Genetic and Molecular Analysis of Aging in Yeast

by
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Abstract

In the yeast, *Saccharomyces cerevisiae*, aging is defined as the number of daughter cells that one mother cell can produce. Aging mother cells undergo a number of morphological changes, yet they retain the capacity to give rise to young daughter cells throughout much of their life span. As they become old, however, the daughters they produce have depleted division potential. Full life span is eventually restored in cells generated by the short-lived daughter. These findings suggest a mechanism of aging in which a toxic cytoplasmic factor accumulates in old mother cells, eventually inhibiting their division potential.

The life span potential of many strains correlates with stress resistance. By screening for stress resistant mutants in one such strain, mutants were identified in four genes which had significantly longer life spans. Two of these genes have been cloned and characterized. The first gene identified was identical to the *SIR4* gene, which participates in transcriptional silencing. The mutation in *SIR4*, *SIR4-42*, was recessive for most phenotypes, including stress resistance and transcriptional silencing at telomeres and silent mating type loci. Yet life span extension by *SIR4-42* was a dominant phenotype. These and other findings have led to a model whereby the *SIR4-42* mutant allele extends life span by causing a re-direction of the Sir complex from telomeres and silent mating type loci to an unidentified Aging locus. This would result in increased silencing of the Aging locus. Expression of a gene or genes at the Aging locus would accelerate aging resulting in shorter life span potential.

The second gene isolated from the stress resistance screen, *UTH4*, extends life span in a similar manner. Life span extension by *UTH4* is absolutely dependent on the presence of intact *SIR3* and *SIR4* genes. Moreover *UTH4* interferes with telomere silencing. Much as with *SIR4-42*, *UTH4* likely extends life span by redirecting silencing factors away from telomeres and silent mating type loci, to the Aging locus.

Thesis Supervisor: Leonard Guarente
Title: Professor of Biology
This work is dedicated to my wife, Brenda, whose love, support, encouragement, and suggestions have been immeasurable, and to my parents, James and Betty Kennedy, who inspired me to pursue a career in science and provided the love and support that made that pursuit possible.
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Chapter 1: Introduction to the Genetics of Aging

Portions of this chapter have been submitted for publication. The authors are Brian Kennedy and Leonard Guarente.
Introduction

Aging is manifest by an exponential increase in the rate of mortality over time (Gompertz, 1825). This feature is found in organisms as complex as humans (Gompertz, 1825) to those as simple as the single-celled yeast, *Saccharomyces cerevisiae* (Pohley, 1987).

While aging research has broadened significantly in recent years, very little is known about the molecular determinants of aging in any organism. Several theories have been put forth to explain the phenomenon of aging. The primary question addressed in these theories is whether aging is somehow genetically programmed or if aging results from an increase in damage to an organism over time. To date, there is no compelling biological evidence to distinguish between these two possibilities. The answer to this fundamental question awaits a better understanding of the molecular determinants of aging.

The aging phenomenon is presently being addressed at the molecular level in several organisms. The observation that the mortality rate increases exponentially over time in most organisms does not constitute proof that determinants of aging within organisms will be conserved. However, the striking similarities observed at the cellular level from one organism to another suggest that at least some elements of aging are closely related. At present, there is not enough understanding to effectively compare the knowledge learned in one organism to that learned in another. Therefore, I will briefly address aging research in several different organisms saving yeast for last. In addition, I will detail the phenomenon of cell senescence and how it may relate to aging at the organismal level.
The Study of Aging in Lower Organisms

The method of choice to study aging in lower organisms has been to identify and study mutants that alter the rate of aging. Most screens are designed to isolate mutants with longer life spans (Kennedy et al., 1995; Klass, 1983; Munkres and Furtek, 1984; Roberts and Iredale, 1985). Mutations that shorten life span potential are generally avoided since it is assumed that many mutations that reduce life span potential may not be directly related to the aging process. Such mutations could decrease the general health of the organism or interfere with processes that repair cellular damage.

Another approach involves selective breeding programs to increase life span potential. Although laborious, this approach has been very successful in defining the variability in life span potential within an organism (Luckinbill et al., 1984; Rose, 1984). The selective breeding approach has limitations because there is no direct way to identify and clone the polymorphic genes responsible for differences in life span potential. Other experimental approaches are aimed at studying the relationship between known genes and aging (e.g., superoxide dismutase) (Jazwinski et al., 1993; Reveillaud et al., 1992). Finally, researchers have attempted to isolate important aging genes by examining age-specific phenotypes such as transcript prevalence (Egilmez et al., 1989; Fabian and Johnson, 1995). These last two methods are more limited than direct screens for aging mutants since they require the researcher to make several assumptions about the aging process.
The only published screen for longer-lived mutants of *Drosophila* proved unsuccessful in turning up such mutations (Roberts and Iredale, 1985). Instead, most efforts to understand the *Drosophila* aging process have focused on selective breeding experiments. Several groups have been successful at breeding flies with longer life spans (Clare and Luckingbill, 1985; Luckinbill, et al., 1984; Rose, 1984). The identification of the polymorphic genes responsible for delayed senescence has proven elusive. The extended life span may occur through the extended effects of several genes (Hutchinson and Rose, 1990).

