

DNA Repair and Genomic Instability in Yeast Aging

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ABSTRACT

Aging is a universal process that occurs in eukaryotic organisms. Many features of aging, including the genetic pathways involved in aging, appear to be evolutionarily conserved. Extrachromosomal rDNA circles (ERCs) have been identified to be a cause of replicative aging in budding yeast *S. cerevisiae*. Genomic instability can cause ERCs to excise from chromosomal rDNA arrays, containing 35S and 5S rRNA genes. At each cell division, ERCs replicate via an origin of replication present in each rDNA repeat, accumulate asymmetrically in the mother cells due to their segregation bias, and ultimately cause nucleolar fragmentation and senescence. Introduction of an ERC into young cells shortens life span and accelerates the onset of age-associated sterility.

ERCs are excised from the rDNA locus by homologous recombination. *rad52* mutant cells, defective in DNA repair through homologous recombination, do not accumulate ERCs with age; likewise, mutations in other genes of the *RAD52* class that have varying effects on homologous recombination have corresponding effects on ERC formation. *rad52* mutation leads to a progressive delocalization of a silencing and DNA repair protein Sir3p from telomeres to other nuclear sites with age and, surprisingly, shortens life span despite the absence of ERCs. Spontaneous DNA damage, perhaps double-strand breaks (DSBs), are likely the cause of lethality in mutants of the *RAD52* class and may be an initial step in aging in wild-type cells.

Replication fork pausing in *E. coli* can cause DSBs. Consistently, a *fob1* mutation, which abolishes unidirectional replication fork barrier (RFB) at the rDNA, reduces rDNA recombination, decreases ERC accumulation with age, and thus extends life span. Therefore, reduction of ERC formation in *fob1* cells is likely the consequence of an absence of DSBs caused by replication fork pausing at RFB, again suggesting that DSBs are the initial step in yeast aging.

Fob1p is also required for transcription enhancement from an ectopic RNA polymerase I (Pol I) promoter outside of the rDNA, but not at rDNA. The role of *Fob1p* in this enhancement may be to facilitate interactions between rDNA repeats and the ectopic promoter, thus allowing recruitment of Pol I transcriptional machinery to the ectopic promoter. Consistent with this notion, *Fob1p* influences the structural dynamics at rDNA. *Fob1p* antagonizes *Sir2p*-dependent rDNA silencing and thus affects rDNA chromatin structure. *fob1* mutation partially suppresses the aberrant rDNA structure in topoisomerase I-deficient (*top1*) cells, likely by relieving torsional stress created during rDNA replication. In addition, a *fob1* mutation was able to rescue the synthetic lethality in *top1 trf4* double mutant cells, due to the failure of mitotic condensation at rDNA. *fob1* mutation complemented several defects caused by *trf4* mutation, suggesting that *Trf4p*, DNA polymerase σ , may have a specialized function at rDNA. *FOB1* and likely RFB may play a direct role in the macromolecular rDNA structure that influences chromosome transmission and indirectly affects aging in yeast.

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**This thesis is dedicated to my mother Yeong Soon and my late father
Sun Ok who by coming to this country sacrificed everything
for their children's education.**

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Chapter 1

Introduction

Genetic and Molecular Analyses of Aging in Yeast

The portion of this chapter was published in *Current Opinion in Microbiology*, Volume 1, Pages 707-711 in 1998. The authors were Pierre-Antoine Defossez, Peter U. Park, and Leonard Guarente.

Aging is a process that can be defined by two criteria: statistical definition and phenotypic definition. The statistical definition is based on the fact that the probability of death of individuals within a population increases with the age of the organism (Gompertz 1825). The phenotypic definition is based on the fact that characteristic changes in phenotypes occur in all individuals with the age of the organism. This phenotypic definition is distinguished from diseases associated with aging, such as heart disease and cancer, which only affect a subset of the population. For example, recent advances in the treatment of heart disease have increased the life span of those afflicted with the disease, but have not substantially altered the maximum life span because these interventions have not changed the aging process itself.

Like other great strides in understanding the causes of human diseases, recent advances in understanding the basics underlying mechanisms of aging have been made possible by the development of molecular tools, as well as progress in human genetics. Physiological experiments in different organisms indicate that caloric restriction appears to be a universal mechanism of slowing the pace of aging (reviewed in (Masoro 2000)). Genetic studies of heritable diseases that mimic the appearance of premature aging have allowed the identification of genes responsible for the diseases (Weeda et al. 1990; Sung et al. 1993; Henning et al. 1995; Yu et al. 1996). Most strikingly, studies in model organisms, such as yeast *S. cerevisiae*, nematode *C. elegans*, fruit fly *Drosophila*, and mouse have shown that mutation of a single gene can dramatically extend the life span of that organism (reviewed in (Tissenbaum and Guarente 2002)). Furthermore, several pathways and many key genes involved in aging are conserved from yeast to humans.

This chapter will focus on the molecular mechanisms and genetic pathways of aging in yeast. The recent advances in the studies of aging in humans and other model organisms will be discussed in the context of yeast aging.

TWO DEFINITIONS OF LIFE SPAN IN YEAST

Life span in most eukaryotic organisms is determined by time. Life span in *Saccharomyces cerevisiae* can be defined in two ways: chronological life span and replicative life span.

Chronological Life Span

Chronological life span in yeast measures the length of time that yeast cells survive after they have stopped dividing. Most of studies done on chronological life span in yeast measure the rate of survival under nutrient deprivation, usually glucose limitation. Yeast cells grown on a medium with limiting glucose divide by fermentation of glucose until glucose becomes limiting. Once glucose becomes limiting, cells undergo a metabolic change, called "post-diauxic shift," in which they stop fermentation and grows slowly by respiration. When cells cease respiratory growth, they enter stationary phase. Cells in stationary-phase have characteristic changes that allow long-term survival, including thermotolerance, alteration of cell wall surface, and changes in transcriptional and translational activities (reviewed in (Werner-Washburne et al. 1993)). After several months in stationary phase, the viability of the cells, tested by their ability to give rise to colonies in rich medium, starts to decline and ultimately leads to senescence.

Stationary-phase aging appears to be caused by accumulation of reactive oxygen species (ROS) because mutation in superoxide dismutase or catalase reduces stationary-phase life span (Longo et al. 1996; Longo et al. 1999). ROS can be produced in the mitochondria as a byproduct of oxidative phosphorylation.

Superoxide dismutase and catalase convert ROS into non-reactive products.

Also, mutations that increase resistance to paraquat, a free radical generator, increase stationary-phase life (Fabrizio et al. 2001).

One theory of aging proposed that ROS cause aging by producing cumulative damage over a lifetime (Harman 1981). Evidence that ROS also plays a role in aging in higher organisms suggests that ROS might be a universal cause of aging. In *Drosophila*, life span has been extended by counteracting oxidative damage through overexpression of superoxide dismutase and catalase (Orr and Sohal 1994). In addition, overexpression of human superoxide dismutase *SOD1* in motor neuron lengthens the fly life span by 40% (Parkes et al. 1998). Furthermore, flies with the partial loss of function allele of *methuselah*, a gene encoding a putative G-protein-coupled receptor, have extended life span and are resistant to number of different stresses, including starvation, high temperature, and oxidative damage (Lin et al. 1998). The treatment of *C. elegans* with synthetic compounds that mimic catalase extends life span by about 50% (Melov et al. 2000).

No strong correlation exists between chronological life span and replicative life span in yeast. Muller and coworkers first showed that the cellular life span is strictly determined by the number of divisions undergone, not by chronological time (Muller et al. 1980). Cells grown at lower temperatures take longer to divide, and thus live a longer time than cells grown at higher temperatures; however, they produce the same number of progeny, and thus have the same replicative life span. However, recent study showed that yeast cells that have been passed through stationary phase have reduced replicative

life span potential (Ashrafi et al. 1999), suggesting a link between chronological and replicative life spans.

Reproductive Life Span

The second way to define yeast life span is reproductive life span.

Saccharomyces cerevisiae exhibits asymmetric division. A bud emerges from a mother cell during the late G₁ stage of the cell cycle and continues to grow until late nuclear division and cytokinesis, leading to a daughter cell. At every round of mitosis, it is possible to distinguish the mother cell from the smaller daughter cell. By continuously micromanipulating daughters away as they appear, one can determine how many times a specific mother cell can divide. By this procedure, Mortimer and Johnston established in a seminal paper that yeast cells, like mammalian cells, have a limited division potential (Mortimer and Johnston 1959). Their replicative life span is defined as the number of daughter cells they produce before ceasing to divide. This life span varies from strain to strain and is typically in the order of 20 to 40 generations (for complete procedures for the life span analysis, see Appendix A). By determining the life span of numerous cells in a cohort, a mortality curve can be obtained (Fig. 1A).

Most of the recent advances in yeast aging have been made in replicative aging. The rest of this chapter will discuss replicative aging. From this point, reference to life span will mean replicative life span, unless stated otherwise.

BIOMARKERS ASSOCIATED WITH YEAST AGING

Studies in replicative aging were first carried out by investigating the characteristic changes that occur with age. Many biomarkers of yeast aging have been identified including slowing of the cell cycle to the loss of silencing at *HM* loci. These biomarkers have been an important tool in understanding the mechanism of aging in yeast.

Visible exterior changes

Some of the first characterized biomarkers were the exterior changes that are visible through microscope. The size of yeast cells increases with age. Mortimer and Johnson proposed that cells senesce because they reach a critical upper limit in size (Mortimer and Johnston 1959). Evidence that refutes this hypothesis is that artificially increasing the cell size by arresting the cells transiently with α -factor does not affect life span. Another change that occurs in the cell exterior with age is bud scar accumulation. When a daughter cell buds off from a mother cell, a circular chitin-containing remnant, called the bud scar, is left at the bud site. It was also proposed that bud scar accumulation might be the cause of senescence (Mortimer and Johnston 1959). Two lines of evidence refute this hypothesis. First, the size of one bud scar occupies about 1% of the cell surface. Thus, the cell surface should be able to accommodate about 100 bud scars; yet, average life span of laboratory strains is about 20-40 generations. Second, the same evidence that refutes the increase in cell size with age as a cause of aging applies to the bud scar accumulation as a cause of aging. Artificially increasing the cell size should increase the cell surface, which can

accommodate more bud scars. Again, the increase in cell size does not extend life span.

Loss of Asymmetry

Another visible change that occurs at the end of yeast life span is the loss of asymmetry in size between mother and daughter cells. As yeast cells age they grow in size but continue to bud off small daughter cells throughout most of their life span. However, very old mother cells often undergo symmetric divisions, giving rise to daughter cells that are equivalent in size to mother cells. Importantly, these daughters from very old mothers have shorter life span than the daughters from young mothers. It was proposed that this breakdown of asymmetry could be accounted by a diffusible senescence factor (Müller 1985; Egilmez and Jazwinski 1989). When divisions cease to be asymmetrical, the daughters would receive more of this diffusible senescence factor, thus accounting for the reduced life span potential in daughters from symmetric division. This hypothetical senescence factor was later shown to be extrachromosomal rDNA circles (ERCs) (Sinclair and Guarente 1997). The characteristics of ERC are discussed below.

Sterility Caused by Loss of Transcriptional Silencing

Two types of haploid yeast cells, α and α cells, exist in *S. cerevisiae*. α and α cell types are determined by the mating type loci, *MAT α* and *MAT α* respectively. Haploid cells of opposite mating type can mate with each other to form a diploid zygote. Diploid cells do not mate with other haploid or diploid

cells because they express both α and α information. Haploid cells do have both α and α mating type information at *HM* loci, *HML α* and *HMR α* , but they are fertile because *HML α* and *HMR α* remain in a transcriptionally silent state, which is maintained, in part, by the Sir2/3/4p silencing complex (reviewed by (Laurenson and Rine 1992)). Muller observed that haploid cells lose their ability to mate as they get old (Müller 1985) (Fig. 1A). Old cells not yet sterile, when mated to a young cell, yield a zygote with a limited life span (Müller 1985), again supporting the idea that there must be a dominant 'senescence factor' building up in old cells.

It was later found that the sterility is caused by the loss of transcriptional silencing at *HM* loci, resulting in the expression of both α and α information (Smeal et al. 1996). α cells with *HMR α* deletion do not become sterile, thus indicating that the loss of silencing at *HM* loci, not another step in the mating pathway, is the cause of age-dependent sterility. However, since *HMR α* deletion in α cells did not affect the life span or the age-associated phenotypes, the loss of silencing at *HM* loci is an effect, rather than the cause, of aging. In addition, the silencing at telomeres is lost in old cells (Kim et al. 1996). The loss of silencing at telomeres and likely *HM* loci in old cells is caused by the movement of the Sir3p away from the telomeres and *HM* loci and to the nucleolus (Kennedy et al. 1997) (Fig. 1B).

Nucleolar Fragmentation

Along with Sir3p relocalization to the nucleolus, the nucleolus becomes enlarged and fragmented in old yeast cells (Kennedy et al. 1997; Sinclair et al.

1997) (Fig. 1B). The nucleolus is a specialized compartment of the nucleus, where the ribosomal DNA (rDNA) resides and the ribosome biogenesis occurs (reviewed in (Shaw and Jordan 1995; Leary and Huang 2001)). The nucleolus in yeast is localized at the periphery of nuclear envelope as a crescent-shaped structure. It has been suggested that the Sir complex might relocalize to the nucleolus to delay or slow the changes that eventually lead to fragmentation. Consistent with this hypothesis, *SIR2* regulates the transcriptional silencing (Bryk et al. 1997; Smith and Boeke 1997) and the recombination rate (Gottlieb and Esposito 1989) at the rDNA loci. Furthermore, Sir proteins play a role in repair of double-stranded DNA breaks (Lee et al. 1999; Martin et al. 1999; McAinsh et al. 1999; Mills et al. 1999).

EXTRACHROMOSOMAL rDNA CIRCLES AS A CAUSE OF YEAST AGING

As more biomarkers were experimentally characterized and the description of cellular senescence became more precise, their underlying causes still remained obscure. Recent studies have helped to provide a molecular interpretation of yeast senescence. The 'senescence factor' has been identified as autonomously replicating extrachromosomal ribosomal DNA circle that excise from the repeated arrays and amplify as cells age, eventually leading to death.

ERCs Cause Aging

The nucleolus in *S. cerevisiae* contains rDNA, made of 100-200 tandem arrays located on chromosome XII (reviewed in (Warner 1989)). Each repeat of rDNA is about 9.1 kb in size and encodes the large precursor 35S rRNA gene transcribed by RNA polymerase I (Pol I) and the small 5S rRNA transcribed by RNA polymerase III (Pol III) (Fig. 2). Because the nucleolus contains a large array of repeated rDNA units, Sinclair and Guarente investigated whether the nucleolar fragmentation is due to the change in the status of this locus during senescence (Sinclair and Guarente 1997). Extrachromosomal rDNA circles (ERCs) were found to appear in old cells and to accumulate as they grew older. By two-dimensional electrophoresis, these circles were shown to contain one or several 9.1 kb rDNA units. Such species had already been described but, curiously, only in stationary phase cells (Larionov et al. 1980), in cells devoid of mitochondria function (Conrad-Webb and Butow 1995), and in cells with defective topoisomerases (Kim and Wang 1989).

The excision of an ERC from the genome must be a fairly rare event, because logarithmically growing cells seem almost devoid of them (Sinclair and Guarente 1997). Once an initial ERC is formed, however, two causes contribute to its accumulation (Figure 2). First, each rDNA unit, and thus each circle, carries an origin of replication (ARS) and can be duplicated during S phase (Larionov et al. 1984). The replication event may be the molecular clock that times the aging of the cells. Second, ERCs show biased segregation and tend to stay in the mother cell during mitosis, a phenomenon previously reported for plasmids containing a replication origin but lacking a centromere (Murray and Szostak 1983). It is likely that the amplified ERCs clump together and act as organizers to form the nucleolar fragments observed in old cells. The ability of rRNA-encoding plasmids to 'seed' nucleolar structures has indeed been reported (Nierras et al. 1997; Oakes et al. 1998).

Importantly, the build up of ERCs in old cells could be a cause of aging or a mere correlate of growing old. A crucial experiment proved that release of an artificial ERC in the young cell decreased longevity, making ERCs a likely cause of yeast aging (Sinclair and Guarente 1997). Moreover, the asymmetrical inheritance pattern of ERCs breaks down in old cells resulting in leakage of ERCs into daughter cells (Sinclair and Guarente 1997), thus accounting for the symmetrical division observed in old cells and for the reduced replicative potential in daughter cells from old mothers.

Why Do ERCs Kill Cells?

It is not yet known how the accumulation of ERCs leads to cellular senescence and death; however, several hypotheses are possible. It has been

shown that when yeast cells lose their mitochondria, they form ERCs that become transcribed, oddly enough, not by RNA polymerase I but by polymerase II (Conrad-Webb and Butow 1995). Whether the ERCs that appear in old cells are transcribed, and by which polymerase, has not been studied. If transcription of the ERCs does take place, it could produce a vast excess of rRNA over ribosomal proteins that would disturb ribosome assembly. Sinclair and Guarente showed, however, that an ERC-like plasmid devoid of any rDNA sequences, but contains ARS sequence can also shorten life span of yeast cells (Sinclair and Guarente 1997). Furthermore, they showed that cells with ERC-like plasmids lost silencing at *HM* loci prematurely. If indeed ERC-like plasmids can accelerate age-associated phenotypes, this would imply that this mechanism of ribosome disruption is not necessary for senescence to occur.

It is also conceivable that the ERCs titrate limiting transcription factors away from the genome. The TATA-box binding protein TBP, which is required for transcription by all three RNA polymerases and whose cellular abundance is limiting for growth (Patterson et al. 1998), could be such a limiting factor. Finally, the DNA replication machinery itself may be titrated by the sheer number of replication origins carried by the ERCs, and become unavailable for replication of the genome. In this regard, senescence might be similar to the situation described in certain yeast strains where runaway amplification of 2-micron circles occurs, eventually leading to cell death (Holm 1982).

How Do ERCs Form?

Since the replication of ERCs may be the molecular clock that times the age of yeast cells, it is important to determine what triggers the ERC formation.

It is likely that ERC arises by homologous recombination between the rDNA repeats. Indeed, we founded that *rad52* mutant cells, defective for DNA repair through homologous recombination, did not accumulate ERCs with age. Furthermore, *rad52* mutation leads to a progressive delocalization of Sir3p from telomeres to other nuclear sites with age, and, surprisingly, shortens life span. These results and a potential role for spontaneous DNA damage, perhaps double strand breaks, as an initial step of yeast aging will be discussed in Chapter 2.

GENES INVOLVED IN YEAST AGING

Many genes that affect aging in yeast have been characterized. Some of genes have been shown to affect aging by influencing ERC level, while others influence ERC-independent mechanisms of aging. The effects of some genes on ERC have not been fully characterized.

CDC6

CDC6 is important for initiation of DNA replication in yeast. A *cdc6-1* mutation decreases the efficiency of ARS initiation at the nonpermissible temperature, resulting in high rate of plasmid loss (Hogan and Koshland 1992). Addition of extra ARSs on a plasmid suppresses the high rate of plasmid loss. Sinclair and Guarente reasoned that there might be a semipermissive temperature at which the rate of ARS initiation within ERCs would be reduced without affecting ARS initiation elsewhere in the genome. At 27°C, they found that *cdc6-1* mutation significantly increased mean life span and increased the maximum life span by 25% (Sinclair and Guarente 1997).

LAG1 and LAG2

A differential hybridization approach was used to identify genes whose transcript level changes with replicative age (Egilmez et al. 1989). Two of the identified genes, *LAG1*, and *LAG2* (Longevity Assurance Gene), which showed a decrease in transcript level with age, have an affect on life span. *lag1* deletion extends life span by 50 % (D'Mello N et al. 1994). *Lag2p* is a putative transmembrane protein that plays a role in ceramide biosynthesis (Guillas et al.

2001) and transport of GPI-anchored proteins (Barz and Walter 1999). Deletion of *LAG2* gene shortens life span by 50% while overexpression of *LAG2* gene lengthened life span by 36% (Childress et al. 1996). Lag2p also appears to be a transmembrane protein. The function of Lag1p or Lag2p in aging process is not known. It will be interesting to investigate whether *LAG1* or *LAG2* affects ERC-dependent mechanism of aging.

SGS1, a Homologue of WRN, Werner Syndrome Gene

Werner Syndrome is a rare, autosomal recessive disorder. Patients die prematurely and display many features of aging early in adulthood, including loss of skin elasticity, premature loss and graying of hair, and development of cataracts (reviewed in (Shen and Loeb 2000)). Their condition is brought about by the mutation of single nuclear gene named *WRN*. *WRN* encodes a DNA helicase related to the bacterial protein *RecQ* (Yu et al. 1996), and has a one close homologue in the yeast genome *SGS1*. Mutation in *BLM* or *REQL4*, other *RecQ* homologues in human, causes Bloom's Syndrome (Ellis et al. 1995) or some cases of Rothmund-Thomson's Syndromes (Kitao et al. 1999), respectively. All of these diseases display genetic instability (reviewed in (Mohaghegh and Hickson 2001)).

In yeast, mutation in *SGS1*, like other *RecQ* homologues, also causes genomic instability. *SGS1* was originally identified as a suppressor of slow growth in topoisomerase III-deficient (*top3*) cells (Gangloff et al. 1994). *Sgs1p* interacts physically with *Top3p* or *Top2p* (Gangloff et al. 1994; Watt et al. 1995). *sgs1* mutation suppresses high levels of rDNA recombination in *top3* mutant

cells, and the *sgs1* mutation, by itself, increases the rate of rDNA recombination (Gangloff et al. 1994).

sgs1 mutant cells have a life span 60% shorter than wild type cells and display premature sterility, nucleolar fragmentation, and movement of Sir3p to the nucleolus (Sinclair et al. 1997). These results suggested that mutation of *SGS1*, like *WRN*, causes premature aging. However, subsequent study has shown that the majority of *sgs1* cells do not senesce due to the premature aging, but die because of stochastic arrest at G₂ phase of cell cycle (McVey et al. 2001).

TOP1, TOP2, and TOP3 (Topoisomerases)

Mutation in *TOP1*, *TOP2*, or *TOP3* highly elevates the rDNA recombination rate (Christman et al. 1988; Gangloff et al. 1994; Gangloff et al. 1996). The elevated recombination of tandem arrays in *top1* or *top2* mutation is specific to rDNA arrays since the recombination frequency of another tandem arrays, the *CUP1* locus, was unaffected in *top1* or *top2* cells (Christman et al. 1988). Top1p is concentrated in the nucleolus (Giroux et al. 1989; Edwards et al. 2000). In *top1 top2* double mutant cells, about half of the rDNA is present as ERCS at semipermissive temperature (Kim and Wang 1989), suggesting *top1 top2* should have a short life span. rRNA transcription is also inhibited in *top1 top2* cells (Brill et al. 1987; Schultz et al. 1992). Thus, yeast topoisomerases perform a crucial aspect of DNA metabolism at rDNA. Other aspects of *TOP1* in DNA metabolism at rDNA will be discussed in Chapter 3.

UTH Genes

Exploiting a correlation between stress resistance and long life span, Kennedy and coworkers carried out a screen for longer-lived yeast mutants (Kennedy et al. 1995). Four complementation group, *UTH1-4*, that when mutated, showed increased stress resistance and life span extension were isolated. One long-lived mutant carried a mutation in *SIR4* (*UTH4*), truncating the carboxyl terminus of the protein. This mutation, named *SIR4-42*, eliminated silencing at *HML* and *HMR*, causing sterility. It also abolished telomeric silencing, yet extended life span in a semi-dominant fashion. This led the authors to speculate that Sir4-42p might leave the normally silenced loci in the telomeres and redistribute to novel locations to turn down the expression of some senescence-causing genes (Kennedy et al. 1995). The direct examination of Sir4-42p localization by immunofluorescence led to the identification of this novel locus as the rDNA (Kennedy et al. 1997).

Two lines of evidence support the idea that Sir4p relocalization to the nucleolus extends life span. First, overexpression of a Sir4p carboxy-terminal fragment dislodges the endogenous Sir4p from the telomeres, making it free to migrate to the nucleolus, and extends life span (Kennedy et al. 1994). Second, another mutation promoting long life recovered in the same screen is a gain of function in the *UTH4* (*MPT5*) gene. *UTH4* is a post-transcriptional regulator (Tadauchi et al. 2001), which promotes cell wall integrity (Kaeberlein and Guarente 2002). Moreover, overexpression of *UTH4* extends life span by 40% (Kennedy et al. 1997). It was shown that the products of *UTH4* and its homologue *YGL023W* are necessary for the movement of Sir4p to the rDNA (Gotta et al. 1997; Kennedy et al. 1997). The shift of the Sir complex away from

the telomeres and the silent mating-type cassettes to the rDNA was also observed in wild-type cells as they aged, explaining the onset of sterility (Fig. 1B). The *SIR4-42* mutation thus mimics a naturally occurring life-extending mechanism. The longevity extension gained by the movement of Sir4p to the nucleolus is dependent on the function of both Sir2p and Sir3p (Kennedy et al. 1997).

SIR2/3/4 Silencing Factors

The silent information regulator (*SIR*) genes encode a set of proteins involved in transcriptional silencing. Sir1p, Sir2p, Sir3p and Sir4p are all required for efficient repression of the silent mating-type cassettes *HML* and *HMR* in haploid cells (review in (Laurenson and Rine 1992)). Silencing was also shown to occur at the telomeres (Gottschling et al. 1990) and the ribosomal DNA (rDNA) (Bryk et al. 1997; Smith and Boeke 1997). Telomeric silencing depends on Sir2p, Sir3p and Sir4p, whereas rDNA silencing only requires Sir2p.

Role of Sir complex in yeast longevity was first characterized in the screen for *UTH* genes (Kennedy et al. 1995; Kennedy et al. 1997), as described above. More careful investigation on the effects of mutations in *SIR2*, *SIR3*, and *SIR4* on the life span of wild type yeast cells revealed that *SIR2* affects life span through two different mechanisms (Kaeberlein et al. 1999). First, along with *sir3* or *sir4* mutation, *sir2* mutation modestly shortens life span caused by coexpression of a α and α information. Coexpression of a and α information indirectly increases the recombination rate at rDNA and likely increases the production of ERCs. Second, *SIR2* has a direct role in regulation of life span through the repression of recombination generated by Fob1p-mediated replication block in the rDNA.

Furthermore, increasing the dosage of Sir2p extends life span in wild type cells by 30%, indicating Sir2p is a limiting component in promoting yeast longevity.

Possible roles for the movement of Sir complex from HM loci and telomeres to rDNA

The discovery of ERCs may be the key to explaining the life-extending role of the movement of Sir proteins from HM loci and telomeres to rDNA. In fact, there are several nonexclusive reasons why the Sir complex could hinder the formation of ERCs or their accumulation (Fig. 3). First, it is known that Sir2p suppresses rDNA recombination, in a fashion independent of Sir1p, Sir3p and Sir4p (Gottlieb and Esposito 1989; Fritze et al. 1997). As the rDNA and the telomeres compete for a limited amount of Sir2p (Smith et al. 1998), the movement of the Sir complex from the telomeres to the nucleolus might simply make more Sir2p available to suppress rDNA recombination and reduce ERC formation. Second, the rDNA arrays is the location of high levels of RNA polymerase I transcription within strict topological constraints. This transcription may induce DNA breaks that elicit repair mechanisms, including gene conversion between repeats, single-strand annealing and nonhomologous end joining (Ozenberger and Roeder 1991; Gangloff et al. 1996). If associated with a crossing-over, gene conversion can generate a circle of DNA, whereas the other repair pathways cannot. It has been shown that Sir4p binds the yeast Ku homologue Hdflp and is involved in nonhomologous end joining, together with Sir2p and Sir3p (Tsukamoto et al. 1997; Boulton and Jackson 1998). Furthermore, Sir proteins move from telomeres to the site of damage (Lee et al. 1999; Martin et al. 1999; McAinsh et al. 1999; Mills et al. 1999). The redirected Sir complex may

thus affect the balance of usage of the different repair mechanisms to reduce the probability of forming an ERC.

Another possibility is that the Sir complex influences the partitioning of ERCs. Indeed, tethering Sir4p to a plasmid devoid of centromere significantly increased its mitotic stability by promoting equal segregation of the plasmid between mother and daughter cell (Ansari and Gartenberg 1997). This probably explains why inserting telomeric sequences into a plasmid lacking a centromere lessens its segregation bias (Longtine et al. 1992). It is a particularly interesting finding in light of the recent suggestion that the Sir complex represses transcription by attracting the silenced locus to a perinuclear location (Andrulis et al. 1998). It is possible that the Sir complex similarly binds the ERCs and tethers them to a nuclear structure that drives equal partition.

Enzymatic activity of conserved Sir2p

Recently, it was discovered that Sir2p is a novel deacetylase requiring nicotinamide adenine dinucleotide (NAD) as a cofactor (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). Its enzymatic activity is required for its function in recombination, silencing, and life span (Imai et al. 2000; Armstrong et al. 2002). The requirement of this enzymatic activity is consistent with previously finding that the silencing is associated with reduced acetylation (Braunstein et al. 1993; Braunstein et al. 1996). Sir2p also has phosphoribosyltransferase activity (Tsang and Escalante-Semerena 1998; Frye 1999; Tanny et al. 1999), which appears to be one of intermediate steps in deacetylation reaction (Tanner et al. 2000; Tanny and Moazed 2001).

From bacteria to humans, Sir2p is evolutionarily conserved (Brachmann et al. 1995; Frye 2000). Strikingly, just as increased Sir2p dosage can extend life span in yeast (Kaeberlein et al. 1999), overexpression of *Sir-2.1* protein, a closest *SIR2* homologue, can extend life span in *C. elegans* (Tissenbaum and Guarente 2001). This life span extension requires *daf-16*, suggesting a link between Sir2 protein and known aging pathway in worm. *daf-16* is a forkhead transcription factor in a *daf-2*-insulin-like signaling pathway that positively regulates dauer formation in larvae and longevity in adult worm (reviewed in (Tissenbaum and Guarente 2002)). Mutations that weakly inactivate the *daf-2* pathway extend life span and increase resistance to oxidative stress. In addition, mammalian *SIRT1* protein, the closest *SIR2* homologue in mammals, regulates p53 protein activity via deacetylation (Luo et al. 2001; Vaziri et al. 2001). Interestingly, p53 and activation of p53 pathway by oxidative damage have been implicated in mice aging (Migliaccio et al. 1999; Tyner et al. 2002), suggesting a possible link between Sir2 protein and aging in mammals.

Caloric Restriction--PKA pathway, Respiration, and SIR2

One conserved mechanism for life span extension is caloric restriction (CR) (reviewed in (Masoro 2000)). Restricting food intake in rodents can lengthen life span of rodents by 30-40% (Weindruch et al. 1986), and reduction of caloric intake by glucose or amino acid limitation also extends life span in yeast (Jiang et al. 2000; Lin et al. 2000) and *C. elegans* (Klass 1977; Hosono et al. 1989). One common hypothesis that has been proposed to explain this life span extension is that CR slows metabolism, thereby slowing the production of reactive oxygen species (ROS). Although this hypothesis is very plausible, no

concrete experimental evidence has been shown to support the hypothesis.

Recently, the mechanism by which CR extends life span of yeast has been characterized.

Lin and coworkers showed that by reducing the glucose concentration from 2% to 0.5%, yeast life span could be lengthened by up to 35% (Lin et al. 2000). Furthermore, they showed that genetic manipulation of cyclic AMP-dependent protein kinase (PKA) pathway that mimics caloric restriction could extend life span. The PKA pathway senses glucose level in the environment. The PKA pathway is activated when the environmental glucose level increases. Mutation of *CDC25*, encoding GTP-GDP exchange factor, or *CDC35*, encoding adenylate cyclase, decreases PKA activity and extends life span up to 75% (Lin et al. 2000). Thus, both mutations mimic low glucose environment. In addition, Sir2p, NAD-dependent deacetylase, and Npt1p, an enzyme in the yeast NAD biosynthesis pathway, are required for CR (Lin et al. 2000), suggesting that CR activates Sir2p activity by increasing NAD level (Guarente and Kenyon 2000). Moreover, shunting of carbon metabolism from fermentation to respiration by overexpression of Hap4p, a transcription factor that regulates metabolic switch, or deletion of Hxk2p, hexose kinase, extends life span (Lin et al. 2002). These life span extensions require *SIR2* and fail to synergize with CR, suggesting that CR extends life span by shifting carbon flow toward respiration.

Other evidence that the PKA pathway regulates life span comes from studies that show that *RAS1* and *RAS2* have antagonistic roles in life span (Sun et al. 1994). Deletion of *RAS1* extends life span, while overexpression of *RAS2* extends life span. The mechanism by which *RAS* genes regulate life span is not reported. Since *RAS* genes influence PKA pathway, it is tempting to speculate

that the regulation of longevity by *RAS* genes occurs by modulating PKA activity.

Retrograde Pathway.

Yet another pathway that regulates the longevity in yeast is the retrograde response pathway. Retrograde response allows yeast cells to respond to the changes in mitochondria by altering gene expression in nucleus (Parikh et al. 1987). In some strains, the deletion of mitochondrial DNA (ρ^- mutation) extends life span (Kirchman et al. 1999). This extension was dependant on Rtg2p, a key transcription factor in retrograde response, thus indicating that retrograde response is necessary for life span extension in ρ^- mutant cells. These results suggest that the presence of mitochondrial function is deleterious to longevity. This is contrary to the result that the increase in respiration in mitochondria extends life span (Lin et al. 2002). The effects of mitochondria and ρ^- mutation on longevity are far from clear since it has been reported that ρ^- mutations cause an increase in ERC formation (Conrad-Webb and Butow 1995), which should shorten life span of yeast cells. The effect of retrograde pathway on life span and ERC accumulation merits further investigation.

FOB1—Replication Fork Barrier

DNA replication at rDNA is unique. It is the only locus in the yeast genome where DNA replication occurs unidirectionally. In addition to 35S and 5S rRNA sequences, each rDNA unit also contains two nontranscribed regions, NTS1 and NTS2 (Fig. 2). NTS1 contains replication fork barrier (RFB), located

just 3' to the termination of 35S rRNA. RFB blocks the bidirectional replication in one direction at RFB, the direction that is opposite as transcription of 35S rRNA (Brewer and Fangman 1988; Brewer et al. 1992; Kobayashi et al. 1992). NTS2 contains an origin of replication (ARS), located just 5' to the promoter 35S rRNA. About one in five ARS sites in rDNA is fired for replication during each round of DNA replication (Brewer and Fangman 1988; Linskens and Huberman 1988).

One of the genes required for RFB at rDNA is *FOB1* (Kobayashi and Horiuchi 1996). *fob1* mutation abolishes RFB and causes bidirectional replication. *FOB1* was originally identified as a gene required for HOT1 recombinational activity (Lin and Keil 1991; Kobayashi and Horiuchi 1996). HOT1 is an rDNA element, consists of an E element, containing enhancer for Pol I transcription and RFB, and an I element, the promoter for Pol I transcription (Voelkel-Meiman et al. 1987) (Fig. 2). HOT1 enhances recombination and Pol I-dependent transcription of nearby marker genes when inserted at an ectopic site. Mutation in *FOB1* also decreases the recombination rate at rDNA (Lin and Keil 1991; Kobayashi and Horiuchi 1996), likely a consequence of the absence of replication fork pausing at RFB. Replication fork pausing in *E. coli* leads to the formation of a Holliday junction, which can lead to double-strand DNA breaks (Michel et al. 1997; Seigneur et al. 1998). Consistent with this model, *fob1* mutation reduces ERC accumulation with age, accounting for its effect on life span extension (Defossez et al. 1999). These results are consistent with the hypothesis proposed in Chapter 2 that double-strand DNA breaks are the stochastic events that trigger ERC formation in yeast aging.

