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Loss of Transcriptional Silencing Causes Sterility in Old Mother Cells of S. cerevisiae

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Summary

We show that sterility is an aging-specific phenotype in S. cerevisiae and, by genetic and physical means, demonstrate that this phenotype results from a loss of silencing in most old cells by the SIR complex at the *HM* loci. This loss of silencing is specific because transcription of genes, such as *MEI4* and *DCM1*, normally induced by sporulation, is not observed, while transcription of *HMRa* is observed. These findings pinpoint the molecular cause of an aging-specific phenotype in yeast. Further, they provide direct evidence for a breakdown of silencing in old cells, as predicted from earlier findings that SIR4 is a determinant of life span in this organism.

Introduction

Aging is characterized by an increase in mortality rate over time and by accompanying phenotypic changes. These changes can be observed at the level of the entire organism (Finch, 1990) or at the level of individual cells (Hayflick, 1965). In the latter case, a decrease in the length of telomeres has been described in cells from humans as they grow older or in primary cultured fibroblasts as they are passaged (Harley et al., 1990; Allsopp et al., 1992; Vaziri et al., 1993; Schwartz et al., 1993). In paramecium (Gilley and Blackburn, 1994) and in yeast (D'Mello and Jazwinski, 1991), telomere shortening has not been observed in old cells.

In Caenorhabditis elegans, there is an intriguing association between life span and the Dauer pathway, which enables nutritionally starved larvae to enter a dormant state for extended periods of time. Once animals are past the larval stage of development, the Dauer morphological pathway cannot be induced. However, the activation of genes in this pathway in adults by use of conditional mutations extends their life span substantially (Kenyon et al., 1993).

In the case of the yeast Saccharomyces cerevisiae, aging has also been observed in mother cells, which can be distinguished from daughter cells because of their larger size after cell division (Mortimer and Johnston, 1959). The mortality rate of mother cells increases exponentially with the number of cell divisions undergone (Pohley, 1987; Jazwinski et al., 1989). Mean and maximum life spans vary from one strain to another, with means ranging from 13 to 30 (Kennedy et al., 1995). Mother cells also undergo phenotypic changes as they grow old, most notably an increase in size accompanied by a slowing of the cell cycle (Mortimer and Johnston, 1959), and the accumulation of intracellular blebs, as viewed by Nomarski optics (Kennedy et al., 1994).

Establishing causes of aging-related phenotypes or increased mortality rates presents a challenge to modern biology. In Neurospora (Bertrand et al., 1985) and Podospora (Wright et al., 1982), clonal senescence can occur due to changes in mitochondrial DNA. Theories on aging range from the notion that the process is genetically programmed, i.e., a part of development, to the idea that it results from the accumulation of damage. Consistent with the notion of a genetic program is the rapid senescence of numerous organisms (Finch, 1990). For example, the Pacific salmon and American shad exhibit a rapid senescence program, which is triggered by spawning. However, there is also correlative data consistent with the damage hypothesis, for example, the cross-linking of proteins in old cells (Molnar et al., 1986; Reiser et al., 1987).

We have initiated a genetic study of aging in yeast (Kennedy et al., 1995). By noticing a correlation between life span and stress resistance in several lab strains, we used the latter phenotype to generate stress-resistant mutants that had increased mean and maximum life spans. One mutation to emerge was an unusual allele of *SIR4*. This gene, along with *SIR2* and *SIR3*, silences the unexpressed copies of α and **a** mating-type information at *HML* and *HMR* (Rine and Herskowitz, 1987) and also silences genes positioned at telomeres (Gottschling et al., 1990; Aparicio et al., 1991). The *SIR4-42* mutation behaved like a null allele with respect to silencing at *HM* loci and telomeres; silencing was abolished. However, only the *SIR4-42* allele extended life span and was dominant to *SIR4* for this effect. A null mutation in *SIR4* actually shortened life span. These findings indicated that extension in life span by *SIR4-42* was not due to phenomena occurring at *HM* loci or telomeres, but involved some other *SIR4*-regulated locus or loci.

The extension in life span by *SIR4-42* required *SIR2* and *SIR3*, indicating that it was a property of the SIR complex. The *SIR4-42* mutation deletes the carboxyl 121 residues of SIR4. We suggested that this region of the protein might be involved in recruiting the SIR complex to *HM* loci and telomeres. Consistent with this surmise, expression of only the carboxyl 154 residues of SIR4 abolished silencing at *HM* loci (Ivy et al., 1986). Moreover, this fragment also extended life span in a strain expressing the wild-type SIR4 (Kennedy et al., 1995). Further, Cockell et al. (1995) have recently demonstrated that this region of SIR4 binds to RAP1, a protein found at *HM* loci and telomeres. We proposed that by preventing recruitment of the complex to *HM* loci and telomeres, the *SIR4-42* mutation strengthened silencing at one or more novel loci that cause mortality. A primary prediction of this model is that mortality is due, at least in part, to the loss of silencing by the SIR complex in old cells.

Thus, it was critical to obtain direct evidence for the idea that silencing by the SIR complex is lost in old cells and that this can cause mortality and possibly, other aging-related phenotypes. In this report, we employ genetic and physical analyses to demonstrate a loss of

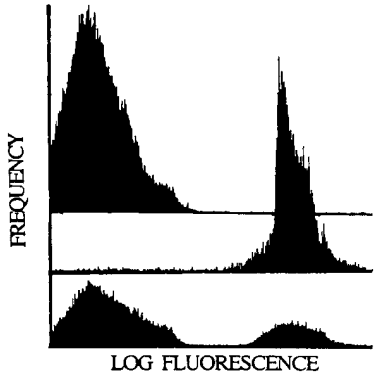

Figure 1. Sorting of Biotinylated Cells by FACS

PSY142 cells were biotinylated and treated with fluorescein-conjugated avidin as described in Experimental Procedures. A mixture of these cells with a 4-fold excess of nonfluorescent cells (bottom) was subjected to sorting by FACS, and a fluorescent population of cells (middle) and nonfluorescent population (top) were obtained. The purity of the fluorescent population was estimated to be >99%.

silencing in old cells that causes the aging-specific phenotype of sterility. Our findings provide a molecular explanation for this aging-related phenotype and are consistent with the earlier prediction that silencing is a key determinant of life span in this organism.