In one study, Rose et al. (1984) found that long-lived flies had a reduced fertility rate early in life. Although the reduction in fertility was not great, this finding provided correlative evidence in favor of the hypothesis that rapid aging and early fertility are coordinated. The basis of this aging theory is that organisms experience a trade-off between reproductive capacity and life span. In other words, reproduction requires diversion of limited resources that would otherwise be used for maintenance of somatic cells.

Long-lived strains also showed increased resistance to environmental stresses such as starvation (Service et al., 1985). This result was strengthened by reversing the phenotypes. Strains were selectively bred for starvation resistance and then subjected to life span analysis (Rose et al., 1992). The resultant strains displayed a 40% increase in mean longevity. Longer-life and stress resistance seem to correlate in a variety of organisms. However, the link between these phenotypes remains fragile and a molecular understanding of this relationship is required before this relationship is demonstrated to be significant at the inter-organismal level.
Unlike fruit flies, a screen for extended life span in *C. elegans* has proven successful (Klass, 1983). One mutation in the *age-l* gene was identified in this screen which extended the mean life span of the nematode by 60%. Otherwise, the mutant appears normal. The *age-l* mutant does eventually undergo senescence (Johnson, 1990).

Several other mutations demonstrably delay the rate of aging on *C. elegans*. The best characterized set of these mutants lies in the dauer pathway. In conditions of high population density and low-food, *C. elegans* can enter an alternate developmental pathway termed dauer (Cassada and Russell, 1975). Entry into this pathway can only occur after the second larval stage of development. Dauer formation involves several morphological changes which arrest the molting cycle, make the animal unable to feed and confer resistance to a variety of stresses including starvation and desiccation. The animal can remain suspended in this state for months, only emerging when it encounters food (Golden and Riddle, 1984). After exiting dauer, the animal resumes normal development and ages at a normal rate.

In general, two types of mutations have been isolated in the dauer pathway. Dauer defective mutations (*daf-d*) block dauer formation and dauer constitutive mutations (*daf-c*) inappropriately enter dauer at elevated temperatures (Riddle et al., 1981). Extensive epistasis analysis has defined two parallel genetic pathways that regulate dauer formation (Gottlieb and Ruvkun, 1994; Riddle, et al., 1981; Thomas et al., 1993; Vowels and Thomas, 1992)(Figure 1). As presently understood, the signal for dauer induction begins with secretion of a pheromone (Golden and Riddle, 1982) which is in turn recognized by sensory neurons (Albert et al., 1981; Lewis and Hodgkin, 1977;
Perkins et al., 1986). Other sensory neuron classes are important for negative regulation of dauer formation (Bargmann and Horvitz, 1991). Sensory neuron recognition then presumably activates a signal transduction pathway which leads to regulation of transcription factors. Consistent with this hypothesis, two of the cloned dauer mutants have significant homology to signal transduction proteins (Estevez et al., 1993; Georgi et al., 1990).

Mutations in daf-2 or daf-23, both having the daf-c phenotype, double the C. elegans life span (Kenyon et al., 1993; Larsen et al., 1995). To determine the life span, these mutants must be raised at low temperatures during early development to avoid dauer induction. Mutation in daf-16 does not substantially alter life span, but the prolongation of life span in daf-2 and daf-23 mutants is completely abrogated in strains also mutant for daf-16 (Larsen, et al., 1995) (Figure 1). This finding is consistent with the proposed genetic pathway for dauer formation which places daf-16 downstream of daf-2 and daf-23. Mutant alleles of daf-12 do not affect life span but act in an allele-specific manner to enhance life span potential dramatically in combination with some alleles of daf-2. Surprisingly, daf-c mutants in the other branch of the dauer pathway do not alter life span potential.

The age-1 mutant may be increasing life span in a manner similar to that of the daf-c mutants. The primary evidence for this theory is that daf-16 mutations suppress the life span effects of the age-1 mutant allele (Dorman et al., 1995). Moreover, the age-1 mutation enhances some dauer phenotypes in a daf-2 mutant background. Together, these results strongly suggest that life span extension by these two mutants occurs through a common pathway.