Additionally, along with rDNA *cis*-elements, *FOB1* is required for expansion and contraction of rDNA arrays (Kobayashi et al. 1998; Kobayashi et

al. 2001). Most recently, Wai and coworkers showed that the Pol I enhancer is not required for rRNA transcription or cell growth. Deletion of the Pol I enhancer from every copy of rDNA repeats had no effect on the transcription of the chromosomal rRNA gene and for cell growth. Furthermore, *FOB1* is only required for transcription enhancement from an ectopic promoter outside of rDNA. The authors suggested that Fob1p may play a role in facilitating interactions between rDNA repeats through the E elements and that RFB may be the result of particular macromolecular structures created by these interactions. The recruitment of Pol I transcriptional machinery to the ectopic promoters may be the result of interactions between the rDNA repeats and the E element in HOT1. This hypothesis is consistent with the results that Pol I transcription is not necessary for RFB (Brewer et al. 1992) and most recently, RFB is not required for HOT1 activity (Ward et al. 2000). Ward and coworkers showed that only one orientation of the E element relative to the origin of replication created the RFB, but HOT1 recombination is elevated in both orientations (Ward et al. 2000). Therefore, the authors concluded that RFB is not necessary for HOT1 recombination. If the E element in HOT1 is required for interactions with other E elements at the rDNA repeats through Fob1p, the orientation of E element would not matter for Pol I recruitment, as previously described (Voelkel-Meiman et al. 1987). However, the orientation of the E element may matter for the unidirectional RFB activity, thus explaining the shared sequences but independent activities between RFB and HOT1. Therefore, Fob1p may have a structural function at rDNA that allows interactions between rDNA repeats. The evidence for the function of Fob1p in modulating rDNA structure and mitotic condensation will be described in Chapter 3.

SUMMARY

The first molecular cause of aging in yeast has been identified. Extrachromosomal rDNA circles excise from the chromosomal rDNA arrays, replicate, segregate asymmetrically, and ultimately cause senescence. From nucleolar fragmentation to the reduced replicative potential in daughters of very old mothers, the ERC model of aging can account for most of the biomarkers of yeast aging. Several genetic pathways that affect the ERC model of aging have been characterized, and the influence of other genetic pathways on the ERC model of aging still needs to be investigated.

This thesis focuses on the mechanism and the genes involved in the ERC model of aging. Chapter 2 will concentrate on the role of DNA repair genes on the formation of ERC and life span in yeast. Most importantly, it will show that Rad52p-mediated homologous recombination is absolutely necessary for ERC formation. Double-strand DNA breaks as an initial step in aging will be discussed. Chapter 3 will show the role of Fob1p, which promotes ERC formation, on rDNA structure and mitotic condensation in *top1/trf4* mutants. Finally, Appendix A will outline a step-by-step description of two of the most important techniques in studying replicative yeast aging: life span analysis by micromanipulation and large-scale sorting for old cells.

Although no evidence has been found that rDNA, ERCs, or extrachromosomal DNA circles have a role in aging in higher eukaryotes, several genetic pathways that govern the ERC model of aging appears to be conserved. It will be exciting to uncover how these genetic pathways influence aging in humans. The last ten years have been a very fruitful time for the aging

biologists. Perhaps the next ten years will bring advances that uncover the molecular mechanism of aging in humans.

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REFERENCES

- Andrulis, E.D., A.M. Neiman, D.C. Zappulla, and R. Sternglandz. 1998. *Perinuclear localization of chromatin facilitates transcriptional silencing.* *Nature* 394: 592-5.
- Ansari, A. and M.R. Gartenberg. 1997. *The yeast silent information regulator Sir4p anchors and partitions plasmids.* *Mol Cell Biol* 17: 7061-8.
- Armstrong, C.M., M. Kaeberlein, S.I. Imai, and L. Guarente. 2002. *Mutations in Saccharomyces cerevisiae Gene SIR2 Can Have Differential Effects on In Vivo Silencing Phenotypes and In Vitro Histone Deacetylation Activity.* *Mol Biol Cell* 13: 1427-38.
- Ashrafi, K., D. Sinclair, J.I. Gordon, and L. Guarente. 1999. *Passage through stationary phase advances replicative aging in Saccharomyces cerevisiae.* *Proc Natl Acad Sci U S A* 96: 9100-5.
- Barz, W.P. and P. Walter. 1999. *Two endoplasmic reticulum (ER) membrane proteins that facilitate ER-to- Golgi transport of glycosylphosphatidylinositol-anchored proteins.* *Mol Biol Cell* 10: 1043-59.
- Boulton, S.J. and S.P. Jackson. 1998. *Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing.* *Embo J* 17: 1819-28.
- Brachmann, C.B., J.M. Sherman, S.E. Devine, E.E. Cameron, L. Pillus, and J.D. Boeke. 1995. *The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability.* *Genes Dev* 9: 2888-902.
- Braunstein, M., A.B. Rose, S.G. Holmes, C.D. Allis, and J.R. Broach. 1993. *Transcriptional silencing in yeast is associated with reduced nucleosome acetylation.* *Genes Dev* 7: 592-604.
- Braunstein, M., R.E. Sobel, C.D. Allis, B.M. Turner, and J.R. Broach. 1996. *Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern.* *Mol Cell Biol* 16: 4349-56.
- Brewer, B.J. and W.L. Fangman. 1988. *A replication fork barrier at the 3' end of yeast ribosomal RNA genes.* *Cell* 55: 637-43.
- Brewer, B.J., D. Lockshon, and W.L. Fangman. 1992. *The arrest of replication forks in the rDNA of yeast occurs independently of transcription.* *Cell* 71: 267-76.
- Brill, S.J., S. DiNardo, K. Voelkel-Meiman, and R. Sternglandz. 1987. *Need for DNA topoisomerase activity as a swivel for DNA replication for transcription of ribosomal RNA.* *Nature* 326: 414-6.

- Bryk, M., M. Banerjee, M. Murphy, K.E. Knudsen, D.J. Garfinkel, and M.J. Curcio. 1997. *Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast.* *Genes Dev* 11: 255-69.
- Childress, A.M., D.S. Franklin, C. Pinswasdi, and S. Kale. 1996. *LAG2, a gene that determines yeast longevity.* *Microbiology* 142: 2289-97.
- Christman, M.F., F.S. Dietrich, and G.R. Fink. 1988. *Mitotic recombination in the rDNA of *S. cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II.* *Cell* 55: 413-25.
- Conrad-Webb, H. and R.A. Butow. 1995. *A polymerase switch in the synthesis of rRNA in *Saccharomyces cerevisiae*.* *Mol Cell Biol* 15: 2420-8.
- D'Mello N. P., A.M. Childress, D.S. Franklin, S.P. Kale, C. Pinswasdi, and S.M. Jazwinski. 1994. *Cloning and characterization of LAG1, a longevity-assurance gene in yeast.* *J Biol Chem* 269: 15451-9.
- Defossez, P.A., R. Prusty, M. Kaeberlein, S.J. Lin, P. Ferrigno, P.A. Silver, R.L. Keil, and L. Guarente. 1999. *Elimination of replication block protein Fob1 extends the life span of yeast mother cells.* *Mol Cell* 3: 447-55.
- Edwards, T.K., A. Saleem, J.A. Shaman, T. Dennis, C. Gerigk, E. Oliveros, M.R. Gartenberg, and E.H. Rubin. 2000. *Role for nucleolin/Nsr1 in the cellular localization of topoisomerase I.* *J Biol Chem* 275: 36181-8.
- Egilmez, N.K., J.B. Chen, and S.M. Jazwinski. 1989. *Specific alterations in transcript prevalence during the yeast life span.* *J Biol Chem* 264: 14312-7.
- Egilmez, N.K. and S.M. Jazwinski. 1989. *Evidence for the involvement of a cytoplasmic factor in the aging of the yeast *Saccharomyces cerevisiae*.* *J Bacteriol* 171: 37-42.
- Ellis, N.A., J. Groden, T.Z. Ye, J. Straughen, D.J. Lennon, S. Ciocci, M. Proytcheva, and J. German. 1995. *The Bloom's syndrome gene product is homologous to RecQ helicases.* *Cell* 83: 655-66.
- Fabrizio, P., F. Pozza, S.D. Pletcher, C.M. Gendron, and V.D. Longo. 2001. *Regulation of longevity and stress resistance by Sch9 in yeast.* *Science* 292: 288-90.
- Fritze, C.E., K. Verschueren, R. Strich, and R. Easton Esposito. 1997. *Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA.* *Embo J* 16: 6495-509.
- Frye, R.A. 1999. *Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity.* *Biochem Biophys Res Commun* 260: 273-9.

- 2000. *Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins.* *Biochem Biophys Res Commun* **273**: 793-8.
- Gangloff, S., J.P. McDonald, C. Bendixen, L. Arthur, and R. Rothstein. 1994. *The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase.* *Mol Cell Biol* **14**: 8391-8.
- Gangloff, S., H. Zou, and R. Rothstein. 1996. *Gene conversion plays the major role in controlling the stability of large tandem repeats in yeast.* *Embo J* **15**: 1715-25.
- Giroux, C.N., M.E. Dresser, and H.F. Tiano. 1989. *Genetic control of chromosome synapsis in yeast meiosis.* *Genome* **31**: 88-94.
- Gompertz, B. 1825. *On the nature of the function expressive of the law of human mortality, and on a new mode of determining life contingencies.* *Philos. Trans. R. Soc.* **115**: 513-85.
- Gotta, M., S. Strahl-Bolsinger, H. Renauld, T. Laroche, B.K. Kennedy, M. Grunstein, and S.M. Gasser. 1997. *Localization of Sir2p: the nucleolus as a compartment for silent information regulators.* *Embo J* **16**: 3243-55.
- Gottlieb, S. and R.E. Esposito. 1989. *A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA.* *Cell* **56**: 771-6.
- Gottschling, D.E., O.M. Aparicio, B.L. Billington, and V.A. Zakian. 1990. *Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription.* *Cell* **63**: 751-62.
- Guarente, L. and C. Kenyon. 2000. *Genetic pathways that regulate ageing in model organisms.* *Nature* **408**: 255-62.
- Guillas, I., P.A. Kirchman, R. Chuard, M. Pfefferli, J.C. Jiang, S.M. Jazwinski, and A. Conzelmann. 2001. *C26-CoA-dependent ceramide synthesis of *Saccharomyces cerevisiae* is operated by Lag1p and Lac1p.* *Embo J* **20**: 2655-65.
- Harman, D. 1981. *The aging process.* *Proc Natl Acad Sci U S A* **78**: 7124-8.
- Henning, K.A., L. Li, N. Iyer, L.D. McDaniel, M.S. Reagan, R. Legerski, R.A. Schultz, M. Stefanini, A.R. Lehmann, L.V. Mayne, and et al. 1995. *The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH.* *Cell* **82**: 555-64.
- Hogan, E. and D. Koshland. 1992. *Addition of extra origins of replication to a minichromosome suppresses its mitotic loss in cdc6 and cdc14 mutants of *Saccharomyces cerevisiae*.* *Proc Natl Acad Sci U S A* **89**: 3098-102.
- Holm, C. 1982. *Clonal lethality caused by the yeast plasmid 2 mu DNA.* *Cell* **29**: 585-94.

- Hosono, R., S. Nishimoto, and S. Kuno. 1989. *Alterations of life span in the nematode Caenorhabditis elegans under monoxenic culture conditions*. *Exp Gerontol* **24**: 251-64.
- Imai, S., C.M. Armstrong, M. Kaeberlein, and L. Guarente. 2000. *Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase*. *Nature* **403**: 795-800.
- Jiang, J.C., E. Jaruga, M.V. Repnevskaya, and S.M. Jazwinski. 2000. *An intervention resembling caloric restriction prolongs life span and retards aging in yeast*. *Faseb J* **14**: 2135-7.
- Kaeberlein, M. and L. Guarente. 2002. *Saccharomyces cerevisiae MPT5 and SSD1 Function in Parallel Pathways to Promote Cell Wall Integrity*. *Genetics* **160**: 83-95.
- Kaeberlein, M., M. McVey, and L. Guarente. 1999. *The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms*. *Genes Dev* **13**: 2570-80.
- Kennedy, B.K., N.R. Austriaco, Jr., and L. Guarente. 1994. *Daughter cells of Saccharomyces cerevisiae from old mothers display a reduced life span*. *J Cell Biol* **127**: 1985-93.
- Kennedy, B.K., N.R. Austriaco, Jr., J. Zhang, and L. Guarente. 1995. *Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae*. *Cell* **80**: 485-96.
- Kennedy, B.K., M. Gotta, D.A. Sinclair, K. Mills, D.S. McNabb, M. Murthy, S.M. Pak, T. Laroche, S.M. Gasser, and L. Guarente. 1997. *Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in S. cerevisiae*. *Cell* **89**: 381-91.
- Kim, R.A. and J.C. Wang. 1989. *A subthreshold level of DNA topoisomerases leads to the excision of yeast rDNA as extrachromosomal rings*. *Cell* **57**: 975-85.
- Kim, S., B. Villeponteau, and S.M. Jazwinski. 1996. *Effect of replicative age on transcriptional silencing near telomeres in Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* **219**: 370-6.
- Kirchman, P.A., S. Kim, C.Y. Lai, and S.M. Jazwinski. 1999. *Interorganelle signaling is a determinant of longevity in Saccharomyces cerevisiae*. *Genetics* **152**: 179-90.
- Kitao, S., A. Shimamoto, M. Goto, R.W. Miller, W.A. Smithson, N.M. Lindor, and Y. Furuichi. 1999. *Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome*. *Nat Genet* **22**: 82-4.
- Klass, M.R. 1977. *Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span*. *Mech Ageing Dev* **6**: 413-29.

- Kobayashi, T., D.J. Heck, M. Nomura, and T. Horiuchi. 1998. Expansion and contraction of ribosomal DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (*Fob1*) protein and the role of RNA polymerase I. *Genes Dev* 12: 3821-30.
- Kobayashi, T., M. Hidaka, M. Nishizawa, and T. Horiuchi. 1992. Identification of a site required for DNA replication fork blocking activity in the rRNA gene cluster in *Saccharomyces cerevisiae*. *Mol Gen Genet* 233: 355-62.
- Kobayashi, T. and T. Horiuchi. 1996. A yeast gene product, *Fob1* protein, required for both replication fork blocking and recombinational hotspot activities. *Genes Cells* 1: 465-74.
- Kobayashi, T., M. Nomura, and T. Horiuchi. 2001. Identification of DNA cis elements essential for expansion of ribosomal DNA repeats in *Saccharomyces cerevisiae*. *Mol Cell Biol* 21: 136-47.
- Landry, J., A. Sutton, S.T. Tafrov, R.C. Heller, J. Stebbins, L. Pillus, and R. Sternglanz. 2000. The silencing protein *SIR2* and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A* 97: 5807-11.
- Larionov, V., N. Kouprina, and T. Karpova. 1984. Stability of recombinant plasmids containing the *ars* sequence of yeast extrachromosomal rDNA in several strains of *Saccharomyces cerevisiae*. *Gene* 28: 229-35.
- Larionov, V.L., A.V. Grishin, and M.N. Smirnov. 1980. 3 micron DNA - an extrachromosomal ribosomal DNA in the yeast *Saccharomyces cerevisiae*. *Gene* 12: 41-9.
- Laurenson, P. and J. Rine. 1992. Silencers, silencing, and heritable transcriptional states. *Microbiol Rev* 56: 543-60.
- Leary, D.J. and S. Huang. 2001. Regulation of ribosome biogenesis within the nucleolus. *FEBS Lett* 509: 145-50.
- Lee, S.E., F. Paques, J. Sylvan, and J.E. Haber. 1999. Role of yeast *SIR* genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. *Curr Biol* 9: 767-70.
- Lin, S.J., P.A. Defossez, and L. Guarente. 2000. Requirement of NAD and *SIR2* for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* 289: 2126-8.
- Lin, S.J., M. Kaeberlein, A. Andalis, L.A. Sturtz, P.A. Defossez, V.C. Culotta, G.R. Fink, and L. Guarente. 2002. Caloric restriction extends life span by shifting carbon flow toward respiration. *Nature* in press.

- Lin, Y.H. and R.L. Keil. 1991. *Mutations affecting RNA polymerase I-stimulated exchange and rDNA recombination in yeast*. *Genetics* 127: 31-8.
- Lin, Y.J., L. Seroude, and S. Benzer. 1998. *Extended life-span and stress resistance in the Drosophila mutant methuselah*. *Science* 282: 943-6.
- Linskens, M.H. and J.A. Huberman. 1988. *Organization of replication of ribosomal DNA in Saccharomyces cerevisiae*. *Mol Cell Biol* 8: 4927-35.
- Longo, V.D., E.B. Gralla, and J.S. Valentine. 1996. *Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae*. *Mitochondrial production of toxic oxygen species in vivo*. *J Biol Chem* 271: 12275-80.
- Longo, V.D., L.L. Liou, J.S. Valentine, and E.B. Gralla. 1999. *Mitochondrial superoxide decreases yeast survival in stationary phase*. *Arch Biochem Biophys* 365: 131-42.
- Longtine, M.S., S. Enomoto, S.L. Finstad, and J. Berman. 1992. *Yeast telomere repeat sequence (TRS) improves circular plasmid segregation, and TRS plasmid segregation involves the RAP1 gene product*. *Mol Cell Biol* 12: 1997-2009.
- Luo, J., A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, and W. Gu. 2001. *Negative control of p53 by Sir2alpha promotes cell survival under stress*. *Cell* 107: 137-48.
- Martin, S.G., T. Laroche, N. Suka, M. Grunstein, and S.M. Gasser. 1999. *Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast*. *Cell* 97: 621-33.
- Masoro, E.J. 2000. *Caloric restriction and aging: an update*. *Exp Gerontol* 35: 299-305.
- McAinsh, A.D., S. Scott-Drew, J.A. Murray, and S.P. Jackson. 1999. *DNA damage triggers disruption of telomeric silencing and Mec1p-dependent relocation of Sir3p*. *Curr Biol* 9: 963-6.
- McVey, M., M. Kaeberlein, H.A. Tissenbaum, and L. Guarente. 2001. *The short life span of Saccharomyces cerevisiae sgs1 and srs2 mutants is a composite of normal aging processes and mitotic arrest due to defective recombination*. *Genetics* 157: 1531-42.
- Melov, S., J. Ravenscroft, S. Malik, M.S. Gill, D.W. Walker, P.E. Clayton, D.C. Wallace, B. Malfroy, S.R. Doctrow, and G.J. Lithgow. 2000. *Extension of life-span with superoxide dismutase/catalase mimetics*. *Science* 289: 1567-9.
- Michel, B., S.D. Ehrlich, and M. Uzest. 1997. *DNA double-strand breaks caused by replication arrest*. *EMBO J* 16: 430-38.

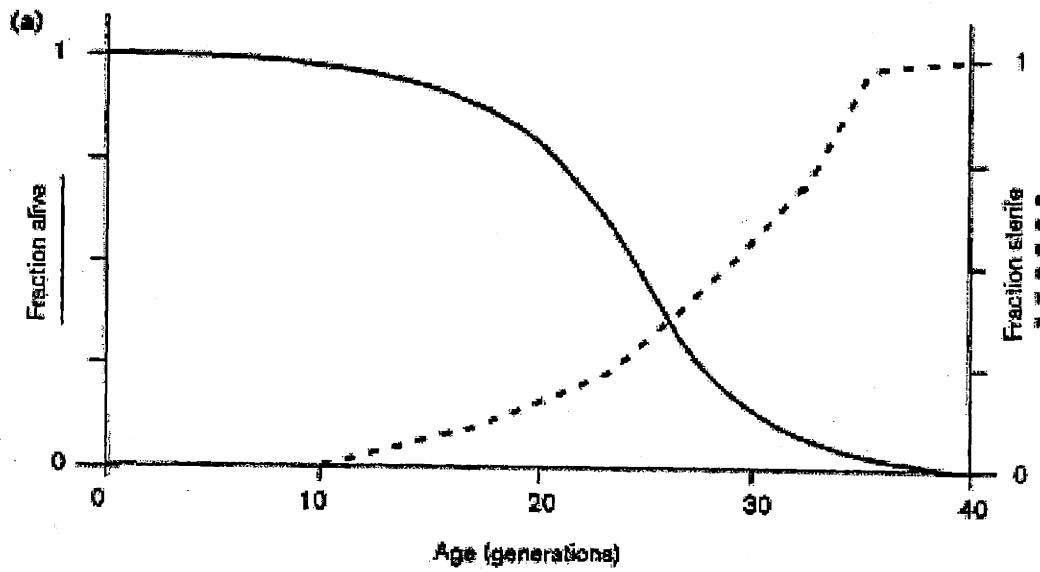
- Migliaccio, E., M. Giorgio, S. Mele, G. Pelicci, P. Rebaldi, P.P. Pandolfi, L. Lanfrancone, and P.G. Pelicci. 1999. *The p66shc adaptor protein controls oxidative stress response and life span in mammals*. *Nature* **402**: 309-13.
- Mills, K.D., D.A. Sinclair, and L. Guarente. 1999. *MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks*. *Cell* **97**: 609-20.
- Mohaghegh, P. and I.D. Hickson. 2001. *DNA helicase deficiencies associated with cancer predisposition and premature ageing disorders*. *Hum Mol Genet* **10**: 741-6.
- Mortimer, R.K. and J.R. Johnston. 1959. *Life span of individual yeast cells*. *Nature* **183**: 1751-1752.
- Müller, I. 1985. *Parental age and the life-span of zygotes of Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* **51**: 1-10.
- Muller, I., M. Zimmermann, D. Becker, and M. Flomer. 1980. *Calendar life span versus budding life span of Saccharomyces cerevisiae*. *Mech Ageing Dev* **12**: 47-52.
- Murray, A.W. and J.W. Szostak. 1983. *Pedigree analysis of plasmid segregation in yeast*. *Cell* **34**: 961-70.
- Nierras, C.R., S.W. Liebman, and J.R. Warner. 1997. *Does Saccharomyces need an organized nucleolus?* *Chromosoma* **105**: 444-51.
- Oakes, M., J.P. Aris, J.S. Brockenbrough, H. Wai, L. Vu, and M. Nomura. 1998. *Mutational analysis of the structure and localization of the nucleolus in the yeast Saccharomyces cerevisiae*. *J Cell Biol* **143**: 23-34.
- Orr, W.C. and R.S. Sohal. 1994. *Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster*. *Science* **263**: 1128-30.
- Ozenberger, B.A. and G.S. Roeder. 1991. *A unique pathway of double-strand break repair operates in tandemly repeated genes*. *Mol Cell Biol* **11**: 1222-31.
- Parikh, V.S., M.M. Morgan, R. Scott, L.S. Clements, and R.A. Butow. 1987. *The mitochondrial genotype can influence nuclear gene expression in yeast*. *Science* **235**: 576-80.
- Parkes, T.L., A.J. Elia, D. Dickinson, A.J. Hilliker, J.P. Phillips, and G.L. Boulianne. 1998. *Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons*. *Nat Genet* **19**: 171-4.
- Patterson, G.H., S.C. Schroeder, Y. Bai, A. Weil, and D.W. Piston. 1998. *Quantitative imaging of TATA-binding protein in living yeast cells*. *Yeast* **14**: 813-25.

- Schultz, M.C., S.J. Brill, Q. Ju, R. Sternglanz, and R.H. Reeder. 1992. *Topoisomerases and yeast rRNA transcription: negative supercoiling stimulates initiation and topoisomerase activity is required for elongation.* *Genes Dev* 6: 1332-41.
- Seigneur, M., V. Bidnenko, S.D. Ehrlich, and B. Michel. 1998. *RuvAB acts at arrested replication forks.* *Cell* 95: 419-30.
- Shaw, P.J. and E.G. Jordan. 1995. *The nucleolus.* *Annu Rev Cell Dev Biol* 11: 93-121.
- Shen, J.C. and L.A. Loeb. 2000. *The Werner syndrome gene: the molecular basis of RecQ helicase-deficiency diseases.* *Trends Genet* 16: 213-20.
- Sinclair, D.A. and L. Guarente. 1997. *Extrachromosomal rDNA circles--a cause of aging in yeast.* *Cell* 91: 1033-42.
- Sinclair, D.A., K. Mills, and L. Guarente. 1997. *Accelerated aging and nucleolar fragmentation in yeast sgs1 mutants.* *Science* 277: 1313-6.
- Smeal, T., J. Claus, B. Kennedy, F. Cole, and L. Guarente. 1996. *Loss of transcriptional silencing causes sterility in old mother cells of *S. cerevisiae*.* *Cell* 84: 633-42.
- Smith, J.S. and J.D. Boeke. 1997. *An unusual form of transcriptional silencing in yeast ribosomal DNA.* *Genes Dev* 11: 241-54.
- Smith, J.S., C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai, J.L. Avalos, J.C. Escalante-Semerena, C. Grubmeyer, C. Wolberger, and J.D. Boeke. 2000. *A phylogenetically conserved NAD⁺-dependent protein deacetylase activity in the Sir2 protein family.* *Proc Natl Acad Sci U S A* 97: 6658-63.
- Smith, J.S., C.B. Brachmann, L. Pillus, and J.D. Boeke. 1998. *Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p.* *Genetics* 149: 1205-19.
- Sun, J., S.P. Kale, A.M. Childress, C. Pinswasdi, and S.M. Jazwinski. 1994. *Divergent roles of RAS1 and RAS2 in yeast longevity.* *J Biol Chem* 269: 18638-45.
- Sung, P., V. Bailly, C. Weber, L.H. Thompson, L. Prakash, and S. Prakash. 1993. *Human xeroderma pigmentosum group D gene encodes a DNA helicase.* *Nature* 365: 852-5.
- Tadauchi, T., K. Matsumoto, I. Herskowitz, and K. Irie. 2001. *Post-transcriptional regulation through the HO 3'-UTR by Mpt5, a yeast homolog of Pumilio and FBF.* *Embo J* 20: 552-61.

- Tanner, K.G., J. Landry, R. Sternglanz, and J.M. Denu. 2000. Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proc Natl Acad Sci U S A* 97: 14178-82.
- Tanny, J.C., G.J. Dowd, J. Huang, H. Hilz, and D. Moazed. 1999. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. *Cell* 99: 735-45.
- Tanny, J.C. and D. Moazed. 2001. Coupling of histone deacetylation to NAD breakdown by the yeast silencing protein Sir2: Evidence for acetyl transfer from substrate to an NAD breakdown product. *Proc Natl Acad Sci U S A* 98: 415-20.
- Tissenbaum, H.A. and L. Guarente. 2001. Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* 410: 227-30.
- . 2002. Model organisms as a guide to mammalian aging. *Dev Cell* 2: 9-19.
- Tsang, A.W. and J.C. Escalante-Semerena. 1998. CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase activity in cobT mutants during cobalamin biosynthesis in *Salmonella typhimurium* LT2. *J Biol Chem* 273: 31788-94.
- Tsukamoto, Y., J. Kato, and H. Ikeda. 1997. Silencing factors participate in DNA repair and recombination in *Saccharomyces cerevisiae*. *Nature* 388: 900-3.
- Tyner, S.D., S. Venkatachalam, J. Choi, S. Jones, N. Ghebranious, H. Igelmann, X. Lu, G. Soron, B. Cooper, C. Brayton, S. Hee Park, T. Thompson, G. Karsenty, A. Bradley, and L.A. Donehower. 2002. p53 mutant mice that display early ageing-associated phenotypes. *Nature* 415: 45-53.
- Vaziri, H., S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, and R.A. Weinberg. 2001. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107: 149-59.
- Voelkel-Meiman, K., R.L. Keil, and G.S. Roeder. 1987. Recombination-stimulating sequences in yeast ribosomal DNA correspond to sequences regulating transcription by RNA polymerase I. *Cell* 48: 1071-9.
- Wai, H., K. Johzuka, L. Vu, K. Eliason, T. Kobayashi, T. Horiuchi, and M. Nomura. 2001. Yeast RNA polymerase I enhancer is dispensable for transcription of the chromosomal rRNA gene and cell growth, and its apparent transcription enhancement from ectopic promoters requires Fob1 protein. *Mol Cell Biol* 21: 5541-53.
- Ward, T.R., M.L. Hoang, R. Prusty, C.K. Lau, R.L. Keil, W.L. Fangman, and B.J. Brewer. 2000. Ribosomal DNA replication fork barrier and HOT1 recombination hot spot: shared sequences but independent activities. *Mol Cell Biol* 20: 4948-57.

- Warner, J.R. 1989. *Synthesis of ribosomes in Saccharomyces cerevisiae*. *Microbiol Rev* 53: 256-71.
- Watt, P.M., E.J. Louis, R.H. Borts, and I.D. Hickson. 1995. *Sgs1: a eukaryotic homolog of E. coli RecQ that interacts with topoisomerase II in vivo and is required for faithful chromosome segregation*. *Cell* 81: 253-60.
- Weeda, G., R.C. van Ham, W. Vermeulen, D. Bootsma, A.J. van der Eb, and J.H. Hoeijmakers. 1990. *A presumed DNA helicase encoded by ERCC-3 is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome*. *Cell* 62: 777-91.
- Weindruch, R., R.L. Walford, S. Fligiel, and D. Guthrie. 1986. *The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake*. *J Nutr* 116: 641-54.
- Werner-Washburne, M., E. Braun, G.C. Johnston, and R.A. Singer. 1993. *Stationary phase in the yeast Saccharomyces cerevisiae*. *Microbiol Rev* 57: 383-401.
- Yu, C.E., J. Oshima, Y.H. Fu, E.M. Wijsman, F. Hisama, R. Alisch, S. Matthews, J. Nakura, T. Miki, S. Ouais, G.M. Martin, J. Mulligan, and G.D. Schellenberg. 1996. *Positional cloning of the Werner's syndrome gene*. *Science* 272: 258-62.

FIGURE 1. Age-related phenotypic changes in *S. cerevisiae*. (a) As a cell gets older its probability of dying increases exponentially. The occurrence of sterility also increases with age, with almost every very old cell being sterile. (b) Changes in nuclear morphology with age. In a young cell, the nucleolus (light gray oval) is small, and the Sir complex (dark gray) resides at telomeric foci and silent mating-type cassettes. In middle age, the Sir complex leaves the previously silenced loci, causing sterility, and concentrates in the enlarged nucleolus. In old cells, the nucleolus enlarges further and fragments.



