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Extrachromosomal rDNA Circles—A Cause of Aging in Yeast

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Summary

Although many cellular and organismal changes have been described in aging individuals, a precise, molecular cause of aging has yet to be found. A prior study of aging yeast mother cells showed a progressive enlargement and fragmentation of the nucleolus. Here we show that these nucleolar changes are likely due to the accumulation of extrachromosomal rDNA circles (ERCs) in old cells and that, in fact, ERCs cause aging. Mutants for *sgs1*, the yeast homolog of the Werner's syndrome gene, accumulate ERCs more rapidly, leading to premature aging and a shorter life span. We speculate on the generality of this molecular cause of aging in higher species, including mammals.

Introduction

Aging is a process in which individuals undergo an exponential decline in vitality, leading to death. In the budding yeast Saccharomyces cerevisiae, cell division is asymmetric; a mother cell gives rise to a small daughter cell each division. Life span can thus be determined by following the fate of a mother cell through multiple rounds of division until cessation of cell division and eventual senescence (Mortimer and Johnston, 1959). Yeast mother cells display a relatively fixed life span in which the probability of senescence increases exponentially with age (Pohley, 1987; Jazwinski, 1996). As yeast cells grow old, they become sterile (Müller, 1985), and this phenotype is currently the most reliable marker for yeast aging.

One aging theory proposes that cells senesce from accumulated DNA damage. However, for haploid organisms, this model predicts an irreversible loss of genetic information. Clearly, this theory can not explain yeast aging since haploid yeast mother cells typically give rise to daughter cells with full life span potential. The exceptions are daughters from old mothers, which have a shortened life span potential and appear to be prematurely old themselves (Emilgez and Jazwinski, 1989; Kennedy et al., 1994). Even this shortening of life span is reversible: by the second or third generation of daughters from old mothers, life span potential is fully restored. Another theory, invoked to explain these observations, proposes that "cytoplasmic senescence factor" accumulates in yeast cells, causing them to age and eventually senesce (Emilgez and Jazwinski, 1989). The eventual "dilution" of the senescence factor through successive cell divisions would explain the restoration of full life span potential. The asymmetry of segregation of this substance appears to break down in the last few divisions of a mother cell, resulting in its leakage into daughters. As in metazoan systems such as Caenorhabditis elegans (Dorman et al., 1995; Larsen et al., 1995; Lakowski and Hekimi, 1996; Ewbank et al., 1997; Kimura et al., 1997), yeast genes that influence life span have also been identified. However, their precise role in aging remains to be determined (Chen et al., 1990; D'Mello et al., 1994; Kennedy et al., 1995; Childress et al., 1996).

Two recent studies have implicated a role for the nucleolus in yeast aging (reviewed in Guarente, 1997). The yeast nucleolus is a crescent-shaped region of the nucleus containing the ribosomal DNA (rDNA) and the components for ribosome assembly (Warner, 1990; Shaw and Jordan, 1995). The yeast rDNA locus on chromosome XII consists of a directly repeated array of 100–200 copies of a 9.1 kb repeat. Although each rDNA repeat contains an autonomously replicating sequence (*ARS*), fewer than one-third of these are used as origins in a given S phase (Brewer and Fangman, 1988; Liskens and Huberman, 1988).

Approximately half of the rDNA repeats are transcriptionally active at any one time (Dammann et al., 1993). The rest are maintained in a "silent" state, in part, by the action of the Sir2 silencing protein (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997). In concert with Sir3p and Sir4p, Sir2p also silences chromatin at telomeres and the two repositories of mating-type information (*HMR* and *HML*) (Rine and Herskowitz, 1987; Gottschling et al., 1990).

Smeal and colleagues (1996) showed that age-associated sterility is caused by a loss of silencing at the mating-type loci and the simultaneous expression of both **a** and α information. This event is also accompanied by a loss of silencing at telomeres (Kim et al., 1996). It was postulated that during aging, Sir proteins relocalize to another site termed the "age locus" (Kennedy et al., 1995). The age locus was identified by immunofluorescence: Sir3p relocalized to the nucleolus apparently during the latter half of the yeast life span (Kennedy et al., 1997). Since deletion of either *SIR2*, *SIR3*, or *SIR4* results in a shortened life span (Kennedy et al., 1994), the Sir complex functions to promote longevity in wild-type cells. We suggested that the redistribution of Sir proteins to the nucleolus delays senescence by countering some defect accruing at this site.

Further evidence for the role of Sir proteins in aging comes from the semidominant *SIR4-42* mutation, which was isolated in a genetic screen for long-lived yeast mutants (Kennedy et al., 1994). The resulting C-terminal truncated Sir4 protein localizes to the nucleolus even in young cells. Thus, *SIR4-42* is a constitutive mimic of an aging-regulated process that may delay senescence by redirecting Sir proteins to the nucleolus of young cells.

Another study implicating the nucleolus in yeast aging focused on the yeast homolog of the human *WRN* gene, *SGS1*. Both *WRN* and *SGS1* are members of a broad family of RecQ-like DNA helicases (Gangloff et al., 1994; Ellis et al., 1995; Watt et al., 1995; Yu et al., 1996; Stewart

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et al., 1997) and have DNA helicase activity in vitro (Lu et al., 1996; Gray et al., 1997). *SGS1* physically interacts with topoisomerases II and III (Gangloff, 1994; Watt et al., 1995), and deletion of *SGS1* results in increased recombination at the repeated ribosomal DNA (rDNA) locus and other loci (Gangloff et al, 1994; Watt et al., 1996).

Mutations eliminating *WRN* function result in Werner's syndrome, a disease characterized by a shortened life span and an earlier onset of many symptoms that resemble aging such as gray hair, wrinkled skin, cataracts, atherosclerosis, osteoporosis, and certain cancers (Salk et al., 1981; Martin, 1985). Likewise, deletion of *SGS1* reduces the average life span of yeast strains by about 40% and accelerates the onset of yeast aging phenotypes including cell enlargement, the relocalization of the Sir complex to the nucleolus, and sterility (Sinclair et al., 1997). This finding suggested a conserved mechanism of aging in a wide range of organisms, which occurs more rapidly in the absence of this DNA helicase.

