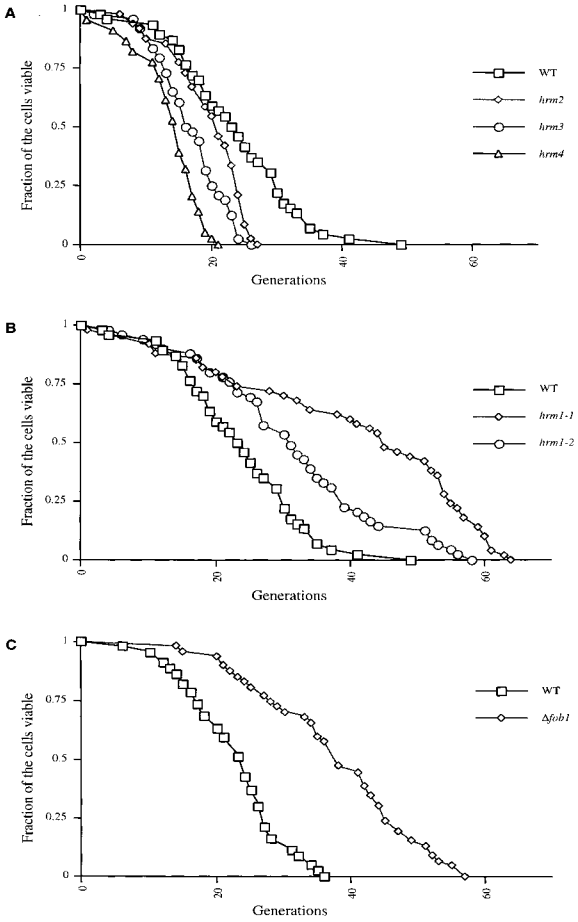

Figure 2. Life Spans of *hrm* Mutants in Strain K2307

Life spans of yeast mother cells are shown. Between 45 and 50 cells were assayed in each case.

(A) The *hrm2*, *hrm3*, and *hrm4* mutants exhibit shorter life spans than wild type (average life spans: wild type, 23.7; *hrm2*, 19.2; *hrm3*, 16.5; and *hrm4*, 13.4).

(B) The *hrm1-1* and *hrm1-2* mutants exhibit longer life spans than wild type (average life spans: wild type, 23.7; *hrm1-1*, 40.7; and *hrm1-2*, 31.4).

(C) The *hrm1/fob1* deletion mutant exhibits a life span longer than wild type (average life spans: wild type, 22.2; Δ*fob1*, 37.0). When compared in the same experiment, the life span of the deletion strain is slightly longer than *hrm1-1* (data not shown).

Life spans were determined for an isogenic set of strains consisting of the wild type and mutants in *HRM1–4*. The life spans of *hrm2*, *3*, and *4* mutants were actually shortened compared to the wild type (Figure 2A). These strains all exhibit a reduced growth rate,

Table 1. *HOT1* Activity in *hrm1*(*fob1*) Mutant Strains

Strain	Recombination Rate ($\times 10^5$)	
	HOT1	non-*HOT1*
HRM1(*FOB1*)	75.3	3.4
hrm1-1	5.4[a]	2.4
hrm1-2	8.1[a]	2.4
*hrm1*Δ(*fob1*Δ)	3.4[a]	5.3

The strain K2307 (Lin and Keil, 1991) contains two templates, *his4::URA3::his4::HOT1* and *lys2::CAN1::LYS2*, that permit the measurement of *HOT1*-dependent or independent excisive recombination, respectively. Fluctuation analysis was used to determine the median recombination rates in K2307, which has a wild-type *HRM1/FOB1*, and in its isogenic derivatives *hrm1-1*, *hrm1-2*, and *fob1*Δ. The experiments were conducted as in Lin and Keil, 1991.
[a] $p < 0.01$ as compared to *HRM1*(*FOB1*).