(b) Young Middle Age Old

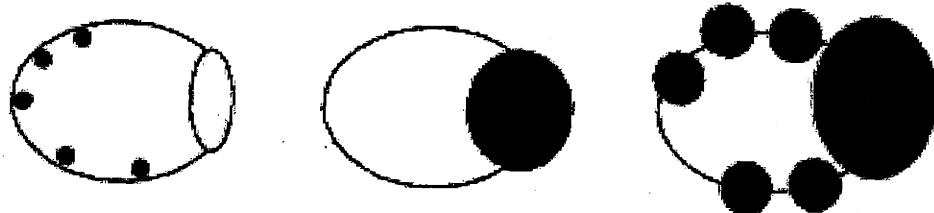
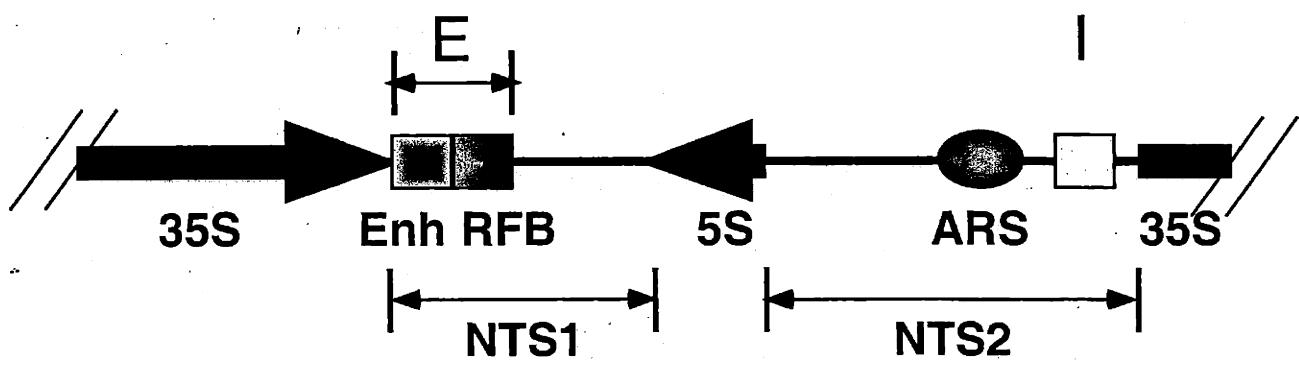




FIGURE 2. Structure of rDNA repeats in *S. cerevisiae*. The yeast rDNA is organized in an array of 150-200 directly repeated units. One repeat unit of rDNA is 9,137 bp. The locations of the 35S and 5S rRNA genes (thick black arrows; the direction of transcription is indicated by the direction of arrows), nontranscribed spacer regions (NTS1 and NTS2), replication fork barrier (RFB), RNA polymerase I (Pol I) enhancer (Enh), and origin of replication (ARS) are shown. Fragments containing the 35S rDNA transcriptional enhancer and initiator (called E and I elements, respectively) (Voelkel-Meiman et al. 1987), essential to HOT1-stimulated recombination, are indicated. E element consists of RFB and Pol I enhancer (Enh). Pol I enhancer (Enh) is dispensable for transcription of the native rRNA gene and cell growth, but not for transcription and recombination enhancement from ectopic promoters (initiators) (Wai et al. 2001).



rDNA repeat unit (9.1kb)

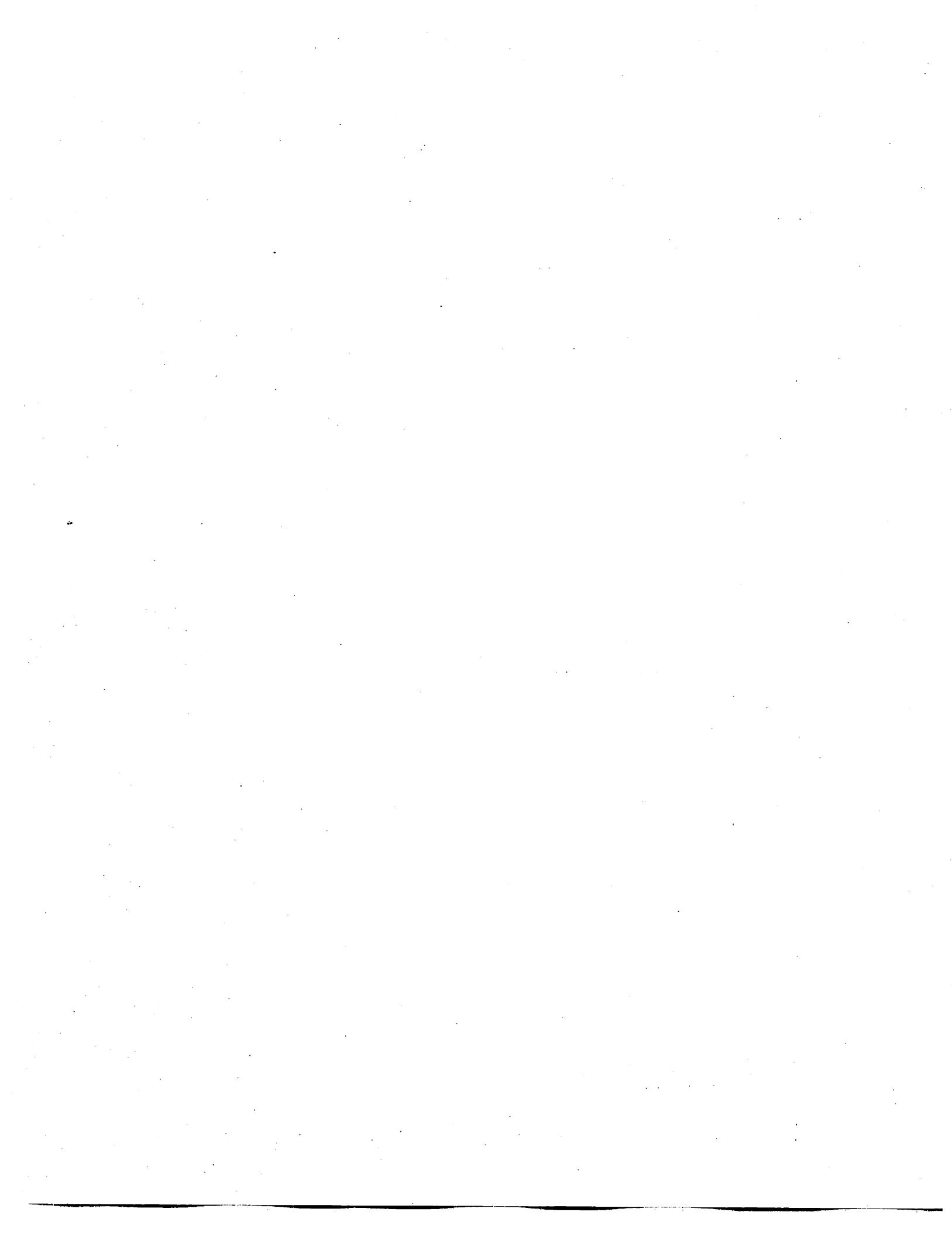
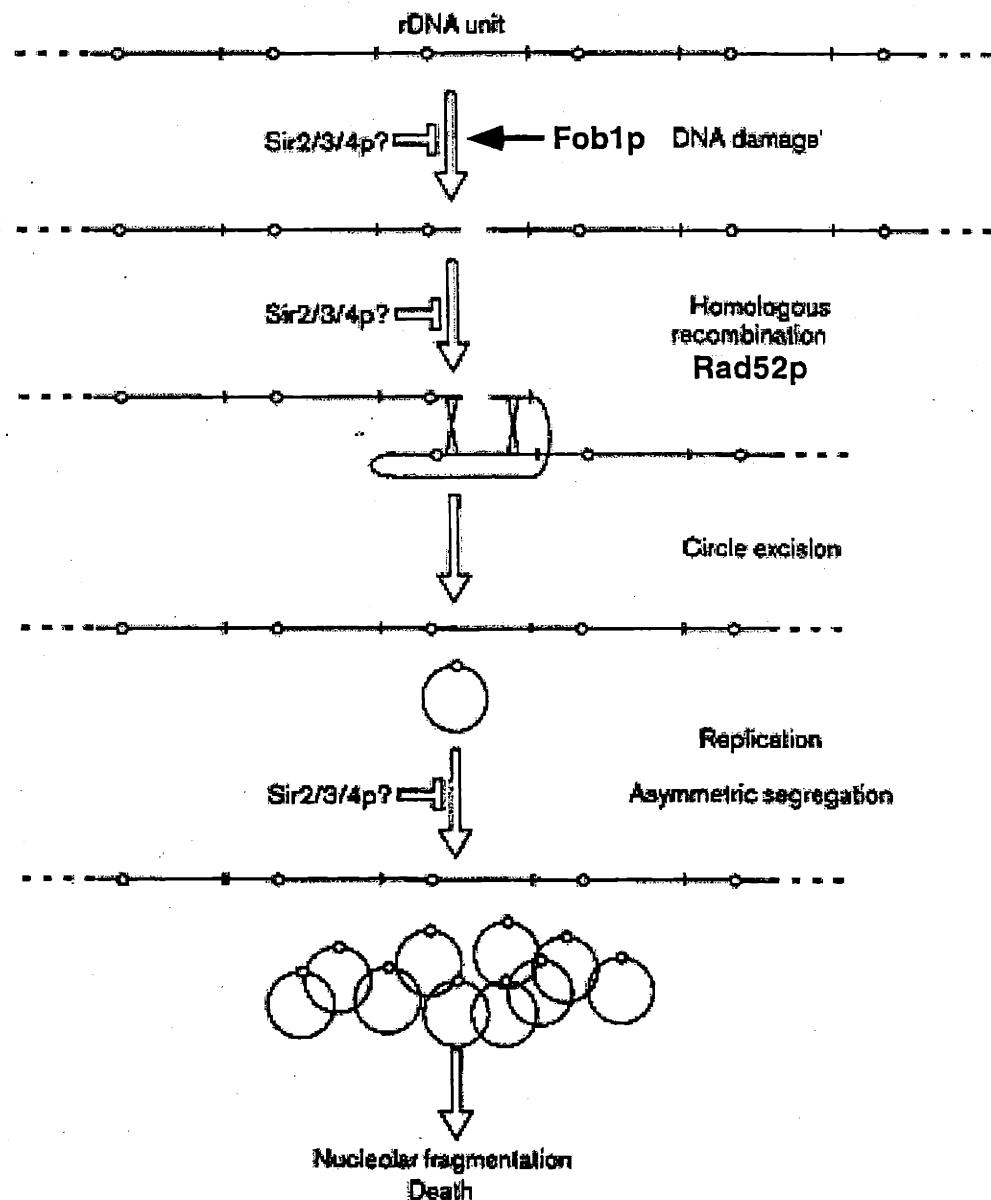
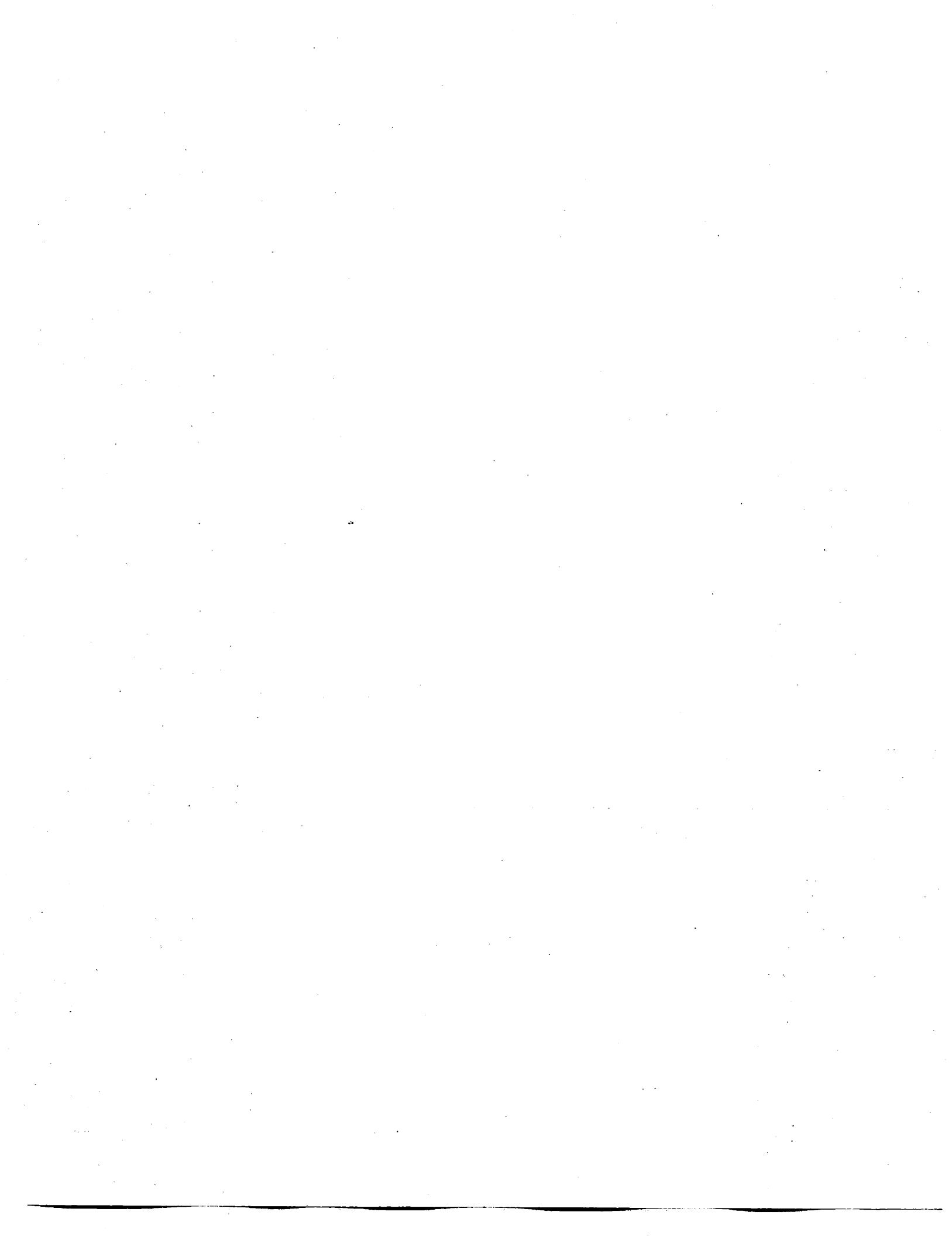


FIGURE 3. A hypothetical senescence pathway in yeast. Extrachromosomal rDNA circles (ERCs) can excise from the rDNA repeats through Rad52p-mediated homologous recombination. They can then replicate by virtue of the replication origin present in each repeat (small circle), and display a segregation bias leading to their accumulation in the mother cell, eventually causing nucleolar fragmentation and death. Double-strand DNA breaks could trigger this recombination event. A mutation in the *FOB1*, which is required for replication fork barrier (RFB), reduces ERC accumulation and thus extends life span, a likely consequence of absence of double-strand breaks caused by the replication fork pausing at RFB. Additionally, the redistribution of the Sir2/3/4p complex to the nucleolus might act to decrease DNA damage, inhibit recombination, or decrease circle segregation bias.







Chapter 2

Effects of Mutations in DNA Repair Genes on the Formation of rDNA Circles and Life span in *S.* *cerevisiae*

This chapter was published in *Molecular and Cellular Biology*, Volume 19, Pages 3848-3856 in 1999. The authors were Peter U. Park, Pierre-Antoine Defossez, and Leonard Guarente.

ABSTRACT

A cause of aging in *Saccharomyces cerevisiae* is the accumulation of extrachromosomal rDNA circles (ERCs). Introduction of an ERC into young mother cells shortens life span and accelerates the onset of age-associated sterility. It is important to understand the process by which ERCs are generated. Here, we demonstrate that homologous recombination is necessary for ERC formation. *rad52* mutant cells, defective in DNA repair through homologous recombination, do not accumulate ERCs with age, and mutations in other genes of the *RAD52* class have varying effects on ERC formation. *rad52* mutation leads to a progressive delocalization of Sir3p from telomeres to other nuclear sites with age, and, surprisingly, shortens life span. We speculate that spontaneous DNA damage, perhaps double strand breaks, causes lethality in mutants of the *RAD52* class and may be an initial step of aging in wild type cells.

INTRODUCTION

One of the hallmarks of aging in most organisms is that mortality rate increases exponentially with age (Finch 1990). Because yeast cells divide asymmetrically, mother and daughter cells can be separated microscopically at each cell division, and such experiments reveal that mothers have a fixed division capacity, called their life span. A number of morphological changes occurs as mother cells grow older: slowing of the cell cycle, enlargement of cell size, loss of mating ability, and accumulation of intracellular granules (Mortimer and Johnston 1959; Müller 1985; Smeal et al. 1996). The daughter cells from old mothers have a reduced life span potential, hinting that a dominant "cytoplasmic senescence factor" asymmetrically accumulates in old mother cells and that this factor can leak to daughter cells from old mothers (Egilmez and Jazwinski 1989; Kennedy et al. 1994).

A genetic study has revealed that the allele of *SIR4* affects life span (Kennedy et al. 1995). Null alleles cause a shortened life span, and a gain of function allele gives rise to an extended life span. The *SIR2/3/4* gene products are normally positioned at telomeres and *HM* loci, where they mediate transcriptional silencing (Loo and Rine 1995; Grunstein 1997). *SIR2* also plays a role at the nucleolus, the site of repeated copies of ribosomal DNA (rDNA), to suppress recombination and mediate silencing (Gottlieb and Esposito 1989; Bryk et al. 1997; Smith and Boeke 1997). In aging cells the SIR complex at telomeres and *HM* loci relocates to the nucleolus (Kennedy et al. 1997). This relocalization is mimicked constitutively by the gain of function allele of *SIR4* that extends life

span (Kennedy et al. 1995). Thus, the relocalization of the Sir complex to the nucleolus extends life span in wild type yeast cells.

Studies of the human WRN gene, recessive mutations in which cause the disease Werner Syndrome (Yu et al. 1996), further support the close link between the nucleolus and aging. Individuals with Werner Syndrome show symptoms of accelerated aging, including hair graying and loss, atherosclerosis, bilateral ocular cataracts, diabetes, and osteoporosis (Epstein et al. 1966; Salk 1982). WRN has greatest homology with genes encoding DNA helicases of the RecQ family, including *S. cerevisiae* *SGS1* (Gangloff et al. 1994), *E. coli* *RecQ* (Nakayama et al. 1985; Kowalczykowski et al. 1994), *S. pombe* *rqh1* (Stewart et al. 1997), *Xenopus laevis* FFA-1 (Yan et al. 1998), and human *BLM* and *RecQL* (Purānam and Blackshear 1994; Ellis et al. 1995). The WRN protein has been demonstrated to have ATP-dependent DNA helicase activity and 3'→5' exonuclease activity (Gray et al. 1997; Huang et al. 1998; Shen et al. 1998). Importantly, WRN protein is localized in the nucleolus in human cells (Gray et al. 1998; Marciniak et al. 1998), suggesting its role in promoting longevity may be linked to a nucleolar function.

The *sgs1* mutation suppresses the slow growth and hyperrecombination at the rDNA caused by a *top3* mutation and Sgs1p interacts with both Top2p and Top3p (Gangloff et al. 1994; Watt et al. 1995). The *sgs1* mutation also causes genomic instabilities, including hyperrecombination at rDNA and other loci and a reduction in fidelity of both mitotic and meiotic chromosome segregation (Gangloff et al. 1994; Watt et al. 1995; Watt et al. 1996). Interestingly, like the WRN mutation, the *sgs1* mutation accelerates aging: it decreases the average life span of yeast cells by 60% and accelerates the onset of age-associated phenotypes, including sterility and the redistribution of the Sir proteins from

telomeres to the nucleolus (Sinclair et al. 1997). Sgs1p, like WRN protein, is concentrated in the nucleolus (Sinclair et al. 1997). In addition, expression of the WRN protein in the *sgs1* mutant suppresses the hyperrecombination phenotype (Yamagata et al. 1998).

Microscopic analysis has revealed that the nucleolus in old mother cells is enlarged and fragmented (Sinclair et al. 1997). These changes are caused by the genomic instability in the tandem repeats of rDNA. Midway in the life span of mother cells, an extrachromosomal rDNA circle (ERC) is excised from the genome (Sinclair and Guarente 1997). Each ERC contains an ARS sequences, and plasmids containing such sequences autonomously replicate and segregate asymmetrically in mother cells (Murray and Szostak 1983). Thus, mother cells build up ERCs to very high levels and daughters are ERC free (Sinclair and Guarente 1997). The release of a single ERC in young cells is sufficient to shorten life span by 40%, proving that ERCs accumulation causes senescence. ERCs can leak into daughters of very old mothers, consistent with the view that they are the previously described senescence factor (Egilmez and Jazwinski 1989; Kennedy et al. 1994).

Since the *sgs1* mutant displays hyperrecombination at the rDNA, it is possible that cellular recombination mechanisms lead to the formation of ERCs. We thus sought to understand how ERCs were formed. Here, we analyze the effects of mutations that cause a defect in recombination on ERC formation and aging. Our findings show a link between ERC formation and the *RAD52* pathway of homologous recombination. Further, our results suggest that DNA breaks might be an early event in the aging process, which then triggers the

formation of ERCs and the release of the Sir protein complex from telomeres in aging cells.

RESULTS

RAD52 is required for ERC formation and longevity.

To investigate the possibility that ERCs are excised from the rDNA locus through intrachromosomal homologous recombination, we tested whether ERC formation requires *RAD52*, a gene needed for most homologous recombinational events (Petes et al. 1991; Shinohara and Ogawa 1995). Age-matched wild-type and mutant cells that have divided on average 7 to 8 generations were magnetically sorted and their DNA was analyzed using gel electrophoresis. While the old wild-type cells clearly accumulated ERC species, ERCs were undetectable in the old *rad52* cells (Fig. 1A). A small amount of ERCs could have been present in the old *rad52* cells and gone undetected. Thus, we also searched for ERCs in *sgs1 rad52* double mutant cells. Although the old *sgs1* cells accumulated slightly more ERCs than the age-matched wild-type cells, the old *sgs1 rad52* cells showed no detectable ERCs (Fig. 1A). Thus, the *RAD52* gene and, presumably, homologous recombination are required for the formation of ERCs.

Since ERCs are a cause of aging and the old *rad52* cells do not accumulate ERCs, one might predict that the *rad52* mutant will have a long life span. To test this possibility, we performed a life span analysis on *rad52*, *sgs1*, and *sgs1 rad52* cells. Contrary to the prediction, the average life span of *rad52* cells (avg. = 7.1) was about 70% shorter than that of the wild-type strain (avg. = 23.5) (Fig. 1B). It was even shorter than the average life span of *sgs1* cells (avg. = 9.8). Interestingly, the *sgs1 rad52* cells (avg. = 5.5) had a slightly shorter average life

span than that of the *rad52* cells, indicating synthetic shortening of life span by each mutation.

rad52 cells lose chromosomes at an elevated rate (Mortimer et al. 1981). To determine whether chromosome loss was responsible for the premature death of *rad52* cells, we compared the life span of *rad52* haploid cells and homozygous *rad52* diploid cells. Chromosome loss in diploid cells should not lead to lethality. The wild-type diploids showed a similar life span as the wild-type haploids (Fig. 1C), as previously reported (Müller 1971; Kennedy et al. 1995). The homozygous *rad52* diploids (avg. = 7.5) also displayed a similar life span as the *rad52* haploids (Fig. 1C). Thus, *rad52* cells do not appear to die due to loss of essential genes caused by a chromosome loss.

Interestingly, 70 to 80% of both *rad52* haploid and diploid mother cells ceased dividing as large-budded cells, while only 15-25% of wild-type cells arrested as large-budded cells. Therefore, most of the *rad52* cells arrested at the G2/M phase of the cell cycle, perhaps due to a failure to adapt after DNA damage-induced checkpoint arrest (Sandell and Zakian 1993; Lee et al. 1998). These findings suggest that premature death in *rad52* mutant could be caused by double strand breaks (DSBs), which go unrepaired. In wild type cells these breaks would be repaired using sister chromatids through homologous recombination. Moreover, the repair of breaks in the rDNA might also generate ERCs if repaired using another rDNA repeat on the same chromosome.

The *rad52* mutant displays a premature loss of silencing at HMR.

We then investigated whether other age-associated phenotypes are still present in cells that do not accumulate ERCs with age. One of the hallmarks of

yeast aging is the gradual increase in the number of cells that lose silencing at the *HM* loci (Smeal et al. 1996). We investigated the state of silencing in old *rad52* cells. Because the previous assay to determine age-specific phenotype of sterility is laborious (Smeal et al. 1996; Sinclair et al. 1997), we developed an assay to easily detect the state of silencing at the *HM* loci. We replaced the *a1* and *a2* genes at the *HMR* locus with the GFP gene driven by the constitutive *ADH1* promoter (Fig. 2A). We postulated that in young cells GFP expression would be silenced, while in old cells GFP would be expressed, giving rise to green fluorescence.

Indeed, GFP was efficiently silenced in young cells as measured by Fluorescence Activated Cell Sorting (FACS). Young cells with GFP at *HMR* had a similar profile to cells without GFP except for a small sub-population of cells with slightly higher fluorescence (Fig. 2B). GFP expression at *HMR* was, as expected, silenced in a Sir-dependent manner: young *sir3* cells showed about 10-fold higher fluorescence than young wild-type cells. Moreover, in aging cells with GFP (average 7 - 8 generations old), a sub-population of the cells showed higher fluorescence, indicated by the rightward shift of the fluorescence histogram (Fig. 2B). It is known that old cells become enlarged, which might cause an increase in autofluorescence. However, this does not account for the increase in fluorescence in old cells with GFP because the difference between young and old cells *with* GFP (4.95) is more than three-fold higher than that between young and old cells *without* GFP (1.31). This assay is thus effective in determining the age-specific phenotype of loss silencing at *HMR*.

FACS analysis of the old *rad52* cells that were on average 7 - 8 generations-old also showed that a high proportion of cells (avg. fluor. = 26.08)

have lost silencing compared to the *age-matched*, wild-type cells (avg. fluor. = 9.60) (Fig. 2B). Again, the increase in the average fluorescence seen in old *rad52* cells is not due to the enlargement of cells (Fig. 2). Thus, although devoid of rDNA circles, *rad52* cells prematurely lose HMR silencing as they age.

Sir3p redistributes from telomeres to other sites in the nucleus in old *rad52* cells.

Loss of HM silencing in old wild-type cells is likely due to the redistribution of Sir3 proteins from the telomeres and HM loci to the nucleolus (Kennedy et al. 1997). Thus, we examined Sir3p localization in old *rad52* cells by indirect immunofluorescence using anti-Sir3p antibody. The nucleus is stained with DAPI (blue) and the nucleolus with anti-Nop1p (red) in this experiment. In young *rad52* cells, Sir3p was found at 3 to 7 bright perinuclear foci (green), characteristic of telomeres (Fig. 3). This pattern was indistinguishable from that observed in young wild-type cells. Old wild-type cells (about 18 generations-old) showed a nucleolar relocalization of Sir3p (yellow in the merged image), as previously described (Kennedy et al. 1997). Distinct from the old wild type cells, 20 to 30% of sorted, old *rad52* cells (average 7 to 8 generations-old) showed a diffuse, nuclear pattern of Sir3p staining that included the nucleolus. About a third of the cells that displayed a diffuse, nuclear pattern showed many bright foci, some of which could be telomeric foci. The remaining cells showed a telomeric staining like young cells. We believe that those cells that showed a diffuse, nuclear pattern are cells that have reached the end of their life span. They are not likely to be dead cells because very old wild-type cells (18 generations-old) did not give a similar staining. The pattern of nuclear staining is consistent

with the movement of Sir3p away from telomeres and *HM* loci and could explain the premature loss of silencing seen at *HMR* (Fig. 2B). We speculate that the Sir proteins, which play a role in DNA repair through nonhomologous end-joining (Tsukamoto et al. 1997; Boulton and Jackson 1998), leave the telomeres and *HM* loci to repair DNA damage, perhaps DSBs, that occur elsewhere in old *rad52* cells.

Role of other genes in the *RAD52* epistasis group for ERCs formation and longevity.

We then set out to determine if other genes important for homologous recombination played a role in ERC formation. *RAD51* encodes a RecA homolog, and *RAD57* shows RecA homology (Johnson and Symington 1995; Shinohara and Ogawa 1995; Sung 1997). Both display a partial defect in homologous recombination (Aguilera 1995; Rattray and Symington 1995). In contrast to the old *rad52* cells, old *rad51* and *rad57* cells had a detectable level of ERCs, albeit lower than that of the age-matched wild-type cells (Fig. 4A). Also, both *rad51* (avg. = 13.0) and *rad57* (avg. = 12.5) mutations shortened life span by about 40% (Fig. 4B). The less severe shortening of life span seen in *rad51* and *rad57* mutants compared to that of the *rad52* mutant (70% shorter) (Fig. 1A) correlates with their lesser degree of deficiency in homologous recombination. FACS analysis of age-matched *rad51* (avg. fluor. = 20.30) and *rad57* cells (avg. fluor. = 19.56) showed a premature loss of silencing compared to the *age-matched* wild-type cells (Fig. 2B).

We also examined another member of *RAD52* epistasis group, *RAD50*, which plays a role in resection of broken ends by a 5' to 3' exonuclease activity during DSB-induced homologous recombination (Ivanov et al. 1994; Shinohara

and Ogawa 1995). Strikingly, the *rad50* mutant had a life span (avg. = 7.3) similar to the *rad52* mutant (avg. = 7.1) (Fig. 4B, 1B), and showed a low, but visible amount of ERCs (Fig. 4A). While indirect immunofluorescence performed on young *rad50* cells showed a similar pattern of staining as young wild-type cells, old *rad50* cells showed a diffuse, nuclear localization of Sir3p (Fig. 3). As in the *rad52* mutant, a defect in homologous recombination may play a role in the shortening of life span in the *rad50* mutant. Since *RAD50* also has roles in illegitimate recombination, telomeric maintenance, and checkpoint function (Allen et al. 1994; Moore and Haber 1996; Boulton and Jackson 1998; Nugent et al. 1998), it is also possible that a disruption in these functions contributes to the shortened life span in the mutant.

Effects of mutations in other DNA repair genes on longevity.

We then investigated if the effects of mutations in DNA repair genes on longevity are restricted to mutants defective in homologous recombination. Another form of repair that applies to repeated DNA sequences is single-strand annealing (SSA) (Ozenberger and Roeder 1991; Haber 1995). SSA occurs between homologous regions flanking a DSB, by annealing of complementary DNA after extensive 5' to 3' degradation extending away from the break (Fishman-Lobell and Haber 1992; Baumann and West 1998). *RAD1* encodes an endonuclease that can remove nonhomologous single-strand ends of a DSB, and it is required for SSA (Ivanov and Haber 1995; Ivanov et al. 1996). The *rad1* mutation did not have a significant effect on life span (Fig. 5), indicating that SSA is not necessary for normal life span.

Since *RAD1* is also required for nucleotide-excision repair (Prakash et al. 1993; Aboussekha and Wood 1994), we infer that this form of repair is also not germane to aging. Consistent with this conclusion, mutation in another gene involved in nucleotide-excision repair, *RAD7* (Miller et al. 1982; Prakash et al. 1993), did not affect life span (Fig. 5). Finally, the *rad26* mutation, which causes a defect in transcription-coupled repair (van Gool et al. 1994), also had no effect on life span (Fig. 5). In summary, the shortening of life span by mutations in the DNA repair genes examined is specific to those affecting homologous recombination.

DISCUSSION

Effects of mutations in DNA repair genes on ERC formation and life span in mother cells.

In this paper we have determined the effects of mutations in various DNA repair genes on the formation of extrachromosomal rDNA circles (ERCs) and life span. Interestingly, mutations in *RAD52*, *RAD50*, and *RAD51* (or *RAD57*), all of which affect homologous recombination, gave a total, severe, or partial reduction, respectively, in the formation of ERCs. Thus, the formation of ERCs requires the activity of the *RAD52*-dependent pathway of homologous recombination. Surprisingly, these mutations did not extend life span of mother cells, but, rather, shortened their life span.

Since ERCs are a *cause* of aging in wild type mother cells, how can we explain the shortened life spans of these mutants? For the *rad52*, *rad51*, and *rad57* mutants, the degree of shortening correlates well with the severity in the reduction of homologous recombination in these mutants. In fact, it is this reduction in homologous recombination that governs the lower rate of generation of ERCs in these mutants.

Surprisingly, the *rad50* mutation, which has a modest or no effect on the intrachromosomal recombination rate (Game 1993; Haber 1995; Rattray and Symington 1995), had a severe reduction in ERC accumulation. Heteroallelic interchromosomal recombination is increased by 10-fold in the *rad50* mutant (Malone and Esposito 1981; Malone et al. 1990). It is possible that *RAD50*, along with *MRE11* and *XRS2*, regulates the balance of intrachromosomal vs. interchromosomal recombination events. In the absence of *RAD50* function, the

frequency of the interchromosomal events could increase at the expense of reduction in intrachromosomal events. If so, the severe reduction in ERC formation observed in the *rad50* mutant may be due to such dysregulation. Since *RAD50* also has roles in illegitimate recombination, telomeric maintenance, and checkpoint function (Allen et al. 1994; Moore and Haber 1996; Boulton and Jackson 1998; Nugent et al. 1998), it is possible that a disruption in these functions contributes to the reduction in ERC formation and shortening of life span, in addition to the disruption in homologous recombination.

Since DNA lesions, such as DSBs are not repaired efficiently in the mutants defective in homologous recombination, these mutants are likely to be dying prematurely due to unrepaired DSBs. Consistent with this idea, unlike wild-type cells, most of the *rad52* cells ceased dividing as large budded cells (G2/M). The premature death of *rad52* cells does not appear to be due to chromosome loss since the *rad52* haploid cells lived as long as the *rad52* diploid cells. Introduction of two unrepairable DSBs that cannot be repaired through homologous recombination in wild-type cells causes an adaptation failure and a permanent G2/M arrest (Lee et al. 1998). We speculate that old *rad52* cells cease dividing and permanently arrest at G2/M because of multiple unrepaired DSBs.

In wild type cells, these DSBs and other lesions are repaired efficiently so that cells escape early death. However, as a by-product of those repair events, ERCs can be generated by homologous recombination in the rDNA, and these ERCs then carry out the proposed gradual aging program (Fig. 6). By this view, the generation of an ERC in a mother cell at once corrects the acute problem of a DNA break in the rDNA at the price of establishing the mortality of that mother cell lineage.

Mutations in other *RAD* genes were also examined but did not affect life span. These include mutants defective in single strand annealing (*rad1*), nucleotide-excision repair (*rad1* and *rad7*), and transcription-coupled repair (*rad26*). Thus, the only DNA repair genes examined that shortened life span were a part of the *RAD52* pathway of homologous recombination. Finally, mutation in the *RAD52* homolog, *RAD59* (Bai and Symington 1996), had a little effect on life span (data not shown).

Redistribution of Sir3p away from telomeres in old *rad* mutant cells defective in homologous recombination.

In wild type cells, the Sir complex bound at telomeres redistributes to the nucleolus approximately mid-way in the life span of mothers (Kennedy et al. 1997). This relocalization leads to the appearance of the sterile phenotype because of a loss of silencing at *HML* and *HMR*, from which the Sir complex has departed (Kennedy et al. 1995). We have speculated that the generation or accumulation of ERCs is slowed down by the redirected Sir complex, explaining the life span extension (Sinclair and Guarente 1997; Defossez et al. 1998). In *rad* mutants defective in homologous recombination, immunostaining with anti-Sir3p antibodies showed that the Sir complex, in contrast with old wild type cells, is present diffusely throughout the nucleus including the nucleolus. This relocalization is probably responsible for the loss of silencing with age, as determined by the expression of a GFP marker inserted at *HMR* (Fig. 2).

Why is there a redistribution of the Sir complex away from telomeres and *HM* loci with age in *rad* mutants? We infer that the relocalization of the Sir complex in *rad* mutant cells is caused by DSBs at the rDNA and elsewhere in the

genome, most likely during DNA replication (Fig. 6) (Michel et al. 1997; Zou and Rothstein 1997; Seigneur et al. 1998). The Sir proteins, along with Ku70/80, have been implicated in the repair of DSBs by an end-joining reaction in yeast (Tsukamoto et al. 1997). Recent findings show that the induction of DSBs by EcoRI endonuclease also elicits the movement of the Sir complex away from telomeres (Mills et al. 1999). We speculate that diffuse nuclear staining is not observed in the wild type because DSBs are repaired efficiently by homologous recombination between sister chromatids in the S or G2 phases of the cell cycle.

The redistribution of the Sir complex to the nucleolus in wild type cells may be triggered by DSBs that occur specifically in the rDNA. The recruitment of the Sir complex to the nucleolus in aging wild type cells may be an attempt to employ end-joining to supplement the repair of rDNA breaks by homologous recombination (Fig. 6). The repair of such damage by other repair pathways, including SSA and end-joining pathways, would avoid the possibility of generating ERCs by homologous recombination in the tandemly repeated rDNA. A DSB occurring at rDNA has been shown to be efficiently repaired in *rad52* cells through SSA pathway (Ozenberger and Roeder 1991). The deletion of the *RAD1* gene does not have a significant effect on life span (Fig. 5), suggesting that either the contribution of SSA in DSB repair in the rDNA is small, or *RAD1* is not required for SSA in the rDNA.

However, it is also possible that the redistributed Sir complex extends life span of wild type cells through other mechanisms (Defossez et al. 1998): by bolstering Sir2p-mediated suppression of rDNA recombination and thus reducing ERC formation (Gottlieb and Esposito 1989; Fritze et al. 1997); by slowing the replication of ERCs that have already formed; or by reducing the

bias in the segregation of these plasmids for mother cells (Ansari and Gartenberg 1997).

DSBs in aging - a general mechanism?

Our results argue that DNA damage, probably DSBs, occurs throughout the genome of a wild-type yeast cells during its life span, and is normally repaired efficiently through homologous recombination. Two lines of evidences suggest that yeast rDNA is particularly prone to DSBs. First, the continuous activity of topoisomerases, perhaps along with Sgs1p, is required for the transcription of rDNA (Schultz et al. 1992), and for the maintenance of stability of the rDNA repeats in the genome (Christman et al. 1988; Kim and Wang 1989; Christman et al. 1993; Gangloff et al. 1994). These findings suggest that a high rate of rDNA transcription may pose unusual topological problems. Second, yeast cells accumulate arrested replication forks at rDNA (Brewer and Fangman 1988; Brewer et al. 1992), which in *E. coli*, are known to generate DSBs (Michel et al. 1997; Seigneur et al. 1998). Concordantly, mutation in the *FOB1* gene (Lin and Keil 1991; Kobayashi and Horiuchi 1996; Kobayashi et al. 1998), which is required for replication fork blocking and HOT1-recombination activities, extends life span of mother cells (Defossez et al. 1999).

The identification of a DNA lesion that triggers the formation of ERCs may be important in the larger context of aging in higher organisms. In mammals, the repair of DNA breaks by homologous recombination is less avid than in yeast (Sargent et al. 1997; Liang et al. 1998). Rather, nonhomologous end-joining pathway involving DNA-PK and Ku appears to be as important as homologous recombination pathway (Takata et al. 1998). Any break in

mammalian rDNA therefore, may be resolved to yield not an ERC but a deletion within the genomic rDNA array.