Strikingly, the nucleolus of old *sgs1* cells is enlarged and fragmented, and the following findings indicate that these changes may represent a cause of aging. Sgs1p is concentrated in the nucleolus (Sinclair et al., 1997), supporting the idea that its absence might expose the rDNA to some form of damage. Further, nucleolar fragmentation also occurs in very old wild-type cells, showing that this phenomenon is not an aberration of the *sgs1* mutant. Nucleolar fragmentation occurs earlier in a *sir3* mutant than in wild-type cells, implying that the Sir complex delays senescence, perhaps by forestalling nucleolar fragmentation (Sinclair et al., 1997).

We thus sought to identify the molecular events embodied by the enlarged and fragmented nucleoli of old cells. Here we show that extrachromosomal rDNA circles (or ERCs) excise from the yeast rDNA locus and replicate via the *ARS* present in each rDNA repeat. Highly asymmetrical segregation of ERCs leads to their exponential accumulation in aging mother cells, thus providing a structural basis for the expanded and fragmented nucleoli. We further demonstrate that the accumulation of ERCs is more rapid in *sgs1* strains and that ERCs cause aging in yeast mother cells. We suggest that ERCs form due to the inherent instability of the tandemly repeated rDNA locus, and accumulation of such agents may be a general cause of aging.

Results

ERCs Accumulate in Aging Cells

One plausible explanation for the nucleolar fragments observed in old yeast cells (Sinclair et al., 1997) is that they are formed by circular rDNA molecules that have been excised from the rDNA array. To investigate this possibility, we performed two-dimensional chloroquine gel analysis of DNA from young and old yeast cells (Figure 1). In this system, DNA is electrophoresed successively in two different concentrations of chloroquine (Brill and Sternglanz, 1988). Since the mobilities of both linear genomic and nicked circular DNA are unaffected by chloroquine concentration, they align along the diagonal of the gel (Brill and Sternglanz, 1988). Closed circular DNA species appear as arcs that lie off the diagonal;

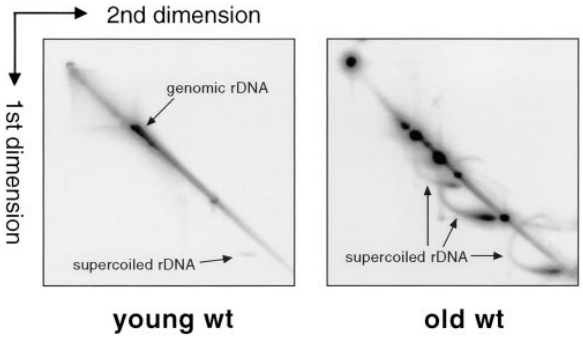

Figure 1. Aging Yeast Cells Accumulate ERCs

Total DNA was isolated from 1×10^8 magnetically sorted young and old wild-type cells. To minimize shearing and nicking of DNA, cells were spheroplasted prior to lysis and DNA was extensively purified. For two-dimensional chloroquine gel analysis, 15 μg of DNA was electrophoresed in TAE containing 0.6 μg/ml chloroquine. The gel was turned 90°, electrophoresed in 3.0 μg/ml chloroquine, and subsequenctly probed for rDNA 25S sequences by Southern blotting. In this gel system, sheared genomic DNA migrates along the diagonal of the gel (as shown). Closed circular topoisomers appear as arcs that bisect the diagonal; the highly negatively supercoiled species migrate in the lower region of each arc. Intense spots on the diagonal typically represent nicked circular species whose mobility is unaffected by the chloroquine concentration. Average bud scar count (ABSC) for young and old cell preparations was 1.1 ± 2.1 and 20.3 ± 4.1, respectively.

each arc represents an array of closed circular topoisomers, with the highly negatively supercoiled species migrating in the lower region of each arc. Intense spots on the diagonal represent nicked rDNA circles whose mobilities are unaffected by the chloroquine concentration.

The same amount of DNA from young and old cells was subjected to two-dimensional chloroquine-gel analysis and probed for rDNA sequences. The rDNA from young cells consisted almost exclusively of linear DNA fragments that migrated along the diagonal of the gel (Figure 1). A low level of a highly negatively supercoiled circular rDNA molecule was observed as an arc to the left of the diagonal (as indicated). Old wild-type cells were found to contain an abundance of such molecules of varying size. Large rDNA circles, possibly concatamers, did not migrate into the gel. We have named these extrachromosomal rDNA circles ERCs.

Because the short lived *sgs1* mutant undergoes normal aging phenotypes such as sterility, we proposed that *sgs1* cells age by the same mechanisms as those of normal yeast cells (Sinclair et al., 1997). By this model, we would expect ERCs to accumulate more rapidly in *sgs1* cells, at a rate inversely proportional to their shortened life span. We define old *sgs1* cells as those beyond the average life span of the strain (nine generations) which is only 40% that of the wild-type strain. Total DNA from young and old *sgs1* cells was isolated and examined by two-dimensional chloroquine-gel analysis as above (Figure 2). As for wild-type cells, young *sgs1* cells contained a low abundance of ERCs. A proportion of the rDNA did not migrate into the gel. In old *sgs1* cells, there was an abundance of ERC species identical

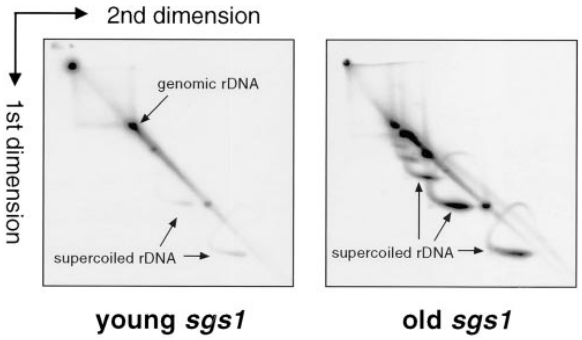

Figure 2. ERCs Accumulate More Rapidly in the *sgs1* Mutant

Total DNA was isolated from 2×10^8 magnetically sorted young and old *sgs1* cells and examined by two-dimensional chloroquine gel analysis (see Figure 1). Sheared genomic DNA migrates along the diagonal of the gel whereas closed circular topoisomers appear as arcs that bisect the diagonal; the highly negatively supercoiled species migrate in the lower region of each arc. Young cell ABSC = 0.9 ± 1.1; old cell ABSC = 9.9 ± 4.4.

to those observed in old wild-type cells. Thus, the accumulation of ERCs is a general phenomenon that occurs as cells age.