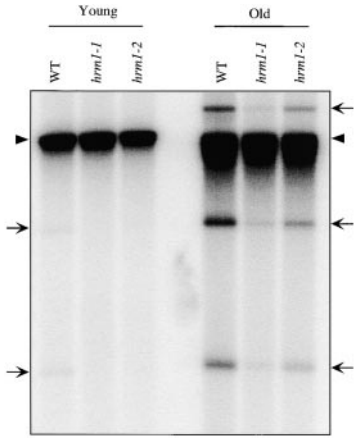

Figure 3. ERCs in *hrm1* Mutants

DNA from unsorted young cells and sorted eight-generation-old mother cells was isolated as described and electrophoresed on an agarose gel without ethidium bromide (Sinclair and Guarente, 1997). The gel was transferred and blotted with an rDNA probe. The chromosomal band is shown by the arrowhead, and ERC species by arrows. A trace of the oligomeric ladder of ERC species is seen in young wild-type cells but not *hrm1* mutants. In the old cells, wild type shows a greater abundance of ERCs than the *hrm1* mutants. The ratio of circular species to total rDNA in old cells was: wild type, 0.273; *hrm1-1*, 0.073; and *hrm1-2*, 0.168.

and the shortening of life spans probably results from a general effect on cell robustness, as observed in other slow-growing mutants (Sinclair et al., 1997). The life spans of two strains with different mutant alleles of *HRM1*, however, were substantially extended compared to the wild type (Figure 2B). Interestingly, the *hrm1-1* mutant, which showed a 70% increase in life span, also showed a greater reduction in *HOT1*-promoted recombination than the *hrm1-2* mutant, which showed a 35% increase in life span (see Table 1 and Figure 2B). We show later that the life span of *hrm1-1* is comparable to that of a null mutant in that gene; *hrm1-2* is thus likely to be a leaky mutation.

ERC Levels in Aging *hrm1* Mutants

The longer life spans of *hrm* mutants could result from a reduction in the accumulation of ERCs in aging mother cells. Thus, the *hrm1-1* and *hrm1-2* mutants, along with the wild-type strain were all grown for eight generations and sorted as described (Smeal et al., 1996; Sinclair and Guarente, 1997). In young, unsorted cells, a small quantity of ERC species (arrows) was visible in the wild type but not *hrm1* mutants (Figure 3). The levels of chromosomal rDNA were comparable in all three samples (arrowhead). In the eight-generation-"old" cells, ERC species were quite apparent in the wild type, but much less abundant in the *hrm1* mutants. The levels of ERC species in the *hrm1-1* mutant were less than in the *hrm1-2* mutant, consistent with the notion that *hrm1-2* is a weaker allele.

Cloning of *HRM1*

Because the *hrm1* mutants reduce the accumulation of ERCs and extend life span of yeast mother cells, the identity of this gene may shed light on the mechanism of ERC production (or replication) and the initiation of the aging process. Since the cloning of *HRM1* had proved problematic in the past, a modified procedure to isolate the gene, detailed in the Experimental Procedures, was used. A candidate clone was obtained that restored a normal level of *HOT1*-stimulated recombination as visualized by a colony sectoring assay (Lin and Keil, 1991). This clone was deemed to be authentic *HRM1* by several criteria (see Experimental Procedures for details). First, the isolated plasmid DNA was capable of restoring colony sectoring when transformed into the *hrm1-1* mutant. Second, the cloned sequence was linked to the *hrm1-1* mutation in 40 of 40 tetrads. Third, deletion of the cloned gene in a wild-type strain evoked the *hrm* hyporecombination phenotype, which was not complemented by *hrm1-1*. Fourth, the deletion mutant had a life span similar to the *hrm1-1* mutant (see below). Fifth, the *hrm1-1* and *hrm1-2* mutations were identified within the sequence of the clone (see below).

HRM1 is identical to the *FOB1* gene. The sequence of the *hrm1-1* and *hrm1-2* mutations were identified and change Cys-246 to Arg and Thr-277 to Pro, respectively. The *FOB1* sequence was precisely deleted from the genome of the parent strain, and the resulting derivative showed a reduced level of *URA3* loss in the rDNA as compared to wild type (0.3×10^{-3} and 1.9×10^{-3}, respectively). The life span of the Δ*fob1* strain was extended about 70% compared to wild type, like the *hrm1-1* mutant (Figure 2C). The rate of *HOT1*-stimulated recombination in the Δ*fob1* strain was also similar to that in the *hrm1-1* mutant (Table 1). The deletion of *FOB1* reduced ERC accumulation in old cells to a similar extent as the *hrm1-1* mutation (data not shown). Thus, these findings show clearly that inactivation of the replication barrier protein, Fob1p, elicits a very long life span, most likely by reducing rDNA recombination and the accumulation of ERCs.