A human homolog of *SGS1*, *WRN*, is defective in people with the premature aging disease Werner Syndrome. It is of interest that *WRN* protein, like *Sgs1p*, is concentrated in the nucleolus in human cells (Sinclair et al. 1997; Gray et al. 1998; Marciniak et al. 1998). Moreover, deletions in genomic DNA occur at an elevated frequency in WS cells (Fukuchi et al. 1989; Fukuchi et al. 1990), although a specific affect on rDNA has yet to be demonstrated. It will be of interest to determine whether deletions resulting from DSBs accumulate with age in mammalian rDNA and whether a progressive loss in functional rDNA copies is a plausible explanation of aging-related changes.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study are listed in Table 1. All strains are isogenic. Strains were cultured at 30°C using standard media (Sherman et al. 1986). For isolation of old cells, YPD with 2.5% glucose was used as previously described (Sinclair and Guarente 1997). PPY74 (*RDN1::ADE2*) was constructed by transforming PSY316α with pDS40 (Sinclair and Guarente 1997). PPY16 and PPY103 were constructed by transforming PSY316a and PPY74 with pBS/SK-E1/E2-I3/I4-3ARU (a gift of A. Lau and S. Bell) cut with KpnI/NotI. This transformation replaced the region from *HMR-I* to *HMR-E* silencer with the *URA3* gene (*hmrΔ1::URA3*), which is not silenced. PPY27 was constructed by transforming with PPY16 with a gel-purified, BamHI/PstI *ADE2* fragment from pURADE2 (Sinclair and Guarente 1997). PPY35 and PPY143 were made by transforming PPY27 and PPY103 with pPP46 cut with PshAI/AatII and uncut pRS315 (Sikorski and Hieter 1989). The transformed cells were first grown on Leu⁻ plate to select for cells that had acquired pRS315 and replica-plated onto Leu⁻ 5-FOA plate to select for Ura⁻ cells among the Leu⁺ cells. Ura⁻ Leu⁺ cells were screened by PCR to check for the correct transformants (*hmrΔ2::ADH1-GFP*). PPY56 (*sir3Δ::URA3*) was constructed by transforming PPY35 with pDM42 (Mahoney and Broach 1989). To construct PPY70 (*sgs1Δ::hisG*), after transforming PPY35 with pPP69 cut with NotI, a correct transformant was passed over 5-FOA to eliminate the *URA3* gene (Boeke et al. 1987). PPY98 (*rad52::LEU2*) was constructed by transforming PPY35 with pSM20 (D. Schild).

Disruption of *rad* genes was carried out by one-step transplacement method (Rothstein 1991; Baudin et al. 1993). The following regions within coding sequences of *rad* genes were replaced with *HIS3*: from +40 to +3285 for *rad1Δ*; from +21 to +1648 for *rad7Δ*; from +49 to +3253 for *rad26Δ*; from +165 to +3843 for *rad50Δ*; from +1 to +1196 for *rad51Δ*; from +63 to +1488 for *rad52Δ*; from +43 to +1379 for *rad57Δ*; and from +39 to +668 for *rad59Δ*.

Plasmids

pPP46 was constructed by following procedures: 1) pJR1426 (a gift of M. Foss and J. Rine) contains a 5.1 kb fragment of *HMRα* in a pRS316 backbone (Sikorski and Hieter 1989). The *HMRα* fragment contains the *α1* and *α2* genes replaced with the *α1* and *α2* genes, but contains intact *HMR-E* and *HMR-I* silencers. pJR1426 was first cut with SacI/SmaI, and the ends were blunted with the Klenow Fragment and ligated, resulting in pPP21. These procedures removed the linker sequences, including *Xba*I site, between the two restriction sites that are outside of the *HMRα* fragment; 2) pPP21 was digested with BclI/*Xba*I, which removed *α1* and *α2* genes, and ligated with polylinker insert cut with BclI/*Xba*I, resulting in pPP33. This polylinker insert, containing many restriction sites, was created by annealing and extending following two oligonucleotides with *Taq* DNA polymerase: 5'-GCGCGTCGGCCGCTGATCAG TCGACTCGCGATCGATCCTAGGCT AGCGAATT CAGATCTTCCGGA-3' and 5'-GCTGGCTCTAGAGCATGCGGCCGGTTAACCCGCGGTCCGGAAGATCT GAATT CGCTAGCCTAGGA-3'; 3) pPP16 was constructed by inserting HindIII fragment containing sGFP (S65T and V163A mutant) gene amplified by PCR from pJK19-1 (Kahana and Silver 1996) into pSP400. pSP400 was constructed by

moving the entire promoter and terminator of *ADH1* in pDB20 (Becker 1991) into pRS306 (Sikorski and Hieter 1989); and 4) pPP46 was made by inserting entire promoter and terminator of *ADH1* including sGFP of pPP16 cut with SmaI/XbaI into pPP33 cut with HpaI/XbaI.

pSGS12 μ was constructed by inserting NotI fragment containing *SGS1* coding sequence amplified by PCR from a cosmid into the NotI site of pDB20. pMM2 was created by replacing the region between base pair +481 and base pair +4026 of the coding region with *hisG::URA3::hisG* fragment (Alani et al. 1987). NotI fragment of pMM2 was cloned into the NotI site of pTKS(+) (Ichihara and Kurosawa 1993), producing pPP69.

Life span analysis

Life span analysis was performed by counting the number of daughter cells that bud off from a virgin mother cell before cessation of cell division, as previously described (Kennedy et al. 1994). The sample size for each life span analysis was 43-51 cells. Each life span analysis was carried out at least two independent times.

Isolation of old cells

Old cells were obtained as previously described (Sinclair and Guarente 1997), except that for some experiments sulfo-NHS-LC-LC-biotin (Pierce, Rockford, IL) was used for biotinylation, instead of sulfo-NHS-LC-biotin. They both gave a similar yield of old cells.

Immunofluorescence

Immunofluorescence experiments were performed as described (Gotta et al. 1997; Kennedy et al. 1997), except that anti-Sir3p used in this study was generated by immunizing a rabbit with the full-length Sir3p (unpublished reagent, K. Mills and L. Guarente). Optical sections of images were obtained using the CELLscan System (Scanalytics, Billerica, MA) as previously described (Kennedy et al. 1997). Strains that do not contain the GFP gene at HMR were used for these studies.

One-dimensional gel analysis

DNA used for gel analysis was prepared as previously described (Sinclair and Guarente 1997), except that no phenol extraction was performed. Total DNA (5 µg) for each sample was electrophoresed without ethidium bromide at 1V/cm for 24 - 30 hrs. Young cells used in this experiment were free cells that were removed when old cells were magnetically sorted.

FACS analysis

Young or old cells (about 2×10^6 cells) were resuspended in 1 ml of PBS containing 5 µg/ml of propidium iodide (PI) (Sigma, MO) and sonicated briefly to separate the clumped cells. To ensure appropriate comparison with old cells, young cells were biotinylated and incubated with streptavidin-coated magnetic beads (PerSeptive Biosystems, MA). The only exception was *sir3* cells that were from a log-phase culture. The magnetic beads present along with the cells did not interfere with the FACS analysis; young cells with and without beads gave a similar result. The level of green fluorescence of each cell was determined by using FACScan (Becton Dickinson, CA). Dead cells were first excluded from the

analysis by staining with propidium iodide (PI), which was measured using 650 long pass filter (Carter et al. 1993; Deere et al. 1998). PI preferentially stains dead cells that have porous membranes and will not diffuse appreciably into intact cells. Then, the level of green fluorescence of 10^5 live cells was measured using 530/30 band pass filter, and their fluorescence was displayed in a histogram using CellQuest Analysis program (Becton Dickinson, San Jose, CA).

Statistical Analysis

The significance of differences in mean life span between two strains was determined as previously described (Kennedy et al. 1995).

REFERENCES

- Aboussekra, A. and R.D. Wood. 1994. Repair of UV-damaged DNA by mammalian cells and *Saccharomyces cerevisiae*. *Curr Opin Genet Dev* 4: 212-20.
- Aguilera, A. 1995. Genetic evidence for different RAD52-dependent intrachromosomal recombination pathways in *Saccharomyces cerevisiae*. *Curr Genet* 27: 298-305.
- Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics* 116: 541-545.
- Allen, J.B., Z. Zhou, W. Siede, E.C. Friedberg, and S.J. Elledge. 1994. The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes Dev* 8: 2401-15.
- Ansari, A. and M.R. Gartenberg. 1997. The yeast silent information regulator Sir4p anchors and partitions plasmids. *Mol Cell Biol* 17: 7061-8.
- Bai, Y. and L.S. Symington. 1996. A Rad52 homolog is required for RAD51-independent mitotic recombination in *Saccharomyces cerevisiae*. *Mol Cell Biol* 16: 4832-41.
- Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, and C. Cullin. 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* 21: 3329-3330.
- Baumann, P. and S.C. West. 1998. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. *Trends Biochem Sci* 23: 247-51.
- Becker, D.M., Fikes, J. D., and Guarente, L. 1991. A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. *Proc Natl Acad Sci U S A* 88: 1968-1972.
- Boeke, J.D., J. Trueheart, G. Natsoulis, and G.R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* 154: 164-75.
- Boulton, S.J. and S.P. Jackson. 1998. Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. *Embo J* 17: 1819-28.
- Brewer, B.J. and W.L. Fangman. 1988. A replication fork barrier at the 3' end of yeast ribosomal RNA genes. *Cell* 55: 637-43.
- Brewer, B.J., D. Lockshon, and W.L. Fangman. 1992. The arrest of replication forks in the rDNA of yeast occurs independently of transcription. *Cell* 71: 267-76.

- Bryk, M., M. Banerjee, M. Murphy, K.E. Knudsen, D.J. Garfinkel, and M.J. Curcio. 1997. *Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast.* *Genes Dev* 11: 255-69.
- Carter, E.A., F.E. Paul, and P.A. Hunter. 1993. Cytometric evaluation of antifungal agents. In *Flow Cytometry in Microbiology* (ed. E. Llyod), pp. 111-120. Springer-Verlag, London, U.K.
- Christman, M.F., F.S. Dietrich, and G.R. Fink. 1988. *Mitotic recombination in the rDNA of S. cerevisiae is suppressed by the combined action of DNA topoisomerases I and II.* *Cell* 55: 413-25.
- Christman, M.F., F.S. Dietrich, N.A. Levin, B.U. Sadoff, and G.R. Fink. 1993. *The rRNA-encoding DNA array has an altered structure in topoisomerase I mutants of Saccharomyces cerevisiae.* *Proc Natl Acad Sci U S A* 90: 7637-41.
- Deere, D., J. Shen, G. Vesey, P. Bell, P. Bissinger, and D. Veal. 1998. *Flow cytometry and cell sorting for yeast viability assessment and cell selection.* *Yeast* 14: 147-60.
- Defossez, P.-A., P.U. Park, and L. Guarente. 1998. *Vicious circles: a mechanism of yeast aging.* *Curr Opin Microbiol* 1: 707-11.
- Defossez, P.-A., R. Prusty, M. Kaeberlein, S.J. Lin, P. Ferrigno, P.A. Silver, R.L. Keil, and L. Guarente. 1999. *Elimination of replication block protein Fob1 extends the life span of yeast mother cells.* *Mol Cell* 3: 447-55.
- Egilmez, N.K. and S.M. Jazwinski. 1989. *Evidence for the involvement of a cytoplasmic factor in the aging of the yeast Saccharomyces cerevisiae.* *J Bacteriol* 171: 37-42.
- Ellis, N.A., J. Groden, T.Z. Ye, J. Straughen, D.J. Lennon, S. Ciocci, M. Proytcheva, and J. German. 1995. *The Bloom's syndrome gene product is homologous to RecQ helicases.* *Cell* 83: 655-66.
- Epstein, C.J., G.M. Martin, A.L. Schultz, and A.G. Motulsky. 1966. *Werner's syndrome a review of its symptomatology, natural history, pathologic features, genetics and relationship to the natural aging process.* *Medicine* 45: 177-221.
- Finch, C. 1990. *Longevity, Senescence, and the Genome.* The University of Chicago Press, Chicago.
- Fishman-Lobell, J. and J.E. Haber. 1992. *Removal of nonhomologous DNA ends in double-strand break recombination: the role of the yeast ultraviolet repair gene RAD1.* *Science* 258: 480-4.
- Fritze, C.E., K. Verschueren, R. Strich, and R. Easton Esposito. 1997. *Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA.* *Embo J* 16: 6495-509.

- Fukuchi, K., G.M. Martin, and R.J. Monnat, Jr. 1989. *Mutator phenotype of Werner syndrome is characterized by extensive deletions.* Proc Natl Acad Sci U S A 86: 5893-7.
- Fukuchi, K., K. Tanaka, Y. Kumahara, K. Marumo, M.B. Pride, G.M. Martin, and R.J. Monnat, Jr. 1990. *Increased frequency of 6-thioguanine-resistant peripheral blood lymphocytes in Werner syndrome patients.* Hum Genet 84: 249-52.
- Game, J.C. 1993. *DNA double-strand breaks and the RAD50-RAD57 genes in Saccharomyces.* Semin Cancer Biol 4: 73-83.
- Gangloff, S., J.P. McDonald, C. Bendixen, L. Arthur, and R. Rothstein. 1994. *The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase.* Mol Cell Biol 14: 8391-8.
- Gotta, M., S. Strahl-Bolsinger, H. Renauld, T. Laroche, B.K. Kennedy, M. Grunstein, and S.M. Gasser. 1997. *Localization of Sir2p: the nucleolus as a compartment for silent information regulators.* Embo J 16: 3243-55.
- Gottlieb, S. and R.E. Esposito. 1989. *A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA.* Cell 56: 771-6.
- Gray, M.D., J.C. Shen, A.S. Kamath-Loeb, A. Blank, B.L. Sopher, G.M. Martin, J. Oshima, and L.A. Loeb. 1997. *The Werner syndrome protein is a DNA helicase.* Nat Genet 17: 100-3.
- Gray, M.D., L. Wang, H. Youssoufian, G.M. Martin, and J. Oshima. 1998. *Werner helicase is localized to transcriptionally active nucleoli of cycling cells.* Exp Cell Res 242: 487-94.
- Grunstein, M. 1997. *Molecular model for telomeric heterochromatin in yeast.* Curr Opin Cell Biol 9: 383-7.
- Haber, J.E. 1995. *In vivo biochemistry: physical monitoring of recombination induced by site-specific endonucleases.* Bioessays 17: 609-20.
- Huang, S., L. Baomin, M.D. Gray, J. Oshima, I.S. Mian, and J. Campisi. 1998. *The premature ageing syndrome protein, WRN, is a 3'-5' exonuclease.* Nat Genet 20: 114-16.
- Ichihara, Y. and Y. Kurosawa. 1993. *Construction of new T vectors for direct cloning of PCR products.* Gene 130: 153-4.
- Ivanov, E.L. and J.E. Haber. 1995. *RAD1 and RAD10, but not other excision repair genes, are required for double-strand break-induced recombination in Saccharomyces cerevisiae.* Mol Cell Biol 15: 2245-51.

- Ivanov, E.L., N. Sugawara, J. Fishman-Lobell, and J.E. Haber. 1996. *Genetic requirements for the single-strand annealing pathway of double-strand break repair in Saccharomyces cerevisiae*. *Genetics* 142: 693-704.
- Ivanov, E.L., N. Sugawara, C.I. White, F. Fabre, and J.E. Haber. 1994. *Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in Saccharomyces cerevisiae*. *Mol Cell Biol* 14: 3414-25.
- Johnson, R.D. and L.S. Symington. 1995. *Functional differences and interactions among the putative RecA homologs Rad51, Rad55, and Rad57*. *Mol Cell Biol* 15: 4843-50.
- Kahana, J.A. and P.A. Silver. 1996. Use of the *A. victoria* green fluorescent protein to study protein dynamics in vivo. In *Current Protocols in Molecular Biology* (ed. F.M. Ausuble, R. Brent, R.E. Kingston, D.E. Moore, J.G. Seidman, J.A. Smith, and K. Struhl), pp. 9.7.22-9.7.28. John Wiley & Sons, New York, NY.
- Kennedy, B.K., N.R. Austriaco, Jr., and L. Guarente. 1994. *Daughter cells of Saccharomyces cerevisiae from old mothers display a reduced life span*. *J Cell Biol* 127: 1985-93.
- Kennedy, B.K., N.R. Austriaco, Jr., J. Zhang, and L. Guarente. 1995. *Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae*. *Cell* 80: 485-96.
- Kennedy, B.K., M. Gotta, D.A. Sinclair, K. Mills, D.S. McNabb, M. Murthy, S.M. Pak, T. Laroche, S.M. Gasser, and L. Guarente. 1997. *Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in S. cerevisiae*. *Cell* 89: 381-91.
- Kim, R.A. and J.C. Wang. 1989. *A subthreshold level of DNA topoisomerases leads to the excision of yeast rDNA as extrachromosomal rings*. *Cell* 57: 975-85.
- Kobayashi, T., D.J. Heck, M. Nomura, and T. Horiuchi. 1998. *Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I*. *Genes Dev* 12: 3821-30.
- Kobayashi, T. and T. Horiuchi. 1996. *A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities*. *Genes Cells* 1: 465-74.
- Kowalczykowski, S.C., D.A. Dixon, A.K. Eggleston, S.D. Lauder, and W.M. Rehrauer. 1994. *Biochemistry of homologous recombination in Escherichia coli*. *Microbiol Rev* 58: 401-65.
- Lee, S.E., J.K. Moore, A. Holmes, K. Umezu, R.D. Kolodner, and J.E. Haber. 1998. *Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage*. *Cell* 94: 399-409.

- Liang, F., M. Han, P.J. Romanienko, and M. Jasin. 1998. *Homology-directed repair is a major double-strand break repair pathway in mammalian cells*. Proc Natl Acad Sci U S A 95: 5172-7.
- Lin, Y.H. and R.L. Keil. 1991. *Mutations affecting RNA polymerase I-stimulated exchange and rDNA recombination in yeast*. Genetics 127: 31-8.
- Loo, S. and J. Rine. 1995. *Silencing and heritable domains of gene expression*. Annu Rev Cell Dev Bio 11: 519-48.
- Mahoney, D.J. and J.R. Broach. 1989. *The HML mating-type cassette of Saccharomyces cerevisiae is regulated by two separate but functionally equivalent silencers*. Mol Cell Biol 9: 4621-30.
- Malone, R.E. and R.E. Esposito. 1981. *Recombinationless meiosis in Saccharomyces cerevisiae*. Mol Cell Biol 1: 891-901.
- Malone, R.E., T. Ward, S. Lin, and J. Waring. 1990. *The RAD50 gene, a member of the double strand break repair epistasis group, is not required for spontaneous mitotic recombination in yeast*. Curr Genet 18: 111-6.
- Marciniak, R.A., D.B. Lombard, F.B. Johnson, and L. Guarente. 1998. *Nucleolar localization of the Werner syndrome protein in human cells*. Proc Natl Acad Sci U S A 95: 6887-92.
- Michel, B., S.D. Ehrlich, and M. Uzest. 1997. *DNA double-strand breaks caused by replication arrest*. EMBO J 16: 430-38.
- Miller, R.D., L. Prakash, and S. Prakash. 1982. *Defective excision of pyrimidine dimers and interstrand DNA crosslinks in rad7 and rad23 mutants of Saccharomyces cerevisiae*. Mol Gen Genet 188: 235-9.
- Mills, K.D., D.A. Sinclair, and L. Guarente. 1999. *MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks*. Cell 97: 609-20.
- Moore, J.K. and J.E. Haber. 1996. *Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae*. Genes Dev 10: 1310-26.
- Mortimer, R.K., R. Contopoulou, and D. Schild. 1981. *Mitotic chromosome loss in a radiation-sensitive strain of the yeast Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 78: 5778-82.
- Mortimer, R.K. and J.R. Johnston. 1959. *Life span of individual yeast cells*. Nature 183: 1751-1752.
- Müller, I. 1971. *Experiments on Ageing in Single Cells of Saccharomyces cerevisiae*. Arch. Mikrobiol. 77: 20-25.

- 1985. Parental age and the life span of zygotes of *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* 51: 1-10.
- Murray, A.W. and J.W. Szostak. 1983. Pedigree analysis of plasmid segregation in yeast. *Cell* 34: 961-70.
- Nakayama, K., N. Irino, and H. Nakayama. 1985. The *recQ* gene of *Escherichia coli* K12: molecular cloning and isolation of insertion mutants. *Mol Gen Genet* 200: 266-71.
- Nugent, C.I., G. Bosco, L.O. Ross, S.K. Evans, A.P. Salinger, J.K. Moore, J.E. Haber, and V. Lundblad. 1998. Telomere maintenance is dependent on activities required for end repair of double-strand breaks. *Curr Biol* 8: 657-60.
- Ozenberger, B.A. and G.S. Roeder. 1991. A unique pathway of double-strand break repair operates in tandemly repeated genes. *Mol Cell Biol* 11: 1222-31.
- Petes, T.D., R.E. Malone, and L.S. Symington. 1991. Recombination in Yeast. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis and Energetics*, pp. 407-521. Cold Spring Harbor Laboratory Press.
- Prakash, S., P. Sung, and L. Prakash. 1993. DNA repair genes and proteins of *Saccharomyces cerevisiae*. *Annu Rev Genet* 27: 33-70.
- Puranam, K.L. and P.J. Blackshear. 1994. Cloning and characterization of RECQL, a potential human homologue of the *Escherichia coli* DNA helicase RecQ. *J Biol Chem* 269: 29838-45.
- Rattray, A.J. and L.S. Symington. 1995. Multiple pathways for homologous recombination in *Saccharomyces cerevisiae*. *Genetics* 139: 57-66.
- Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* 194: 281-301.
- Salk, D. 1982. Werner's syndrome: a review of recent research with an analysis of connective tissue metabolism, growth control of cultured cells, and chromosomal aberrations. *Human Genetics* 62: 1-5.
- Sandell, L.L. and V.A. Zakian. 1993. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell* 75: 729-39.
- Sargent, R.G., M.A. Brenneman, and J.H. Wilson. 1997. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol Cell Biol* 17: 267-77.

- Schultz, M.C., S.J. Brill, Q. Ju, R. Sternglandz, and R.H. Reeder. 1992. *Topoisomerase and yeast rRNA transcription: negative supercoiling stimulates initiation and topoisomerase activity is required for elongation.* *Genes Dev* 6: 1332-41.
- Seigneur, M., V. Bidnenko, S.D. Ehrlich, and B. Michel. 1998. *RuvAB acts at arrested replication forks.* *Cell* 95: 419-30.
- Shen, J.C., M.D. Gray, J. Oshima, and L.A. Loeb. 1998. *Characterization of Werner syndrome protein DNA helicase activity: directionality, substrate dependence and stimulation by replication protein A.* *Nucleic Acids Res* 26: 2879-85.
- Sherman, F., G. Fink, and J. Hicks. 1986. *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shinohara, A. and T. Ogawa. 1995. *Homologous recombination and the roles of double-strand breaks.* *Trends Biochem Sci* 20: 387-91.
- Sikorski, R.S. and P. Hieter. 1989. *A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae.* *Genetics* 122: 19-27.
- Sinclair, D.A. and L. Guarente. 1997. *Extrachromosomal rDNA circles--a cause of aging in yeast.* *Cell* 91: 1033-42.
- Sinclair, D.A., K. Mills, and L. Guarente. 1997. *Accelerated aging and nucleolar fragmentation in yeast sgs1 mutants.* *Science* 277: 1313-6.
- Smeal, T., J. Claus, B. Kennedy, F. Cole, and L. Guarente. 1996. *Loss of transcriptional silencing causes sterility in old mother cells of S. cerevisiae.* *Cell* 84: 633-42.
- Smith, J.S. and J.D. Boeke. 1997. *An unusual form of transcriptional silencing in yeast ribosomal DNA.* *Genes Dev* 11: 241-54.
- Stewart, E., C.R. Chapman, F. Al-Khadairy, A.M. Carr, and T. Enoch. 1997. *rqh1+, a fission yeast gene related to the Bloom's and Werner's syndrome genes, is required for reversible S phase arrest.* *Embo J* 16: 2682-92.
- Sung, P. 1997. *Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase.* *Genes Dev* 11: 1111-21.
- Szostak, J.W., T.L. Orr-Weaver, R.J. Rothstein, and F.W. Stahl. 1983. *The double-strand-break repair model for recombination.* *Cell* 33: 25-35.
- Takata, M., M.S. Sasaki, E. Sonoda, C. Morrison, M. Hashimoto, H. Utsumi, Y. Yamaguchi-Iwai, A. Shinohara, and S. Takeda. 1998. *Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells.* *Embo J* 17: 5497-5508.

- Tsukamoto, Y., J. Kato, and H. Ikeda. 1997. *Silencing factors participate in DNA repair and recombination in Saccharomyces cerevisiae*. *Nature* 388: 900-3.
- van Gool, A.J., R. Verhage, S.M. Swagemakers, P. van de Putte, J. Brouwer, C. Troelstra, D. Bootsma, and J.H. Hoeijmakers. 1994. *RAD26, the functional S. cerevisiae homolog of the Cockayne syndrome B gene ERCC6*. *Embo J* 13: 5361-9.
- Watt, P.M., I.D. Hickson, R.H. Borts, and E.J. Louis. 1996. *SGS1, a homologue of the Bloom's and Werner's syndrome genes, is required for maintenance of genome stability in Saccharomyces cerevisiae*. *Genetics* 144: 935-45.
- Watt, P.M., E.J. Louis, R.H. Borts, and I.D. Hickson. 1995. *Sgs1: a eukaryotic homolog of E. coli RecQ that interacts with topoisomerase II in vivo and is required for faithful chromosome segregation*. *Cell* 81: 253-60.
- Yamagata, K., J. Kato, A. Shimamoto, M. Goto, Y. Furuichi, and H. Ikeda. 1998. *Bloom's and Werner's syndrome genes suppress hyperrecombination in yeast sgs1 mutant: implication for genomic instability in human diseases*. *Proc Natl Acad Sci U S A* 95: 8733-8.
- Yan, H., C.Y. Chen, R. Kobayashi, and J. Newport. 1998. *Replication focus-forming activity 1 and the Werner syndrome gene product*. *Nat Genet* 19: 375-8.
- Yu, C.E., J. Oshima, Y.H. Fu, E.M. Wijsman, F. Hisama, R. Alisch, S. Matthews, J. Nakura, T. Miki, S. Ouais, G.M. Martin, J. Mulligan, and G.D. Schellenberg. 1996. *Positional cloning of the Werner's syndrome gene*. *Science* 272: 258-62.
- Zou, H. and R. Rothstein. 1997. *Holliday junctions accumulate in replication mutants via a RecA homolog- independent mechanism*. *Cell* 90: 87-96.

TABLE 1. Yeast strains used in this study

Strain names	Genotypes	Source
PSY316a	<i>MATα ura3-52 leu2-3,112 his3-Δ200 ade2-101 lys2-801</i>	Laboratory strain
PSY316α	<i>MATα ura3-52 leu2-3,112 his3-Δ200 ade2-101 lys2-801</i>	Laboratory strain
PPY16	PPY316a <i>hmrΔ1::URA3</i>	This study
PPY27	PPY16 <i>ADE2</i>	This study
PPY35	PPY27 <i>hmrΔ2::ADH1-GFP</i>	This study
PPY56	PPY35 <i>sir3Δ::URA3</i>	This study
PPY70	PPY35 <i>sgs1Δ::hisG</i>	This study
PPY74	PPY316α <i>RDN1::ADE2</i>	This study
PPY92	PPY35 <i>rad1Δ::HIS3</i>	This study
PPY93	PPY35 <i>rad7Δ::HIS3</i>	This study
PPY95	PPY35 <i>rad26Δ::HIS3</i>	This study
PPY96	PPY35 <i>rad51Δ::HIS3</i>	This study
PPY97	PPY35 <i>rad57Δ::HIS3</i>	This study
PPY98	PPY35 <i>rad52Δ::LEU2</i>	This study
PPY103	PPY74 <i>hmrΔ1::URA3</i>	This study
PPY111	PPY35 <i>rad52Δ::HIS3</i>	This study
PPY113	PPY70 <i>rad52Δ::HIS3</i>	This study
PPY143	PPY103 <i>hmrΔ2::ADH1-GFP</i>	This study
PPY170	PPY143 <i>rad52Δ::HIS3</i>	This study
PPY172	PPY27 <i>rad52Δ::HIS3</i>	This study
PPY182	PPY98 X PPY170	This study
PPY190	PPY35 X PPY143	This study
PPY217	PPY143 <i>rad50Δ::HIS3</i>	This study
PPY219	PPY143 <i>rad59Δ::HIS3</i>	This study
PPY276	PPY27 <i>rad50Δ::HIS3</i>	This study
PPY278	PPY27 <i>rad51Δ::HIS3</i>	This study
PPY280	PPY27 <i>rad57Δ::HIS3</i>	This study

FIGURE 1. A *rad52* mutant does not accumulate ERCs and has a very short life span. (A) Gel electrophoresis was performed on genomic DNA isolated from young and old wild-type, *sgs1*, *rad52*, and *sgs1 rad52* cells (see Materials and methods). Various ERCs species (arrowheads) similar to the ones previously observed (Sinclair and Guarente 1997) were seen in old wild-type and were slightly more abundant in old *sgs1* cells. ERCs were undetectable in old *rad52* and *sgs1 rad52* cells. They were undetectable even after longer exposure time. Average bud scar counts of old cells were: wild-type, 7.9 ± 1.6 ; *sgs1*, 7.6 ± 1.8 ; *rad52*, 7.4 ± 2.2 ; and *sgs1 rad52*, 7.4 ± 2.0 . (B) Life span analysis was performed by standard methods as previously described (Kennedy et al. 1994). Average life spans were: wild-type, 23.5; *sgs1*, 9.8; *rad52*, 7.1; and *sgs1 rad52*, 5.5. (C) A homozygous *rad52* diploid cells have a similar life span as the *rad52* haploid cells. Average life spans were: wild-type haploid, 24.7; wild-type diploid, 23.9; *rad52* haploid, 7.5; and *rad52* diploid, 7.2.

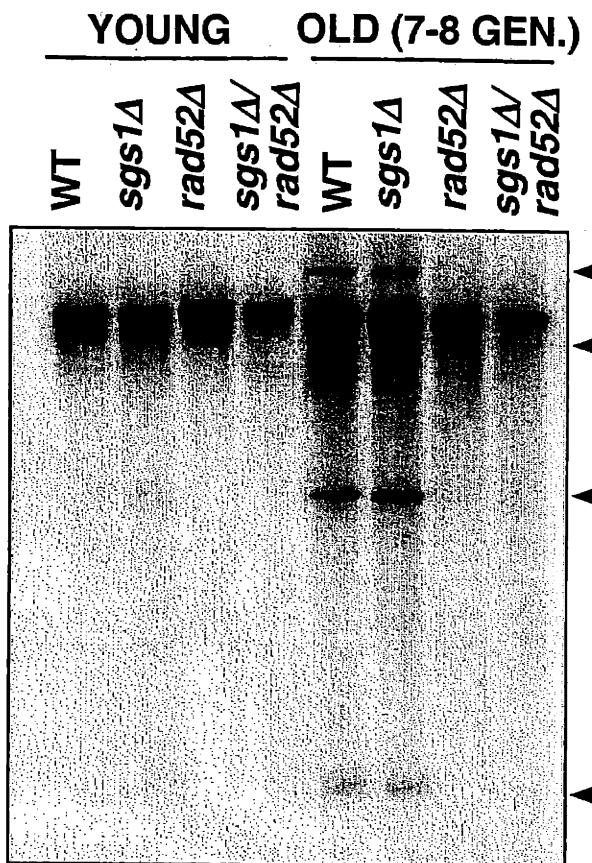
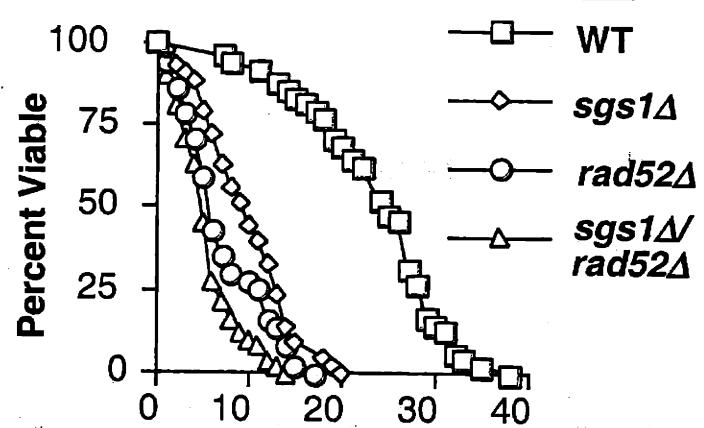
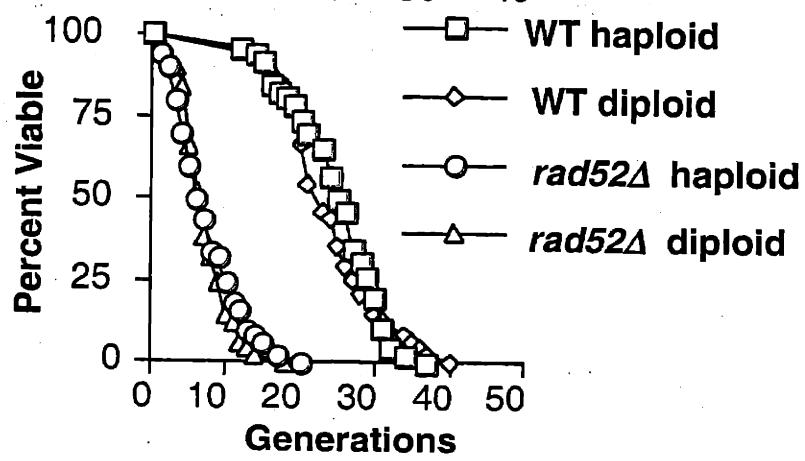
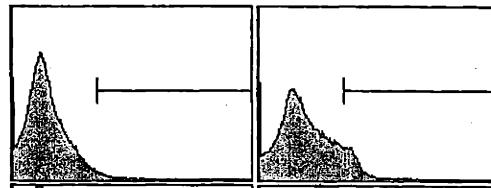
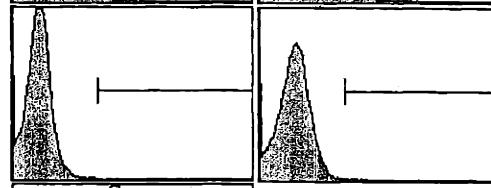
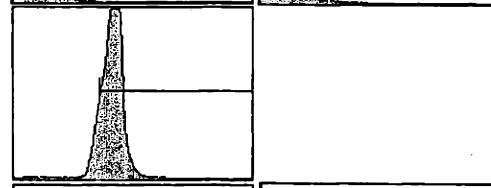
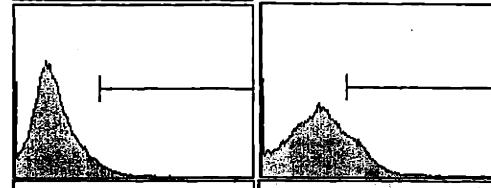
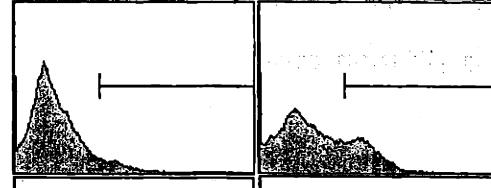
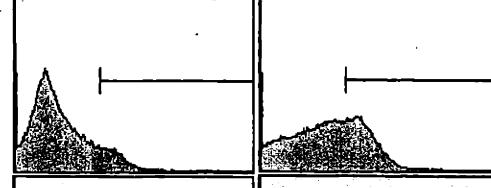
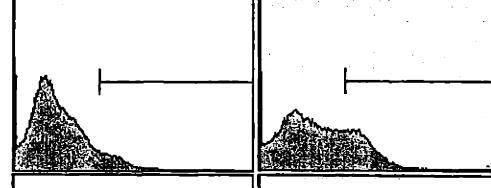
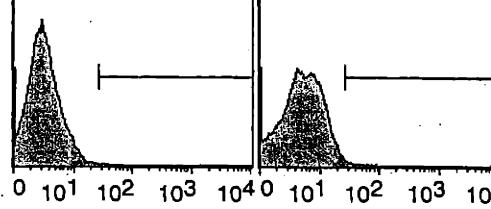
A**B****C**



FIGURE 2. Mutants in the *RAD52* epistasis group show a varying degree of premature loss of silencing at *HMR*. (A) A schematic diagram of *hmrΔ2::ADH1-GFP* construct present in +GFP cells. A GFP gene driven by the constitutive *ADH1* promoter was inserted between the *HMR-E* and *HMR-I* silencers. E and I represent *HMR-E* and *HMR-I*. (B) GFP expression was efficiently silenced in a Sir-dependent manner, and silencing at *HMR* was lost in an age-dependent fashion. Green fluorescence level of 10^5 live cells was measured by FACS. The Y-axis indicates the number of cells, and the X-axis indicates the level of green fluorescence intensities in a log-scale. Average green fluorescence intensities were as follows (average bud scar counts of old cells are given in parenthesis): young wild-type +GFP, 4.65; old wild-type +GFP, 9.60 (8.9 ± 0.9); young wild-type -GFP, 2.85; old wild-type -GFP, 4.16 (8.7 ± 0.9); young *sir3* +GFP, 45.16; young *rad50* +GFP, 6.33; old *rad50* +GFP, 16.44 (7.6 ± 1.0); young *rad51* +GFP, 7.81; old *rad51* +GFP, 20.30 (7.7 ± 0.8); young *rad52* +GFP, 10.35; old *rad52* +GFP, 26.08 (7.2 ± 0.8); young *rad57* +GFP, 9.15; old *rad57* +GFP, 19.56 (8.0 ± 1.0); young *rad52* -GFP cells, 3.65 ; and old *rad52* -GFP cells, 6.32 (7.5 ± 0.9).