Several experiments were performed to investigate whether the circular rDNA molecules were oligomers of the 9.1 kb rDNA repeat. The rDNA molecules had a faster mobility relative to linear DNA size standards in the absence of ethidium bromide, a known property of circular DNA (Figure 3, between the 19.3 and 40.4 kb size markers). Digestion of old genomic DNA with KpnI (which cleaves once within the rDNA repeat unit) prior to electrophoresis reduced the ladder of bands to a single 9.1 kb species, demonstrating that the circular rDNA molecules consist of directly repeated multiples of the rDNA unit (Figure 3). Further, cleavage with BamHI had no effect on the banding pattern since rDNA is refractory to this enzyme. A partial KpnI digest reduced the abundance of the large circular species, while increasing the abundance of smaller ones. This implies that the large species are comprised of interlocked smaller circles (i.e., concatamers) that are released during digestion. Finally, treatment of the DNA with topoisomerase I, an enzyme known to relax supercoiled DNA, caused the mobility of these DNA species to migrate more slowly, coincident with the linear genomic rDNA (Figure 3). By quantitating the levels of total linear and extrachromosomal DNA from old wild-type cells (see Experimental Procedures), we estimate that the amount of ERC DNA in old cells can reach levels equal to the total linear yeast genome.

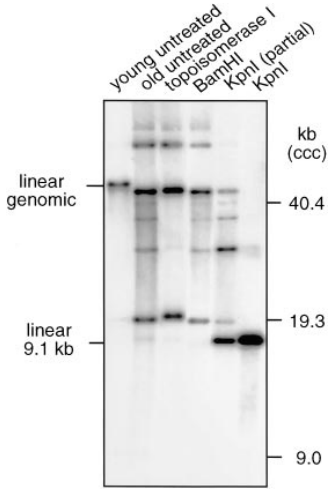

Figure 3. ERCs Are Circular Multimers of the rDNA Repeat

Total DNA (15 μg) from either young or old *sgs1* cells electrophoresed without ethidium bromide at 1 V/cm. Old cell DNA was treated with topoisomerase I, BamHI, or KpnI using standard conditions (Kim and Wang, 1995) and probed for rDNA. Sizes represent covalently closed circular markers (ccc). ABSC = 9.9 ± 4.4.

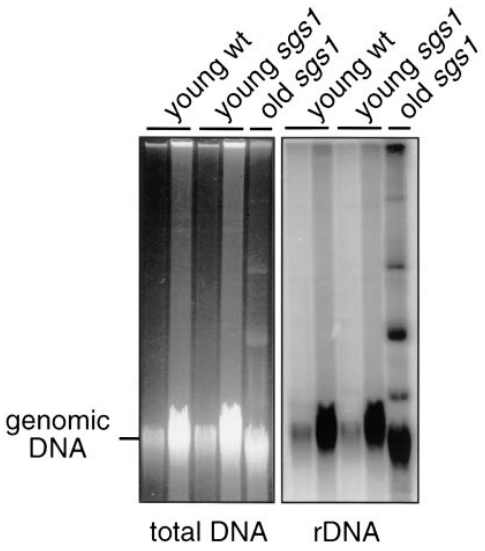

Figure 4. ERCs Are the Only Major Form of Extrachromosomal DNA in Old Cells

Total DNA (5 or 20 μg) from young wild-type cells, young *sgs1* cells, and old *sgs1* cells was electrophoresed in TAE buffer containing 0.5 μg/ml ethidium bromide (left half). DNA was probed for rDNA 25S sequences by Southern blotting (right half). The only extrachromosomal DNA that could be detected by ethidium bromide staining also hybridized to the rDNA probe. These species correspond to circular oligomers of rDNA of increasing size. The shear sizes of the linear genomic DNA from old cells were consistently smaller than those from young cells for reasons that are not clear at this time. Wild-type ABSC = 1.1 ± 2.4; young *sgs1* ABSC = 0.9 ± 1.1; old *sgs1* cell ABSC = 8.7 ± 4.9.

ERCs were the only chromosomally derived circular DNA species we could detect by ethidium bromide staining of total DNA (Figure 4). It was possible that other minor species of circular DNA were present but were unable to be detected. Because we reasoned that tandemly repeated sequences were more likely to excise as circular molecules, we investigated whether other tandemly repeated DNA sequences could also accumulate during aging. The other highly repeated DNA sequences in yeast are found within Y′ elements of telomeres. In fact, earlier work has shown that circular Y′ sequences can excise from the genome and replicate autonomously (Horowitz and Haber, 1985). Southern analysis of DNA showed a 3-fold increase in the level

of Y′ circles between young and old *sgs1* cells (data not shown) to reach a level that is extremely low compared to the level of ERCs in old cells. This finding, however, does demonstrate that other chromosomally derived circular DNAs can accumulate as cells age.

ERC Accumulation Due to Mother-Cell Bias

Three events are necessary to promote accumulation of ERCs (or other circular DNAs) in old mother cells: one or more circular DNA molecules must be excised from the genome or be inherited from a mother cell, the molecules must have a likelihood of replicating in subsequent S phases of the cell cycle, and the molecules must segregate to the mother cell in a biased fashion during cell division. For ERCs, there is some evidence for the first two of these events: low levels of 3μ circles containing yeast rDNA have been observed (Clark-Walker and Azad, 1980; Larionov et al., 1980), and mutations in the topoisomerase genes, *TOP1* and *TOP2*, stimulate their formation (Kim and Wang, 1989); also, the rDNA *ARS* has been shown to support the replication of recombinant plasmids (Larionov et al., 1984).

To test the properties of ERCs in more detail, we constructed a plasmid containing one copy of the rDNA and the *ADE2* marker gene (ERC::*ADE2*). *ade2* cells that lose a copy of the plasmid can be readily identified by their accumulation of a red pigment and their failure to grow on media lacking adenine. Transformation of the *ade2* strain W303-1A with the ERC::*ADE2* plasmid gave rise to colonies with two distinct morphologies on complete media without adenine (Figure 5A). Southern analysis showed that large white colonies (Ade^+) were derived from cells in which the plasmid stably integrated into the genomic rDNA locus, whereas pink minicolonies ($Ade^{+/-}$) carried the plasmid as an extrachromosomal molecule (data not shown). No pink colonies were observed following transformation with the same plasmid lacking rDNA, indicating that the rDNA was responsible for plasmid replication. At a low but observable frequency, each class of transformant gave rise to the other; thus, ERC::*ADE2* integrants can excise from the genome, and circles can integrate into the genome.