Mutation of *FOB1* Reduces the Formation of ERCs

The reduction in accumulation of ERCs in the *fob1* mutants could either result from a reduction in the frequency of formation of ERCs, an impaired ability of ERCs to replicate or segregate to mother cells after they have formed, or an increased degradation or reintegration into the genome of ERCs. To distinguish between these

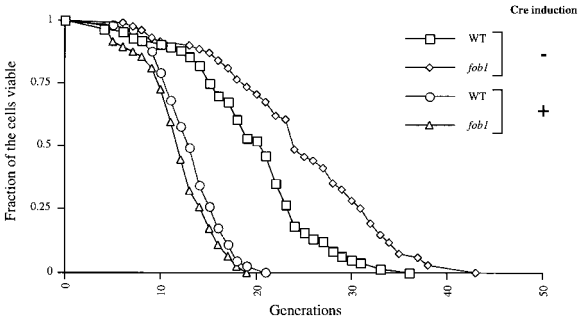

Figure 4. Induction of ERCs in Wild-Type and *fob1* Mutant Cells

The Cre–lox system was used to generate ERCs as described (Sinclair and Guarente, 1997), and life spans were determined. In this strain, W303R, in the absence of induction of ERCs, the *fob1* mutation extends life span, but to a lesser degree than in strain K2307. After induction of ERCs on galactose/raffinose (+), the *fob1* mutant actually had a shorter average life span (12.0), comparable to that of wild type (13.0). Without induction (−) the *fob1* mutant had a longer life span (24.4) than wild type (19.5). At least 47 cells were included in each of the four life span assays. In another control using W303R not bearing the Cre recombinase, the *fob1* deletion extended life span by about 20% on galactose/raffinose compared to the *FOB1* wild type (data not shown).

possibilities, we used the site-specific recombinase Cre to create ERCs, as previously described. This method employs two plasmids, one with an *ADE2*-marked ERC flanked by loxP sites and a second with Cre under Gal-inducible control (Sinclair and Guarente, 1997). If the *fob1* mutant extends life span because it reduces the frequency of formation of ERCs from the rDNA array, then both the mutant and wild-type strains should have similar, short life spans when ERCs are generated ectopically by Cre. Induction of the Gal promoter was not sufficiently strong in the K2307 strain background to efficiently produce ERCs, so the W303R (*RAD5*) strain was used. In this strain background, the *fob1* deletion results in a 30% life span extension (see Figure 6).

As shown in Figure 4, induction of the marked ERC gave rise to a similarly short life span in both the wild-type parent and the *fob1* mutant. In the uninduced control, the wild type showed the normal life span, and the *fob1* deletion gave an extended life span. This experiment demonstrates that the ability of ERCs to replicate and to arrest the growth of cells is not impaired in the *fob1* mutant relative to the wild type. We also observed a comparably strong segregation bias of the ERC for mother cells in *FOB1* and *fob1* strains. In more than 600 cell divisions monitored for each strain, ERCs failed to segregate to daughter cells in 81% of cases for the wild type, and 83% for the *fob1* mutant. We conclude therefore that the reduction in ERC accumulation and the extension of life span in the *fob1* mutant must be due to a reduction in the formation of ERCs. This finding suggests that a blocked replication fork is the structure that elicits the formation of ERCs and aging in wild-type strains. A model for how this may occur is presented in the Discussion.

Fob1 Is a Nucleolar Protein

To gain additional evidence that the effect of Fob1p on life span occurs via the rDNA, the intracellular localization of the protein was determined. The *FOB1* gene was fused to the GFP-coding sequence on a centromeric plasmid and transformed into *hrm1-1* mutant cells. The plasmid fully complemented the hyporecombination phenotype (data not shown). Fluorescence microscopy performed on live cells revealed Fob1p–GFP to be concentrated in a crescent-shaped structure beside the nucleus reminiscent of the nucleolus (Figure 5A). Cells transformed with a control GFP plasmid failed to show this pattern (data not shown). The localization persisted throughout the cell cycle (see Figure 5A). We performed indirect immunofluorescence on cells expressing the chimeric protein with an antibody directed to the nucleolar protein Nop1p, as well as with an anti-GFP antibody (see Figure 5B). The signals colocalize exactly, and we conclude that Fob1p is concentrated in the nucleolus.