A*hmrΔ2::ADH1-GFP construct***B**

YOUNG OLD (7-8 GEN.)

WT
+GFPWT
-GFP*sir3Δ*
+GFP*rad50Δ*
+GFP*rad51Δ*
+GFP*rad52Δ*
+GFP*rad57Δ*
+GFP*rad52Δ*
-GFP0 10¹ 10² 10³ 10⁴ 0 10¹ 10² 10³ 10⁴

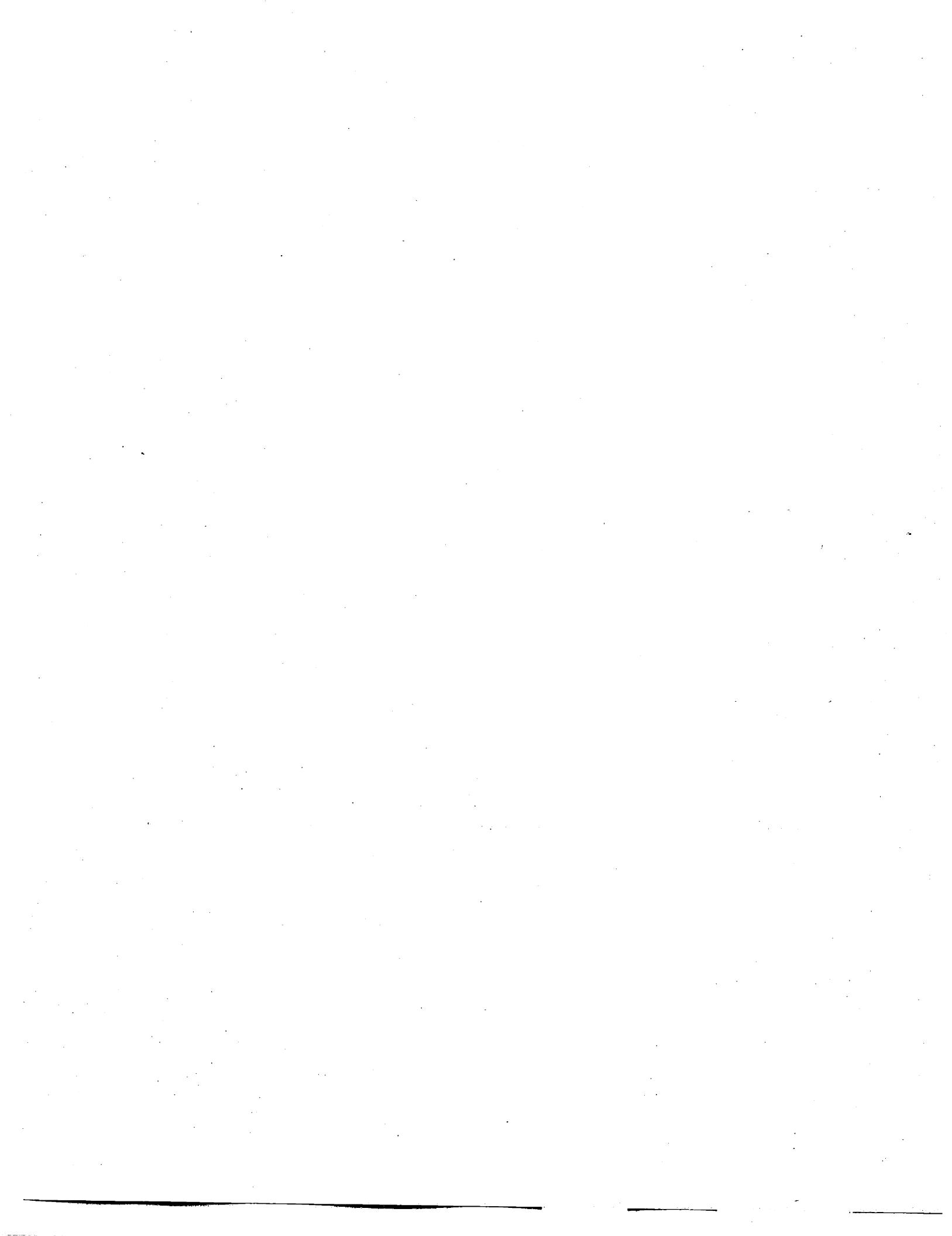


FIGURE 3. Redistribution of Sir3p from telomeres to other sites in the nucleus in old *rad52* and *rad50* cells. Young and old wild-type, *rad52*, and *rad50* cells were subjected to a double immunolabeling with a mouse monoclonal antibody against Nop1p and affinity-purified rabbit antibodies against Sir3p (Gotta et al. 1997; Kennedy et al. 1997). Optical sections were acquired using CCD microscopy and the CELLscan System (Scanalytics, MA). The green stain represents Sir3p; the red stain represents Nop1p, a nucleolar marker; and the blue stain (DAPI) represents nuclei. In all young cells, Sir3p staining displayed perinuclear foci, indicative of telomeric localization. In old wild-type cells, Sir3p staining was observed in the nucleolus as previously observed (Kennedy et al. 1997). In old *rad52* and *rad50* cells, Sir3p staining showed a diffuse, nuclear pattern, most evident in the absence of DAPI staining. Average bud scar count of old cells: wild-type, 17.9 ± 1.3 ; *rad50*, 7.9 ± 0.8 ; and *rad52*, 7.9 ± 1.5 .

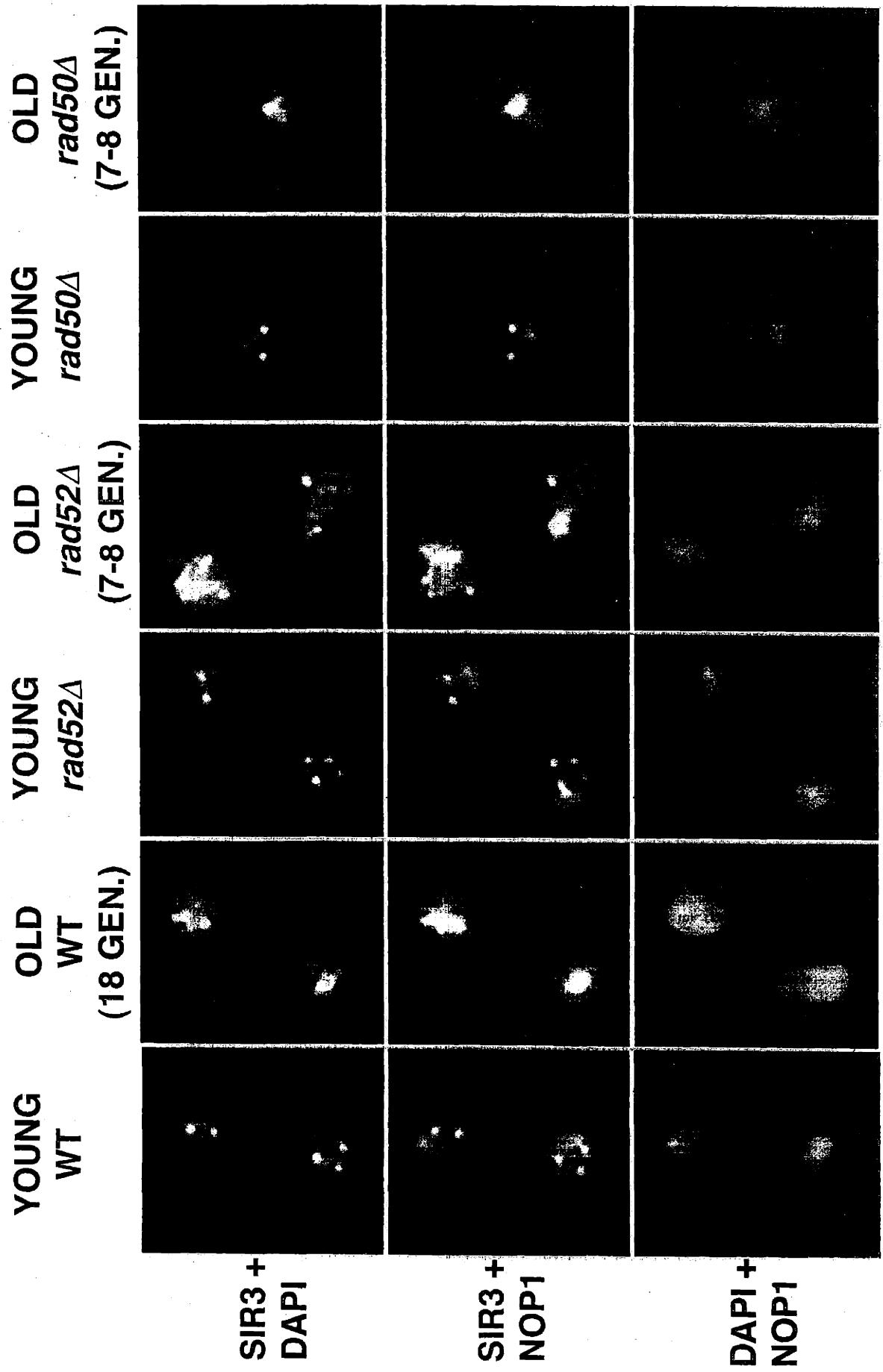




FIGURE 4. Role of other members of the *RAD52* epistasis group on ERC formation and life span. (A) Old *rad50*, *rad51*, and *rad57* cells accumulated different levels of ERCs (arrowheads) that are lower than the age-matched wild-type cells. Average bud scar count of old cells were: wild-type, 8.2 ± 1.0 ; *rad50*, 7.9 ± 0.8 ; *rad51*, 7.8 ± 0.8 ; and *rad57*, 7.8 ± 0.8 . (B) *rad51* and *rad57* mutants had a similar life span, which is shorter than wild type, but longer than the *rad52* mutant. Average life spans were: wild-type, 22.0; *rad51*, 13.0; and *rad57*, 12.5. The *rad50* mutant had a very short life span similar to that of *rad52* mutant. Average life spans were: wild-type, 22.3 and *rad50*, 7.3.

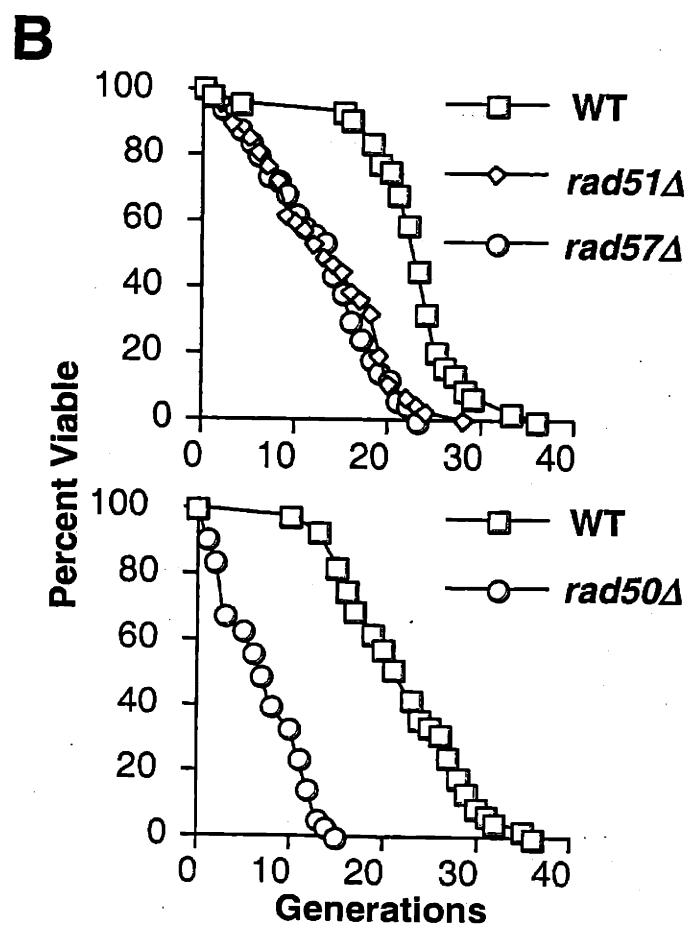
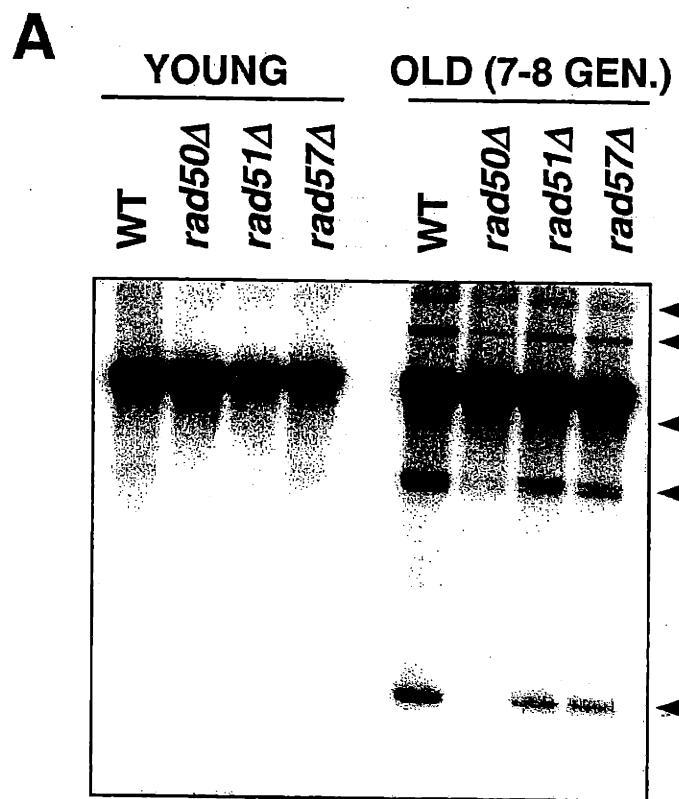




FIGURE 5. Single-strand annealing, nucleotide excision, and transcription-coupled repair are not necessary for wild-type life span. Neither *rad1*, *rad7*, nor *rad26* mutation had a significant affect on life span. Average life spans were: wild-type, 22.0; *rad1*, 20.9; *rad7*, 22.1; and *rad26*, 21.1.

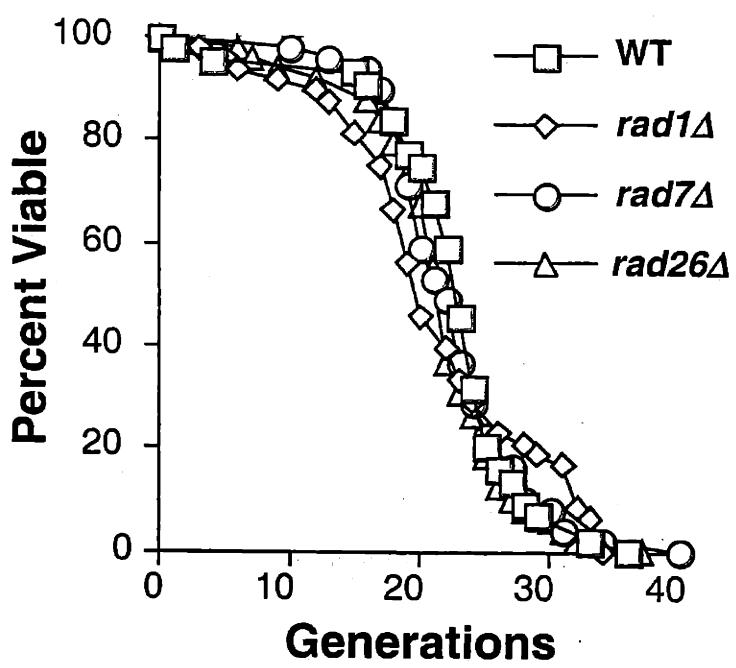
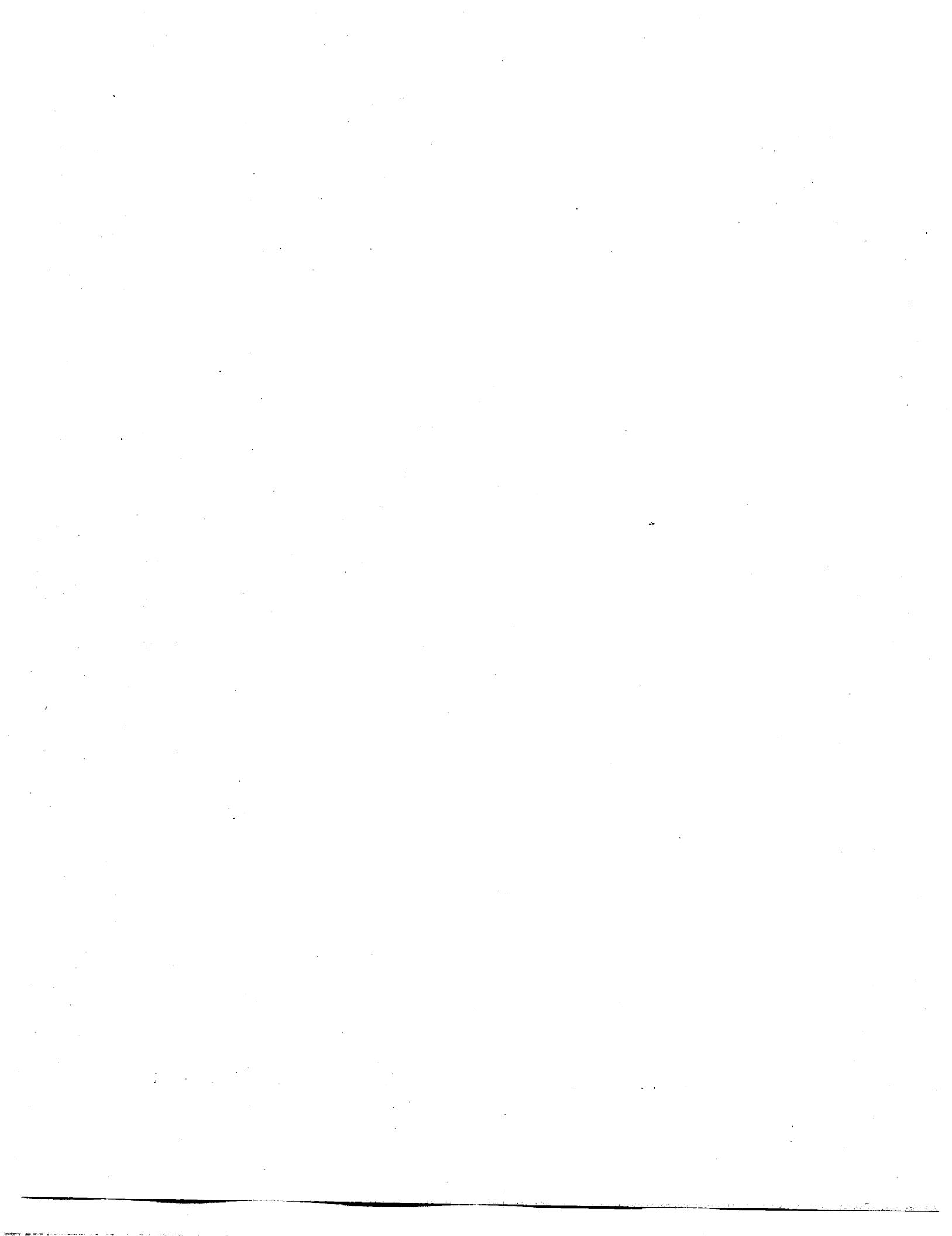


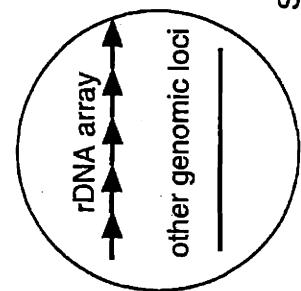


FIGURE 6. Model of yeast aging in presence and absence of DNA repair through homologous recombination. (A) As a young haploid cell divides, spontaneous DNA damage events, such as double-strand breaks (DSBs), occur throughout the genome including rDNA, most likely during DNA replication (Michel et al. 1997; Zou and Rothstein 1997; Seigneur et al. 1998). (B) DSBs can efficiently be repaired through homologous recombination. DSBs that occur in the S and G2-phases of the cell cycle can be repaired using sister chromatids through interchromosomal gene conversion, by a mechanism similar to the model proposed by Szostak et al (Szostak et al. 1983). In repeated loci, including rDNA, single-strand annealing and intrachromosomal recombination can also be used for repair. The repair event at rDNA occurring through intrachromosomal recombination, if associated with a reciprocal crossover, forms a ERC. (C) As previously proposed (Sinclair and Guarente 1997), the excised ERC propagates in the mother cell with age through replication and asymmetric segregation, eventually leading to nucleolar fragmentation and death. (D) In the *rad52* mutant, other DNA repair pathways, including Ku-mediated illegitimate recombination and *RAD52*-independent single strand annealing, may try to compensate for the absence of homologous recombination and repair the DSBs. Movement of Sir proteins from telomeres to other sites in the nucleus might be linked to their involvement in such repair processes. (E) When these other repair pathways are overwhelmed, *rad52* cells die due to multiple DSBs.



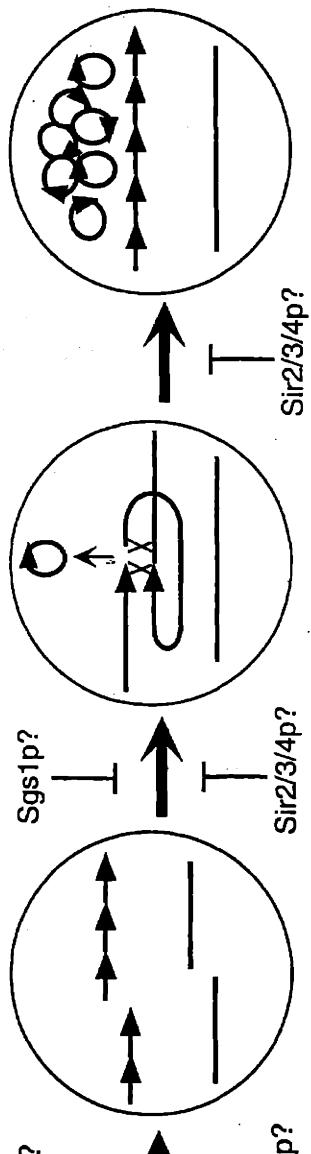
A

DNA damage
(Double strand breaks)
during replication?



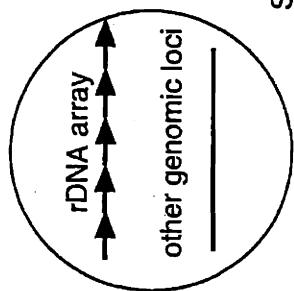
Sir2/3/4p?

Sgs1p?



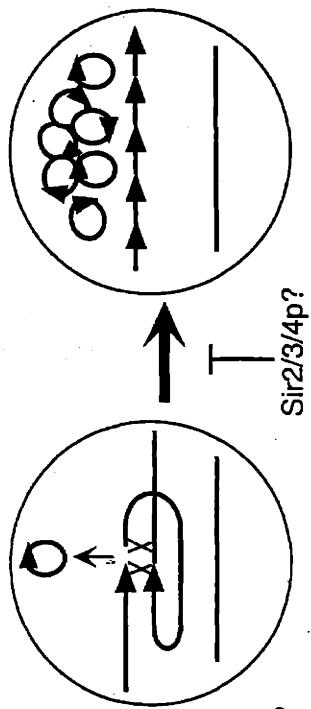
B

DNA repair through
homologous
recombination
+RAD52



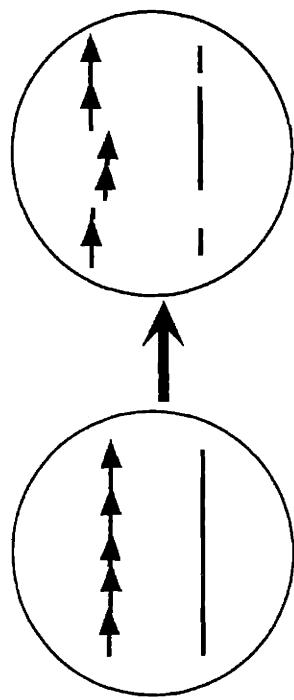
C

ERC replication
and asymmetric
segregation
Death



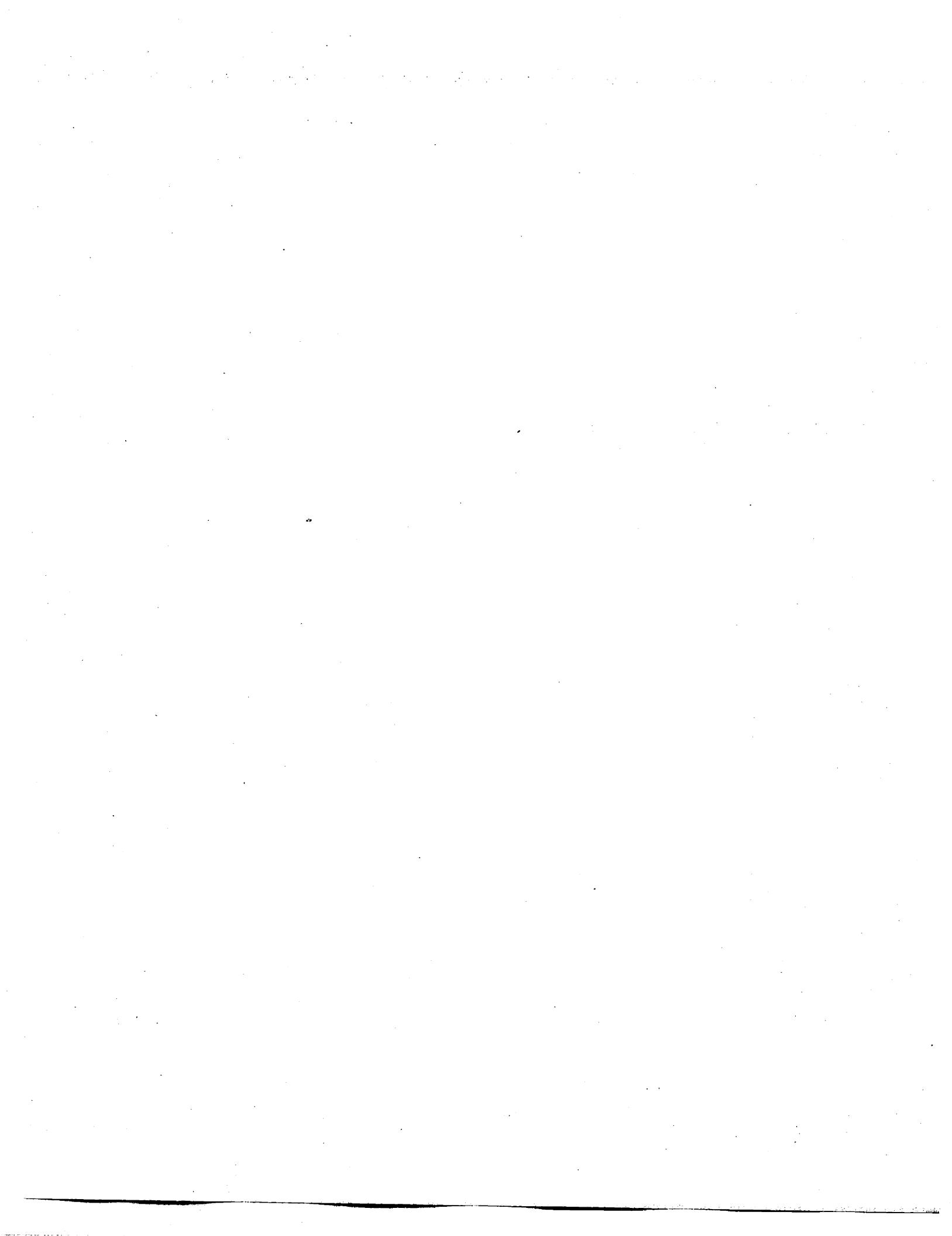
Other DNA repair
pathways including
Sir/Ku-mediated
end-joining

D



More
double-strand
breaks
Death

E



Chapter 3

***FOB1* modulation of rDNA structure and condensation in *top1/trf4* mutants**

This chapter will be submitted for publication. The authors are Peter U. Park,
Greg Liszt, and Leonard Guarente.

ABSTRACT

In *S. cerevisiae*, *FOB1* is required for the formation of replication fork barrier (RFB) at the rDNA, which causes unidirectional DNA replication. *FOB1* is also necessary for the transcription enhancement from ectopic RNA polymerase I (pol I) promoter outside of the rDNA, but not at rDNA. Here, we demonstrate that *FOB1* antagonizes *SIR2*-dependent rDNA silencing and thus influences rDNA chromatin structure. *fob1* mutation partially suppresses the aberrant rDNA structure in *top1* cells, likely by relieving torsional stress created during rDNA replication. In addition, a *fob1* mutation was able to rescue the synthetic lethality in *top1 trf4* mutant cells, due to the failure of mitotic condensation at rDNA. Furthermore, *fob1* mutation complemented several defects caused by *trf4* mutation, suggesting *TRF4*, DNA polymerase σ , may have a specialized function at rDNA. We suggest that *FOB1* and likely RFB plays a direct role in the macromolecular rDNA structure that influences chromosome transmission.

INTRODUCTION

In most eukaryotic cells, ribosome biogenesis occurs in the nucleolus, a specialized compartment of the nucleus, which contains the ribosomal DNA (rDNA) (reviewed in (Shaw and Jordan 1995; Leary and Huang 2001)). RNA polymerase I (Pol I) transcribes large precursor ribosomal RNA (rRNA) from rDNA and is important for maintaining the integrity of nucleolar structure (Oakes et al. 1998). rRNA maturation and ribosome assembly also take place in the nucleolus. The nucleolus in yeast is localized at the periphery of nuclear envelope as a crescent-shaped structure. Recent studies showed that other important cellular processes take place in the nucleolus (reviewed in Pederson 1998; Olson et al. 2000; Visintin and Amon 2000), including cell cycle regulation in mitosis (Shou et al. 1999; Straight et al. 1999) and DNA damage response (Weber et al. 1999).

In the yeast *Saccharomyces cerevisiae*, rDNA consists of 100-200 tandem repeats located on chromosome XII. Each repeat of rDNA is about 9.1 kb in size and encodes the large precursor 35S rRNA gene transcribed by Pol I and the small 5S rRNA transcribed by RNA polymerase III (Pol III). In addition to 35S and 5S rRNA sequences, each rDNA unit also contains two nontranscribed regions, NTS1 and NTS2, which contain two DNA elements related to DNA replication. NTS1 contains a replication fork barrier (RFB), located just 3' to the termination of 35S rRNA, while NTS2 contains an origin of replication (ARS), located just 5' to the promoter 35S rRNA. About one in five ARS sites in rDNA fires during each round of DNA replication (Brewer and Fangman 1988; Linskens and Huberman 1988). Bidirectional replication proceeds until it is

halted in one direction at RFB, the direction that is opposite as transcription of 35S rRNA (Brewer and Fangman 1988; Brewer et al. 1992; Kobayashi et al. 2001). Thus, rDNA is the only place in the yeast genome where replication is unidirectional.

FOB1 is required for the formation of RFB since *fob1* mutant cells have bidirectional replication within the rDNA (Kobayashi and Horiuchi 1996). *FOB1* was originally identified in the screen for mutations that suppress the HOT1 recombination activity (Lin and Keil 1991; Kobayashi and Horiuchi 1996). HOT1 is an rDNA element, composed of the I element, which corresponds to the Pol I promoter, and the E element, which includes Pol I enhancer and the RFB (Keil and Roeder 1984; Voelkel-Meiman et al. 1987). HOT1 enhances recombination and Pol I-dependent transcription of nearby marker genes when inserted at a non-rDNA site. *fob1* cells have reduced recombination at rDNA (Lin and Keil 1991; Kobayashi and Horiuchi 1996), likely a consequence of the absence of replication fork pausing at RFB. Replication fork pausing can lead to a double strand break in *E. coli* (Michel et al. 1997; Seigneur et al. 1998). Consistently, *fob1* cells accumulate lower levels of extrachromosomal rDNA circles (ERCs) with age, accounting for their life span extension (Defossez et al. 1999). Furthermore, *FOB1* is required for expansion and contraction of rDNA repeats (Kobayashi et al. 1998), which is thought to be important for adaptation to changes in environment.

Mitotic recombination within the rDNA array was found to be substantially lower than what is expected for such highly repeated sequences (Szostak and Wu 1980). A tight control mechanism exists in rDNA repeats to balance the propensity toward high levels of recombination. Several genes other

than *FOB1* that are involved in the controlling the rate of recombination specific to rDNA have been characterized. Topoisomerase I (*TOP1*) and II (*TOP2*) are required for the suppression of rDNA recombination (Christman et al. 1988). This suppression of recombination of repeated sequences by *TOP1* and *TOP2* appears to be specific to rDNA because the recombination frequency at another tandem array, the *CUP1* locus, is not elevated. In *top1 top2* double mutant cells, as much as half of the rDNA is present as extrachromosomal rDNA circles (ERCs) (Kim and Wang 1989). ERCs are a cause of yeast aging and are a product of homologous recombination at rDNA (Sinclair and Guarente 1997; Park et al. 1999). rRNA transcription is also inhibited in *top1 top2* cells (Brill et al. 1987; Schultz et al. 1992). Furthermore, *top1* mutant cells have an altered rDNA structure that causes chromosome XII to fail to migrate into the pulsed-field gel, suggesting that *TOP1* is important for macromolecular structure of rDNA (Christman et al. 1993).