As an assay for the segregation properties of the ERC::*ADE2* plasmid, we determined the number of bud scars on the surface of cells within colonies arising from the integrated or extrachromosomal forms of the plasmid. The bud scar count is a direct measure of the number of cell divisions a given cell has undergone. As expected, the bud scar distribution of cells bearing the integrated plasmid was similar to a mitotic culture of yeast cells (50% have no bud scars, 25% have 1, 12.5% have 2, etc.). Among 100 cells counted, no cells had greater than 6 bud scars. In contrast, minicolonies bearing extrachromosomal plasmids had a much higher proportion of cells with no bud scars, presumably corresponding to Ade^- daughters (70%), and a high proportion of older cells with 6–9 bud scars, presumably corresponding to Ade^+ mothers (7%), consistent with a mother cell-biased inheritance pattern of the plasmid.

To further assay the segregation properties of the ERC::*ADE2* plasmid, cells from pink minicolonies were subjected to pedigree analysis. The inheritance of an ERC was determined by assaying for growth on complete medium without adenine. Some cells divided only a few times after separation from their mothers, indicating that they did not inherit an ERC and were Ade^- cells (open circles) utilizing the *ADE2* mRNA inherited from their mothers (Murray and Szostak, 1983). Segregation frequency, as defined by Murray and Szostak (1983), expresses the degree of inheritance bias of a plasmid during cell division. Mother divisions (M) are those where only the mother receives the plasmid (i.e., can grow into colonies or produce daughters that grow into colonies). Daughter divisions (D) result in only the daughter inheriting the plasmid, and symmetrical divisions (S) result in both cells inheriting the plasmid. Segregation frequency is thus calculated according to the equation: SF = (M + D)/(M + D + S). A circular *ARS1* plasmid has an SF of 0.38, indicating a strong inheritance bias.

As shown in three typical pedigrees in Figure 5B, mother cells carrying the ERC::*ADE2* plasmid had a pronounced tendency to maintain the plasmid each cell

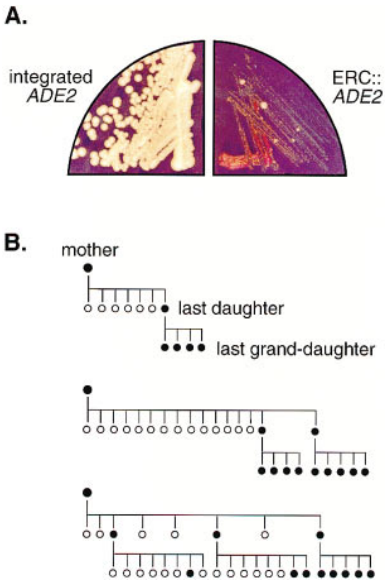

Figure 5. Asymmetric Inheritance of ERCs Breaks Down in Old Cells

Wild-type W303-1A was transformed with an *ADE2*-marked ERC plasmid (ERC::*ADE2*) to form large white colonies (integrated ERC::*ADE2*) or pink minicolonies (extrachromosomal ERC::*ADE2*) on complete medium without adenine (A). At a low frequency, the two colony types interconverted. Large white (Ade^+) colonies produced by integration of the ERC::*ADE2* plasmid can been seen among the background of minicolonies. Pedigree analysis was performed on mother cells from pink minicolonies carrying extrachromosomal ERCs. Three typical examples are shown (B). Daughter cells were moved away from mother cells to determine their ability to grow on adenine-lacking medium. Mother cells carrying ERCs are represented by the uppermost closed circle. Inheritance of an ERC was scored by the ability to inititate a colony or to produce daughters able to initiate a colony. The first daughter removed from a mother is represented below her, and subsequent daughters from that same mother are shown to the right. Daughters from these daughters (granddaughters) were also removed and scored for growth on this medium. Cells carrying an ERC are denoted by closed circles, and cells lacking an ERC are denoted by open circles. The segregation frequency (Murray and Szostak, 1983) for the ERC::*ADE2* plasmid was 0.78, indicating a strong mother cell bias of inheritance.

division, whereas daughters rarely inherited it. Based on the first generation of daughters, the segregation frequency of the ERC plasmid is 0.78. This pattern of inheritance explains the slow growth and pink color of ERC minicolonies, which were presumably comprised primarily of Ade$^-$ daughter cells that failed to inherit the plasmid.

The exception to this segregation pattern were daughters from old mothers, which displayed an increased tendency to inherit the plasmid. In these late-arising daughters, granddaughters also had a high probability of inheriting the plasmid (Figure 5B). By the last division, ERC segregation was almost always symmetrical. This breakdown in asymmetry in old cells parallels the inheritance pattern of the "senescence factor", which has been proposed to cause daughters from old mothers to be prematurely old themselves (Emilgez and Jazwinski, 1989).

ERC Accumulation Can Cause Aging

The above findings were consistent with the hypothesis that an exponential accumulation of ERCs causes aging in yeast. To test whether ERCs could shorten life span, we constructed a two-plasmid system in which asymmetrically inherited ERCs could be released at a specified time. As shown in Figure 6A, one plasmid carries the ERC::*ADE2* sequence with a 500 bp yeast *ARS-CEN* (centromeric) sequence flanked by *loxP* sites. Southern analysis showed that this plasmid is stably maintained as a single copy per cell (data not shown). On glucose medium, this plasmid gave rise to large white colonies similar to plasmid integrants (Figure 6B, upper half). The other plasmid carries the Cre recombinase under control of the galactose-inducible *GAL1* promoter (Güldener et al., 1996). When cells carrying both plasmids were transferred from glucose to galactose/raffinose medium, the Cre-*loxP* recombinase system efficiently removed the *ARS-CEN* sequence from the ERC::*ADE2* plasmid, giving rise to asymmetrically inherited plasmids, as indicated by the appearance of pink minicolonies (Figure 6B, lower half). A time-course study showed that within 4 hr of galactose induction, over 97% of *ARS-CEN* sequences are excised (data not shown).