Deletion of *FOB1* Does Not Extend Life Span in the Δ*sgs1* Strain

Mutation of the yeast *WRN* homolog, *SGS1*, causes accelerated aging in mother cells (Sinclair et al., 1997). We wished to determine whether the blocked replication fork was the substrate for the formation of the high level of ERCs in the *sgs1* mutant. If this were so, then the life span in the *fob1 sgs1* double mutant would approximate the long life span of the *fob1* single mutant. Thus, the life span of isogenic strains in which *SGS1*, *FOB1*, or both *SGS1* and *FOB1* were deleted was determined. This experiment was carried out in the W303R (*RAD5*) strain. Figure 6A shows that the short life span of the *sgs1* mutant was hardly affected by deleting *FOB1*. This finding suggests that the ERCs that are produced prematurely in the *sgs1* mutant are generated by a mechanism that does not depend upon blocked replication forks. In addition to its failure to suppress the short life span of the *sgs1* mutant, the *fob1* deletion did not reduce the levels of rDNA circles or the elevated rate of recombination in the rDNA, as assayed by marker loss rates (Figure 6B; Table 2). This finding reinforces the claim that the *sgs1* mutation stimulates rDNA recombination and the formation of ERCs by a mechanism that does not involve blocked replication forks. The nature of this mechanism, which is also likely to cause the observed elevated rates of recombination outside of the rDNA in *sgs1* mutants (Gangloff et al., 1994; Watt et al., 1995), is considered in the Discussion. These findings thus suggest that the earliest steps leading to the generation of ERCs and aging may differ between wild type and the *sgs1* mutant. However, these pathways would quickly converge because the ERCs, which are produced by either mechanism, elicit subsequent steps in aging.

Discussion

In this paper, we show that mutation of *FOB1* reduces the formation of extrachromosomal rDNA circles (ERCs) in mother cells and impressively extends their life span. The known molecular function of *FOB1* is the creation of a unidirectional block in replication of the rDNA (Kobayashi and Horiuchi, 1996). This block assures that rDNA is replicated in one direction, that is, in the direction of transcription of 35S rRNA. In *fob1* mutants, rDNA replication is bidirectional, with no evident disadvantage to cells (Kobayashi and Horiuchi, 1996). The extended

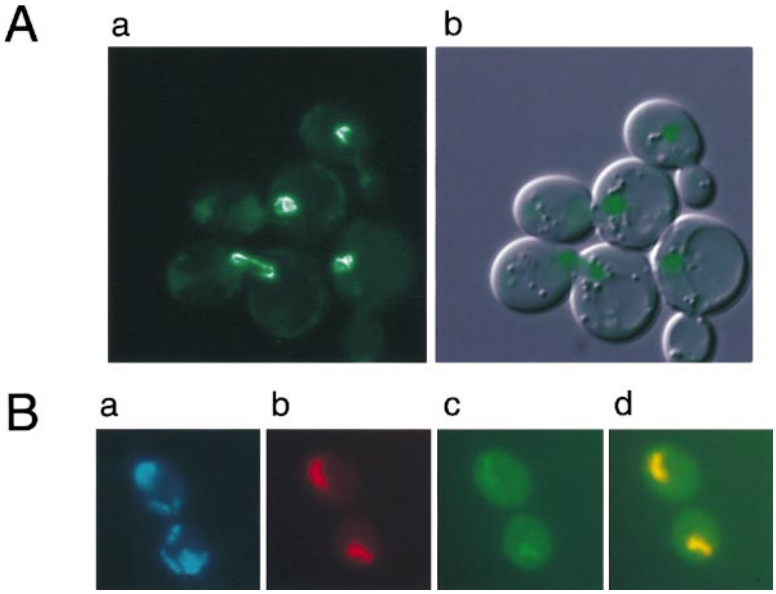

Figure 5. Fob1p Is a Nucleolar Protein

The *FOB1* ORF under its natural promoter was fused to the GFP-coding sequence on a centromeric plasmid. The plasmid was transformed into *hrm1-1* cells and fully complemented the hyporecombination phenotype.

(A) Direct observation of GFP fluorescence in live cells (a), and overlay with the Nomarski image (b). Fob1p is concentrated in a crescent-shaped region on the side of the nucleus.

(B) Immunofluorescence on cells harboring the *FOB1–GFP* plasmid was carried out with antibodies directed to Nop1p, a nucleolar protein (red signal in [b]), or GFP (green signal in [c]). Cells were then treated with DAPI, which stains nuclear and mitochondrial DNA (blue signal in [a]). Nop1p and Fob1p–GFP colocalize in the nucleolus, as evidenced by the yellow signal in the merged picture (d).

life span of *fob1* mutants suggests that a blocked replication fork may be a vulnerable structure in the rDNA that initiates the aging process. We can not eliminate the possibility, however, that some other, yet to be determined, function of *FOB1* is the basis of the effects on ERC formation and life span.