Along with *TRF4*, *TOP1* is also implicated in the mitotic condensation of rDNA. *TRF4* was originally identified in a genetic screen to find gene products that perform overlapping or dependent functions with Top1p (Sadoff et al. 1995). *trf4 top1* cells are inviable and fail to establish and maintain mitotic rDNA condensation (Castano et al. 1996). Recently, *TRF4* and its homologue, *TRF5*, have been characterized as members of novel DNA polymerase family, Pol σ, and are required for the establishment and maintenance of sister-chromatid cohesion (Wang et al. 2000).

The product of the *SIR2* gene is another regulator that controls mitotic stability at the rDNA; *sir2* cells have an elevated rate of rDNA recombination (Gottlieb and Esposito 1989). In complex with Sir1p, Sir3p, and Sir4p, Sir2p

represses transcription at the silent mating type loci, *HML* and *HMR* (Ivy et al. 1986; Rine and Herskowitz 1987). In addition, *SIR2*, *SIR3*, and *SIR4* are required for transcriptional silencing at telomeres, called telomeric positional effect (Gottschling et al. 1990). Only *SIR2* is necessary for transcriptional silencing at rDNA (Bryk et al. 1997; Smith and Boeke 1997), and Sir2p is concentrated at the nucleolus (Gotta et al. 1997). Using *in vivo* psoralen cross-linking experiments, Sogo and coworkers demonstrated that chromatin structures of inactive and actively transcribed rDNA repeats are different; inactive repeats including adjacent nontranscribed spacers (NTS) are packaged in regular nucleosomal arrays, while active repeats are nucleosome-free, and NTS of active repeats show an unusual cross-linking patterns suggestive of a complex structure (Dammann et al. 1993; Dammann et al. 1995). The accessibility of rDNA chromatin is responsive to Sir2p dosage (Fritze et al. 1997; Smith and Boeke 1997). Recently, it was discovered that Sir2p is a novel deacetylase requiring NAD as a cofactor (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). Its enzymatic activity is required for its function in recombination, silencing, and life span (Imai et al. 2000).

Recently, it was shown that the Pol I enhancer is not necessary for transcription of the chromosomal rRNA gene and for cell growth (Wai et al. 2001). Fob1p is only required for transcription enhancement from ectopic promoters outside of the rDNA. The authors suggested that the role of Fob1p protein is to facilitate interaction between different rDNA repeats through the E elements and that the RFB may be the result of a particular macromolecular structures created by these interactions. In this paper, we present evidence that *FOB1* does influence macromolecular chromosome structure at rDNA. *FOB1*

regulates *SIR2*-dependent transcriptional silencing at rDNA. We found that *fob1* mutation suppresses many changes in the structural dynamics at rDNA caused by *top1* and *trf4* mutations.

RESULTS

fob1 mutation increases SIR2-dependent rDNA silencing

If the function of Fob1p is to facilitate interactions between rDNA repeats (Wai et al. 2001), it may have an influence on the heterochromatin structure at rDNA. To investigate this possibility, we tested whether the *fob1* mutation has any effect on the transcriptional silencing at rDNA. *fob1* mutation increased the silencing of a Ty1-*mURA3* reporter gene (Smith and Boeke 1997) at rDNA about 10-fold, while it did not have effect on the silencing outside of rDNA (Fig. 1). The increase in silencing in *fob1* mutant cells was *SIR2*-dependent: the silencing in *fob1* mutant was completely abolished when *sir2* mutation was introduced. We next investigated whether the observed increase in silencing was dependent on *TOP1*. *top1* mutation, another mutation that decreases silencing at rDNA (Smith et al. 1999), had an opposite effect in *fob1* cells. Unlike *sir2* mutation, *top1* mutation did not significantly decrease the silencing in *fob1* mutant cells (Fig.1), suggesting that the silencing mediated by Sir2p and Top1p occurs through two different genetic pathways. This result is consistent with the previous result indicating that *top1* mutant cells are unresponsive to the increased *SIR2* dosage (Smith et al. 1999).

fob1 mutation suppresses the elevated recombination rate and the altered structure in top1 mutant

Since *fob1* mutation suppresses the increased recombination rate at rDNA in *sir2* mutant cells (Kaeberlein et al. 1999), we checked whether the elevated recombination rate in *top1* cells is suppressible by *fob1* mutation. *top1* mutation

increased the rate of rDNA recombination about 30-fold, similar to the published result (Christman et al. 1988) (Fig. 2). *fob1* mutation lowered the high level of rDNA recombination in *top1* cells.

top1 cells not only have the elevated rate of rDNA recombination, but also have an aberrant rDNA structure (Christman et al. 1993). Chromosome XII in *top1* cells fails to migrate into the pulsed-field gel and instead remains in the well, while all the other chromosomes have similar mobility on the gel as isogenic *TOP1* strain. This altered structure is caused by rDNA repeats and not by another loci on chromosome XII (Christman et al. 1993), and is thought to be caused by torsional stress generated by the unrelieved supercoils created during rDNA replication or transcription. Since the *fob1* mutation was able to partially suppress both the high level of recombination rate and the decrease in silencing in *top1* mutant, we tested whether *fob1* mutation has an effect on the formation of the altered rDNA structure in *top1* cells. A fraction of chromosome XII from *top1 fob1* cells migrated properly into the gel (Fig. 3). The quantitative analysis of ethidium bromide stained bands of chromosomes XII in *top1* and *top1 fob1* mutant indicated that *top1 fob1* mutant had a significantly higher level of chromosome XII that properly migrated into the gel. Thus, *fob1* mutation suppressed the aberrant rDNA structure in *top1* cells.

fob1 mutation rescues the synthetic lethality in *top1 trf4* cells

top1 trf4 cells are inviable and fail to establish and maintain chromosome condensation of rDNA at mitosis (Castano et al. 1996). It is possible that the failure to condense rDNA in *top1 trf4* cells may be influenced by the aberrant structure in rDNA caused by the absence of Top1p. Because *fob1* mutation

suppressed the rDNA structural defect caused by *top1* mutation, we tested whether *fob1* mutation could suppress *top1 trf4* synthetic lethality. Strains were constructed containing chromosomal *top1* deletion and ARS/CEN plasmid with *TOP1* (pTOP1.URA3). Each strain also has *trf4*, *fob1*, *trf4 fob1*, or no additional mutation. As previously reported (Castano et al. 1996), the combination of *top1* and *trf4* mutations leads to the synthetic lethality (Fig. 4). *top1 trf4* pTOP1 cells fail to grow when cells that have lost pTOP1 were selected for growth by selecting on 5-fluoroorotic acid (5-FOA) medium. *fob1* mutation was found to complement the synthetic lethality between *trf4* and *top1* mutations. *top1 trf4 fob1* cells survived following the loss of pTOP1.URA3 and gave rise to 5-FOA-resistant segregants (Fig. 4). These 5-FOA- resistant segregants formed smaller colonies than the 5-FOA-resistant segregants from *TRF4* control cells. Therefore, *fob1* mutation does not appear to suppress completely the deleterious phenotype observed in *top1 trf4* mutant cells. Since *top1 trf4 fob1* cells are viable, the *fob1* mutation must allow *top1 trf4* cells for better condensation of their rDNA at mitosis.

fob1 mutation complements the cold sensitivity in *trf4* cells

Next, we check whether *fob1* mutation can suppress other phenotypes associated with *trf4* mutation. We tested whether *fob1* mutation can suppress the cold sensitivity in *trf4* cells (Sadoff et al. 1995). As reported, *trf4* cells grew slowly at 15°C. The cold-sensitivity was suppressed in *trf4 fob1* cells (Fig. 5). Since *trf4* mutation causes chromosome segregation defect (Castano et al. 1996), the suppression of cold sensitivity in *trf4 fob1* cells may be caused by an unrelated genetic change that occurred during the strain construction. This

possibility is not likely since homozygous *fob1* mutations complemented the cold sensitivity in the homozygous *trf4* diploid cells (data not shown). Furthermore, the elevated recombination rate at rDNA in *trf4* cells (Sadoff et al. 1995) was also partially suppressed by *fob1* mutation (Fig. 2). However, slow growth at 30°C and MMS-sensitivity (Sadoff et al. 1995; Walowsky et al. 1999) in *trf4* cells were not suppressed by *fob1* mutation (data not shown). Along with its homologue *TRF5*, *TRF4* is a DNA polymerase σ that plays a role in sister-chromatid cohesion. *trf4 trf5* double mutant cells are inviable. This synthetic lethality of *trf4* and *trf5* mutations was not rescued by *fob1* mutation (data not shown). This is not surprising since *TRF4* and *TRF5* is required for sister-chromatid cohesion not only in rDNA but also in other genomic loci (Wang et al. 2000).

Since the cold sensitivity of *trf4* cells is suppressed by *fob1* mutation, we reasoned that this phenotype might be the result of improper sister-chromatid cohesion at rDNA. If so, *trf4* mutation might not cause the cold sensitivity in cells that have the chromosomal rDNA deletion and have *RDN1* on multi-copy plasmids. Surprisingly, when we tried to isolate haploid segregants that are *rdn1Δ trf4 2μ-RDN1* by sporulation of diploid cells that are homozygous for *rdn1* deletion, are heterozygous for *trf4* and *fob1* mutations, and carry *2μ-RDN1* plasmids, none of the *rdn1Δ trf4 2μ-RDN1* spores were viable (Table 2). *fob1* mutation did not rescue this inviability. Since *trf4* mutation does not affect on *2μ* plasmid segregation (Castano et al. 1996), *TRF4* function must be required for *2μ-RDN1* segregation or amplification in chromosomal *rdn1Δ* strain. Interestingly, *top1* mutation did not lead to the lethality in *rdn1Δ 2μ-RDN1* spores. *rdn1Δ top1 2μ-RDN1* spores were viable (data not shown).

fob1 mutation lowers the chromosome III-rDNA loss rate

To investigate further into the function of *FOB1*, *TRF4*, and *TOP1* in rDNA condensation and segregation, we examined the effects of mutations on the rate of chromosome III-rDNA loss. Chromosome III-rDNA is the product of translocation between chromosome III and rDNA locus in chromosome XII and is marked with *ADE2* and *LEU2* genes (Fig. 6A). Unfortunately, we could not directly assess the rate of chromosome XII loss because chromosome XII aneuploidy is most likely to be lethal (Granot and Snyder 1991). We constructed a diploid strain that is heterozygous for chromosome III-rDNA translocation, but homozygous for *trf4* or *fob1* mutation. The chromosome III-rDNA loss rate was determined by modified half-sector assay, described in (Freeman et al. 2000). Number of half-red/half-white sector colonies with cells in red sector that have also lost *LEU2* and *MATa* markers was counted to determine the chromosome-III-rDNA loss rate. *fob1* cells displayed about 6-fold lower chromosome loss rate than the wild-type cells while *trf4* cells had about 3-4-fold higher loss rate than the wild-type cells (Fig. 6B). Thus, the presence of Fob1p likely antagonizes proper chromosome XII condensation and segregation. (The experiment to see if the elevated chromosome loss in *trf4* cells is suppressed by *fob1* mutation could not be performed in time for the writing of this thesis. This experiment will be included in the future publication. Also, we will assay the chromosome III-rDNA loss rate in *top1* and *top1 fob1* cells. In addition, the control experiment testing the effects of *trf4*, *fob1*, *trf4 fob1*, *top1*, and *top1 fob1* mutations on the loss rate chromosome III without rDNA translocation will be included in the future publication.)

DISCUSSION

Role of *FOB1* in rDNA chromatin structure.

In this paper, we present evidence that *FOB1* plays a direct role in macromolecular rDNA structure. *fob1* mutation increases *SIR2*-dependent rDNA silencing. *FOB1* is required for the formation of unidirectional RFB. Recently, Wai and coworkers showed that pol I enhancer is not required for transcription of the chromosomal rRNA gene (Wai et al. 2001). They proposed that the reason for the requirement of pol I enhancer and *FOB1* in *HOT1* transcription is that pol I enhancer allows interaction between ectopic promoter and rDNA repeats through Fob1p. This interaction may allow the recruitment of Pol I transcriptional machinery, which is normally found in nucleolus, to the ectopic promoter. They further proposed that the interactions of rDNA repeats through the E element facilitated by *FOB1* might play a role in forming a macromolecular structure that would allow RFB function. If indeed *FOB1* plays a role forming this structure, *FOB1* may have more general influence on rDNA structure. We found that *FOB1* antagonizes transcriptional silencing at rDNA, suggesting it plays a role in rDNA chromatin structure. Perhaps the formation of structure required for RFB requires unwrapping of the packaged nucleosomes at rDNA array, resulting in the reduced transcriptional silencing (Fig. 7). Sir2p is the limiting component in accessibility of the rDNA array (Fritze et al. 1997; Smith and Boeke 1997). Sir2p is thought to reduce this accessibility by increasing the number of rDNA repeats that are bound to nucleosomes or regulating an aspect of higher-order chromatin configuration. Since the increased silencing in *fob1* mutant cells is abolished by *sir2* mutation, absence of RFB may allow rDNA

chromatin to be packaged more tightly in *SIR2*-dependent manner. Consistent with this idea, *SIR2*-Responsive Region (SSR1) defined by MNase sensitivity assay at rDNA is directly adjacent to RFB site in NTS1 (Fritze et al. 1997). It would be interesting to see whether there is a change in MNase sensitive sites near or at SSR1 in *fob1* mutant cells.

***FOB1*-dependent RFB may act as an anchor that cause torsional stress during rDNA replication**

Evidence that *FOB1* plays a role in high-order rDNA structure is that *fob1* mutation was able to partially suppress the altered rDNA structure in *top1* cells. Chromosome XII in *top1* cells fails to migrate into a pulsed-field gel. Christman and coworkers showed that the failure of chromosome XII is caused by rDNA and not by another loci on chromosome XII, because when only rDNA locus was transferred from chromosome XII to chromosome III, the chromosome III failed to migrate into the gel (Christman et al. 1993). Furthermore, the failure of chromosome XII to migrate into the gel occurs in preparations from exponentially growing *top1* cultures, and not in preparations from stationary-phase *top1* cultures. The authors postulated that since this altered structure only occurs in exponentially growing *top1* cells, but not in stationary-phase *top1* cells, perhaps this altered structure is caused a defect in rDNA replication or transcription. They suggested that the *top1* defect in DNA replication might occur only at rDNA because of the unique, unidirectional replication caused by RFB at rDNA. Consistent with this idea, we show that the *fob1* mutation, which abolishes the RFB, partially suppresses the aberrant structure at the rDNA. Perhaps *FOB1*-dependent RFB acts as an anchor in higher-order rDNA structure

that creates excessive torsional stress during DNA replication, which needs to be relieved by Top1p. Absence of RFB may allow rDNA to rotate more freely during replication and thus allow superhelical stress to be relieved by diffusion. This relaxation of supercoils by diffusion might reduce the likelihood of forming the altered rDNA structure. This idea is consistent with the result that *fob1* mutation did not completely suppress the altered structure at rDNA. Absence of RFB will not likely to completely compensate for the loss of *TOP1* function since relaxation of supercoils by diffusion would be slow. Consistently, the elevated rDNA recombination in *top1* cells, which is likely caused by the torsional stress, is suppressed by *fob1* mutation.

RFB structure and mitotic condensation at rDNA.

Another evidence that *FOB1* plays an important function in structural dynamics at rDNA comes from the result that *fob1* mutation complements the defect in rDNA condensation in *top1 trf4* cells. *top1 trf4* cells are inviable and fail to establish and maintain chromosome condensation at rDNA at mitosis (Castano et al. 1996). Castano and coworkers proposed that *top1 trf4* cells are also very likely to have a failure of condensation at non-rDNA loci because *top1* and *trf4* single mutants display a centromere-mediated plasmid segregation defect. We found that the lethality in *top1 trf4* cells is most likely caused by the failure to condense rDNA and not another loci, since *fob1* mutation suppresses the synthetic lethality caused by *top1 trf4* double mutations. Thus, proper condensation of rDNA at mitosis must be the critical process that is limiting in *top1 trf4* cells.

Unless *FOB1* has unknown function outside of rDNA, which is very unlikely since Fob1p is concentrated in the nucleolus (Defossez et al. 1999) and all of its known functions are specific to the rDNA (Lin and Keil 1991; Kobayashi and Horiuchi 1996), *FOB1* and perhaps RFB must antagonize the proper condensation of rDNA in *top1 trf4* cells. *FOB1*-dependent, high-order rDNA structure that is required for the formation of RFB may exacerbate torsional stress generated during condensation (Fig. 7). Without Trf4p, the absence of Top1p to relieve such torsional stress may be detrimental to proper rDNA condensation. This hypothesis is supported by our result that *FOB1* directly affects the rDNA chromosome transmission by the chromosome loss study. *fob1* mutation decreased the loss rate of chromosome III with rDNA translocation. Therefore, most likely RFB antagonizes stable rDNA chromosome transmission. It is unlikely that *FOB1* is directly involved in the regulation of mitotic condensation since *fob1* mutation did not suppress the temperature-sensitive phenotype of condensation-defective *smc2-8* or *smc4-1* mutant cells (our unpublished results).

In most cases, topoisomerase II is the only topoisomerase required to carry out mitotic chromosome condensation and for separating intertwined, replicated chromosomes at the time of mitosis (DiNardo et al. 1984; Holm et al. 1985; Uemura et al. 1987; Adachi et al. 1991). Perhaps because rDNA is highly transcribed and has a unique structure that allows unidirectional DNA replication, rDNA, unlike other genomic loci, may require Top1p and other proteins to cope with many changes in its structural dynamics, including condensation. This might explain why Top1p is concentrated in nucleolus (Giroux et al. 1989; Edwards et al. 2000). Bolstering this hypothesis, Smc4p, one

of the proteins in condensin complex, is concentrated at rDNA *in vivo* (Freeman et al. 2000), suggesting that condensin complex has a specialized function in mitotic segregation of the rDNA locus.

rDNA and sister-chromatid cohesion

Since Trf4p is a DNA polymerase σ that is required for sister-chromatid cohesion, the requirement for Trf4p in mitotic condensation at rDNA is likely to be linked to its role in sister-chromatid cohesion. A little is known about how sister-chromatid cohesion occurs at highly repeated rDNA. We found that *TRF4* is required for proper segregation of a plasmid containing rDNA in *rdn1 Δ* cells, suggesting that *TRF4* plays a role in sister-chromatid cohesion at rDNA. While *fob1* mutation was not able to complement the *RDN1* plasmid segregation defect caused by *trf4* mutation (Table 2), *fob1* mutation suppressed other phenotypes associated with *trf4* mutation: the increased level of rDNA recombination and cold sensitivity. The cold sensitivity in *trf4* cells might be caused by defect in sister-chromatid cohesion at rDNA since *fob1* mutation was able to complement the sensitivity. Since *FOB1* function is not likely to be directly linked to the regulation of sister-chromatid cohesion, perhaps RFB antagonizes the formation of sister-chromatid cohesion at rDNA.

RFB in other organisms

Although Fob1p does not appear to be evolutionarily conserved, the replication fork stalling at the 3' end of the rRNA transcription unit is conserved (Hernandez et al. 1993; Gerber et al. 1997; Lopez-estrano et al. 1998; Sanchez et al.

1998). It will be interesting to investigate whether the polar replication fork barrier at rDNA affects chromosome structure and transmission in other organisms.

MATERIALS AND METHODS

Yeast strains, plasmids, and media.

Yeast strains used in this study are listed in Table 1. All strains are isogenic except indicated otherwise. Strains were cultured at 30°C with standard media (Sherman et al. 1986), except indicated otherwise. Strains carrying *top1-7::LEU2* disruption was constructed by transforming CB25 cut with HindIII (Christman et al. 1988). Strains carrying *top1-7::TRP1* disruption was constructed by replacing from +74 to +1033 of *LEU2* coding sequence in *top1-7::LEU2* with *TRP1* marker by one-step transplacement (Rothstein 1991; Baudin et al. 1993). pNL2 (gift from G. Fink) was constructed by cloning HindIII/NruI fragment containing *TOP1* into YCP50, a yeast shuttle vector containing ARS/CEN and *URA3*. SMY186 (gift of S. Ma) with *rdn1Δ* and pRDN-wt-U was generated by procedures described in (Chernoff et al. 1994). PPY853 was generated by crossing SMY186 with PPY789 and isolating *rdn1Δ fob1 2μ-RDN1* haploid spore after sporulation.

Disruption of following genes was carried out by one-step transplacement method (Rothstein 1991; Baudin et al. 1993). Disruption using *kanR* marker was carried out as described in (Guldener et al. 1996). The following regions within coding sequences were replaced with indicated markers: from +36 to +1643 for *fob1Δ:HIS3* and *fob1Δ:kanR*; from +222 to +1531 for *trf4Δ:TRP1* and *trf4Δ:kanR*; and from +41 to +1611 for *sir2Δ:kanR*.

Quantitative growth spot assay.

Freshly grown overnight-cultured cells were serially diluted in five or ten-fold increments. 7 µl of each dilution was spotted onto either nonselective or selective SC agar plates. Plates were incubated either at 30°C or 15°C depending on the assay. Plates were incubated for 2 to 7 days. The image of the plates was taken using Image Store 7500 gel documentation system (UVP, Upland, CA).

Synthetic lethality assay.

Strains that have chromosomal *top1* mutation and carry pTOP1.URA3 (pNL2) were constructed. Each strain also has *trf4*, *fob1*, *trf4 fob1*, or no additional mutation. The test for synthetic lethality was performed by the ability of each strain to grow after losing the plasmid containing *TOP1*. Each strain was grown non-selectively for pTOP1.URA3 on YPD, and then streaked on either SC (Synthetic Complete) or SC+5-FOA (SC plus 5-fluoroorotic acid) plates. 5-FOA medium provides counterselection for *URA3*⁺ cells (Boeke et al. 1987). Plates were incubated at 30°C for 3 days before the image of the plates were taken.

Pulse-field gel conditions.

Yeast chromosomes were prepared from exponentially growing cells as described in (Carle and Olson 1987) with following modifications. The experiment was carried out using Bio-Rad CHEF DR II system (Hercules, CA). The DNA plugs were made with 0.66% low melting agarose (SeaPlaque GTG agarose, FMC Bioproducts, Rockland, ME) using Bio-Rad Reusable Plug Mold. Pulse-field gel (21 x 14 cm) made of 0.5 x TBE, pH 8.0/1% agarose was prepared using provided casting stand. Plugs of equal size were inserted into well, and

the wells were sealed with 1% low melting agarose. Pulse-field gel was electrophoresed in 0.5 x TBE buffer, pH 8.0 at 3.5 V/cm for 20 hrs with gradual increase in switch time from 100 sec to 200 sec and then for 40 hrs with gradual increase in switch time from 200 sec to 350 sec. The buffer was kept cooled by circulation using provided cooling system at 10.3°C. The gel was stained with ethidium bromide and the image was taken using Image Store 7500 gel documentation system. Intensity of bands was quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA). Intensity of a low or no chromosome XII band in *top1* mutant cells was used as background intensity. Background intensity was first subtracted from the intensity of chromosome XII and IV bands. Ratios of background-subtracted chromosome XII and IV bands were determined.

Determination of rDNA recombination rate.

Freshly grown overnight-cultured cells carrying *ADE2* marker integrated into the rDNA array were plated onto YPD plates with low adenine. Colonies were allowed to grow for 2 days at 30°C, except for colonies carrying *trf4* mutation, which were allowed to grow for 3 days at 30°C. Then, the plates were stored at 4°C for 2 days. The number of half-red/half-white colonies was counted along with the total number of colonies, excluding entirely red colonies. The half-red/half-white colonies were assumed to represent a marker loss event during the first cell division after plating. The recombination rate was determined by dividing the number of half-red/half-white colonies by the total number of colonies. At least 20,000 colonies were counted for each strain.

Determination of chromosome III-rDNA loss rate.

Modified half-sector assay were performed on isogenic strains carrying *ADE2* and *LEU2*-marked rDNA on chromosome III as described in (Freeman et al. 2000). Experiments were carried similarly as describe above in the procedure for determining the rDNA recombination rate. The number of half-red/half-white colonies that contained red (*ade2*⁻) cells that have also lost *LEU2* and *MATa* markers were determined. These half-red/half-white colonies were assumed to represent a chromosome loss event during the first cell division after plating. The chromosome loss rate was determined by dividing the number of these half-red/half-white colonies by the total number of colonies. At least 20,000 colonies were counted for each strain.

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REFERENCES

- Adachi, Y., M. Luke, and U.K. Laemmli. 1991. *Chromosome assembly in vitro: topoisomerase II is required for condensation.* *Cell* 64: 137-48.
- Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, and C. Cullin. 1993. *A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*.* *Nucleic Acids Res* 21: 3329-3330.
- Boeke, J.D., J. Trueheart, G. Natsoulis, and G.R. Fink. 1987. *5-Fluoroorotic acid as a selective agent in yeast molecular genetics.* *Methods Enzymol* 154: 164-75.
- Brewer, B.J. and W.L. Fangman. 1988. *A replication fork barrier at the 3' end of yeast ribosomal RNA genes.* *Cell* 55: 637-43.
- Brewer, B.J., D. Lockshon, and W.L. Fangman. 1992. *The arrest of replication forks in the rDNA of yeast occurs independently of transcription.* *Cell* 71: 267-76.
- Brill, S.J., S. DiNardo, K. Voelkel-Meiman, and R. Sternglanz. 1987. *Need for DNA topoisomerase activity as a swivel for DNA replication for transcription of ribosomal RNA.* *Nature* 326: 414-6.
- Bryk, M., M. Banerjee, M. Murphy, K.E. Knudsen, D.J. Garfinkel, and M.J. Curcio. 1997. *Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast.* *Genes Dev* 11: 255-69.
- Carle, G.F. and M.V. Olson. 1987. *Orthogonal-field-alternation gel electrophoresis.* *Methods Enzymol* 155: 468-82.
- Castano, I.B., P.M. Brzoska, B.U. Sadoff, H. Chen, and M.F. Christman. 1996. *Mitotic chromosome condensation in the rDNA requires TRF4 and DNA topoisomerase I in *Saccharomyces cerevisiae*.* *Genes Dev* 10: 2564-76.
- Chernoff, Y.O., A. Vincent, and S.W. Lieberman. 1994. *Mutations in eukaryotic 18S ribosomal RNA affect translational fidelity and resistance to aminoglycoside antibiotics.* *Embo J* 13: 906-13.
- Christman, M.F., F.S. Dietrich, and G.R. Fink. 1988. *Mitotic recombination in the rDNA of *S. cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II.* *Cell* 55: 413-25.
- Christman, M.F., F.S. Dietrich, N.A. Levin, B.U. Sadoff, and G.R. Fink. 1993. *The rRNA-encoding DNA array has an altered structure in topoisomerase I mutants of *Saccharomyces cerevisiae*.* *Proc Natl Acad Sci U S A* 90: 7637-41.

- Dammann, R., R. Lucchini, T. Koller, and J.M. Sogo. 1993. *Chromatin structures and transcription of rDNA in yeast Saccharomyces cerevisiae*. Nucleic Acids Res 21: 2331-8.
- 1995. *Transcription in the yeast rRNA gene locus: distribution of the active gene copies and chromatin structure of their flanking regulatory sequences*. Mol Cell Biol 15: 5294-303.
- Defossez, P.A., R. Prusty, M. Kaeberlein, S.J. Lin, P. Ferrigno, P.A. Silver, R.L. Keil, and L. Guarente. 1999. *Elimination of replication block protein Fob1 extends the life span of yeast mother cells*. Mol Cell 3: 447-55.
- DiNardo, S., K. Voelkel, and R. Sternglanz. 1984. *DNA topoisomerase II mutant of Saccharomyces cerevisiae: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication*. Proc Natl Acad Sci U S A 81: 2616-20.
- Edwards, T.K., A. Saleem, J.A. Shaman, T. Dennis, C. Gerigk, E. Oliveros, M.R. Gartenberg, and E.H. Rubin. 2000. *Role for nucleolin/Nsr1 in the cellular localization of topoisomerase I*. J Biol Chem 275: 36181-8.
- Freeman, L., L. Aragon-Alcaine, and A. Strunnikov. 2000. *The condensin complex governs chromosome condensation and mitotic transmission of rDNA*. J Cell Biol 149: 811-24.
- Fritze, C.E., K. Verschueren, R. Strich, and R. Easton Esposito. 1997. *Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA*. Embo J 16: 6495-509.
- Gerber, J.K., E. Gogel, C. Berger, M. Wallisch, F. Muller, I. Grummt, and F. Grummt. 1997. *Termination of mammalian rDNA replication: polar arrest of replication fork movement by transcription termination factor TTF-I*. Cell 90: 559-67.
- Giroux, C.N., M.E. Dresser, and H.F. Tiano. 1989. *Genetic control of chromosome synapsis in yeast meiosis*. Genome 31: 88-94.
- Gotta, M., S. Strahl-Bolsinger, H. Renauld, T. Laroche, B.K. Kennedy, M. Grunstein, and S.M. Gasser. 1997. *Localization of Sir2p: the nucleolus as a compartment for silent information regulators*. Embo J 16: 3243-55.
- Gottlieb, S. and R.E. Esposito. 1989. *A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA*. Cell 56: 771-6.
- Gottschling, D.E., O.M. Aparicio, B.L. Billington, and V.A. Zakian. 1990. *Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription*. Cell 63: 751-62.

- Granot, D. and M. Snyder. 1991. *Segregation of the nucleolus during mitosis in budding and fission yeast*. *Cell Motil Cytoskeleton* 20: 47-54.
- Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J.H. Hegemann. 1996. *A new efficient gene disruption cassette for repeated use in budding yeast*. *Nucleic Acids Res* 24: 2519-24.
- Hernandez, P., L. Martin-Parras, M.L. Martinez-Robles, and J.B. Schwartzman. 1993. *Conserved features in the mode of replication of eukaryotic ribosomal RNA genes*. *Embo J* 12: 1475-85.
- Holm, C., T. Goto, J.C. Wang, and D. Botstein. 1985. *DNA topoisomerase II is required at the time of mitosis in yeast*. *Cell* 41: 553-63.
- Imai, S., C.M. Armstrong, M. Kaeberlein, and L. Guarente. 2000. *Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase*. *Nature* 403: 795-800.
- Ivy, J.M., A.J. Klar, and J.B. Hicks. 1986. *Cloning and characterization of four SIR genes of Saccharomyces cerevisiae*. *Mol Cell Biol* 6: 688-702.
- Kaeberlein, M., M. McVey, and L. Guarente. 1999. *The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms*. *Genes Dev* 13: 2570-80.
- Keil, R.L. and G.S. Roeder. 1984. *Cis-acting, recombination-stimulating activity in a fragment of the ribosomal DNA of S. cerevisiae*. *Cell* 39: 377-86.
- Kim, R.A. and J.C. Wang. 1989. *A subthreshold level of DNA topoisomerases leads to the excision of yeast rDNA as extrachromosomal rings*. *Cell* 57: 975-85.
- Kobayashi, T., D.J. Heck, M. Nomura, and T. Horiuchi. 1998. *Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I*. *Genes Dev* 12: 3821-30.
- Kobayashi, T. and T. Horiuchi. 1996. *A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities*. *Genes Cells* 1: 465-74.
- Kobayashi, T., M. Nomura, and T. Horiuchi. 2001. *Identification of DNA cis elements essential for expansion of ribosomal DNA repeats in Saccharomyces cerevisiae*. *Mol Cell Biol* 21: 136-47.
- Landry, J., A. Sutton, S.T. Tafrov, R.C. Heller, J. Stebbins, L. Pillus, and R. Sternglanz. 2000. *The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases*. *Proc Natl Acad Sci U S A* 97: 5807-11.

- Leary, D.J. and S. Huang. 2001. *Regulation of ribosome biogenesis within the nucleolus*. *FEBS Lett* 509: 145-50.
- Lin, Y.H. and R.L. Keil. 1991. *Mutations affecting RNA polymerase I-stimulated exchange and rDNA recombination in yeast*. *Genetics* 127: 31-8.
- Linskens, M.H. and J.A. Huberman. 1988. *Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae**. *Mol Cell Biol* 8: 4927-35.
- Lopez-estrano, C., J.B. Schwartzman, D.B. Krimer, and P. Hernandez. 1998. *Co-localization of polar replication fork barriers and rRNA transcription terminators in mouse rDNA*. *J Mol Biol* 277: 249-56.
- Michel, B., S.D. Ehrlich, and M. Uzest. 1997. *DNA double-strand breaks caused by replication arrest*. *EMBO J* 16: 430-38.
- Mills, K.D., D.A. Sinclair, and L. Guarente. 1999. *MEC1-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks*. *Cell* 97: 609-20.
- Oakes, M., J.P. Aris, J.S. Brockenbrough, H. Wai, L. Vu, and M. Nomura. 1998. *Mutational analysis of the structure and localization of the nucleolus in the yeast *Saccharomyces cerevisiae**. *J Cell Biol* 143: 23-34.
- Olson, M.O., M. Dundr, and A. Szekely. 2000. *The nucleolus: an old factory with unexpected capabilities*. *Trends Cell Biol* 10: 189-96.
- Park, P.U., P.A. Defossez, and L. Guarente. 1999. *Effects of mutations in DNA repair genes on formation of ribosomal DNA circles and life span in *Saccharomyces cerevisiae**. *Mol Cell Biol* 19: 3848-56.
- Pederson, T. 1998. *The plurifunctional nucleolus*. *Nucleic Acids Res* 26: 3871-6.
- Rine, J. and I. Herskowitz. 1987. *Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae**. *Genetics* 116: 9-22.
- Rothstein, R. 1991. *Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast*. *Methods Enzymol* 194: 281-301.
- Sadoff, B.U., S. Heath-Pagliuso, I.B. Castano, Y. Zhu, F.S. Kieff, and M.F. Christman. 1995. *Isolation of mutants of *Saccharomyces cerevisiae* requiring DNA topoisomerase I*. *Genetics* 141: 465-79.
- Sanchez, J.A., S.M. Kim, and J.A. Huberman. 1998. *Ribosomal DNA replication in the fission yeast, *Schizosaccharomyces pombe**. *Exp Cell Res* 238: 220-30.
- Schultz, M.C., S.J. Brill, Q. Ju, R. Sternnganz, and R.H. Reeder. 1992. *Topoisomerases and yeast rRNA transcription: negative supercoiling stimulates*

- initiation and topoisomerase activity is required for elongation.* *Genes Dev* 6: 1332-41.
- Seigneur, M., V. Bidnenko, S.D. Ehrlich, and B. Michel. 1998. *RuvAB acts at arrested replication forks.* *Cell* 95: 419-30.
- Shaw, P.J. and E.G. Jordan. 1995. *The nucleolus.* *Annu Rev Cell Dev Biol* 11: 93-121.
- Sherman, F., G. Fink, and J. Hicks. 1986. *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shou, W., J.H. Seol, A. Shevchenko, C. Baskerville, D. Moazed, Z.W. Chen, J. Jang, H. Charbonneau, and R.J. Deshaies. 1999. *Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex.* *Cell* 97: 233-44.
- Sinclair, D.A. and L. Guarente. 1997. *Extrachromosomal rDNA circles—a cause of aging in yeast.* *Cell* 91: 1033-42.
- Smith, J.S. and J.D. Boeke. 1997. *An unusual form of transcriptional silencing in yeast ribosomal DNA.* *Genes Dev* 11: 241-54.
- Smith, J.S., C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai, J.L. Avalos, J.C. Escalante-Semerena, C. Grubmeyer, C. Wolberger, and J.D. Boeke. 2000. *A phylogenetically conserved NAD⁺-dependent protein deacetylase activity in the Sir2 protein family.* *Proc Natl Acad Sci U S A* 97: 6658-63.
- Smith, J.S., E. Caputo, and J.D. Boeke. 1999. *A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors.* *Mol Cell Biol* 19: 3184-97.
- Straight, A.F., W. Shou, G.J. Dowd, C.W. Turck, R.J. Deshaies, A.D. Johnson, and D. Moazed. 1999. *Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity.* *Cell* 97: 245-56.
- Szostak, J.W. and R. Wu. 1980. *Unequal crossing over in the ribosomal DNA of *Saccharomyces cerevisiae*.* *Nature* 284: 426-30.
- Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. *DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*.* *Cell* 50: 917-25.
- Visintin, R. and A. Amon. 2000. *The nucleolus: the magician's hat for cell cycle tricks.* *Curr Opin Cell Biol* 12: 372-7.
- Voelkel-Meiman, K., R.L. Keil, and G.S. Roeder. 1987. *Recombination-stimulating sequences in yeast ribosomal DNA correspond to sequences regulating transcription by RNA polymerase I.* *Cell* 48: 1071-9.