Cells harboring both plasmids were shifted from glucose to raffinose/galactose medium (YPRafGal) where life span analysis was performed (Figure 7A). As controls, we employed strains lacking either the Cre recombinase or the ERC::*ADE2* plasmid, or both. Life span was similar to the wild-type control when either the *GAL1*-Cre or the ERC::*ADE2* plasmid was substituted with marker-matched centromeric plasmids. All control strains had average and maximum life spans that were almost identical (avg. = 23; max. = 37). However, the experimental strain carrying both plasmids had an average and maximum life span approximately 40% shorter than the controls (avg. = 15; max. = 21). Thus, the ectopic introduction of an ERC shortens yeast life span.

The same experiment as above was performed, except that at division 14, mother cells were challenged with α-mating factor to determine if they displayed the age-specific phenotype of sterility. Of the cells carrying both ERC::*ADE2* and *GAL1*-Cre, 40% were sterile, compared to the age-matched control strain (ERC::*ADE2* only) in which only 3% were sterile (data not shown). Thus, introduced ERCs shorten yeast life span and accelerate the onset of sterility.

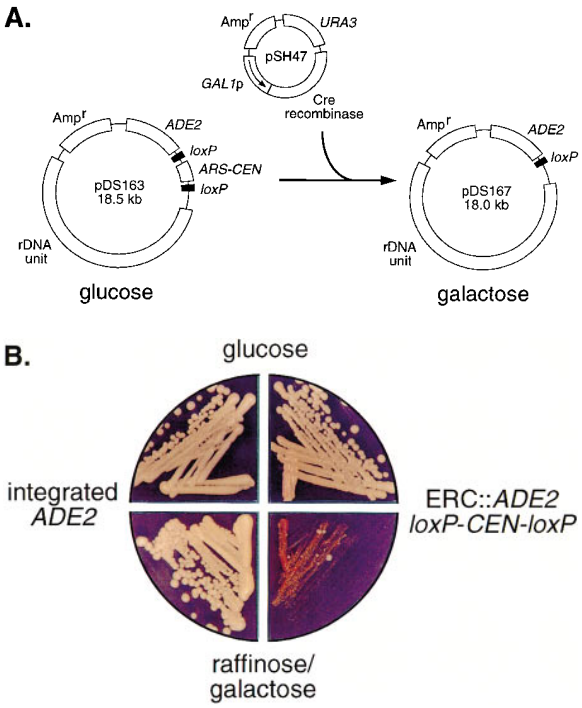

Figure 6. The Two-Plasmid Cre-*loxP* ERC System

(A) A two-plasmid system was devised to permit the controlled release of an ERC. One plasmid, pSH47 (Güldener et al., 1996), contains the *URA3* marker for selection, and the Cre recombinase gene under *GAL1* promoter control. The other plasmid (pDS163) contains the *ADE2* marker for selection and red/white (Ade$^-$/Ade$^+$) detection, as well as an *ARS-CEN* (centromeric) sequence flanked by *loxP* sites.

(B) On complete glucose medium, pDS163 is stably maintained as a single copy per cell, producing large white colonies (upper half). By shifting cells to complete raffinose/galactose medium, the *ARS-CEN* sequence is efficiently removed within 4 hr, resulting in pink minicolony formation (lower half). The morphology of the minicolonies results from the asymmetry of ERC::*ADE2* inheritance, where the majority of daughter cells fail to inherit the plasmid and fail to divide. Only those cells that arise from old mothers have a high probablility of inheriting the plasmid. At low frequency, the ERC::*ADE2* plasmid integrated into the rDNA locus produce to white (Ade$^+$) colonies (lower half). This process was reversible as indicated by the reversion of the Ade$^+$ strain to the pink minicolony morphology (not shown).

The above experiments defined two of the properties required for a molecule to cause aging. The sequence must autonomously replicate and it must segregate asymmetrically to mother cells. *ARS* plasmids possess both basic properties (Murray and Szostak 1983). Thus, we tested whether any such *ARS* plasmid could limit life span. Cells containing an *ARS1* plasmid, or a marker-matched *ARS-CEN* plasmid, were subjected to life span analysis. As shown in Figure 7B, the life span of the cells containing the control *ARS-CEN* plasmid had a wild-type life span, whereas the *ARS1* containing plasmid had a life span approximately 40% shorter than the control. This shortening was similar to that observed for the released ERC construct above.

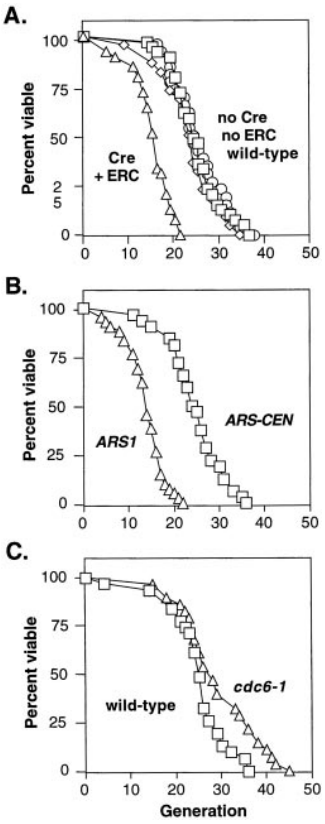

Figure 7. ERCs Limit Yeast Life Span

(A) Life span analysis was performed on W303-1A cells carrying the two-plasmid system in Figure 6: a *loxP-ARS-CEN-loxP* ERC::*ADE2* plasmid (pDS163) and plasmid-borne Cre recombinase gene driven by the galactose-inducible *GAL1* promoter (pSH47). Life span was determined by scoring the number of daughter cells produced by each mother cell before cessation of cell division (Kennedy et al., 1995). Cells were pregrown for a minimum of 48 hr on complete glucose medium before transferring them to rich YPRafGal medium, where life span analysis was immediately performed. Mortality curves for cells carrying the two-plasmid ERC system (open triangles), pDS163 (open squares), pSH47 (open diamonds), or neither plasmids (open circles) are shown. Average life spans (± standard deviation within the population) were 15.0 ± 4.2, 24.1 ± 6.0, 23.2 ± 6.0, and 23.8 ± 7.5, respectively. As further controls, pSH47 was substituted with the *URA3* centromeric plasmid pRS316, and pDS163 was substituted with either the *ADE2* centromeric plasmid pDS38 or an integrated ERC::*ADE2* at rDNA, with no apparent effect on life span (not shown).