Results

Strategy to Isolate Old Cells

We wished to obtain a population of old yeast cells. In a random population of cells, half are daughters, one quarter are mothers that have divided once, one eighth are two-generation mothers, etc. Thus, the fraction of senescent cells in a growing culture of a strain with a mean life span of 20 would be 1 in 2^{20}, or about 1 in 10^6. Our approach to enriching for these old cells comes from the observation that the cell surface of daughters is due to de novo synthesis of the cell wall at the budding site (Ballou, 1982; Scheckman and Novick, 1982). Therefore, if the surfaces of mother cells were labeled, the label would stay associated with those mothers through multiple rounds of cell division. Subsequent recovery of labeled cells would give a pure population of cells that have all divided a predetermined number of times.

Thus, we conjugated the surfaces of cells (strains PSY142 and BKy1-14c) with biotin (see Experimental Procedures), which covalently attaches to primary amines, such as the ϵ-amino of lysine, in proteins on the cell surface. The labeling was assessed by adding fluorescein-conjugated avidin and examining cells in the fluorescence microscope or by sorting in the fluorescence-activated cell sorter (FACS). As shown in Figure 1, cells were efficiently labeled and clearly separable from unlabeled cells by FACS analysis. Starting with a mixture containing 20% fluorescent PSY142 cells and 80% nonfluorescent cells, a single sort produced a fluorescent population that was >99% pure. When cells that had been quantitatively conjugated with biotin were incubated in rich media at 30°C for 6 hr, populations of unlabeled daughters as well as unlabeled buds still attached to fluorescent mothers were now evident (data not shown), indicating that the label was not transferred to daughter cells.

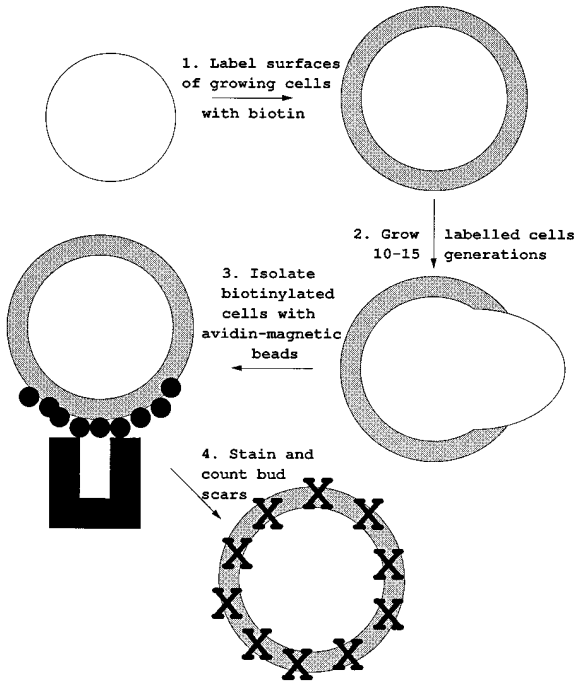

Figure 2. Sorting Old Cells by Biotinylation and Avidin-Conjugated Magnetic Beads

The details of the biotinylation and sorting are described in Experimental Procedures. The stippled ring indicates biotinylated proteins on the cell surface. The emerging daughter cell does not contain biotin because its surface is synthesized de novo at the budding site. Thus, the entire pedigree deriving from the indicated mother cell after 10–15 divisions will contain only one biotinylated cell, the mother originally labeled. Avidin-magnetic beads (small circles) will bind to these biotinylated cells, allowing them to be sorted by a magnet. The age (number of cell divisions undergone) of sorted cells is determined by staining bud scars (indicated by Xs) with calcafluor and counting the number of bud scars in the fluorescence microscope.

We next determined whether the labeling procedure was toxic by carrying out life span analysis of biotinylated and normal cells. The results indicated that maximum life spans were comparable in the two cases, but there was a small reduction in the mean life span of the biotinylated cells due to the sudden death of roughly 20% of labeled cells (data not shown). As will be evident in the analysis below, this fraction of cells that are killed by the procedure does not complicate subsequent analysis.

Sorting by Magnetic Beads Yields Old Cells

While FACS was effective for isolating labeled cells, the large quantity of cells desired for physical characterization necessitated an alternative strategy, outlined in Figure 2 and detailed in Experimental Procedures. Cells were grown in rich media, 10–15 generations after biotinylation, and then avidin-coated magnetic beads were added. Apposition of a magnet to the side of a test tube retained the beads and any associated (biotinylated)

Table 1. Enrichment of Biotinylated Cells by Magnetic Sorting

Cells	Number of Colonies on YPD Plates	Number of Colonies on Minimal Ade⁻ his⁻	Percentage of Biotinylated Cells
Unsorted	680	540	21
First sort	178	24	87
Fourth sort	>1000	0	>99

Biotinylated BKy1-14c and unconjugated PSY142 were mixed and magnetically sorted as detailed in Experimental Procedures. Plating on YPD allows growth of both strains, while plating on minimal medium missing adenine and histidine allows growth of PSY142, but not BKy1-14c, which is auxotrophic for these requirements. The number of BKy1-14c (biotinylated) cells is thus the number of colonies on YPD minus the number on minimal medium. This number divided by the total gives the percentage of biotinylated cells before and after sorting.

cells. The effectiveness of the procedure can be measured by staining and counting bud scars (see below).

We initially determined the efficiency of magnetic sorting by mixing labeled BKy1-14c with unlabeled PSY142, two strains with different nutritional markers. Cells were sorted by addition of beads, apposition of the magnet, and aspiration of culture media. To repeat the process, fresh media was added, cells from the first sort were vortexed, and the magnet was again applied. The identity of cells was then ascertained by plating and determining the nutritional requirements of colonies that arose. After four successive sorts, the fraction of labeled cells rose from 21% of the total to >99% (Table 1).