- Wai, H., K. Johzuka, L. Vu, K. Eliason, T. Kobayashi, T. Horiuchi, and M. Nomura. 2001. *Yeast RNA polymerase I enhancer is dispensable for transcription of the chromosomal rRNA gene and cell growth, and its apparent transcription enhancement from ectopic promoters requires Fob1 protein.* *Mol Cell Biol* 21: 5541-53.
- Walowsky, C., D.J. Fitzhugh, I.B. Castano, J.Y. Ju, N.A. Levin, and M.F. Christman. 1999. *The topoisomerase-related function gene TRF4 affects cellular sensitivity to the antitumor agent camptothecin.* *J Biol Chem* 274: 7302-8.
- Wang, Z., I.B. Castano, A. De Las Penas, C. Adams, and M.F. Christman. 2000. *Pol kappa: A DNA polymerase required for sister chromatid cohesion.* *Science* 289: 774-9.
- Weber, J.D., L.J. Taylor, M.F. Roussel, C.J. Sherr, and D. Bar-Sagi. 1999. *Nucleolar Arf sequesters Mdm2 and activates p53.* *Nat Cell Biol* 1: 20-6.

TABLE 1. Yeast strains used in this study.

<u>Strains</u>	<u>Genotype</u>	<u>Source</u>
W303-1α5	<i>MATα ade2-1 leu2-3,112 can1-100, trp1-1 ura3-52 his3-11,15 RAD5</i>	Laboratory strain; (Mills et al. 1999)
W303-1α5R	W303-1α5 RDN1::ADE2	Laboratory strain; (Mills et al. 1999)
PPY582	W303α5R fob1Δ::HIS3	This Study
PPY519	W303α5R top1-7::LEU2	This Study
PPY540	W303α5R trf4Δ::TRP1	This Study
PPY568	W303α5R top1-7::LEU2 fob1Δ::HIS3	This Study
PPY789	W303α5R trf4Δ::TRP1 fob1Δ::HIS3	This Study
PPY570	W303α5R top1-7::LEU2 pNL2	This Study
PPY571	W303α5R top1-7::LEU2 fob1Δ::HIS3 pNL2	This Study
PPY572	W303α5R top1-7::LEU2 trf4Δ::TRP1 pNL2	This Study
PPY584	W303α5R top1-7::LEU2 trf4Δ::TRP1 fob1Δ::HIS3 pNL2	This Study
PPY951	W303α5 fob1Δ::HIS3	This Study
PPY796	W303α5 trf4Δ::TRP1	This Study
PPY797	W303α5 trf4Δ::TRP1 fob1Δ::HIS3	This Study
L4078	MAT α ade2 leu2 lys2 trp1 ura3 his5 III::(LEU2::ADE2::rDNA::URA3)	G. Fink
PPY912	L4078 fob1Δ::kanR	This Study
PPY914	L4078 trf4Δ::kanR	This Study
PPY919	L4078 x W303-1α5	This Study
PPY922	PPY912 x PPY582	This Study
PPY925	PPY914 x PPY796	This Study
JS122 (R31)	MAT α his3Δ200 leu2Δ1 ura3-167 ????:Ty1- mURA3	J. Boeke; (Smith and Boeke 1997)
JS124 (S2)	MAT α his3Δ200 leu2Δ1 ura3-167 RDN1::Ty1- mURA3	J. Boeke; (Smith and Boeke 1997)
JS151	JS122 (R31) sir2Δ::HIS3	J. Boeke; (Smith and Boeke 1997)
JS155	JS124 (S2) sir2Δ::HIS3	J. Boeke; (Smith and Boeke 1997)
PPY589	JS124 (S2) fob1Δ::HIS3	This Study
PPY611	JS124 (S2) top1-7::LEU2	This Study
PPY612	JS124 (S2) top1-7::LEU2 fob1Δ::HIS3	This Study
PPY943	JS124 (S2) fob1Δ::HIS3 sir2Δ::kanR	This Study
PPY944	JS122 (R31) fob1Δ::HIS3	This Study
PPY945	JS122 (R31) top1-7::LEU2	This Study
PPY946	JS122 (R31) top1-7::LEU2 fob1Δ::HIS3	This Study
PPY947	JS122 (R31) fob1Δ::HIS3 sir2Δ::kanR	This Study
SMY168	W303-1 α rdn1Δ pRDN-wt-U	S. Ma; (Chernoff et

PPY853	<i>W303-1α rdn1Δ pRDN-wt-U fob1Δ::HIS3</i>	al. 1994)
PPY935	<i>SMY168 x PPY853</i>	This Study
PPY937	<i>PPY935 top1-7::LEU2/TOP1</i>	This Study
PPY938	<i>PPY935 trf4Δ::kanR /TRF4</i>	This Study

TABLE 2. Genetic interaction between *rds1Δ 2μ-RDN1* and *trf4*.

<u>Genotype recovered</u>	<u>Number of</u> <u>spores recovered</u>	<u>Total expected</u>
<i>rds1Δ 2μ-RDN1 TRF4 FOB1</i>	17	20
<i>rds1Δ 2μ-RDN1 TRF4 fob1</i>	14	20
<i>rds1Δ 2μ-RDN1 trf4 FOB1</i>	0	20
<i>rds1Δ 2μ-RDN1 trf4 fob1</i>	0	20

PPY938 (W303-1a/W303-1α *rds1Δ/rds1Δ TRF4/trf4 FOB1/fob1 2μ-RDN1*) was sporulated. Tetrads were dissected on YPD plates and incubated at 30°C for 5 days. After the spores had grown into colonies, the plates were replica-plated onto SC--His medium or YPD medium containing G418 to identify the genotypes. All *rds1Δ 2μ-RDN1 trf4* spores stopped dividing after several divisions.

FIGURE 1. *fob1* mutation increases *SIR2*-dependent transcriptional silencing at rDNA. Quantitative growth assays measuring the silencing of weakened *URA3* gene (*Ty1-mURA3*) (Smith and Boeke 1997). S2 strains contain *Ty1-mURA3* at rDNA while R31 strains contain *Ty1-mURA3* outside of rDNA. Ten-fold serial dilutions of freshly grown cells were spotted on either SC (Synthetic Complete) or SC-Ura (SC minus uracil) plate. S2 strains shown are: wild type (JS124), *fob1* (PPY589), *sir2* (JS155), *sir2 fob1* (PPY943), *top1* (PPY611), and *top1 fob1* (PPY612). R31 strains are: wild type (JS122), *fob1* (PPY944), *sir2* (JS151), *sir2 fob1* (PPY947), *top1* (PPY945), and *top1 fob1* (PPY946). Plates were incubated at 30°C for 2 days before plates were photographed.

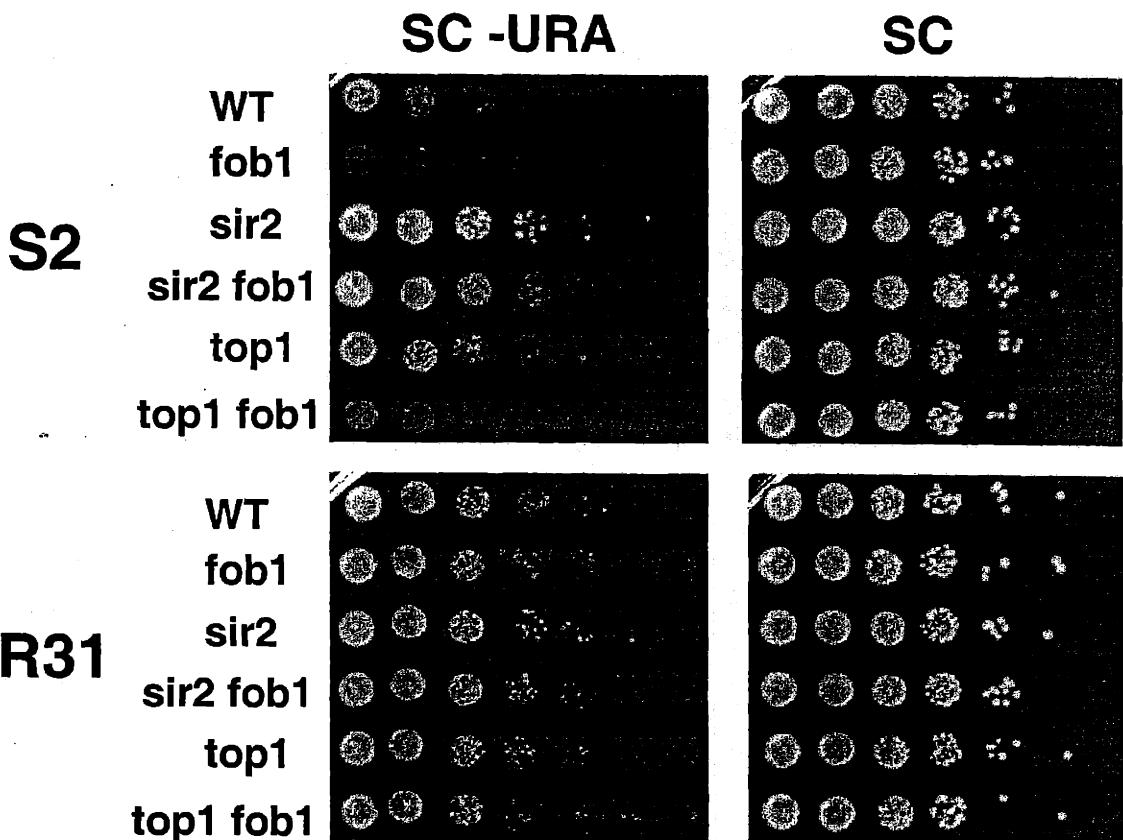
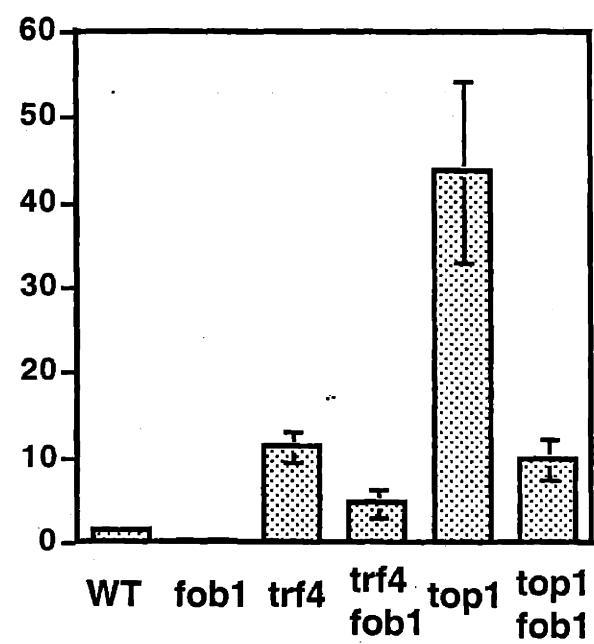




FIGURE 2. *fob1* mutation suppresses the elevated rate of rDNA recombination in *trf4* and *top1* mutant cells. Half-sector assay were performed on isogenic W303R strains containing *ADE2* marker integrated at rDNA. The recombination rate was determined by dividing the number of half-red/half-white colonies by the total number of colonies. At least 20,000 colonies were counted for each strain. Recombination rates were as follows: wild type (W303-1a5R), $1.50 \times 10^{-4} \pm 0.20 \times 10^{-4}$; *fob1* (PPY582), $0.22 \times 10^{-4} \pm 0.38 \times 10^{-4}$; *trf4* (PPY540), $11.31 \times 10^{-4} \pm 1.78 \times 10^{-4}$; *trf4 fob1* (PPY789), $4.73 \times 10^{-4} \pm 1.61 \times 10^{-4}$; *top1* (PPY519), $43.70 \times 10^{-4} \pm 10.58 \times 10^{-4}$; *trf1 fob1* (PPY568), $9.90 \times 10^{-4} \pm 2.43 \times 10^{-4}$.

rate of rDNA marker loss ($\times 10^4$)



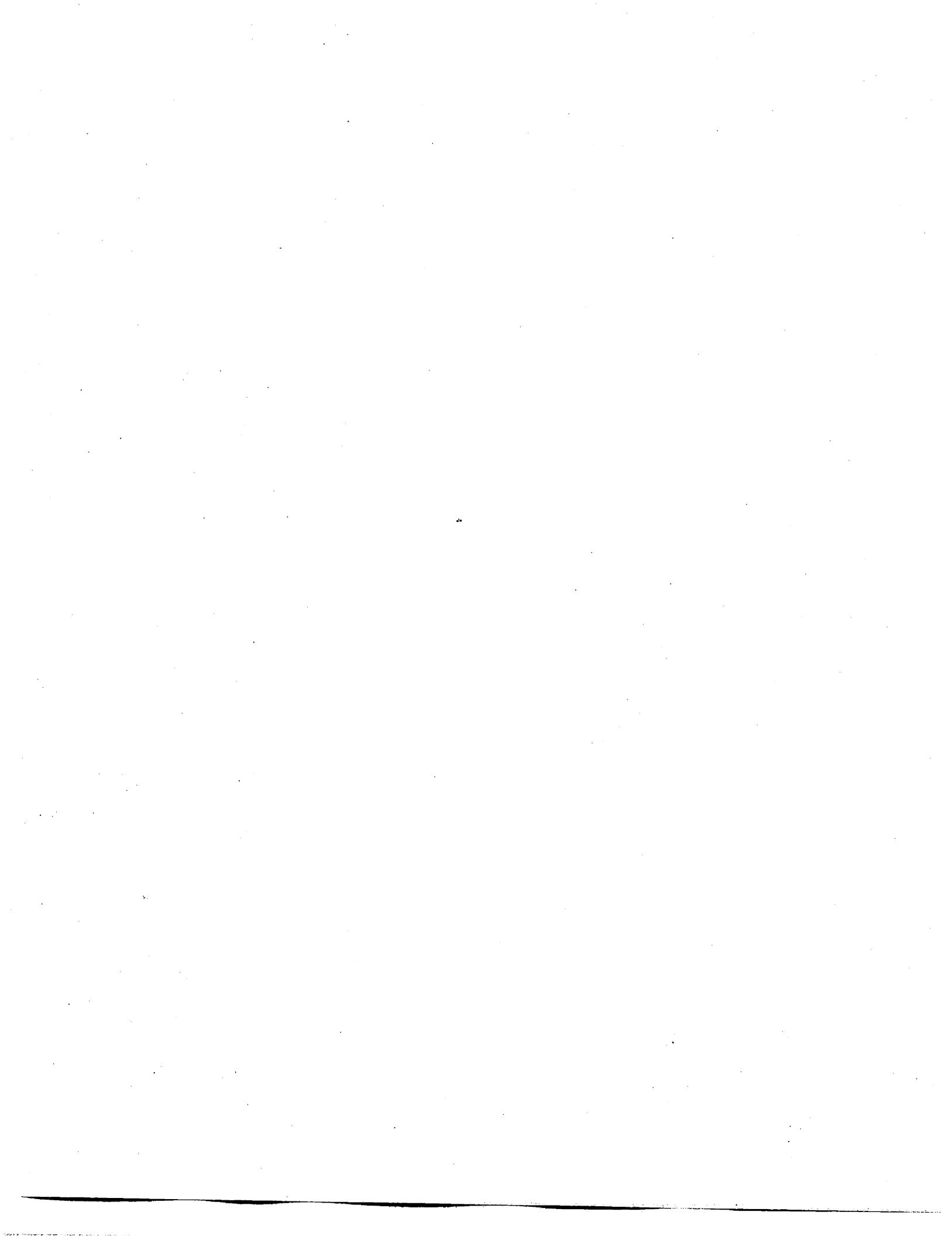


FIGURE 3. *fob1* mutation partially relieves the aberrant rDNA structure in *top1* mutant cells. Inverted image of ethidium bromide-stained chromosomes on pulsed-field gel. Intensity of bands was quantified using MD ImageQuant. Intensity of a low or no chromosome XII band in *top1* mutant cells was used as background intensity. Background intensity was first subtracted from the intensity of chromosome XII and IV bands. Ratios of background-subtracted chromosome XII and IV bands are as follows: wild type (W303-1 α 5R), 1.5120; *top1* (PPY519), 0; *fob1* (PPY582), 1.5128; and *top1 fob1* (PPY568), 0.0850.

top1
WT top1 fob1 fob1

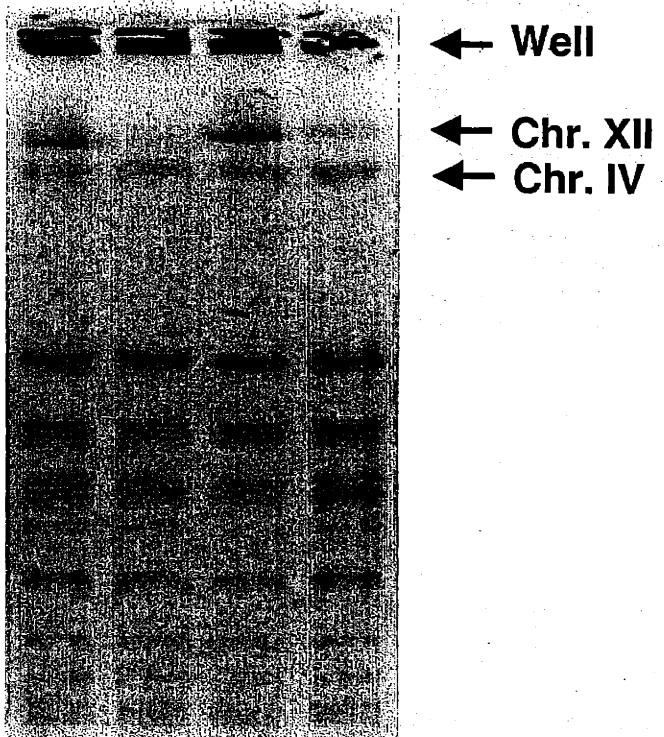
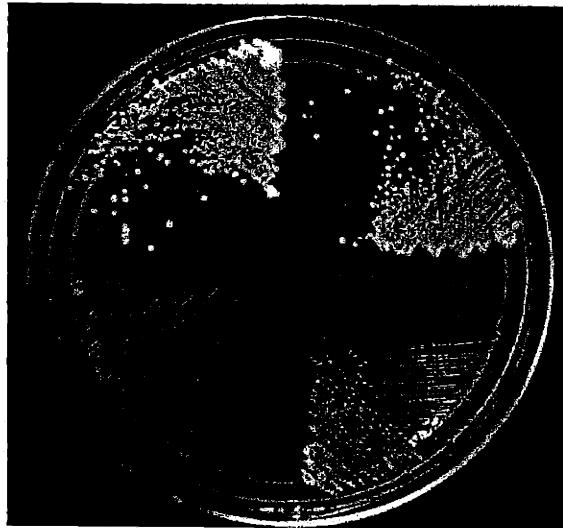




FIGURE 4. *fob1* mutation rescues the synthetic lethality in *top1 trf4* double mutant cells. All the strains shown carry chromosomal *top1* mutation and pTOP1.URA3 (pNL2) plasmid. Each strain also has *trf4* (PPY572), *fob1* (PPY571), *trf4 fob1* (PPY574), or no mutation (PPY570). Each strain was grown non-selectively for pTOP1.URA3 (pNL2) on YPD, and then streaked on either SC (Synthetic Complete) or SC+5-FOA (SC plus 5-fluoroorotic acid) plates. 5-FOA medium provides counterselection for *URA3⁺* cells. Plates were incubated at 30°C for 3 days before photographs were taken.

SC + 5-FOA

**top1 fob1
pTOP1**



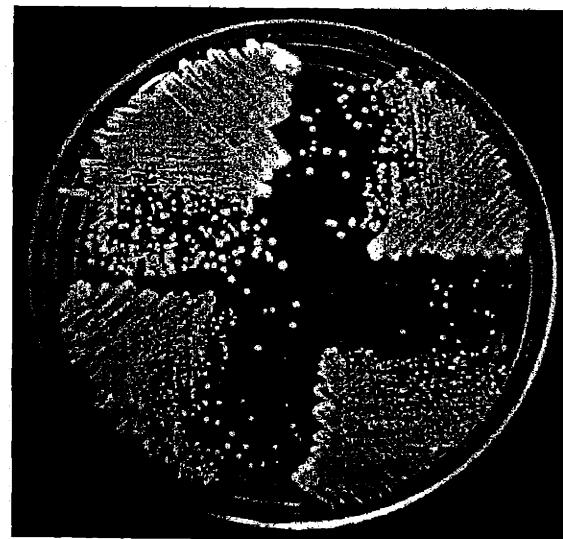
**top1
pTOP1**

**top1 trf4
pTOP1**

**top1 trf4 fob1
pTOP1**

SC

**top1 fob1
pTOP1**



**top1
pTOP1**

**top1 trf4
pTOP1**

**top1 trf4 fob1
pTOP1**

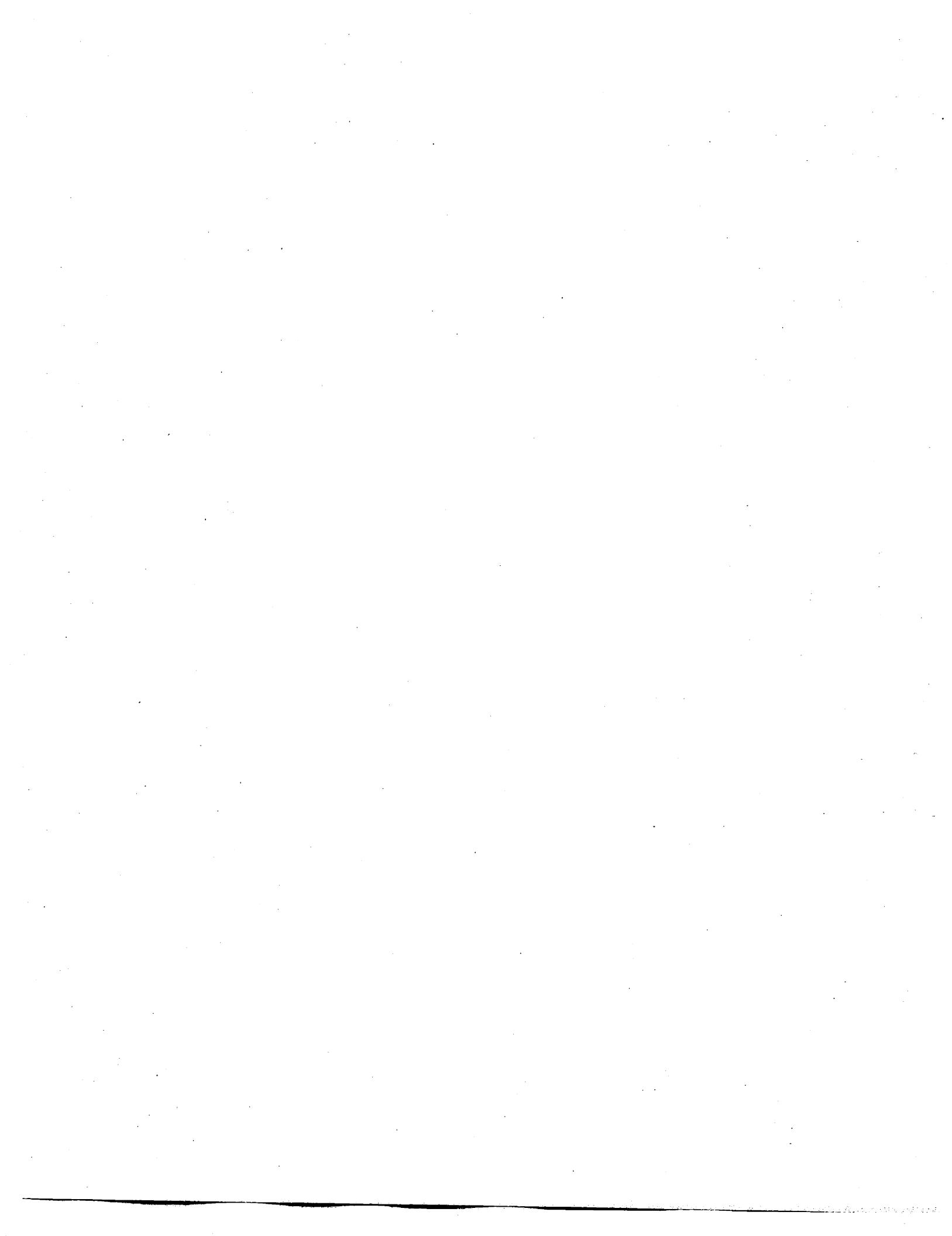
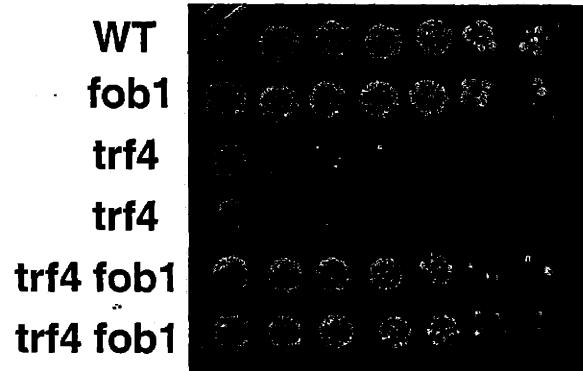


FIGURE 5. Cold sensitivity in *trf4* cells is complemented by *fob1* mutation.
Quantitative assays measuring the growth at 15°C and 30°C. Five-fold serial dilutions of freshly grown cells were plated on SC and incubated either at 15°C or 30°C. Strains shown are: wild type (W303-1 α 5R), *fob1* (PPY582), *trf4* (PPY540), and *trf4 fob1* (PPY789). The photographs were taken after 7 days incubation at 15°C and 3 days incubation at 30°C.

15°C



30°C

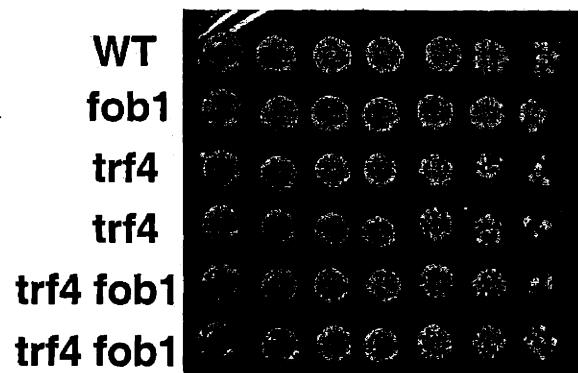
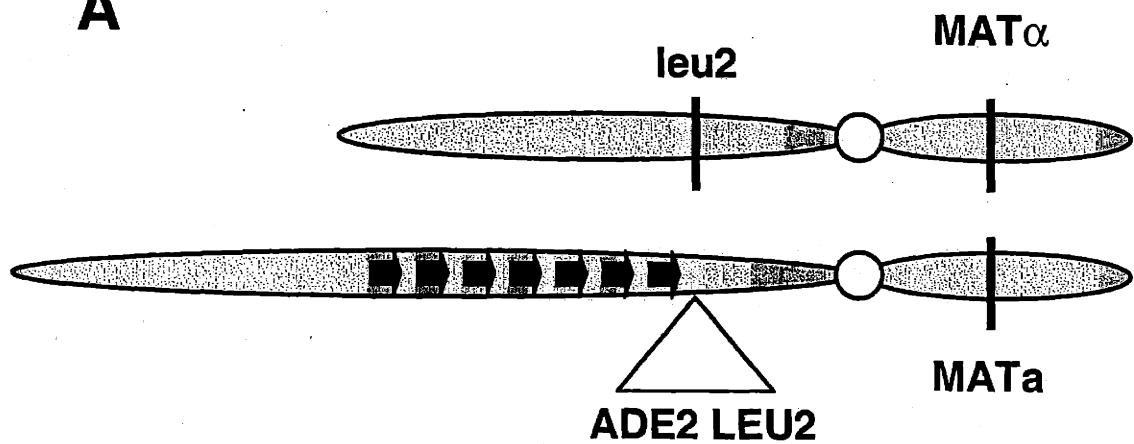


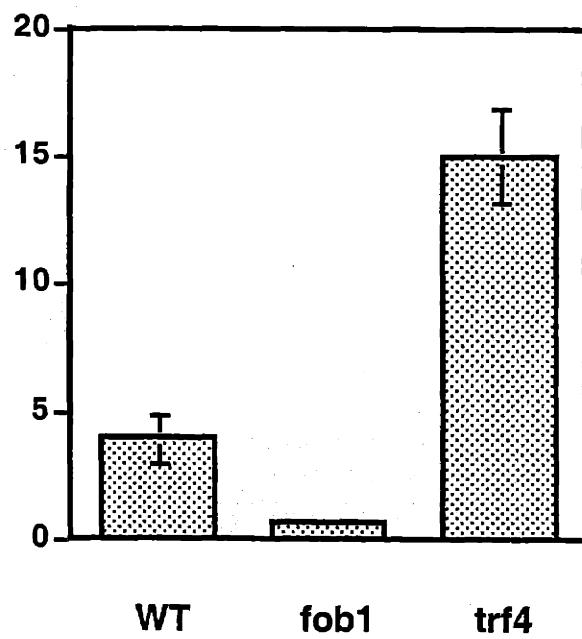


FIGURE 6. Reduced rate of chromosome III-rDNA in *fob1* mutant cells.

Modified half-sector assay were performed on isogenic strains carrying *ADE2* and *LEU2*-marked rDNA on chromosome III. The chromosome loss rate was determined by counting the number of half-red/half-white colonies with red sectors that contain *ade2*⁻ cells that have also lost *LEU2* and *MATa* markers and dividing that number by the total number of colonies. At least 20,000 colonies were counted for each strain. Chromosome loss rates were as follows: wild type (PPY919), $3.94 \times 10^{-4} \pm 0.95 \times 10^{-4}$; *fob1* (PPY922), $0.65 \times 10^{-4} \pm 0.23 \times 10^{-4}$; *trf4* (PPY925), $14.99 \times 10^{-4} \pm 1.88 \times 10^{-4}$.