Relation of Blocked Replication Forks to DSBs and ERCs

In *E. coli*, there is convincing evidence that blocked replication forks lead to double-strand breaks (DSBs) in the bacterial chromosome (Michel et al., 1997; Seigneur et al., 1998). By analogy, we imagine that blocked replication forks in the rDNA of yeast elicit the formation of DSBs that must be repaired by the machinery of homologous recombination. ERCs can then be generated by intrachromosomal recombination. Our own recent data (P. U. Park, P.-A. D., and L. G., unpublished data) show that the machinery for homologous recombination is required for the generation of ERCs. In the absence of this machinery, cells have very short life spans, likely due to the persistence of DSBs.

Figure 7 schematizes our model of the earliest events occurring in the rDNA during the aging process. At the blocked replication fork, the newly synthesized DNA (dotted lines) could pop out of the replication bubble, as proposed to occur at paused forks in bacteria (Seigneur et al., 1998). The resulting structure resembles a Holliday recombinational intermediate, which can then be acted upon by a nuclease to generate a DSB. In *E. coli* this nuclease is the ruvC protein (Seigneur et al., 1998), but in yeast its identity is not known. The DSB will then recruit the machinery of homologous recombination to repair the lesion. This process will generate ERCs if it occurs intrachromosomally and is accompanied by crossing over, which should occur in half the resolution events. Zou and Rothstein (1997) have reported the existence of recombinational intermediates in the rDNA and, importantly, showed that their formation depends upon DNA replication and the machinery of homologous recombination. They further find that mutation in DNA polymerase delta, which is proposed to synthesize the lagging strand during replication, stimulates formation of Holliday structures. By the above model, this could occur because the newly synthesized DNA that pops out of the template and is presented at the end of the resulting DSB would have an extended 3′ single-stranded region, thereby promoting strand invasion and recombination.

Relation to Higher Eukaryotes

It is noteworthy that a unidirectional replication block in the rDNA is a feature conserved in higher eukaryotes, such as plants, *Xenopus*, and humans (Hernandez et al., 1993; Little et al., 1993; Wiesendanger et al., 1994). The possibility that these structures also serve to initiate aging mechanisms in these hosts must therefore be considered. The molecular outcome of a process initiated in this way may differ from organism to organism. For example, if DSBs were generated in mammalian rDNA at the replication block, the machinery of nonhomologous end joining (Critchlow and Jackson, 1998) may be recruited in preference or in addition to the machinery of homologous recombination. In this case, the result of DNA repair may not be ERCs, but rather deletions that emanate away from the replication block sites. These deletions could lead to the gradual inactivation or loss of rDNA copies over time. Loss of rDNA copies in aging has been reported (Strehler et al., 1979), but subsequent studies were unable to confirm these findings (Peterson et al., 1984). A search for recombinational events generated at the site of the replication block in aging mammals appears to be warranted.

Possible Role of the *WRN/SGS1* Helicase

The shortened life span in the *sgs1* mutant is not restored by deletion of *FOB1*. This failure suggests that the stimulation of ERC formation occurring in the absence of the Sgs1p helicase is independent of blocked replication forks. There are several possible mechanisms by which

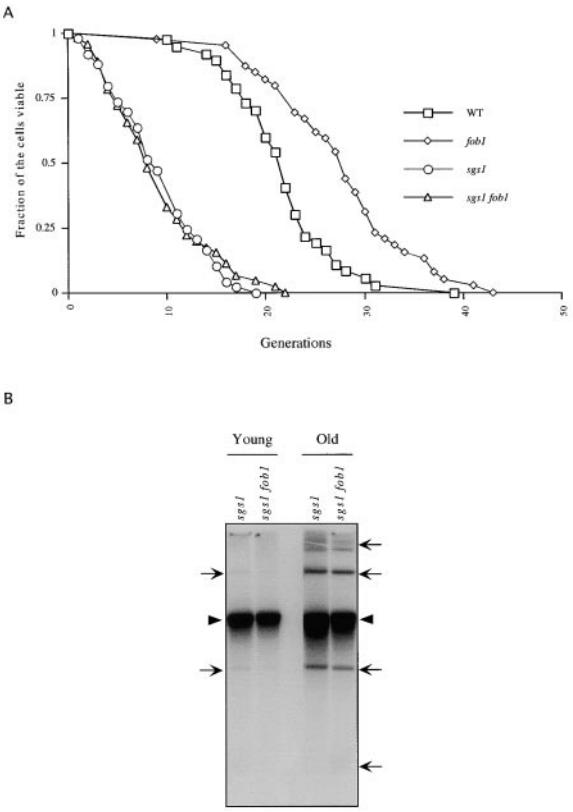

Figure 6. *FOB1* Deletion Fails to Extend Life Span and Decrease ERC Accumulation in *sgs1* Mutant Cells