(B) Mortality curves for W303-1A carrying either the *ARS1* plasmid pRS306-ARS (open triangles) or the *ARS-CEN* plasmid pRS316 (open squares). Average life spans for the two strains were 14.1 ± 4.7 and 24.3 ± 5.8, respectively.

(C) Mortality curves for W303-1A *cdc6-1* (open triangles) and an isogenic wild-type control (open squares) grown on YPD medium at the semipermissive temperature (27°C). Average (and maximum) life spans for the *cdc6-1* and wild-type strains were 29.3 ± 8.0 (45) and 24.2 ± 6.7 (36), respectively. The statistical significance of life span differences were determined as described in Experimental Procedures.

To begin to determine whether native ERCs cause aging in wild-type mother cells, we sought a means to inhibit ERC accumulation. The simple prediction is that this should extend life span. The presence of a conditional thermosensitive allele of *CDC6, cdc6-1* reduces the efficiency of *ARS* initiation at the nonpermissive temperature, leading to a high rate of plasmid loss (Hogan and Koshland, 1992). Since the rDNA *ARS* is weak, we reasoned that there may be a temperature at which chromosomal DNA, but not ERCs, are efficiently replicated. By examining the growth and color of *cdc6* strains with either integrated or extrachromosomal copies of *ADE2* at various temperatures, we determined that at 27°C, ERC::*ADE2* stability was reduced without an appreciable effect on growth rate (data not shown). Thus, we determined the life span of a *cdc6* strain at this semipermissive temperature, along with an isogenic wild-type control. The *cdc6* mutant consistently had a greater average life span compared to the wild-type (24 and 29, respectively) and a maximum life span 25% longer than wild-type cells (Figure 7C).

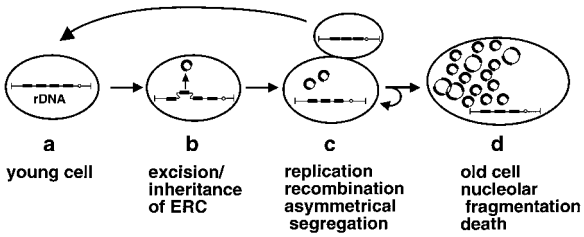

Figure 8. Model of Yeast Aging Due to Accumulation of ERCs

(A) As young mother cells divide, the initiating event of the aging process is the generation of an ERC by homologous recombination between repeats within the rDNA array on chromosome XII.

(B) Most daughter cells do not inherit ERCs and youth is temporarily restored. The excised rDNA repeats are rapidly regenerated by amplifying existing repeats. Daughters from very old mothers can inherit ERCs due to the break down in the asymmetry of inheritance.

(C) ERCs have a high probability of replicating during S phase and are maintained almost exclusively within mother cells.

(D) ERCs accumulate exponentially in mother cells resulting in fragmented nucleoli, cessation of cell division, and cellular senescence.

Discussion

ERCs as the Basis of Nucleolar Fragmentation

In this paper, we show that ERCs accumulate in aging yeast cells and that these circles can cause aging. ERCs that form during a cell's life span (or are inherited from an old mother cell) accumulate in mother cells due to the strong segregation bias at cell division (Figure 8). We presume that the large numbers of ERCs in old cells are the basis for the nucleolar enlargement and fragmentation observed previously (Sinclair et al., 1997). Consistent with this view, strains containing high-copy rDNA plasmids have fragmented nucleoli similar to those of aging yeast cells (Nierras et al., 1997; S. Mah and L. G., unpublished data).

The strong mother cell bias for ERC inheritance is unlikely to be simply due to a passive process, since nuclear volume is partitioned approximately equally to mother and daughter cells (Robinow and Marak, 1966; Gordon, 1977). Rather, an active mechanism may promote the attachment of circular molecules to structures retained in the mother cell. The highly organized association of nucleolar fragments with the nuclear periphery

of aging cells (Sinclair et al., 1997) is consistent with this hypothesis.

Among the plethora of molecular models of the aging process, a model invoking a progressive loss of rDNA from the genome has been suggested previously (Guarente, 1996), based partly on the work of Strehler (1986). However, this model cannot account for the restoration of youth in daughter cells. Instead, the findings here indicate that yeast aging is caused by the dominant effect of multiple extrachromosomal copies of rDNA in old mother cells. Daughter's life spans are restored because these DNA circles are segregated preferentially to mothers.

How Do ERCs Kill Cells?

When an ERC is released into young cells, they divide an average of 15 times prior to senescence. Based on known values for the frequency of rDNA replication initiation (Larionov et al., 1984) and the segregation frequency of ERCs to daughter cells (determined in this study), we estimate that the number of ERCs per mother cell after 15 generations would be between 500 and 1000 (see Experimental Procedures). Although the rDNA *ARS* is relatively weak (Larionov et al., 1984), larger multimeric ERC species arising from recombination would increase the number of *ARS*s per molecule and therefore their replication potential. In fact, we observed a preferential representation of larger multimeric species in old cells compared to species present in younger cells. Similarly, the oligomeric state of the ERC::*ADE2* plasmid was shown by Southern analysis to undergo a transition to a larger species during passaging of the strain (data not shown).

The final arrested state of an old cell, and eventual death, may result from an unbalanced expression of one or more of the encoded RNAs, or an overwhelming number of *cis*-acting DNA elements that could titrate essential factors. The shortening of life span by the *ARS1* plasmid strongly argues for a titration effect. Earlier studies have shown that yeast *nib1* mutants, which overamplify 2μ DNA circles, have phenotypes similar to those of aging cells: a slower S phase, cell enlargement, the breakdown of asymmetry in older cells, and senescence (Holm, 1982; Sweeney and Zakian, 1989). Also, runaway amplification of mitochondrial DNA has been linked to the senescence of the hyphal fungi, Podospora (Jamet-Vierny et al., 1980; Wright et al., 1982; Cummings et al., 1985). It therefore seems likely that the sheer abundance of ERC DNA could titrate components of the replication or transcription machinery, leading to an inability to replicate or transcribe genomic DNA.