The relationship between the dauer pathway and the normal aging process remains controversial. It is unclear whether the daf-c mutations directly regulate the aging process. Caloric restriction clearly promotes dauer
formation (Golden and Riddle, 1984; Klass, 1977) and also extends life span in mammals (McCay, 1947; Osborne et al., 1917; Weindruch et al., 1986). Therefore, daf-c mutations may promote caloric restriction or mislead the worms into thinking they are subject to caloric restriction, thus extending life span indirectly. Evidence against this theory derives from the observation that long-lived adult daf-2 mutants eat normally. On the other hand, it has been reported that daf-2 adults have a decreased amount of pharyngeal pumping before death (Kenyon, et al., 1993). A more complete analysis of dietary intake in adult daf-c mutants must be performed before dietary alterations can be excluded as a mechanism by which these mutants live longer.

Recent reports have linked age-1 and daf-c mutations to stress resistance. age-1 mutants have elevated levels of superoxide dismutase (SOD) and catalase late in life (Larsen, 1992; Vanfleteren, 1993). Worms in the dauer state also have elevated levels of SOD but not catalase (Larsen, 1992). It remains to be determined whether overexpression of SOD increases life span potential in C. elegans. Similar experiments in Drosophila demonstrated no significant increase in life span coordinate with SOD overexpression (Seto et al., 1990; Staveley et al., 1990). Co-overexpression of SOD and catalase in Drosophila, however, increase mean life approximately 30% (Orr and Sohal, 1994). age-1 also confers some degree of thermal resistance, further raising the possibility that aging and stress resistance may be related (Lithgow et al., 1994).

The relationship between reproduction and life span potential has also been examined in C. elegans (Van Voorhies, 1992). Mated males have vastly reduced life span potential relative to unmated males. Mated hermaphrodites do not experience a similar reduction in life span potential relative to unmated
hermaphrodites. A potential clue to this disparity comes from the finding that mating induces sperm production only in males. If increased sperm production was reducing life span potential, then reducing sperm production should eliminate the life span difference observed in mated and unmated males. Van Voorhies (1994) found this hypothesis to be true by examining life span potential in a spe-26 mutant background. The spe-26 mutant has dramatically reduced sperm output, but is otherwise normal (Varkey et al., 1995). Mated spe-26 males do not have reduced life spans (Van Voorhies, 1992). Moreover, hermaphroditic spe-26 mutants enjoy an 80% increase in mean life span. These findings support the hypothesis that reproductive capacity and life span potential may be inversely related. However, it cannot be ruled out that the spe-26 mutation is more pleiotropic in nature and extends life span through some other mechanism. Surprisingly, in the case of C. elegans, the primary drag on energy may occur during the process of spermatogenesis and not oogenesis as commonly predicted.

Another aging mutant has been recovered that seems to extend life span in a manner unrelated to that of dauer mutants (Wong et al., 1995). This mutant, clk-1, seems to slow down the entire developmental process in addition to adult cycles such as defecation and pharyngeal pumping. A thorough analysis of the role of all of these mutants in the nematode life span is one of the most promising avenues toward an understanding of aging in any organism.
The means of senescence in *Podospora anserina* is well understood. Most, but not all, strains of *Podospora* undergo senescence after 1-15 weeks of vegetative growth (Smith and Rubenstein, 1973). The duration of vegetative growth is strain dependent (Kuck et al., 1985). The first clue to the mechanism of senescence came when an infectious agent was discovered which rapidly transferred senescence when senescent and juvenile hyphae were fused (Rizet, 1953). The identity of the infectious agent was later determined to be a \( \alpha \)-senDNA, a 2.6kb plasmid (Jamet-Veirny et al., 1980; Stahl et al., 1978; Wright et al., 1982). The \( \alpha \)-senDNA plasmid is identical to an intron in the cytochrome oxidase subunit 1 gene in the mitochondrial genome (Osiewacz and Esser, 1984). The plasmid is found in a low copy number in the mitochondria of juvenile cells, but at a very high number in senescing mitochondria. At the time of senescence, the mitochondrial genome is no longer detectable. Non-senescent strains do not have this intron in the mitochondrial genome (Belcour and Vierny, 1986; Cummings et al., 1986). The intron has self-splicing capability (Lambowitz, 1989) and an open reading frame that is thought to encode for a viral-type reverse transcriptase (Michel and Lang, 1985; Osiewacz and Esser, 1984). There are also inverted sequence repeats in the adjacent exons (Cummings and Wright, 1983; Osiewacz and Esser, 1983). All of these results suggest the intron may be viral in nature and activated specifically in mitochondria late in vegetative growth.

Senescence in *Neurospora intermedia* occurs by a similar mechanism. In this case a 9kb plasmid is present in the nuclei of senescing strains (Bertrand et al., 1986). Non-senescent strains do not have the plasmid. Senescence is initiated by movement of the plasmid from the nucleus to the
mitochondria and subsequent insertion into one of several sites in the mitochondrial genome (Bertrand et al., 1985). Insertion interferes with mitochondrial functions.

To date, there is no evidence for deleterious, mitochondrially derived plasmids that cause senescence in other organisms. It has been demonstrated that mammalian mitochondrial DNA accumulates specific deletions as the organism ages (Piko et al., 1988). Since the plasmid that instigates senescence is viral-like, it may be more appropriate to consider senescence in these organisms to be a disease state and thus not a good model system for aging in higher organisms.