(A) Life spans of isogenic W303R (*RAD5*) strains of the *FOB1 SGS1*, *fob1*, *sgs1*, or *fob1 sgs1* double mutant genotypes were determined. The *sgs1* mutation shortened life span by about 60%, as previously reported (Sinclair et al., 1997). This shortening was not appreciably affected by deleting *FOB1*. In the *SGS1* wild types, deleting *FOB1* extended life span. Average life spans were: wild type, 21.6; *fob1*, 27.4; *sgs1*, 9.2; and *fob1 sgs1*, 10.3. At least 37 cells were analyzed for each strain.

(B) ERCs accumulate to similar levels in *sgs1* and *fob1 sgs1* cells. DNA from unsorted young yeast cells and sorted seven-generation-old mother cells was isolated and used for a Southern blot as in Figure 3. The gel had a lower agarose concentration than in Figure 3, which resulted in better separation of highest molecular weight ERC species and spreading of the lowest weight ERC species. The chromosomal rDNA band is shown by the arrowhead, and ERC species by arrows. The ratio of circular species to total rDNA in old cells was: *sgs1*, 0.117; *fob1 sgs1*, 0.128.

Table 2. The Elevated Level of rDNA Recombination in an *sgs1* Mutant Strain Is Not Suppressed by Deletion of *FOB1*

Strain	rDNA Marker loss ($\times 10^3$)
FOB1 SGS1	1.0 (0.2)
fob1 SGS1	0.2[a] (0.02)
FOB1 sgs1	3.2[a] (0.4)
fob1 sgs1	3.2[a] (0.7)

ADE2 was inserted in the rDNA of strain W303R. rDNA recombination rates were assessed by determination of the rate of loss of this marker. Cells were plated on YPD and the frequency of half-sectored colonies, reflecting a marker loss event at the first cell division, was measured. More than 25,000 colonies were examined for each strain. Average values are given, with standard deviation in parentheses.

[a] $p < 0.01$ relative to WT.

the absence of Sgs1p may cause DNA lesions that stimulate recombination and the production of ERCs. First, it is possible that the Sgs1p helicase is coupled to the DNA replication machinery itself. It can not be the sole replication helicase, since its absence is not lethal. Its absence, however, could cause pausing of replication forks, which, like the forks blocked by Fob1p, elicits DSBs and recombination. These pauses would stimulate recombination and the production of ERCs were they to occur in the rDNA, and recombination and chromosome loss (Gangloff et al., 1994; Watt et al., 1995) were they to happen outside of the rDNA. Analogously, mutations in the *E. coli* helicase, rep, cause replication pausing and DSBs (Michel et al., 1997). Further, the recent purification of an Sgs1p/WRN homolog required for DNA replication promoted by *Xenopus* extracts is consistent with this model (Yan et al., 1998). Second, the absence of Sgs1p may cause the accumulation of unresolved recombinational intermediates, which themselves block the progression of DNA replication forks. Indeed, the Sgs1p/WRN homolog BLM does appear to resolve recombinational intermediates, since its absence leads to the accumulation of intermediates of sister–chromatid exchange in cells from individuals with Bloom's Syndrome (reviewed in Ellis, 1997). Third, Sgs1p may be a component of the recombinational machinery itself, such that its absence somehow leads to elevated recombination rates and ERC production. Fourth, Sgs1p may be coupled to rDNA transcription, such that its absence leads to a higher frequency of DSBs in the rDNA resulting from an inability to resolve tortional stress induced by transcription. This model is strengthened by the observations that Sgs1p interacts with yeast topoisomerases II and III (Gangloff et al., 1994; Watt et al., 1995) and that mutations in yeast topoisomerases I and II reduce rDNA transcription (Brill et al., 1987).

Since the human WRN protein, like Sgs1p, is concentrated in the nucleolus (Gray et al., 1998; Marciniak et al., 1998), the aging phenocopy in Werner individuals may also be triggered by aberrations in replication, recombination, or transcription in human rDNA.

A Link between rDNA Changes and the Metabolic Rate?