Next, we determined whether magnetic sorting would yield old cells. PSY142 cells were biotinylated and grown for a period of time to allow 12–14 divisions, and cells were magnetically sorted. The sorted cells were grown in fresh media for an additional 12–14 generations and were again sorted. In parallel, BKy1-14c cells were sorted. Owing to the short life span of this strain, it was possible to obtain senescent cells in a single sort.

The ages of cells from both the first and second sorts were determined by staining the bud scars with calcafluor and by counting the number of scars per cell in the fluorescence microscope. Bud scars consist of chitin rings that are deposited on the cell surface of the mother cell each cell division (Cabib and Bowers, 1971). Since each cell division leaves one bud scar, a cycling population of cells will contain 50% cells with no bud scars (daughters), 25% with one bud scar, 12.5% with two bud scars, etc. The vast majority of cells from the first sort of PSY142 contained 10–14 bud scars (Figure 3A), while cells from the second sort had 19–30 bud scars (Figure 3B). Cells from the BKy1-14c sort had a peak of bud scars around 12 (data not shown). There were a few cells from the first sort of PSY142 and from the sort of BKy1-14c with a low number of bud scars. There are two obvious possibilities for this population. First, these may be cells that were damaged by the biotinylation and divided slowly or not at all, as described above. Second, they may derive from daughter cells that separate from old mothers during the sorting procedure.

Examples of cells stained with calcafluor from the second sort of PSY142 are shown in Figure 4. Further confirmation that cells from the second sort of PSY142 and from the BKy1-14c sort were senescent was their very low efficiency of plating (<1%) compared with an efficiency of >50% for cells that are biotinylated and immediately sorted.

Analysis of Telomeres in Old Cells

Having established a method to isolate large quantities of old cells, we began a study of their physical properties. DNA was extracted from about 10^7 old cells, electrophoresed on an agarose gel, and probed for yeast telomeres. As shown in Figure 5A, telomeres of cells obtained from BKy1-14c, which had an average number

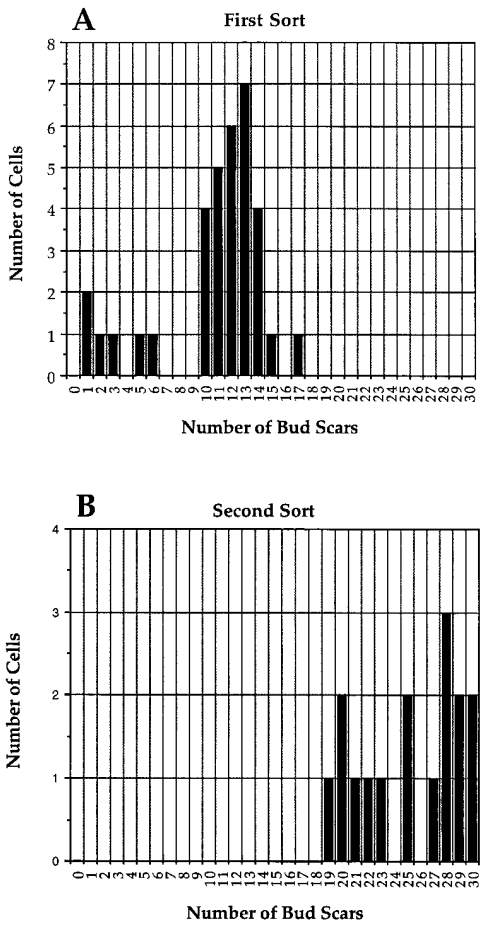

Figure 3. Distribution of Numbers of Bud Scars in Cells Following Magnetic Sorting and Calcafluor Staining of Bud Scars

Cells were sorted as described in Figure 2, and bud scars were counted from entire random fields in the fluorescence microscope. By shifting the plane of focus, it is possible to count bud scars on the undersides of cells. (A) and (B) show a histogram of bud scar number in cells from the first and second sort of the long-lived strain PSY142.

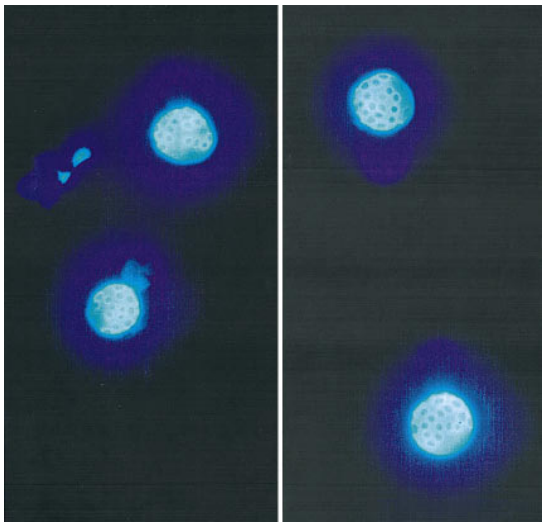

Figure 4. Old Cells after the Second Sort of PSY142

Bud scars are visible as small rings on the cell surface of four typical cells after the second round of sorting. Note that although the number of bud scars is very large, the cells still contain additional surface area that is free of bud scars. Experiments published elsewhere (Kennedy et al., 1994, and references therein) argue that bud scars per se do not cause senescence.

of bud scars of 12, were unaltered (Figure 5A). The shortening seen in cycling cells of Δ*sir4* mutant strains was not observed in old cells. The integrity of the old cell DNA was indistinguishable from cycling cells (data not shown). Notably, the conversion of chromosomal DNA into a ladder of nucleosomal-sized fragments, a property commonly observed in apoptotic cells (Wyllie, 1980; Duke et al., 1983), was not observed.

Loss of Silencing in Old Cells

Since previous findings indicated that the status of silencing was a key determinant of aging in yeast (Kennedy et al., 1995), we determined whether there was a loss of silencing by the SIR complex in old BKy1-14c cells. Note that this strain is *MAT*α. If silencing were abridged in old cells, expression of *HMRa* would result, causing repression of haploid-specific genes, such as *STE12*. As expected, Northern blotting showed that the levels of *STE12* compared with total RNA were reduced 5-fold in **a**/α diploids, or haploids that expressed *HMRa* due to mutation of *SIR4* (Figure 5B). The level of *STE12* RNA in the old cells was also reduced about 3-fold when normalized to the levels of *SPT15* (encoding TBP) RNA.