A**B**

rate of Chr.III-rDNA chromosome loss ($\times 10^4$)



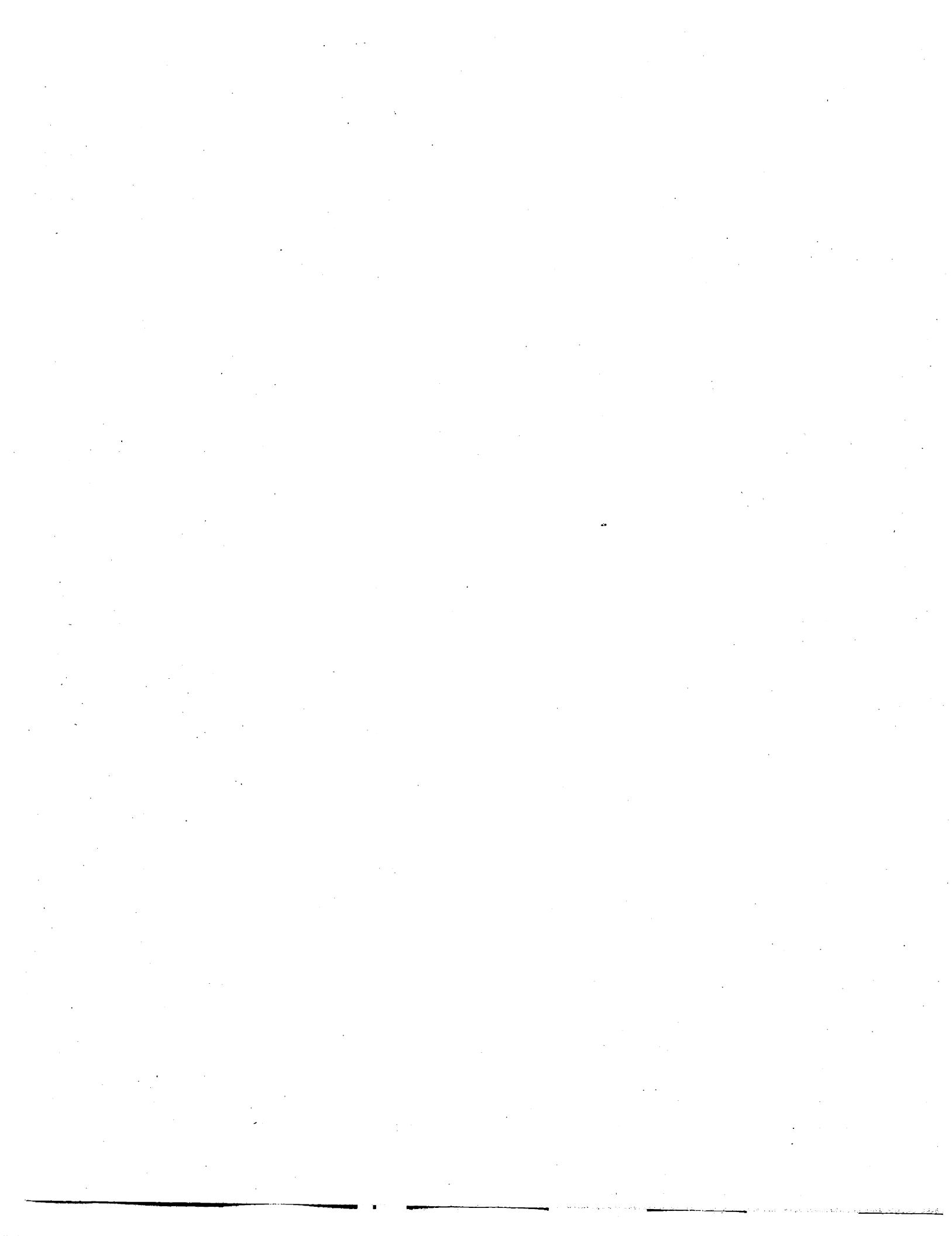
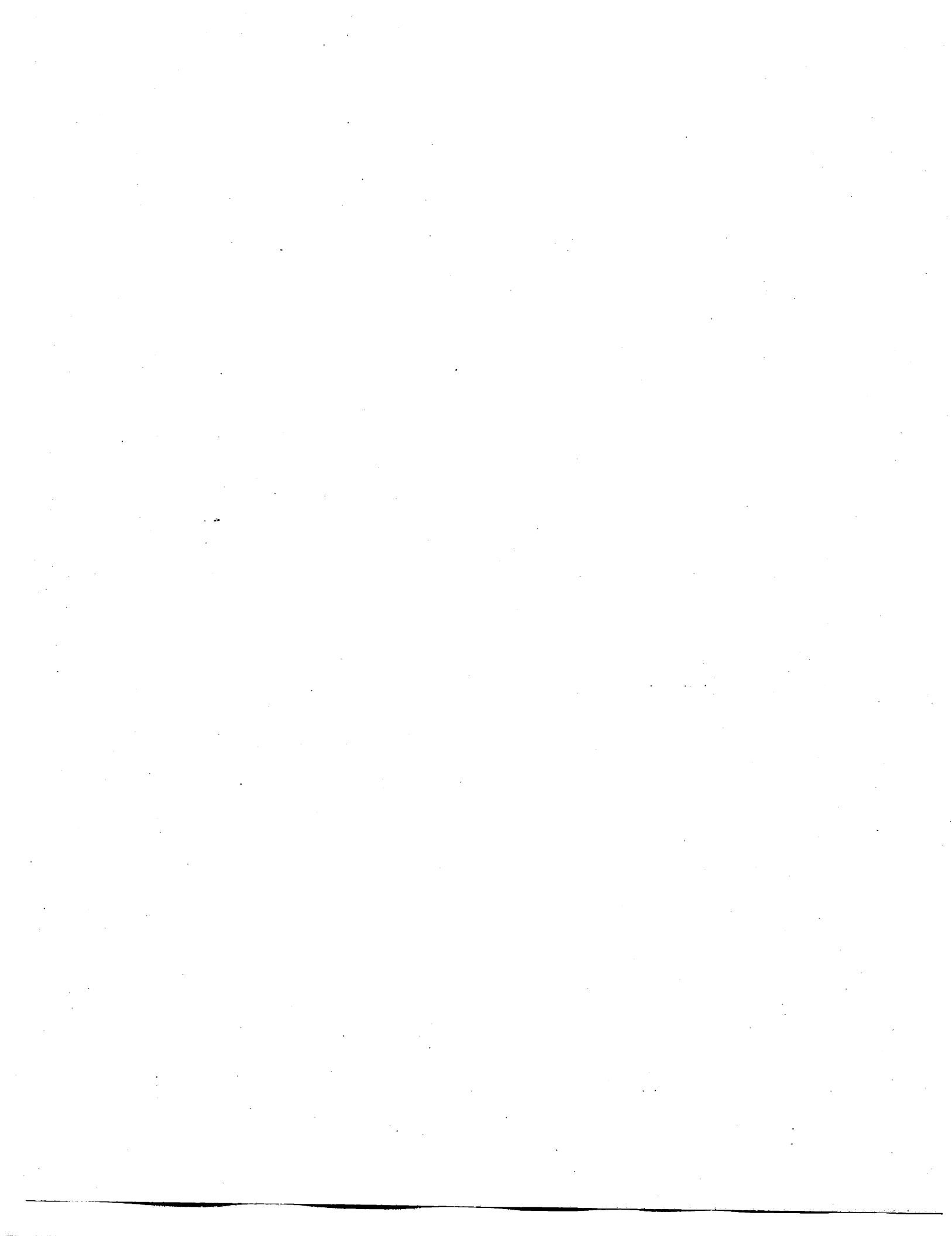
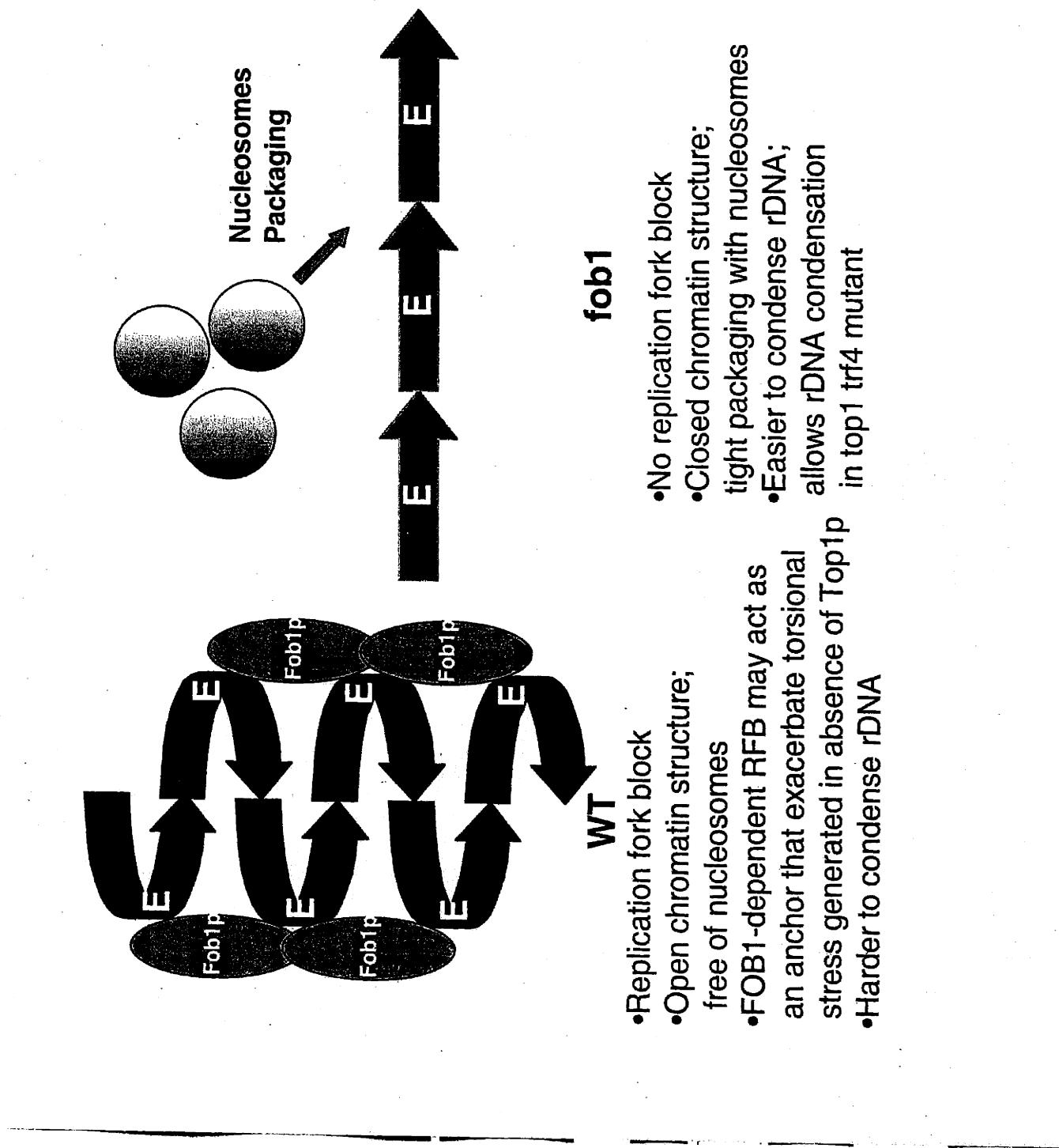


FIGURE 7. Hypothetical model of rDNA structure with or without *F0B1*. In wild type cells, Fop1p may facilitate interactions between rDNA repeats (large arrows) through the E element. These interactions may play a role in forming a macromolecular structure that would allow RFB function. The formation of these rDNA interactions may require unpacking of nucleosomes, thus leading to the decrease in transcriptional silencing. In addition, the mitotic condensation of rDNA may be antagonized by this structure. In *fob1* cells, no Fob1p-mediated interactions exist at rDNA. The rDNA repeats can be packaged more tightly with nucleosomes, leading to the increase in transcriptional silencing. This closed chromatin structure may facilitate the condensation process and even allow rDNA condensation in *top1 trf4* mutant cells. This cartoon is not meant to imply the existence of direct rDNA-Fob1p interactions because such interactions have not been demonstrated.







Appendix A

Separation of Mother and Daughter Cells

This appendix was previously published in *Methods in Enzymology*, Volume 351, Pages 468-477 in 2002. The authors were Peter U. Park, Mitch McVey, and Leonard Guarente.

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* divides asymmetrically. In vegetative growth, yeast cells reproduce by budding, and the position where the bud forms ultimately determines the plane of cell division (Chant 1999). A bud emerges during the late G₁ stage of the cell cycle and continues to grow, first at the tip and then throughout the bud, until late nuclear division and cytokinesis. The fact that budding yeast undergo polarized cell growth and directional cell division has been useful in understanding fundamental processes that are essential for the development of higher eukaryotes, such as early embryogenesis in *Caenorhabditis elegans* and neurogenesis in *Drosophila* (Hyman and White 1987; Kraut et al. 1996). Furthermore, the asymmetric division plays a role in mating type switching and some aspects of cell cycle regulation (Haber 1998). Additionally, this asymmetry has been very useful in studying the aging process in yeast.

A key requirement for tracing aging in an organism is the ability to distinguish and follow individuals over time. Asymmetric cell division in yeast makes it possible to follow the fate of an individual cell throughout many cell divisions. The mother cell buds to give rise to a daughter cell that is clearly smaller in size. By micromanipulating away the daughters from a mother, Mortimer and Johnston found that mother cells divide a relatively fixed number of times before stopping, and the probability of stopping increases exponentially as the number of prior divisions increases (Mortimer and Johnston 1959). These experiments showed that the replicative life span of yeast cells fits the mathematical definition of aging, also seen in humans (Gompertz 1825; Finch

1990). The replicative life span in yeast depends on the number of progressions through the cell cycle, as opposed to the chronological life span, which is measured in stationary-phase cells under conditions of nutrient starvation.

Tracing the fates of individual yeast cells over time by micromanipulation allows for not only the determination of life span, but also the characterization of a number of phenotypic changes that occur during the aging process. These changes include bud scar accumulation, enlargement of cell size, slowing of cell cycle, and loss of fertility (Mortimer and Johnston 1959; Kennedy et al. 1994; Sinclair et al. 1998). However, although micromanipulation allows for the visual characterization of changes associated with aging, it does not allow for the molecular and biochemical analyses of those changes. Such analyses require an isolation of a large number of aged cells. Several methods have been developed that allow for this large-scale isolation. Using these methods, a number of additional changes in old cells have been characterized. These include changes in the expression of *LAG1* and *LAG2*, nucleolar fragmentation, loss of silencing, movement of the Sir complex from telomeres to nucleolus, and rDNA circle accumulation (Egilmez et al. 1989; Smeal et al. 1996; Kennedy et al. 1997; Sinclair and Guarente 1997; Sinclair et al. 1997; Defossez et al. 1998).

This section describes the detailed procedures for separation and isolation of mothers and daughters. These protocols have been used by investigators studying aging, bud site selection, and other aspects of asymmetric cell division. The first part of this chapter describes the procedures for performing life span analysis by micromanipulation, and the second part describes the steps for the large-scale collection of old cells.

ISOLATION OF MOTHERS FROM DAUGHTERS BY MICROMANIPULATION

The most accurate method for isolating mother cells of specific age involves following each mother cell through multiple divisions. Practically, this is accomplished by isolating virgin mother cells on an agar plate and separating daughter cells from each mother by micromanipulation. For life span analysis, this process is continued until the mother cell ceases division and eventually lyses. This technique is of greatest use in experiments where knowledge of the exact age of the mother cells is required. However, the small number of old cells obtained and their isolation on an agar plate preclude their use in either immunofluorescence or biochemical experiments.

Life span Analysis

Prior to beginning a life span, it is essential to ensure that the population of yeast cells is free of petites (which have a longer life span in some strains) (Kirchman et al. 1999) and is growing robustly. Then, the life span begins with cells that have not divided previously. From these virgin mother cells, buds are removed as they form and are discarded. If desired, the daughters can be monitored through additional cell divisions, as in the case of pedigree analysis (Kennedy et al. 1994; Sinclair and Guarente 1997).

Procedure

1. Start with a single colony of the yeast strain whose life span is to be determined. Streak this colony onto a plate containing glycerol as the sole

carbon source (YPG plate) in order to remove any petite cells from the population (Kirchman et al. 1999).

2. From the YPG plate, pick a single colony and patch this onto rich medium (YPD plate). Allow the patch to grow at 30° for 1 day. From the overnight patch, streak cells to a fresh YPD plate and grow for 1 more day at 30°.
3. Transfer several thousand cells (a small dab on the end of a toothpick) to the side of a fresh plate and allow to divide for several hours at 30°.
4. Using a fiber optic glass needle attached to a micromanipulator (see section on tetrad dissection), arrange at least 40 cells in a gridded pattern in the middle of the plate. There should be about 50 μm between each cell to provide enough room to separate mothers from daughters without disturbing neighboring cells. It is often useful to poke holes in the agar, with the fiber optic needle, between every 10 mother cells, thereby providing convenient reference points on the plate (Fig. 1). *Note: Micromanipulation is generally performed at room temperature (20-25°). All incubations are performed at 30°.*
5. Allow the isolated cells to divide at least once. Generally, this will take 1-2 hr, depending on the strain.
6. Separate the mother cell from the daughter cell (the mother is the larger of the two). **Keep the daughter cell** and drag the mother cell back to the patch of cells on the side of the plate. **This daughter cell is now a virgin mother cell.**
7. Incubate the virgin mother cells at 30° for one cell division. At the beginning of the life span, the cells will divide once approximately every 90 min at 30°. Therefore, it is extremely important not to incubate the plates for too long so that the daughters do not divide too many times and obscure the mother.

8. As daughter cells emerge from the mother, separate them by gently placing the needle on the plate just to the side of them and tapping the base of the microscope gently.
9. **Keep the mother cell** and remove each daughter cell to the side of the plate. Each bud that is removed from the mother cell (not from other daughter cells) represents one generation for the mother cell that produced it. After each micromanipulation, record the number of times that each mother cell has divided, using a gridded data sheet.
10. The plates can be incubated overnight at temperatures lower than room temperature (4-10°). Generally, mother cells will bud once during a 12-hr period at 10° and growth ceases at 4°. Incubation at a lower temperature does not significantly affect life span (Kennedy et al. 1994).
11. Continue this procedure until all mother cells cease to divide and lyse. As cells get older, the generation time slows down markedly. Also, cells will often fail to divide for several hours and then resume division. The reasons for this are unknown. Therefore, it is important to follow each mother cell until it lyses.
12. A mortality curve can be constructed that displays the life span of all of the mother cells in the population by graphing the percentage of cells still dividing at each generation point (Mortimer and Johnston 1959).

Useful Hints:

1. Be extremely careful not to contaminate the life span plate with bacteria or other fungi. Any contamination will quickly overtake the plate and ruin the experiment. Contamination, if caught early enough, can be removed from the

agar by sterilizing the round end of a glass pipette and pressing the pipette into the agar, thereby removing the contamination in a round agar plug.

2. To keep the life span plate from drying out and killing the cells, use thick plates and keep them sealed with Parafilm during the 30° incubations.
3. Be careful not to touch the needle into large colonies, as cells can stick to the sides of the needle. During subsequent manipulations, these cells may be transferred accidentally to the part of the plate where the mother cells are dividing. These extra cells will divide and overtake the mother cells rapidly.
4. It is possible to determine the life spans of up to six strains (with 40 cells each) at one time. This is accomplished by performing micromanipulation on one strain while the others are incubating at 30°.
5. When directly comparing the life spans of several strains, it is advisable to only compare mortality curves generated in a single experiment using plates poured at the same time. The life spans of strains have been found to vary slightly using different batches of plates.
6. Life spans can be performed on synthetic complete medium, although we have found that this can affect the life span of certain strains negatively. In addition, it has been shown that altering the nutrient content of the plates can both extend and decrease life span (Jiang et al. 2000; Lin et al. 2000).

Visual Identification of Mother Cells

At the beginning and the end of a life span, it can be difficult to distinguish mothers from daughters. At most points in the life span, daughter cells are smaller than the mothers that produced them. In addition, mother cells will generally bud a second time before their daughter cells form their first bud.

Therefore, if you are unsure which of two cells is a mother cell, you can wait until one of them begins to bud. This cell is usually the mother cell. However, this pattern breaks down as mother cells approach their maximum life span because older cells have a longer generation time than younger cells (Kennedy et al. 1994).

In addition, very old mother cells often produce identically sized daughters during their last few divisions (Kennedy et al. 1994). Therefore, at the end of a life span, it is generally advisable to keep both mother and daughter cells if they are of similar sizes. The cell that stops dividing first can be assumed to be the mother cell.

Finally, we have found it useful after each micromanipulation to make a quick drawing of the positions of the mother cells and buds that cannot be removed. We include these drawings on the same data sheet used to record the number of divisions. This practice aids in the identification of the original mother cell after subsequent incubation.

LARGE-SCALE SEPARATION OF MOTHER AND DAUGHTER CELLS

Although micromanipulation of yeast cells used for life span determination allows isolation of a small number of old mother cells, molecular and biochemical analyses of old mother cells require a large-scale isolation of aged cells. The separation of large quantities of old cells is a difficult task because the proportion of old cells in a random population of exponentially growing cells is minuscule. In a given population, one-half of the cells are virgin daughter cells, one-quarter are cells that have divided once, one-eighth are two divisions old, one-sixteenth are three divisions old, etc. Therefore, exponentially growing cells can be considered as young cells, as more than 87% of exponentially growing cells have divided twice or less. In fact, there are only about 100 twenty-generation-old mother cells in 10^8 cells. Several techniques have been developed for this large-scale separation of old mothers and daughters.

One method for effective isolation of virgin daughter cells from mother cells, but not for recovery of old mothers, is called a "baby machine" (Helmstetter 1991; Grzelak et al. 2001). Mother cells are attached to a membrane and allowed to divide. Daughter cells from these attached cells are eluted continuously by washing the membrane. Two other methods take advantage of the difference in size between mother and daughter cells. One method relies on centrifugal elutriation to continuously separate daughters from mothers (Woldringh et al. 1995). After mother cells are grown in the chamber of elutriation rotor, cells are eluted by centrifugation. Eluted mother cells are kept while daughter cells are discarded. The process can be repeated many times to

enrich for old cells. Another technique uses sucrose gradient centrifugation (Egilmez and Jazwinski 1989; Egilmez et al. 1990). First, virgin daughters and mothers are separated into two distinct bands on a 10-30% sucrose gradient by centrifugation. Daughter cells are recovered and synchronized with mating pheromone. Then, daughters are allowed to grow for several generations and are again separated by centrifugation. This time, mother cells are collected, synchronized, and grown before another round of sucrose gradient separation. A relatively pure population (90%) of old cells can be isolated after repeating the technique many rounds.

Another method for enriching old mother cells comes from the observation that the cell surface of emerging daughter cells is synthesized *de novo* at the budding site (Ballou 1982). The previously synthesized components of the mother cell surface do not contribute to the daughter cell surface. Therefore, if the mother cells are tagged at the surface with a label, then the label will stay with the mother cells and can be used to distinguish them from the daughter cells after many divisions. Subsequent recovery of labeled cells gives a pure population of cells that have divided a predetermined number of times. One method that has been used involves a biotin-avidin labeling system. Proteins on the cell surfaces are first conjugated with biotin and then cells are grown for a desired number of generations. The biotin-labeled cells can be recovered by the addition of either fluorochrome-conjugated avidin or streptavidin-coated magnetic beads. Fluorescence-activated cell sorting (FACS) can be performed to isolated biotin-labeled old cells if fluorochrome-conjugated avidin is used. This technique allows isolation of biotin-labeled old cells with >99% purity, but in relatively small quantities (10^4 cells) (Smeal et al. 1996). Magnetic sorting with

streptavidin-coated paramagnetic iron beads allows procurement of more than 10^8 cells. The magnetic beads are incubated with the bulk culture containing biotin-labeled, old cells (Smeal et al. 1996; Sinclair and Guarante 1997). By placing the culture near a magnet, the old cells coated with beads are separated from the unlabeled, young cells (Fig. 2). Magnetic sorting is the preferred technique for the isolation of old cells in our laboratory because it allows for separation of a high-yield, pure population of old cells with a relatively small number of manipulation steps.

Biotin-Streptavidin Magnetic-Sorting Procedure

1. Grow 5 ml of overnight culture in YPD.
2. Dilute the culture in 50 ml of YPD and grow the cells at least 5 hr to an OD₆₀₀ of 0.7-1.0.
3. Harvest and resuspend the cell pellet with 1 ml of sterile phosphate-buffered saline (PBS). Transfer the cells into a 1.5-ml Eppendorf tube and determine the cell density using a hemocytometer. Transfer 10^8 cells into a new tube and wash with 1 ml of PBS. Resuspend the cells in 0.4 ml of PBS.

Labeling Cells

4. Add 8 mg of sulfo-NHS-LC-biotin (Pierce, Rockford, IL), dissolved in 0.3 ml of PBS just before use, to 10^8 cells. Sulfo-NHS-LC-biotin is very sensitive to moisture. Prewarm the bottle to room temperature before opening the bottle. Batches of sulfo-NHS-LC-biotin were found to vary. The quality of sulfo-NHS-LC-biotin is the most critical element determining the success of this

procedure. Make sure sulfo-NHS-LC-biotin, when opened for the first time, is a fine powder and is not contaminated with moisture. If it is contaminated, try to obtain a different batch of the powder from the supplier. Close the bottle containing sulfo-NHS-LC-biotin immediately after use and wrap the bottle in Parafilm. Store the powder in a -20° freezer.

5. Incubate the cells at room temperature for 15 min with gentle shaking.
6. Wash the cells three times with 1 ml of PBS to remove excess sulfo-NHS-LC-biotin. The cells should be pelleted gently with speed <6000 rpm for 2 min for each wash at room temperature.
7. Resuspend the cells in 1 ml of YPD.
8. Add 10^8 cells per liter of YPD and grow the cells at 30° with shaking. The cells are usually grown for about 11-13 hr. It is important that the cells are **not overgrown to OD₆₀₀ exceeding 1.0** because we have noticed that there is a poor recovery rate of old cells if OD₆₀₀ exceeds 1.0. The YPD used for this procedure contains 2.5% glucose and is made freshly just before use.

Sorting Old Cells

9. Harvest the cells by centrifugation at 5000 rpm for 10 min at 4°. Remove the medium thoroughly.
10. Resuspend the cells into 30-50 ml of prechilled (4°) PBS. From this point, cells must be kept cold to prevent the formation of new daughters.
11. Wash twice 0.3 ml of streptavidin-coated magnetic beads (PerSeptive Biosystems, Framingham, MA) in 1 ml cold PBS. About 0.3 ml of magnetic beads is needed per 10^8 cells.

12. Add the beads to the cells and incubate on ice for 2-3 hr with occasional swirling to bind the cells to the beads.
13. *From this point, perform the sorting steps at 4°.* Add the cells bound to beads into several test tubes and place the test tubes in a magnetic sorter (rack) (PerSeptive Biosystems). Add the appropriate amount of cells into each tube to level with the top of the magnet. Ensure that the test tubes are firmly touching the side surface of the rack. Incubate for 20 min to allow the beads and the cells bound to the beads to move toward the magnetic surface.
14. Gently remove the unlabeled young cells with a 10-ml pipette without disturbing the strip of cells and beads magnetized to the surface of the test tube. Remove the cells by slowly pipetting from the top of the tube and descending. Leave about 1 ml of cells at the bottom of the test tube because some portion of old cells could have settled at the bottom of the tube.
15. Add prechilled (4°) YPD to level with the top of the magnet and incubate for 15 min.
16. Repeat steps 14 and 15.
17. Remove the unbound cells and add more prechilled YPD as before, but this time remove the test tubes from the magnetic sorter and resuspend the bound cells gently by flicking the tubes. Place the tubes back to the sorter and incubate for 15 min. This further separates young and old cells.
18. Repeat step 17 seven to eight more times.
19. Pool the remaining cells in 1.5 ml of cold YPD and check the yield using a hemocytometer.

Counting Bud Scars to Determine Age

20. Dissolve 10 mg of Calcofluor white M2R (fluorescent brightener 28, Sigma) in 1 ml PBS. Centrifuge at the maximum speed and use the top 0.9 ml of supernatant to remove undissolved crystals, which can interfere with bud scar counting.
21. Wash 10^6 cells in 1 ml PBS and spin down the cells at 6000 rpm for 1 min. Resuspend the cells with 0.4 ml of prepared Calcofluor solution and incubate for 5 min.
22. Add 1 ml PBS and spin again at 6000 rpm for 1 min. Wash the cells once with 1 ml of PBS. Resuspend the cells in 20 μ l. Place the cells on a microscopic slide with a coverslip and count the number of bud scars using UV fluorescence. To determine the total number of bud scars, move the microscopic field slightly up and down. Bud scars consist of chitin rings that are deposited on the cell surface of the mother cell and indicate previous sites of cell division, thus indicating age. Old cells from a single sort will have an average bud scar count of 7 to 12, depending on the strain.

Second and Third Sorting

23. To obtain even older cells, second and third sortings must be performed. The remaining cells from the first sort are inoculated at 10^8 cells per liter of YPD and are grown for 11-13 hr. The sorting procedure is then repeated. Because the cells from the first sort are already biotinylated, it is not necessary to biotinylate the cells again. For second and third sorts, it is critical that all the procedures in the previous sort are carried out in a sterile condition. Bacterial

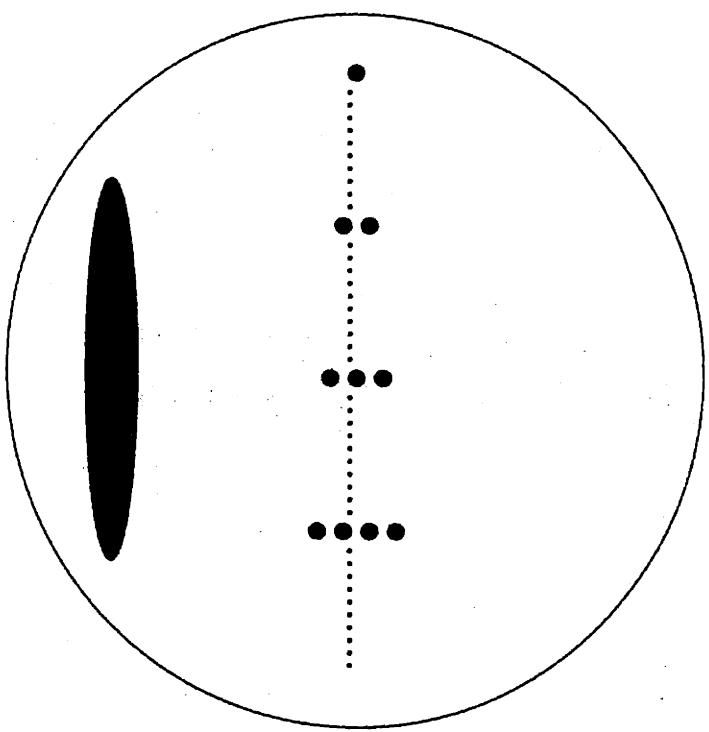
contamination can ruin the separation of old cells. Old cells from two sorts will have an average bud scar count of 14-24.

REFERENCES

- Ballou, C.E. 1982. Yeast cell wall and cell surface. In *"The Molecular Biology of the Yeast Saccharomyces."* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Chant, J. 1999. *Cell polarity in yeast.* Annu Rev Cell Dev Biol 15: 365-91.
- Defossez, P.-A., P.U. Park, and L. Guarente. 1998. *Vicious circles: a mechanism of yeast aging.* Curr Opin Microbiol 1: 707-11.
- Egilmez, N.K., J.B. Chen, and S.M. Jazwinski. 1989. *Specific alterations in transcript prevalence during the yeast life span.* J Biol Chem 264: 14312-7.
- 1990. *Preparation and partial characterization of old yeast cells.* J Gerontol 45: B9-17.
- Egilmez, N.K. and S.M. Jazwinski. 1989. *Evidence for the involvement of a cytoplasmic factor in the aging of the yeast Saccharomyces cerevisiae.* J Bacteriol 171: 37-42.
- Finch, C. 1990. *Longevity, Senescence, and the Genome.* The University of Chicago Press, Chicago.
- Gompertz, B. 1825. *On the nature of the function expressive of the law of human mortality, and on a new mode of determining life contingencies.* Philos. Trans. R. Soc. 115: 513-85.
- Grzelak, A., J. Skierski, and G. Bartosz. 2001. *Decreased antioxidant defense during replicative aging of the yeast Saccharomyces cerevisiae studied using the 'baby machine' method.* FEBS Lett 492: 123-6.
- Haber, J.E. 1998. *Mating-type gene switching in Saccharomyces cerevisiae.* Annu Rev Genet 32: 561-99.
- Helmstetter, C.E. 1991. *Description of a baby machine for Saccharomyces cerevisiae.* New Biol 3: 1089-96.
- Hyman, A.A. and J.G. White. 1987. *Determination of cell division axes in the early embryogenesis of Caenorhabditis elegans.* J Cell Biol 105: 2123-35.
- Jiang, J.C., E. Jaruga, M.V. Repnevskaya, and S.M. Jazwinski. 2000. *An intervention resembling caloric restriction prolongs life span and retards aging in yeast.* Faseb J 14: 2135-7.
- Kennedy, B.K., N.R. Austriaco, Jr., and L. Guarente. 1994. *Daughter cells of Saccharomyces cerevisiae from old mothers display a reduced life span.* J Cell Biol 127: 1985-93.

- Kennedy, B.K., M. Gotta, D.A. Sinclair, K. Mills, D.S. McNabb, M. Murthy, S.M. Pak, T. Laroche, S.M. Gasser, and L. Guarente. 1997. *Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*.* *Cell* 89: 381-91.
- Kirchman, P.A., S. Kim, C.Y. Lai, and S.M. Jazwinski. 1999. *Interorganelle signaling is a determinant of longevity in *Saccharomyces cerevisiae*.* *Genetics* 152: 179-90.
- Kraut, R., W. Chia, L.Y. Jan, Y.N. Jan, and J.A. Knoblich. 1996. *Role of inscuteable in orienting asymmetric cell divisions in *Drosophila*.* *Nature* 383: 50-5.
- Lin, S.J., P.A. Defossez, and L. Guarente. 2000. *Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*.* *Science* 289: 2126-8.
- Mortimer, R.K. and J.R. Johnston. 1959. *Life span of individual yeast cells.* *Nature* 183: 1751-1752.
- Sinclair, D., K. Mills, and L. Guarente. 1998. *Aging in *Saccharomyces cerevisiae*.* *Annu Rev Microbiol* 52: 533-60.
- Sinclair, D.A. and L. Guarente. 1997. *Extrachromosomal rDNA circles--a cause of aging in yeast.* *Cell* 91: 1033-42.
- Sinclair, D.A., K. Mills, and L. Guarente. 1997. *Accelerated aging and nucleolar fragmentation in yeast sgs1 mutants.* *Science* 277: 1313-6.
- Smeal, T., J. Claus, B. Kennedy, F. Cole, and L. Guarente. 1996. *Loss of transcriptional silencing causes sterility in old mother cells of *S. cerevisiae*.* *Cell* 84: 633-42.
- Woldringh, C.L., K. Fluiter, and P.G. Huls. 1995. *Production of senescent cells of *Saccharomyces cerevisiae* by centrifugal elutriation.* *Yeast* 11: 361-9.

FIGURE 1. Schematic of a life span plate. Cells are initially patched onto the plate (oval, left) and allowed to divide for several hours. Mother cells (small circles) are isolated in the middle of the plate in groups of 10 and their positions are marked by holes in the agar (large circles). After incubation, daughters are separated from mothers and moved to the side of the plate (oval).



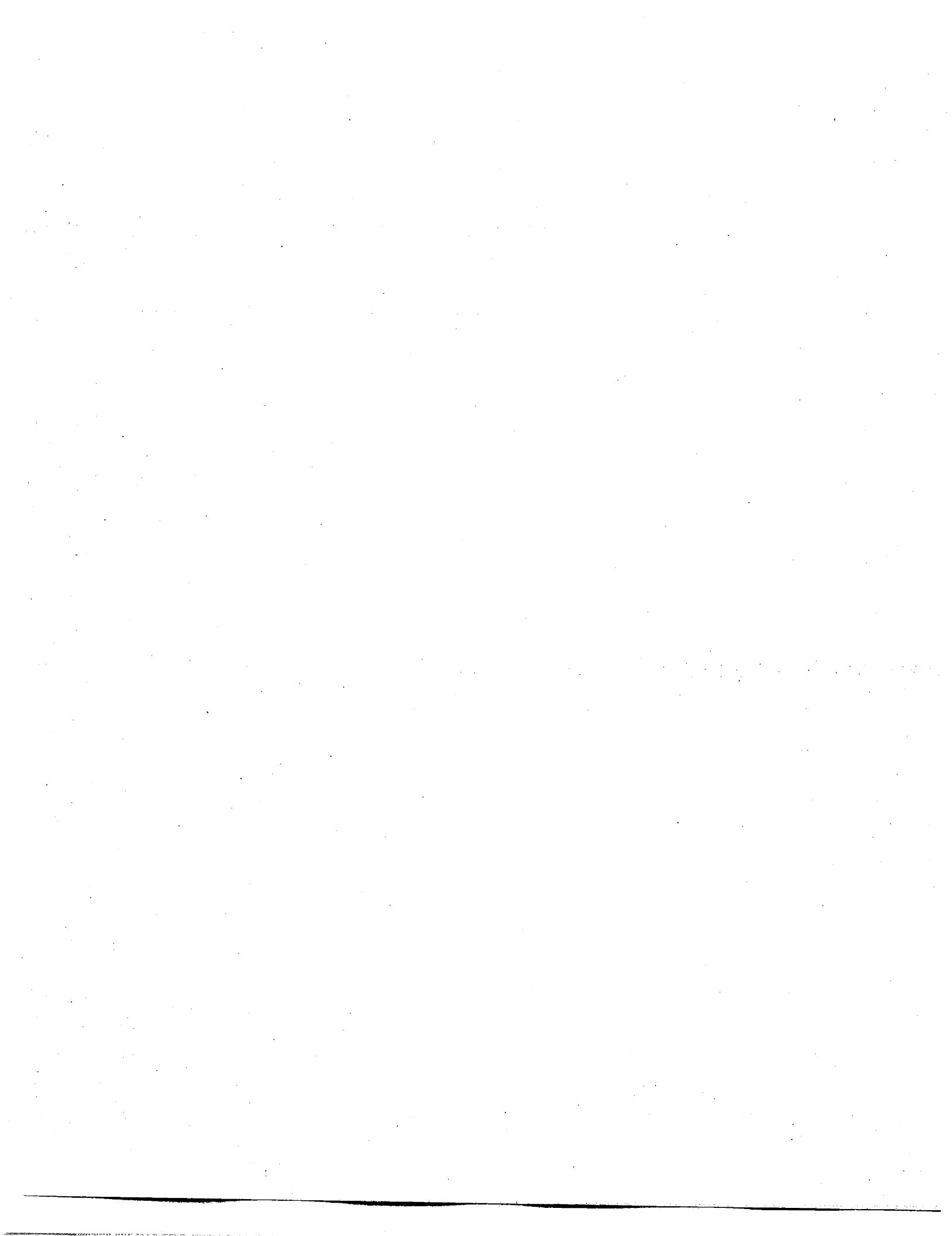


FIGURE 2. Biotin-streptavidin magnetic sorting of old cells. The surface of 10^8 cells is labeled with biotin. Labeled cells are grown for 7-12 generations. The biotin label stays with the original mother cells because the cell surface of emerging daughter cells is synthesized *de novo* at the budding site. The culture now contains the original cells, now old, and a large number of young cells. Labeled, old cells are then bound to streptavidin-coated magnetic beads. Old cells are separated from young cells using a magnetic sorter.