Aging in Mammals

Genetic approaches to the mammalian aging process are very limited. One approach that has recently proven successful is the mapping of mutations thought to cause premature aging in humans. The best known of the premature aging diseases is Werner's syndrome, a rare autosomal recessive disorder. Individuals develop age-related phenotypes shortly after puberty (Epstein et al., 1966; Finch, 1990; Imura et al., 1985). These include premature graying, skeletal muscle atrophy, atherosclerosis, calcification of heart valves, and neoplasms in tissues that are rare in adults. Most individuals die from heart disease before the age of 50. Not all age-related phenotypes appear in Werner's patients. For instance, they do not exhibit an increased frequency of diabetes, age-related dementia, or other neurodegenerative conditions.

The first clue to the nature of the disease came from the study of Werner's fibroblasts in culture. The cells display a ten-fold increase in spontaneous mutations at the HPRT locus (Fukuchi et al., 1989). It has also
been observed that the translocation frequency is increased in Werner's lymphocytes (Salk, 1982). Recently the Werner's syndrome gene (WRN) has been cloned (Yu et al., 1996). The gene encodes a large protein with strong homology to DNA helicases from a number of organisms, including yeast. The finding that WRN may be a DNA helicase is consistent with the "Mutator" phenotype observed in cell cultured from Werner's patients. While WRN is broadly expressed, it is conspicuously absent in brain cells. As previously mentioned, age-related neurological disorders are not observed in Werner's patients.

The identification of the Werner's gene will open up a broad range of aging research (Yu et al., 1996). While WRN is homologous to DNA helicases from a number of organisms, the function of these homologues is poorly understood. The best homologue is the recQ gene from E. coli (Nakayama et al., 1985; Umezu et al., 1990). Loss of recQ only displays DNA metabolism defects when other helicases are also deleted (Mendonca et al., 1995). Similarly, the yeast homologue, SGS1 was identified as a high copy suppresser of a deletion in the topoisomerase TOP3 (Gangloff et al., 1994). WRN is also closely related to a C. elegans gene that is presently uncharacterized (Yu, et al., 1996). In addition to determining the role of WRN in DNA metabolism, it will be interesting to determine if the yeast and C. elegans homologues alter the rate of aging in their respective organisms.

DNA helicases have also been found to be defective in other diseases associated with short life span such as Bloom's syndrome, Cockayne's syndrome and Xeroderma pigmentosum Group D (Ellis et al., 1995; Sung et al., 1993; Weeda et al., 1990). These diseases do not display the broad spectrum of phenotypes associated with premature aging characteristic of Werner's patients. But they are all characterized by progressive tissue degeneration. Together, these
findings point to DNA helicases and their role in DNA metabolism and repair as a major factor in the aging process.

The other focus of aging research in mammals has been directed at correlative studies addressing the molecular changes that occur during the aging process. These studies range broadly and are too numerous to mention. While many correlative studies provide helpful clues about the aging process, they are limited because it is impossible to establish cause and effect.
Senescence in Cell Culture

Non-transformed animal cells have a finite replicative capacity in culture (Hayflick, 1961; Hayflick, 1965). After clonally dividing a given number of times the culture loses its ability to proliferate and becomes senescent. As they approach senescence, cells divide at a slower and slower rate, eventually undergo crisis and arrest in a post-mitotic state (Grove and Cristofalo, 1977). Spontaneous immortalization occurs at a low frequency to generate clones from the senescent culture. The frequency of immortalization varies dramatically, ranging from 1 in $10^4$-$10^6$ in most rodent cells to almost immeasurably low in human cells (McCormick and Maher, 1989).

Does in vitro cell senescence contribute to organismal aging? Although many researchers believe there is a strong relationship between these phenomenon, the evidence remains sparse. Perhaps the best argument for in vitro cell senescence being related to aging is the observation that there is an inverse correlation between donor age and replicative capacity of cultured cells (Martin et al., 1970). Also, fibroblasts recovered from Werner's Syndrome patients senescence after significantly fewer doublings than those from age-matched controls (Goldstein, 1969; Goldstein et al., 1989). In a related series of experiments, interspecies comparisons have been performed. In general, fibroblasts from short-lived species senescence after fewer doublings in culture than those from long-lived species (Rohme, 1981). Finally, there are a number of molecular correlations between aging cells in vivo and senescing cells in vitro (Campisi, 1996). The regulation of several genes is similar in these cells. The most interesting molecular correlate may be the finding that a β-galactosidase activity is activated in senescent human cells and in in vivo skin cells from old individuals (Dimri et al., 1995). This activity is not
associated with quiescence, terminal differentiation or immortality. The authors conclude from this finding that aged individuals accumulate senescent cells.