A more direct experiment is to determine whether *HMRa* itself is expressed in old cells. We developed a polymerase chain reaction PCR-based assay for **a1** RNA, which would be expressed were *HMR* derepressed. In this assay, rTth DNA polymerase is used sequentially under two different buffer conditions that promote reverse transcription in the presence of a dT primer followed by PCR amplification. Two primers were used that bracket an intron in **a1** coding sequences (Miller, 1984) (Figure 6A). Thus, the PCR product resulting from reverse transcription of the RNA will be 33 bases smaller than the product derived from genomic DNA.

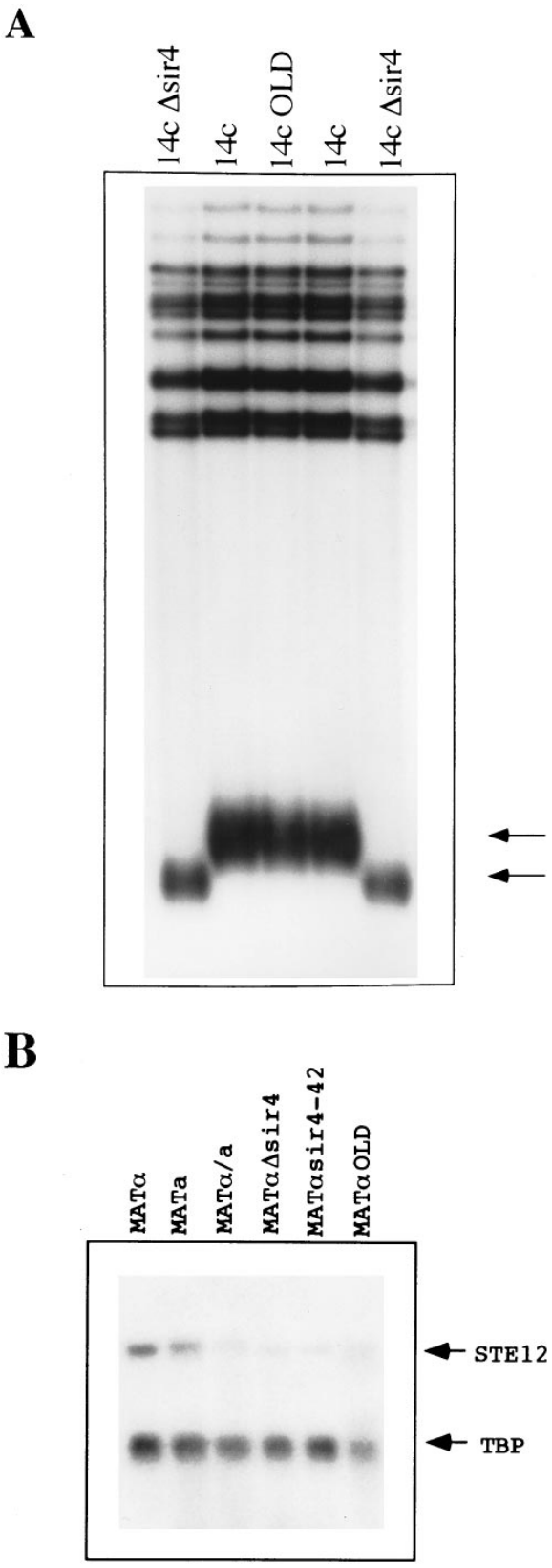

Figure 5. Telomeres and *STE12* Transcription in Old BKy1-14c Cells

(A) DNA was extracted from approximately 10^7 old cells of BKy1-14c and telomeres analyzed using a Y′ probe as described (Kennedy et al., 1995). 14c and 14c Δ*sir4 DNA* were extracted from exponen-

A single fragment was observed using nucleic acid from cycling cells of BKy1-14c, roughly the expected size of the product amplified from **a1** genomic DNA (Figure 6B, lane 1). When we tested nucleic acid from BKy5 (isogenic to 14c, but *MAT***a**) (Figure 6B, lane 2), or BKy6 (an **a**/α diploid of 14c and BKy5) (lane 3), a smaller product predominated. This product was also observed using nucleic acid from BKy1-14c *sir4* mutants, which derepress *HMRa* (lanes 4 and 5). We confirmed that this product was derived from **a1** RNA by demonstrating its sensitivity to RNase (Figure 6B, lane 8) and its requirement for inclusion of the dT primer (lane 10). When nucleic acid from old BKy1-14c cells was examined, the RNA-derived product was clearly evident (Figure 6B, lane 6). There is somewhat less RNA-derived product in this sample than observed in Figure 6B, (lanes 2–4), and, correspondingly, more of the DNA-derived product. This inverse relationship is probably due to competition for the PCR primers between the cDNA and genomic DNA. (A slower migrating fragment observed in lanes 1–6 is probably due to mispriming by the dT primer on the **a1** cDNA.)

Additional control experiments were carried out. First, a similar PCR analysis revealed no trace of **a1** RNA in cells that were biotinylated and immediately sorted (data not shown). Thus, its presence in the old cell preparation must be due to the age of the cells and not the sorting method. Second, PCR analysis of the *MAT* locus in DNA from old cells revealed no trace of **a** information (data not shown). Thus, the **a1** RNA observed above cannot be due to rare HO-independent switching between *HMR* and *MAT*, or to contaminating *MAT***a** cells. In sum, these experiments show that *HMRa1* is transcribed in old cells.