Many studies in a variety of organisms show a link between the rates of metabolism and aging. For example, a calorically restricted diet slows metabolism and can extend life span up to two-fold in rodents (reviewed in Weindruch and Sohal, 1997). This observation has traditionally been explained by proposing a direct relationship between the rates of metabolism and the production of toxic oxygen radicals, which might cause aging. Our findings offer an alternative explanation for the link between aging and metabolism. Only a fraction of the 35S genes in the rDNA is transcribed at any one time, which is proportional to the growth rate of cells. It has been suggested that origins of replication in the rDNA in yeast fire downstream of transcribed 35S genes (Lucchini and Sogo, 1994). Cells growing at a rapid rate would have the greatest demand for synthesis of ribosomes and thus transcribe a large fraction of the 35S genes in the array. This high number of transcribed rDNA copies could trigger the firing of a high fraction of the

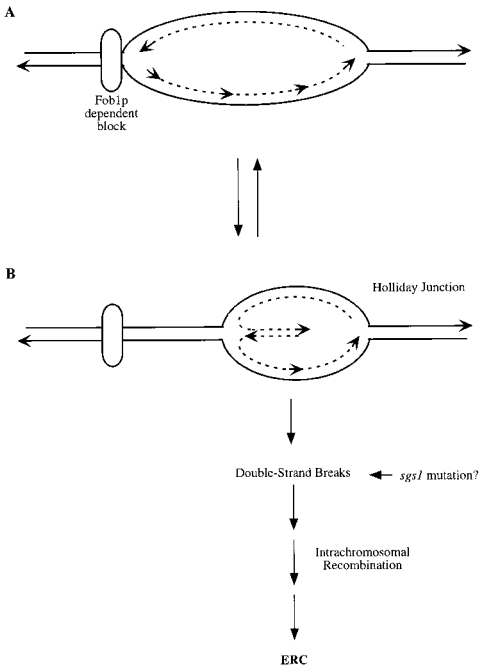

Figure 7. Replication Block Leads to Generation of ERCs

(A) A replication fork is blocked at the RFB by Fob1p. Newly synthesized DNA (leading and lagging strands) is indicated by dotted lines. (B) Isomerization of the replication bubble due to dissociation of new strands at RFB generates a Holliday junction. This structure leads to double-strand breaks and ERCs, if the DSBs are processed by intrachromosomal recombination accompanied by crossing over. In the *sgs1* mutant, double-strand breaks may be generated by a separate mechanism, such as nonspecific pausing of replication in the rDNA.

origins in the rDNA in S phase. Therefore, under these conditions, the probability of DSBs at the fork block sites and the accompanying recombination could be highest, leading to the most rapid production of ERCs and the fastest rate of aging. In contrast, when cells grow at a slow rate, a minimal fraction of 35S genes would be transcribed. The firing of replication origins in the rDNA and the number of fork blockage events and ensuing DSBs would also be at a minimum under these conditions. A slow growth rate indeed has been reported to slow the rate of aging in yeast (Muller et al., 1980).

An alternative model relating metabolism and rDNA changes is that DNA breaks are caused by a high rate of rDNA transcription itself. This would clearly couple the rate of DSBs and ERCs (in yeast) to the metabolic rate. By this model, the effect of the *fob1* mutation in extending life span could possibly result from a small decrease in rDNA transcription in the mutant and not from replication blockage. However, we do not favor this model at present because the *fob1* mutant grows at about the same rate as the wild type, suggesting that the rates of rDNA transcription are comparable in the wild-type and mutant strains.

Conclusion

Fob1p speeds the production of ERCs and aging in yeast mother cells, perhaps by causing the unidirectional blockage of DNA replication forks in the rDNA. What positive role might *FOB1* serve in cells? *fob1* mutants also show a greatly reduced rate of recombination in the rDNA. *FOB1* was recently shown to be required for changes in the rDNA copy number (Kobayashi et al., 1998), which may provide yeast with an advantage in being able to adapt to changes in the environment.

More generally, our results suggest that the blocked replication fork in the rDNA may be the Achilles heel of the aging cell. It will be of interest to determine whether other forms of intervention that inhibit this block also extend life span in yeast and, perhaps, other organisms.