The fundamental recent findings in the study of cell senescence have involved the analysis of telomeres and telomerase. DNA at chromosomal termini is generally composed of several base pair repeats (Blackburn and Gall, 1978). The length of these repeats varies from one organism to another, ranging from several hundred base pairs in yeast (Chan and Tye, 1983) to 50 kb in mouse cells (Kipling and Cooke, 1990). The normal replication machinery is inherently unable to replicate both DNA strands at the telomere resulting in a reduction of telomere length with every cell division (Olovnikov, 1973; Watson, 1972). Telomere length is maintained by telomerase, an enzyme complex composed of an RNA molecule and an unknown number of protein components (Greider and Blackburn, 1985; Greider and Blackburn, 1987).

Telomerase activity is absent in primary human cell cultures (Harley et al., 1990; Hastie et al., 1990). Therefore, telomeres shorten with replicative age. In cells that have been immortalized, telomerase is reactivated and telomere length stabilizes (Counter et al., 1992; Kim et al., 1994). Anti-sense DNA oligonucleotides directed against telomerase RNA inhibit replicative capacity of immortalized cells after several cell divisions (Feng et al., 1995).

Given the rarity of spontaneous immortalization of human cells, it has been proposed that multiple events must occur for a cell to become immortal. The present model is that telomere shortening acts as a counting mechanism to induce senescence (Harley, 1991). The signal to arrest cell division may occur when telomere length is reduced to a point where DNA damage occurs. DNA damage would activate cell cycle inhibitors such as p53 and cause cell cycle

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arrest. Overcoming cell cycle arrest would then be the first requirement for immortalization. Then a second event must occur to activate telomerase for cells to become immortalized.

Mouse cell cultures immortalize at a much higher rate than human cells. Interestingly, most mouse primary cells have active telomerase (Prowse and Greider, 1995). Thus, the second event to activate telomerase may not be required for immortalization. It remains a point of debate whether mouse telomeres shorten with replicative age (Kipling and Cooke, 1990; Prowse and Greider, 1995). In any case, they remain long even in senesced cells. Therefore, it is less plausible that DNA instability due to telomere loss is a signal for senescence in these cells.

Telomeres shorten in human somatic cells as a function of age in vivo (Allsopp et al., 1992). One popular hypothesis is that telomere shortening and inactivation of telomerase in somatic cells is an attempt on the part of long-lived organisms to reduce the rate of cancer (Harley, 1991). Extending this theory further, aging may be the byproduct of this cancer reduction mechanism. There are still several issues to be addressed before this theory becomes widely accepted. Foremost among these is the absence of a direct causal link telomere shortening and senescence, not to mention organismal aging. Another difficulty with this model is the issue of which cells within an organism give rise to cancers. Evidence suggests that some types of tumors primarily arise from stem cells, or self-renewing tissues in adults (Greaves, 1986; Pierce and Speers, 1988; Potten and Loeffler, 1990). It remains unclear whether stem cells have active telomerase or long proliferative potential (Valeriote and Tolen, 1983; Vaziri et al., 1994). Now that telomerase components are being cloned, it will be possible to address more thoroughly many of these questions.
Aging in Yeast

The asymmetrical cell division in *Saccharomyces cerevisiae* makes it possible to follow the fate of an individual cell through many cell divisions (Hartwell and Unger, 1977). During the budding process, the mother cell gives rise to a daughter cell which is clearly smaller in size. By micro-manipulating successive daughter cells away from a mother, it could be determined whether mother cells divide forever or if there are limits to division potential. Mortimer and Johnston addressed this question in 1959 when they determined that mother cells do not divide indefinitely (Mortimer and Johnston, 1959). Rather, they have a fixed life span, defined as the number of daughter cells a mother cell can produce, which varies in a strain-dependent manner. This observation suggested that yeast might serve as a model system in which aging can be studied.

Asymmetric Division and Aging

There are two simple models by which yeast cells could age or be signalled to age. Either old cells accumulate a toxic molecule that causes them to senesce or old cells become more and more depleted for components essential for maintenance of division potential. The first observation suggesting the former model may be correct occurred when Müller mated aged haploid cells with a young cells and then determined the life span of the resultant zygotes (Müller, 1985). The zygotes had life spans which more closely resembled that of the expected remaining life span of the old cell, suggesting that the old cell phenotype was dominant. Diploid clones arising from these zygotes had restored life span potential. Therefore, the toxic
molecule seemed to be specific to the zygote and was diluted out in daughter cells that arise from that zygote.

In another set of experiments, Egilmez and Jazwinski took advantage of a previous observation that old cells divide at a much slower rate (Mortimer and Johnston, 1959) and analyzed the rate of cell cycle progression in daughters from old, slowly dividing mother cells (Egilmez and Jazwinski, 1989). They found that these daughters initially divided slowly, but regained their normal cycling time after approximately two divisions. This observation is also consistent with a proposal that the accumulation of a toxic factor is restricting mitotic potential. After the daughter divided two times, the toxic factor was sufficiently diluted and the daughters resumed normal rates of cell cycle progression.