Transcription of *HMRa1* in old cells could, indeed, reflect a specific loss in silencing by the SIR complex or a general loss of silencing, such as that observed when histone synthesis is shut off (Han and Grunstein, 1988). To ascertain whether the effect were specific, we determined whether the sporulation-specific genes *MEI4* (Menees et al., 1992) and *DCM1* (Bishop et al., 1992) were transcribed in old cells. Transcription of these genes is typically observed in sporulating **a**/α diploids and not in growing haploids. Further, both genes contain introns (88 nt for *MEI4* and 91 nt for *DCM1*), rendering them amenable to PCR analysis, in a manner analogous to *HMRa1* above. Using primers that bracket the introns in *MEI4* and *DCM1* (Figure 7A), we observed that the RNA products of *MEI4* (Figure 7B, lanes 6–10) and *DCM1* (lanes 11–15) were specifically induced in sporulating **a**/α diploids (lanes 10 and 15). Moreover, neither transcript was observed in old cells (Figure 7B, lanes 8 and 13). In the same experiment, the *HMRa1* transcript was observed in old cells as expected (Figure 7B, lane 3) (as well as in *MAT*α, Δ*sir4*, and sporulating **a**/α diploid cells). Thus, we conclude that old cells do not transcribe genes promiscuously and that the derepression of *HMR* is likely due to a loss in silencing by the SIR complex.

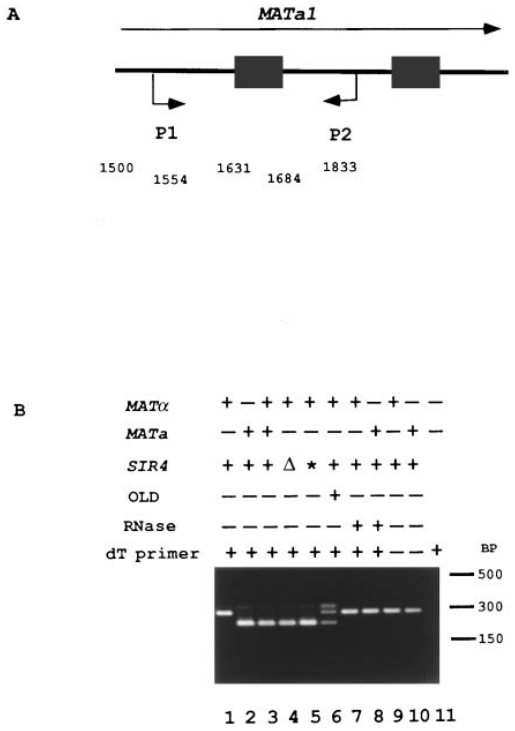

Figure 6. Sequential RT–PCR Amplification Analysis of **a1** RNA from Old BKy1-14c (*MAT*α) Cells

(A) shows the **a1** coding sequence present at *HMR* in BKy1-14c, with the RNA (long arrow) and two introns (stippled boxes) indicated. PCR primers *P1* and *P2* bracket the first intron. Numbering is according to Miller (1984). The fragments amplified by these primers from genomic DNA and RNA-derived cDNA will differ in size by 33 nt.

(B) shows an experiment using these primers in a sequential RT–PCR amplification assay (see Experimental Procedures) with nucleic acid extracted from the indicated strains. (Lane 1) BKy1-14c. This strain does not express **a1** RNA, and the amplification product of **a1** genomic DNA is evident. (Lanes 2 and 3) BKy5 (*MAT***a**) and BKy6 (**a**/α diploid). These strains express **a1**, and the RNA-derived product predominates. (Lanes 4 and 5) BKy1-14c containing a deletion of *sir4*(Δ) or the *SIR4-42* mutation (*). These strains express *HMRa*, giving rise to the RNA-derived product. (Lane 6) Old BKy1-14c cells. The RNA-derived product is evident. Reactions yielding the RNA product all show reduced amounts of the DNA-derived product and a third fragment of slightly larger size. This latter fragment is a product of mispriming in **a1** RNA. (Lanes 7 and 8) controls in which samples were treated with RNase prior to RT–PCR showing only the DNA-derived product. (Lanes 9 and 10) Controls in which the oligo(dT) primer for reverse transcription was omitted prior to RT–PCR also showing only the DNA-derived product.

tially growing cells. The average number of bud scars in the preparation of BK1-14c old cells was 12. The arrows indicate the position of the 1.2 kb telomeric fragment of most chromosomes extending from a XhoI site in the Y′ regions to the ends of the chromosomes. The lower arrow indicates the telomeres shortened in the Δ*sir4* strain. Note that there is no shortening of telomeres in DNA from old cells.

(B) Northern blot of *STE12*, an **a**/α repressed gene, in DNA from old cells of BKy1-14c (*MAT*α). Note that *STE12* is repressed in strains that express both **a** and α mating type information, including an **a**/α diploid, and strains with a deletion of *SIR4* or the *SIR4-42* mutation. The latter two strains express **a** and α information from the derepressed *HM* loci. The amount of *STE12* RNA normalized to *SPT15* (TBP) RNA is repressed about 5-fold in the **a**/α diploid and *sir4* mutant strains compared with the *MAT*α haploid and about 3-fold in the old cell RNA, as determined by quantitation with the phosphorimager.