Role of the Sir Complex and Sgs1 in Aging

As outlined in the Introduction, the redistribution of Sir3p and Sir4p to the nucleolus appears to delay aging. How might this occur? One possibility is that the redirected Sir complex delays the accumulation of ERCs, for example, by forestalling excision, inhibiting ERC replication, or promoting symmetrical inheritance of ERCs. Sir2p has been shown to repress recombination in the rDNA (Gottlieb and Esposito, 1989), and it is possible that this effect is further modulated by the redirection of Sir3p and Sir4p as cells age. This reasoning would suggest that the redirected Sir complex may delay the excision event that generates the first ERC. Another possibility is that the Sir complex delays aging by silencing rDNA transcription. In this regard, Sir2p has been shown to silence a marker gene inserted into the rDNA (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997), and again, this activity may be modulated by Sir3p and Sir4p (Smith and Boeke, 1997; K. Mills, D. A. S, and L. G., unpublished data).

There is some evidence linking DNA repair process with yeast aging. The Sir complex has recently been implicated in illegitimate recombination and double-strand break repair (Tsukamoto et al., 1997). Perhaps the redistribution of Sir proteins to the nucleolus during aging is a response to damage to rDNA. Also, particular domains of the Sgs1 protein are essential for resistance to certain DNA damaging agents, and this resistance correlates with full life span potential (J. Saffi, J. Henriques, D. A. S., and L. G., unpublished data). If the *sgs1* mutation were to compromise DNA repair, then repair by other means, such as homologous recombination, may compensate. This would lead to a greater likelihood of ERC formation and explain the premature aging phenotypes and shortened life span we observe for this strain.

Extrachromosomal Circles May Be the Aging Clock

One appealing aspect of the model that ERCs cause yeast aging is that, once an ERC is formed or inherited, the period of time until a lethal number of ERCs has accumulated may be fixed for each mother cell. Thus, the replication of excised ERCs during S phase may be the clock that determines the life span of the cell. It is clear from the nature of these molecules that they should accumulate in an exponential manner. This rate of accumulation would explain the exponential increase in the mortality rate with every cell division. The duration of time prior to the excision of an ERC from the genome may be either stochastic or dependent on changes that occur earlier in the aging process, such as damage to the rDNA. In either case, the timing of this initial event is likely to be responsible, at least in part, for the substantial variation in the life spans of genetically identical cells in a typical population.

Broader Implications

We imagine that any DNA sequence in a genome bearing an origin of replication has the potential to excise and replicate like ERCs, although tandemly repeated sequences appear to be the most susceptible to circle formation. Because rDNA is the most highly reiterated sequence in the yeast genome carrying an *ARS*, it is the most likely to accumulate in old cells. We do see accumulation of another circular form of repeated DNA, namely Y′ elements of telomeres, but not to any significant level. Y′ circles may form less frequently, or they may lack DNA elements that promote biased inheritance. Presumably, the overwhelming abundance of ERCs stalls cell division before they have a chance to accumulate to high levels.

The accumulation of any such extrachromosomal agent seems to depend on asymmetrical cell division. In this regard, yeast mother cells may be analogous to mammalian stem or progenitor cells, which may undergo asymmetric division to generate differentiated cells. Thus, it will be important to determine whether ERCs or other circular DNAs accumulate in stem cells of aging mice and humans.

It is remarkable that this mechanism of aging in yeast mother cells is so simple at a molecular level. It is conceivable that inhibitors of this process can be found, and if so, such strategies might eventually prove useful in forestalling aging in yeast and, perhaps, higher organisms.

Experimental Procedures

Yeast Strains and Media

All strains used in this study were derived from W303-1A *MAT***a** *leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100* (Thomas and Rothstein, 1989). Strains were propagated using standard media (Sherman et al., 1979) with supplements to aid the growth of old cells. Unless otherwise stated, all strains were cultured at 30°C. Complete glucose medium was SD medium (Sherman et al., 1979) containing 3% glucose and 60 mg/l auxotrophic requirements. Rich glucose medium was YPD containing 2.5% glucose. Complete raffinose/galactose medium consisted of 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% raffinose, and 1% galactose. YPRafGal consisted of 1% yeast extract, 2% bactopeptone, 2% raffinose, and 1% galactose. YPDXtra consisted of 1% yeast extract, 2% bactopeptone, and 3.0% glucose added after autoclaving. W303-1A *sgs1*::*HIS3* (pDS36) was described in Sinclair et al. (1997). W303 *cdc6-1*(pDSY170) was constructed by transforming W303-1A with p306cdc6-1 linearized with BglII (gift of O. Aparicio). Transformants were grown overnight on YPD then on 5-fluoroorotic acid medium to select for loss of the *URA3* marker.

Plasmids

pDS163 was constructed as follows. The *ARS-CEN* sequence of pRS314 was amplified using primers GGGGGGATCCCCGCGCACA TTTCCCCGAAAAGTGCC and GGGGGGAGCTCATCGCTTGCCTGT AACTTACACGCG. The PCR product was cut with BamHI/SacI and ligated to pUG6 (Güldener et al., 1996) cut with BglII/SacI. The *loxP-ARS-CEN-loxP* sequence was amplified by PCR using primers GGGGGAGATCTGCATAGGCCACTAGTGGATCTG and GGGGGGA TCCAGCTGAAGCTTCGTACGC. The product was cut with BamHI/BglII and ligated to the unique BamHI site of pDS113. The efficiency of *ARS-CEN* recombination by Cre was equal for both orientations of the sequence. Plasmid pDB4, constructed by inserting a HindIII-EcoRI fragment containing *LYS2* inserted in to pUC9 (Vieira and Messing, 1982) was a gift of G. Fink. pURADE2 was constructed by inserting an ApaI-PstI fragment of the *URA3* promoter upstream of the *ADE2* gene amplified by PCR (PstI-HindIII) in pRS315 (O. Aparicio and D. Gottschling, personal communication). Plasmid pNL47 was created by cloning the 2.8 kb EcoRI yeast rDNA fragment into the EcoRI site of pDB4 (B. Levin and G. Fink, personal communication). *LYS2* was replaced by *ADE2* by inserting the *URA3p-ADE2* fragment (BamHI-SalI) of pURADE2 (Oscar Aparicio, personal communication) between the unique BamHI and SalI sites of pNL47 to create pDS40. The complete rDNA repeat was obtained by gap repair of pDS40 linearized with KpnI to produce pDS113. pDS38 was constructed by ligating the SalI-BamHI *ADE2* fragment of pURADE2 to pRS314 cut with the same enzymes. pRS306-ARS has a 240 bp SacI-HindIII *ARS1*- wt element (Marahrens and Stillman, 1992) inserted into pRS306 cut with the same enzymes (O. Aparicio and S. Bell, personal communication). pRS vectors are described in Sikorski and Hieter (1989). Plasmid maps are available upon request.