Recent experiments have further cemented the hypothesis that a toxic factor accumulates in old cells. A detailed analysis of life span potential in daughters from aging mothers demonstrated that old mothers are unable to produce young daughters (Chapter 2)(Kennedy et al., 1994). Throughout the first half of the mothers' life span, the daughters have full division potential. As the mothers approach senescence, however, the daughters they create exhibit dramatically decreased life spans. This suggests not only that a toxic factor is accumulating in old mothers, but also that these mothers lose their ability to impede movement of the factor into the daughter. In subsequent experiments, granddaughters, great granddaughters, etc. from old mothers were examined. While the daughters were unable to divide the normal number of times, life span potential steadily increased in subsequent generations with full potential effectively restored in the great, great, great granddaughters. Again, these results suggest are consistent with the model that a toxic factor is diluted after several cell divisions.
Yeast cells increase in volume as much as six-fold during the aging process (Egilmez et al., 1990; Mortimer and Johnston, 1959). It has been suggested that this increase in volume causes the slow cessation of division potential (Mortimer and Johnston, 1959). As the cell increases in size, the volume increases much faster than the surface area. Consequently, transport of metabolites at the cell surface may not proceed at a rate sufficient to satisfy the increasing needs of the voluminous older cell, effectively causing them to starve for nutrients in rich media. Interestingly, the daughters with a short life span potential that are produced by old mothers are nearly always larger than daughters from young mothers (Kennedy et al., 1994). Rarely, the daughters are approximately the same size as the old mother. In these cases, termed symmetric divisions, the life span potential of the daughter is identical to the remaining life span potential of the old mother, usually less than five generations. Thus, there is a inverse correlation between the life span potential and the size of a daughter cell.

If the volume increase observed in aging cells is the agent that eventually prevents progression through the cell cycle, then artificially increasing the volume of a cell should reduce its life span potential. This idea was directly tested by taking mating type a daughter cells from young mothers and exposing them to a factor. This treatment causes them to arrest in the G1 phase of the cell cycle and increase substantially in cell volume. After releasing the cells from arrest, they were monitored for life span potential. These cells enjoyed full life spans, identical in duration to untreated, small daughter cells (Kennedy et al., 1994). While cell volume correlates well with life span potential in yeast cells, it is not the sole determinative agent.

If the cells within a colony are aging and yet the colony will divide indefinitely, then the aging mother cells must be able to divide and produce
daughters with full division potential throughout the early part of their life span. Otherwise the colony would slowly lose division potential. As previously stated, mothers in the first fifty percent of their life span retain the ability to divide symmetrically with regard to life span. The loss of this potential suggests that there is an active mechanism for asymmetry which becomes disassembled with age.

Mating type switching in homothallic yeast strains is another process by which there is clear asymmetry between mother and daughter cells (Strathern and Herskowitz, 1979). Switching is limited specifically to mother cells. Restricting switching in this manner creates a situation whereby one haploid yeast cell or spore is able to generate two diploid cells after only two divisions. Mother cells specifically express the \textit{HO} gene, which encodes a site-specific endonuclease that facilitates the conversion of mating type information at the \textit{MAT} locus from \textit{a} to \textit{\alpha} and \textit{vice versa} (Nasmyth, 1983; Strathern et al., 1982). Only recently has a model for the mechanism by which \textit{HO} expression is restricted to mother cells been suggested. Daughter cells may inherit a repressor of \textit{HO} transcription (Bobola et al., 1996; Sil and Herskowitz, 1996). This repressor (Ash1p) is restricted to daughter cells in a \textit{SHE1/MYO4} dependent manner (Jansen et al., 1996). \textit{SHE1/MYO4} encodes a cytoplasmic protein with homology to myosin that is thought to specifically sort Ash1 to daughters during cell division. It will be interesting to see if the mechanism of asymmetry in mating type switching is similar to the mechanism of asymmetry with regard to aging. It is possible that \textit{ASH1} could repress transcription of a gene in daughter cells which facilitates the aging process. More likely, however, \textit{SHE1/MYO4} may participate in a process that specifically sorts other proteins to daughter cells in addition to \textit{ASH1}. One of
these proteins could positively regulate transcription of genes required to restore youth.

It may be that asymmetric division during aging is of more evolutionary importance than life span potential. It has an obvious bearing on the ability of a single-celled organism to survive since a culture of cells which were unable to rejuvenate daughters would clonally senesce. The life span of the mother cell may be less important, since a cell that divides infinitely would have little obvious advantage over one that only divides twenty times before losing mitotic potential. Twenty divisions would create $2^{19}$ cells or about half of a million cells. Most cells growing in the wild probably arrest cell division due to nutrient depletion long before they die of old age. The mechanism by which yeast cells maintain asymmetry at cell division with regard to aging is one of the critical questions about the yeast aging process.