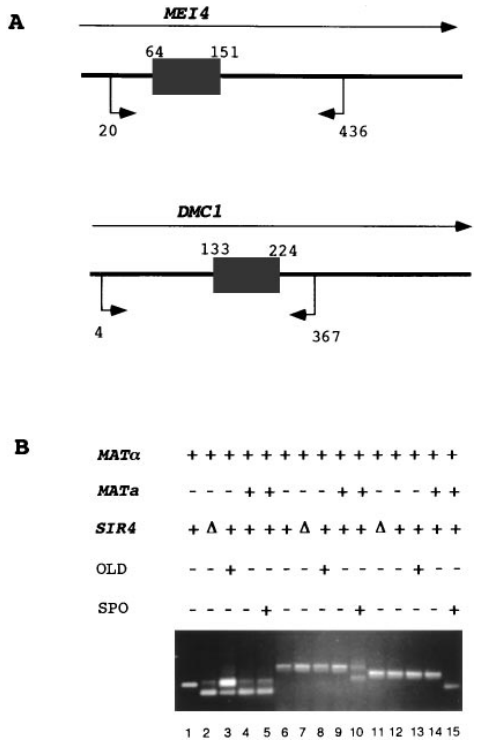

Figure 7. RT–PCR Analysis of *MEI4* and *DCM1* RNA from Old Cells

(A) shows the *MEI4* and *DCM1* genes. The positions of the RNAs (long arrows), introns (boxes), and primers used (small arrows) are indicated. The numbering is from the ATG of each coding sequence. (B) shows the PCR analysis of nucleic acid from the indicated strains. Lanes 1–5 use the *MAT***a***1* primers, as in the previous figure; lanes 6–10 use *MEI4* primers; lanes 11–15 use *DCM1* primers. The strains are as in Figure 6, Old is nucleic acid from old BKy1-14c cells, and SPO is nucleic acid from **a**/α diploid cells sporulated for 20 hr. **a1** RNA is present in cells expressing *MAT***a***1* (lanes 2, 4, and 5) and in old cells (lane 3). *MEI4* and *DCM1* RNA (lower bands) is present in sporulating cells (lanes 10 and 15) but not in old cells (lanes 8 and 13) or in vegatatively grown cells (lanes 6, 7, 9, 11, 12, and 14). The upper bands represent *MEI4* and *DCM1* DNA.

Old Cells Become Sterile Owing to Derepression of *HM* Loci

These findings suggested that old cells might become sterile owing to derepression of *HML and HMR*. To quantitate the degree of sterility in old cells, mother cells of DLy1 (isogenic to PSY142, but *MAT***a**) that had divided various numbers of times were prepared by microscopic manipulation and then exposed to α factor for 4 hr (see Experimental Procedures). Sterile cells will fail to respond in this assay. Cells were scored as responders if they adopted the clear morphological shape termed schmoos (Byers and Goetsch, 1975; Kennedy et al., 1994) in their next passage through G1. Occasional cells responded in their second passage through G1 after exposure. Cells that did not respond typically divided at least twice during exposure to the pheromone. Cells (both responders and nonresponders) were then moved away from the pheromone and allowed to complete their life spans. Note that exposure to α factor does not affect the life spans of cells (Kennedy et al., 1994).

Table 2 shows data plotted as a function of the percentage of their life spans that cells had completed at the time of exposure to α factor. The fraction of cells that did not respond increased steadily with age to about two thirds in cells in the last 10% of their life spans. Thus, as indicated by earlier studies (Müller, 1985) we conclude that sterility is, in fact, an aging-related phenotype and affects the majority of the oldest population of cells.

To determine whether this phenotype was due to derepression of the silent mating–type loci in old cells, we deleted *HML*α in DLy1. We first showed that deleting *HML* did not alter the life span of the strain (mean life spans of about 30 divisions in both the parental and deleted strains; data not shown). Next, we monitored response to α factor as above, and strikingly none of the cells became sterile, regardless of their ages (Table 2). This finding clearly shows that the sterility phenotype in old cells is due to a loss of silencing at *HML*. However, as predicted from earlier genetic studies (Kennedy et al., 1995), the expression **a** and α information of the *HM* loci does not cause aging-related mortality, because deleting *HML* does not itself alter life span.

Discussion

Aging can be assessed by two kinds of changes that occur over time; first, an increase in the mortality rate with time, resulting in a characteristic life span, and second, an alteration in the phenotype of the organism with age. In budding yeast, the two signatures of aging are evident by an increase in mortality with the number of cell divisions undergone by mother cells and by an enlargement of old mother cells.

Our prior study suggested that the status of silencing was one determinant of life span in S. cerevisiae, because loss-of-function mutations in the silencing gene *SIR4* shortened life span and gain–of–function mutations in this gene extended life span. We show here directly that silencing is lost in old cells and that a loss of silencing at the repressed copies of mating-type genes (*HML* and *HMR*) results in a novel aging-specific phenotype, i.e., sterility. The *HM* loci, however, do not cause mortality in old cells because deletion of *HML*, which prevents the simultaneous expression of **a** and α information, does not alter life span.

Our physical studies used a method to isolate old cells involving biotinylation of the cell surface and recovery of old cells after many rounds of cell division using avidin-coated magnetic beads. This approach works because the biotin stays concentrated in mother cells during budding, is stable over many rounds of cell division, and does not significantly interfere with the growth properties of mother cells.

Telomeres and Aging in Yeast

The first issue we addressed in the old cells was whether telomeres had shortened. Telomere shortening correlates with aging in human cells and has been proposed as a possible cause of aging (Harley et al., 1990; Allsopp et al., 1992). We anticipated that telomeres would not be affected in old yeast cells for two reasons. First, it had been reported that telomeres of yeast cells that had been aged to 80% of their mean life span were normal

Table 2. α Factor Responsiveness in Old Cells

	Percent Life Span																	
	0–10		10–20		20–30		30–40		40–50		50–60		60–70		70–80		80–90		90–100	
	R	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N
Wild type	20	0	4	0	4	0	10	0	5	0	8	2	15	4	13	6	6	6	4	8
	(0)		(0)		(0)		(0)		(0)		(20)		(21)		(32)		(50)		(67)	
Δ*HML*α	21	0	6	0	6	0	7	0	12	0	4	0	10	0	16	0	6	0	15	0
	(0)		(0)		(0)		(0)		(0)		(0)		(0)		(0)		(0)		(0)	

DLy1 (*MATa, HML*α) or DLy1 Δ*HML*α cells of different ages were examined for their ability to respond to α factor. After a variable number of divisions (as determined by micromanipulation), cells were exposed to α factor. R indicates that the cells responded by forming schmoos, and N indicates that the cells did not respond to α factor through two complete passages through the cell cycle. A minority of cells in the R class responded in their second passage through G1 (11 in wild-type and 4 in Δ*HML*α). This is probably because the concentration of α factor used was relatively low (see Experimental Procedures). Numbers in parentheses reflect the percentage of sterile cells (nonresponders). The relative ages of cells at the time of exposure to α factor was determined by removing them from the presence of α factor and determining the remaining number of divisions they underwent. All cells tabulated divided at least one time after being removed from the presence of α factor.

(D'Mello and Jazwinski, 1991). Second, our genetic studies indicated that while the *SIR2–SIR3–SIR4* silencing complex was involved in aging (see below), telomeres were not involved (Kennedy et al., 1995).