Micromanipulation and Life Span Analyses

Micromanipulation was performed using an Olympus BX-40 microscope under 10× magnification as previously described (Kennedy et al., 1994). Sterility assays were performed essentially as described in Smeal et al. (1996) using 15 μl of a 30 μg/liter α-factor stock solution (stored at −80°C) on sterilized filter paper (approx. 0.3 cm × 0.5 cm). Cells were challenged for 4 hr, after which the filter paper was removed, and cells were moved at least 0.5 cm to fresh medium before life span analysis was resumed. Life span analysis of strains carrying *ARS1* and *ARS-CEN* plasmids was performed as follows. Strains were grown on complete glucose medium lacking uracil for a minimum of 24 hr before being restreaked onto the same medium. Sixty cells that divided four or more times on the selective medium were used for life span analysis since these cells were likely to contain the plasmid. Daughter cells were moved away from mothers and tested for Ura prototrophy to determine whether the mother cells contained the plasmid. To further ensure that mother cells did not simply die from plasmid loss, a section of agar adjacent to the mother cells was removed and replaced with 200 μl of YPD containing 0.4% uridine. Ura^- cells were streaked on the medium to ensure that such cells would not have a growth disadvantage. Cells that lost the plasmid or divided fewer than four times on the selective medium prior to uridine supplementation were not included in the data set.

Preparation of Old Cell DNA

High yields of old cells were obtained by a modification of a biotin-streptavidin magnetic sorting procedure (Smeal et al., 1996). An overnight YPD culture was diluted in 50 ml YPDXtra and grown for at least 6 hr to an OD_{600} = 0.7. Cells were harvested and washed twice in phosphate-buffered saline (PBS) and resuspended in 1 ml of PBS. Between 8–10 mg of sulfo-NHS-LC-biotin (Pierce, IL) was added to the cells and incubated at room temperature with gentle shaking for 15 min. Sulfo-NHS-LC-biotin is sensitive to moisture and batches were found to vary. Cells were washed seven times in 1 ml PBS and resuspended in 1 ml YPD. Cell density was determined using a hemocytometer, and 1×10^8 cells/liter were added prewarmed YPDXtra. Cells were grown at 30°C for 13 hr with shaking (OD_{600} should not exceed 1.0), harvested by centrifugation, and resuspended in 40 ml of cold PBS. 250–300 μl of PBS-washed streptavidin-coated magnetic beads (PerSeptive Biosystems, MA) were added to the cells and kept on ice for 2 hr with occasional swirling. Cells (10 ml) were added to test tubes and placed in a magnetic sorter (PerSeptive Biosystems, MA) for 20 min at 4°C. Free cells were gently removed with a pipette, and the remaining cells were resuspended gently in 10 ml of cold YPD. After 20 min, the media was withdrawn, and another eight washes were performed before pooling the remaining cells. For double and triple sorts, cells were resuspended at 10^8 cells/liter YPDXtra and grown for 13–15 hr before repeating the sorting procedure.

Average bud scar counts (ABSC) were determined by washing approximately 10^6 cells in 1 ml of PBS. Cells were stained in PBS, and 10 mg/ml calcofluor white M2R (fluorescent brightener 28, Sigma), washed once with 1 ml PBS, and viewed under UV fluorescence using a Zeiss Axioscope microscope. DNA was extracted from old cells within 2 hr, and freezing of cells in YPD + 15% glycerol at −80°C resulted in poor recovery of closed circular DNA.

The following modifications to the spheroplast method were made to isolate total DNA (Ausubel et al., 1994). Cells were incubated in 15 μg/ml zymolyase in 1× TE/1 M sorbitol for 30 min at 37°C with shaking. Cells were gently lysed in 1 M sorbitol/14% SDS at 65°C, and the solution was phenol extracted twice before ethanol precipitation. The pellet was resuspended in 1× TE/0.1 mg/ml RNase and incubated at room temperature for 15 min before ethanol precipitation. DNA was gently resuspended in 1× TE and stored at −80°C.

One and Two-Dimensional Gel Analyses

DNA was heated with loading dye at 55°C for 10 min prior to loading in horizontal 1× TAE (Tris-acetate-EDTA)-agarose slab gels. One dimensional gel electrophoresis was performed at room temperature in 0.7% agarose at 1 V/cm for 30 hr. Two-dimensional electrophoresis was performed in 15 × 15 cm 4.0% (w/v) 1× TAE agarose gels at 1.5 V/cm for 14 hr each dimension (modified from Shure et al., 1977; Pruss, 1985; Brill and Sternglanz, 1988). The first dimension was performed in 0.6 μg/ml chloroquine. The gel was equilibrated in TAE buffer containing 3 μg/ml chloroquine for 5 hr, rotated 90°,

and electrophoresed in fresh TAE buffer containing 3 μg/ml chloroquine. DNA was blotted to a nylon filter and probed with a labeled 2.8 kb EcoRI fragment of yeast rDNA derived from pNL47.

Statistical and Computer Analyses

Determination of the significance of differences in mean life span between two strains was performed using the nonparametric Wilcoxon signed rank test (Systat5 Statistical Software, Systat Inc.). Whenever the mean life spans of two strains are said to be statistically significant, the analysis showed a confidence level greater than 99%.

ERC accumulation was calculated the following way. The chance of an ERC replicating per S phase (s) will depend on the rate of a single rDNA *ARS* firing (r) and the number of rDNA *ARS* sequences per ERC (a). Then, the number of ERCs in the mother cell before each division (N) will depend on the number of generations after ERC excision/release (n), the chance the ERC will replicate per S phase (s), and the proportion of ERCs retained in the mother cell during cell division (m). ERCs will accumulate in mother cells according to the equation: $N = 2^{ns}(1 - m)$, where $s = 1 - (1 - r)^a$.

CA-Cricket Graph III (v1.0) was used for graphical representations. For quantitation of total DNA levels, photographs of ethidium bromide–stained gels were scanned at 600 dpi and analyzed by NIH Image (v.1.60). Quantification of autoradiograms was performed on a PhosphorImager using volume integration (Molecular Dynamics, CA).

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