Genetic Approaches to Yeast Aging

The advantage of studying nearly any biological phenomenon in yeast is the ease of isolating mutants that affect a process. Unfortunately, this advantage does not easily translate to the study of yeast aging. Any growing culture of yeast cells in logarithmic phase will be comprised half of virgin cells, one-quarter of cells that have completed one division, one-eighth of cells that have completed two divisions, etc. Therefore, the number of cells of old age (>20 divisions for example) is extremely small, and genetic approaches based on colonies are not applicable. Further, there is no facile way to examine a large population of old cells, although methods have been recently developed to circumvent this problem (Egilmez et al., 1990; Smeal et al., 1996).
Efforts in the Jazwinski laboratory have been directed at obtaining large numbers of old cells (Egilmez et al., 1990). The method involves allowing a culture of cells to divide a few times and then separating them by rate-zonal sedimentation in sucrose density gradients. As previously stated, old cells are larger than young cells (Mortimer and Johnston, 1959) and can be separated on this basis. Successive repetitions of this protocol make it possible to obtain reasonably pure populations of old cells that have completed almost as many divisions as the mean life span of the strain (Egilmez et al., 1990). RNA prepared from these old cell populations was compared to that of young cells to screen for clones which are differentially expressed (Egilmez et al., 1989). Several genes have been isolated in this manner and one, LAG1, has been characterized further (D'Mello et al., 1994). LAG1 mRNA levels decrease as cells age. Deletion of the LAG1 coding region increases the life span potential of at least one strain by 47% (Table 1). These results suggest that the protein product of LAG1 may restrict life span potential in yeast, making it an interesting candidate for further analysis. Apparently the protein exerts its effect on aging while the cells are young as it is not present in older cells. The protein sequence of LAG1 indicates that it may have membrane-spanning domains but shares no significant homology with other known genes.

Two other genes, RAS1 and RAS2, have also been studied in the Jazwinski laboratory (Sun et al., 1994). These functionally interchangeable homologues are together essential for viability in yeast (Kataoka et al., 1984). Although other functions are possible, the primary role of Ras proteins in yeast is to positively regulate the activity of adenylate cyclase in a GTP-dependent manner (Toda et al., 1985). Surprisingly, mutation of these two homologues have opposite effects on life span (Sun et al., 1994). Deletion of RAS1 lengthens life span potential, whereas deletion of RAS2 diminishes life
span potential (Table 1). Moreover, elevated expression of \textit{RAS2} but not \textit{RAS1} lengthens life span. This is the only known phenotype where the roles of \textit{RAS1} and \textit{RAS2} are separable. Still more puzzling is the observation that increasing cAMP levels by mutating genes downstream of Ras in the adenylate cyclase pathway does not increase life span potential. This last observation suggests that the role of Ras in aging may be independent of the adenylate cyclase pathway.

Another approach to acquire yeast aging mutants takes advantage of an observation that there is a strong correlation between starvation resistance and life span potential within certain strain backgrounds (Chapter 3) (Kennedy et al., 1995). A stress sensitive, short-lived strain was mutagenized and haploid colonies isolated that were better able to maintain viability after prolonged periods of starvation. Cells from each of these colonies were then tested to determine if any clone had significantly longer mean and maximum life spans than the unmutagenized, short-lived strain. Of the 39 starvation resistant colonies obtained, approximately one-quarter displayed mean life span increases between 20 and 55%. Genetic analysis indicated that the aging mutants defined four genes, \textit{UTH1-4}.

To date, the genetic approaches to obtain aging mutants have all been biased. The method used to isolate \textit{LAG1} only screened for genes that were differentially expressed during yeast aging (D’Mello, et al., 1994), and the screen used to identify the \textit{UTH} genes required that aging mutants also be stress resistant (Kennedy et al., 1995). Both of these screens most likely uncovered only a subset of the genes which affect yeast aging. Screening for yeast aging mutants in an unbiased manner would require a method to follow large numbers of mother cells through many divisions. One way to make this possible is to create a system where mothers are able to maintain division, but
the daughters they produce are inviable. Therefore mothers could be followed throughout their life spans without the experiment being overrun by progeny. Two approaches are being attempted. The first involves uses of the \textit{HO} promoter, which is transcriptionally active only in mother cells (Nasmyth, 1983), to express a gene required for cell division. The second approach utilizes strains that conditionally produce inviable daughter cells (McConnell et al., 1990). Using these approaches, it is possible to screen for mutations that lengthen life span and give rise to larger micro-colonies consisting of a mother cell and its daughters. These methods potentiate the isolation of a comprehensive set mutants which extend life span potential.