Telomeres were analyzed in old cells and found to be indistinguishable from telomeres of normal growing cells. The relevance of telomere shortening to aging in mammals is still uncertain. One must entertain the hypothesis that the absence of telomerase in somatic cells of humans (causing the observed shortening) could, rather, be a safeguard against uncontrolled growth due to oncogenic mutations. In long-lived mammals, such as humans, this safeguard might be particularly important, because pathways of tumorigenesis are time-dependent processes.

Silencing and Aging in Yeast

We tested directly whether silencing by the SIR complex was lost by RNA analyses of old cells . First, we showed that a gene repressed in **a**/α diploids, *STE12*, was repressed in old cells of a haploid strain. This was consistent with the possibility that silencing at *HML*α and *HMRa* had been lost in these cells. Second, and more directly, we demonstrated that silencing at *HMRa* was lost in old cells of this *MAT*α strain. Other transcriptionally repressed genes, such as the sporulation-specific genes *MEI4* and *DMC1*, remained silent in old cells. It is noteworthy that the appearance of transcription at *HMR* is a very stringent assay for a reduction in silencing because of redundancy in the *cis*-acting elements causing silencing. Sites for RAP1, ORC, and ABF1 are all present at *HML* and *HMR*, and any two can direct silencing by the SIR complex (Brand et al., 1987; Kimmerly and Rine, 1987; Foss et al., 1993; Bell et al., 1993). Further, silencing at *HML* and *HMR* is strengthened by SIR1 (Pillus and Rine, 1989). Indeed, assays for defects in *trans*-acting mediators of silencing frequently employ *HM* loci that have been weakened by removal of one of the cis-acting elements (Sussel and Shore, 1991).

The loss of silencing at *HMR* in old cells could be due to inactivation of the SIR2–SIR3–SIR4 complex or, perhaps, SIR1. In *sir1* mutants, a fraction of cells transcribe *HM* loci (Pillus and Rine, 1989), which could account for the *HMRa* transcript observed in old cells. However, previous experiments showed that while deletion of *SIR4* shortened life span, deletion of SIR1 did not (Kennedy et al., 1995). If the decay of *SIR1* were the event that occurred in old cells to trigger a loss of silencing, we would expect the Δ*sir1* strain to exhibit a shortened life span similar to the Δ*sir4* strain.

Sterility in Old Cells Due to Loss of Silencing

The simultaneous expression of **a** and α information in growing cells results in sterility. Therefore, we surmised that old cells might have an aging-associated phenotype of sterility because of a loss of silencing at *HML*α and *HMRa*. This would be consistent with earlier findings that the efficiency of zygote formation is reduced when old yeast cells are mated to young cells (Müller, 1985). Cells of various ages of a *MAT***a** strain were thus exposed to α factor. Indeed, the majority of the oldest population of cells (two thirds) did not respond and, thus, were sterile. However, because old cells display other phenotypes of aging, such as enlargement, we could not be certain that sterility resulted specifically from a loss of silencing. To address the issue of the cause of sterility in old cells, we deleted *HML*α and found that the appearance of sterility in old cells was abolished. Thus, we are confident that the aging-related phenotype of sterility is due to a loss of silencing at *HM* loci.

Why do some old cells not display sterility? A possible explanation is that a minority of old cells die not because they have reached the very end of their life spans, but because of the micromanipulation itself. This problem may be exacerbated in cells that are enlarged and fragile. If silencing is lost near the very end of normal life spans, these cells might still be fertile. By this view, the sterility phenotype could well apply to all cells at the ends of their life spans. An alternative possibility is that the loss of silencing is partial, and a minority of old cells maintain enough repression of *HM* loci to be fertile. Any partial loss of silencing, however, would have to cause derepression of loci that cause mortality.

While our physical and genetic studies suggest that a loss of silencing is causally related to mortality, this event may not be sufficient to cause senescence. If it were, deletion of *SIR4* would cause rapid senescence and thus be lethal. We suggest that a loss of silencing is

one of several events occurring in old cells that together cause senescence.

Silencing and Aging in Other Systems

Does the loss of silencing cause aging-related phenotypes in other organisms? In mice, there is evidence that transcription of specific loci residing on the inactive X chromosome becomes active in old animals (Wareham et al., 1987), although it has not been possible to associate a loss of silencing with any phenotype. In mammals, transcriptional silencing is frequently accompanied by DNA methylation (Cedar, 1988). It is interesting to note that treatment of primary fibroblasts with 5–azacytosine, which inhibits DNA-methylase, and thereby causing demethylation in dividing cells, shortens the number of divisions prior to senescence (Holliday, 1987).

One interesting feature of a model proposing that a loss of silencing contributes to aging in higher organisms is that it combines aspects of two schools of thought, that aging is genetically programmed and that it results from accumulated damage. If the machinery mediating silencing were inactivated through accumulated damage, it would result in the activation of gene transcription. One could imagine that a gradual reduction in the activity of a silencing apparatus would activate a discrete set of genes in an invarient sequence over time. Such a hard-wired response can be viewed as a genetic program and may be the basis for the staged pattern of phenotypic changes that are recognized in aging.