Experimental Procedures

Plasmids and Strains

Strain K2307 (*MATa his4-260*::*URA3*::*his4-*Δ::*HOT1 ade2–1 ade5 leu2*::*ADE5*,7::*leu2–3,112 trp1-HIII lys2-DBX*::*CAN1*::*LYS2 can1*) and its isogenic derivatives *hrm1-1*, *hrm1-2*, *hrm2*, *hrm3*, and *hrm4* are described in Lin and Keil (1991). K5582 was derived from K2307 by replacing the entire *FOB1* ORF with the kanamycin-resistance cassette KanMX4 flanked by loxP sites (Wach et al., 1994; Guldener et al., 1996). The *rad5* point mutation was corrected by gene replacement in W303a to generate the strain W303R. The Cre–lox two-plasmid system (Sinclair and Guarente, 1997) was used with minor modifications to remove the CEN sequence from an rDNA–*ADE2* plasmid and thus generate a marked ERC. Cells were pregrown on glucose synthetic media selecting for both plasmids and shifted to galactose/raffinose synthetic media lacking adenine to induce Cre recombinase. Life span assays were performed on mother cells. The daughters of all mothers were analyzed on adenine-lacking media to verify that they failed to inherit the *ADE2*-marked ERC. This segregation bias verified that the *ARS–CEN* sequence had been liberated from the plasmid. Note that this also showed a comparably strong mother cell segregation bias in the wild type and the *fob1* mutant.

To generate a Fob1p–GFP fusion, the whole *FOB1* ORF and its promoter were amplified by PCR on genomic DNA isolated from strain K2307. It was then inserted in frame with GFP in pPS1527, a centromeric, *URA3*-marked plasmid, generating plasmid pPAD146. pPAD146 fully complemented the rDNA recombination defect of *hrm1-1* and *fob1*Δ mutant strains (data not shown).

Life Span Determination

Life span analysis was performed as described (Kennedy et al., 1994). Each experiment involved 40 to 50 cells and was carried out at least twice independently. Experiment-to-experiment variability in the average longevity of a given strain was less than 15%. Statistical significance of life span differences was determined by a Wilcoxon rank sum test. Average life spans are stated to be different when the confidence is higher than 95%.

Purification of Old Cell DNA

The published protocol (Sinclair and Guarente, 1997) was used with slight modifications. Log-phase (10^8) cells were labeled with 6 mg of Sulfo-NHS-LC Biotin or Sulfo-NHS-LC-LC-Biotin (Pierce) for 15 min at room temperature without shaking. They were then washed three times in PBS, resuspended in 1 liter YPD containing 2.5% glucose, and grown to an $OD_{600\,nm}$ less than 1. The phenol/chloroform and chloroform extraction steps were omitted during DNA preparation. The recovered DNA (5 μg) was used for a Southern blot. One-dimensional gel electrophoresis, blotting, and hybridization were performed as described (Sinclair and Guarente, 1997). Circular and linear rDNA species were quantified using a PhosphorImager (Molecular Dynamics).

Cloning of *HRM1*

We sought to clone *HRM1* by complementation of the nonsectoring phenotype of the *hrm1-1* mutant. Complementing clones were not observed in a number of genomic DNA or cDNA libraries that were screened. We therefore screened a library that was transformed into the *hrm1-1* strain directly after the genomic DNA had been ligated to a vector, omitting the amplification step. Briefly, genomic

DNA was isolated from our wild-type strain K2307 and cut to completion with BamHI. The YCplac33 plasmid (*URA3, ARS/CEN*) was cut with BamHI and dephosphorylated. To eliminate uncut or nondephosphorylated vector molecules, it was then ligated upon itself, and the remaining linear molecules were gel-purified (James et al., 1996). Purified linear vector was ligated to cleaved genomic DNA, and the mixture was transformed into the *hrm1-1* mutant using a lithium acetate protocol (Gietz et al., 1995). Five independent complementing clones were recovered out of 12000 screened, and all contained the same 21 kb BamHI fragment harboring *FOB1*. *FOB1* with its promoter and 3′ sequences was PCR amplified and cloned into pRS316 (*URA3, ARS/CEN*). The resulting plasmid complemented the nonsectoring phenotype of *hrm1-1*. Further genetic analysis proved that *FOB1* is *HRM1*, and not an extragenic suppressor (see text).

Microscopy and Immunofluorescence

Detection of GFP fluorescence was as in Ferrigno et al., 1998. Immunofluorescence was carried out as described (Ferrigno and Silver, 1999).

Determination of DNA Recombination Rate

Determination of recombination rate in Table 1 was conducted as previously described (Keil and McWilliams, 1993). Because of jackpot events median, rather than mean, rates are the preferred way to represent the data. A two-tailed statistical test was used to ascertain significance of the rate differences.

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Note Added in Proof

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