Experimental Procedures

Isolation of Old Yeast Cells

Strains BKy1-14c (*MAT*α, *ade1-100*, *his4-519*, *leu2-3*, *2-112*, *lys2-801*, *ura3-52*), the isogenic *MAT***a** derivative BKy5, the isogenic **a**/α diploid BKy6, and PSY142 (*MAT*α, *leu2-3*, *2-112*, *lys2-801*, *ura3-52*) were used in these studies (Kennedy et al., 1995). Cells were grown in YPD rich media to OD_{600} of 0.5. Between 10^7 and 10^8 cells were spun, washed, and resuspended in cold 1× PBS. Cells were then spun and resuspended in 0.33 ml of 10× PBS. Separately, 3.5 mg of NHSLC biotin (Pierce) was dissolved in 0.67 ml of 10× PBS at room temperature and immediately added to the cells. The mixture was shaken for 15 min by vortexing at a slow setting at room temperature. Cells were then spun and washed eight times in 1 ml of cold 1× PBS. The pH was adjusted to 8.0 with NaOH. Cells were then diluted and grown in YPD for 12–14 generations and spun down, washed with water, and resuspended in 10 ml of cold 1× PBS. Cells were sonicated for 30 s at 20% power, and streptavidin paramagnetic beads were added to 50 beads per biotinylated cell. The beads were obtained from Perseptive Diagnostics (formerly Advanced Magnetics; a newer 1994 lot [sorting grade] was found to be much less effective than earlier lots). Cells were placed on ice and swirled every 15 min for 2 hr. The suspension was then placed in a test tube in a magnetic sorter (Perseptive Diagnostics/Advanced Magnetics) in the 4°C room. After 15 min, the supernatent was carefully aspirated, 10 ml of cold YPD was added, and the mixture was vortexed. The cells were again placed in the sorter for 10 min, and the process was repeated seven times.

For early experiments involving FACS, up to 10^8 biotinylated cells were suspended in 1 ml of 1× PBS, and 50 ml of fluorescein-conjugated avidin (5 mg/ml, Pierce) was added. The mixture was vortexed, incubated 10 min on ice covered with foil to avoid bleaching, and washed twice with 1 ml of 1× PBS. Sorting was performed on a Becton–Dickenson FACS.

Determination of α Factor Responsiveness in Old Cells

The mating type of PSY142 (Kennedy et al., 1995) was converted to **a** using a *GAL-HO* expression plasmid. *HMLa* was deleted with a *LEU2* marker as described (White and Haber, 1990) and confirmed by Southern blot analysis. The *LEU2* gene was also integrated into the endogenous locus of the parent to create a matched pair of *LEU2* strains, wild type (DLy1) and Δ*HML*α. Cells were taken from YPD (rich) agar grown 24 hr and replated at low density on YPD agar. After 3 hr, virgin cells (daughters) were isolated with a Zeiss micromanipulator. All subsequent daughters from these cells were removed to generate cells of different ages. Groups of 10 cells were moved to between 50–100 mμ from filters with 0.02 μg/ ml α factor. This relatively low concentration of pheromone afforded the most sensitive identification of sterile cells. Cells were observed hourly for 4 hr, and schmooing was scored (Byers and Goetsch, 1975). This phenotype was unambiguous. Cells were then moved away from the pheromone, and the filters were removed. The remainder of the life spans of cells was determined. Most of the cells were budding when exposed to pheromone. Occasionally, unbudded cells were moved, and it was not possible to know whether they had passed START. These cells were scored as responders if they schmooed in that or the next cell cycle. Any cell that did not continue to divide after being moved away from the pheromone was excluded from the data set because any failure to respond to pheromone could have been due to senescence.

Telomere Analysis

Nucleic acids were isolated by glass bead disruption of cells in phenol–chloroform. Agarose gels and probing for yeast telomeres was as described; a 600 bp fragment located within the conserved Y′ region of yeast telomeres was used as probe (Kennedy et al., 1995).

RNA Isolation

Total RNA was isolated by glass bead lysis and phenol extraction followed by LiCl precipitation (total RNA isolated in this manner is contaminated with low levels of genomic DNA that is detectable by PCR analysis). Total RNA from mitotic cells was isolated from cells growing exponentially in YPD. For meiotic cell RNA preparations, diploid cells from saturated cultures were used to inoculate YEP acetate media at approximately 10^7 cells per ml and grown under well-aerated conditions for 5–10 hr at 30°C, spun down, washed one time with SPM media (0.3% potassium acetate, 0.02% raffinose), and incubated under well-aerated conditions in SPM media for 20 hr at 30°C and then harvested for RNA analysis.

Northern Analysis

Total RNA was fractionated on 1% agarose–formaldehyde gels and transferred to GeneScreen Plus membranes (New England Nuclear Research). Blots were prehybridized in 6× SSC, 5× Denhardt's, 20 mM NaH_2PO_4, and 100 μg/ml salmon sperm DNA for 2 hr at 65°C. Hybridization to random–primed *STE12* or *SPT15* (TBP) DNA probes was in 6× SSC, 0.4% SDS, 20 mM NaH_2PO_4, and 100 μg/ml salmon sperm DNA for 20 hr at 65°C. Filters were washed with 6× SSC and 0.1% SDS for 2 hr at 65°C.

RT–PCR Analysis of RNA

Total RNA was reverse transcribed using the Perkin Elmer GeneAmp system, as per the instructions of the manufacturer. Total RNA and 50 pmol of oligo(dT) 12–18 were incubated at room temperature for 10 min, the reverse transcription components were added, and samples were incubated for 10 min at room temperature, followed by 10 min at 42°C and then 10 min at 70°C (except for MEI4 and DMC1, in which their respective 3′ primers were used instead of oligo [dT], and the reactions after the room temperature step were incubated for 25 min at 50°C and then 15 min at 70°C). The cDNAs were amplified with a thermal cycler set for 35 step cycles of 94°C for 1 min, 60°C for 1 min, followed by a final extention step of 7 min at 60°C (except for *MEI4* and *DCM1* for which the thermal cycler was set for 40 step cycles of 94°C for 1 min, 50°C for 30 s, 60°C for 1 min, followed by a final extension step for 7 min at 60°C). The 5′ primer (5′-GGCGGAAAACATAAACAGAACTCTG-3′) and 3′ primer

(5′-CCGTGCTTGGGGTGATATTGATG-3′) were used to amplify **a1.** *MEI4* was amplified with the 5′ primer (5′ AGATATGGAACAAAAGGA AACATCGG-3′) and the 3′ primer (5′- AAATGATTGCAGCTCCTTTAA ACCAACTGCATCTTC-3′). *DMC1* was amplified with the 5′ primer (5′-TCTGTTACAGGAACTGAGATCGATAGTGATACAGC-3′) and the 3′ primer (5′-CGCTGTCTTATATCTAATTGTACAGTAGCGGGG- 3′). Products were resolved on 1.5% agarose, 1× TAE gel stained with EtBr and photographed under short-wave UV light.

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