The Role of \textit{SIR4} in Aging

All of the starvation resistant colonies were also resistant to a variety of stress conditions, but the \textit{UTH2} mutant had an additional, informative phenotype: sterility (Kennedy et al., 1995). \textit{UTH2} was cloned on the basis of this phenotype and shown to be identical to \textit{SIR4} (Hartwell, 1980; Ivy et al., 1986; Rine and Herskowitz, 1987) (Table 1). \textit{SIR2}, 3, and 4 all encode proteins required for transcriptional silencing at two sets of chromosomal sites: silent mating type loci (HML and HMR) (Rine and Herskowitz, 1987) and telomere proximal loci (Aparicio et al., 1991). Derepression of HM loci leads to expression of both \textit{a} and \textit{\alpha} mating type information, rendering haploid strains sterile (Rine et al., 1979). The mutation isolated in \textit{SIR4}, \textit{SIR4-42}, was recessive for starvation resistance and sterility, yet semi-dominant for life span extension (Kennedy et al., 1995). This bizarre set of phenotypes suggested that the \textit{SIR4-42} mutation alters the property of the protein such that some functions are reduced and others enhanced or created. Strains bearing the
SIR4-42 allele were also unable to silence genes placed near telomeres. These results have led to the hypothesis that SIR4-42 strains have longer life spans because Sir-mediated transcriptional silencing is re-directed from HM loci and telomeres to an as yet unidentified "Aging" locus (Figure 2). Increased repression of this Aging locus would delay aging. Consistent with this view, deletion of SIR4 shortened life span. This hypothesis is also consistent with previous experiments which reflect an inherent competition between HM loci and telomeres for recruitment of Sir proteins (Buck and Shore, 1995). The Aging locus would constitute a third locus or set of loci with affinity for Sirs.

The extension in life span directed by SIR4-42 required SIR2 and SIR3, indicating that it was a property of the Sir complex (Kennedy, et al., 1995). The mutation in SIR4-42 deletes the carboxyl 121 residues of SIR4. Recent experiments have demonstrated that this region of SIR4 binds to RAPI (Cockell et al., 1995; Moretti et al., 1994; Shore and Nasmyth, 1987), a protein present at HM loci and telomeres (Conrad et al., 1990). These results suggested that the SIR4-42 allele is resistant to RAPI-mediated recruitment to HM loci and telomeres, increasing the pool of Sir complexes capable of silencing other loci (Kennedy, et al., 1995). In addition, overexpression of the C-terminal 154 residues of SIR4 not only abolished silencing at HM loci and telomeres, but extended life spans only in strains expressing wild-type SIR4. In this case, the C-terminal fragment presumably interferes with the SIR4 - RAPI interaction allowing the wild-type Sir complex to be directed to the Aging locus. All of the evidence to date suggests that gene(s) at the Aging locus will be important determinants of yeast aging and identifying them is of major importance. However, it remains possible that the Sir complex is altering life span potential through a mechanism unrelated to transcriptional silencing.
Isolating a gene such as *SIR4* in a screen for yeast aging mutants was intriguing given its known function at yeast telomeres. Telomeres have been proposed to be fundamental factors in the mammalian aging process (Olovnikov, 1973; Schwartz et al., 1993; Vaziri et al., 1993) and in the related phenomenon of fibroblast senescence (Harley et al., 1990). Proper maintenance of telomere length in dividing fibroblasts depends on the activity of telomerase (Allsopp et al., 1992). This activity is not present in most primary human cell lines and becomes re-activated only in clones that survive crisis (Counter et al., 1992). This correlative evidence suggests that a reduction in telomere length may be the factor that precipitates senescence. \( \Delta sir4 \) strains display a reduced telomere length (Palladino et al., 1993). Surprisingly, the *SIR4-42* allele also causes a reduction in telomere length even though it extends life span (Kennedy, et al., 1995). This suggests that the ability of mutant alleles of *SIR4* to extend life span is not related to its role in telomere function. Moreover, telomere length is identical in young and old cells (D'Mello and Jazwinski, 1991; Smeal et al., 1996). Together, these experiments would seem to eliminate telomere length as a regulatory factor in yeast aging.

**Loss of Silencing in Old Cells**

The hypothesis that increasing the levels of silencing at an aging locus enhances division potential led to the suggestion that a loss of silencing may be key determinant of aging in yeast. This idea is supported by earlier experiments demonstrating that old cells become sterile (Müller, 1985). If sterility in old cells were related to a loss of silencing at *HM* loci, this would be the first case where a phenotype of old yeast cells could be explained at the
molecular level. It would also reflect a cellular change that was contributing to an age-related increase in the mortality rate.

In a recent study, the hypothesis that silencing of \textit{HM} loci is compromised in old yeast cells was addressed by several independent means (Appendix 1)\cite{Smeal96}. In one line of experiments, a novel method was deployed to obtain large populations of old yeast cells. This method is based on the observation that the cell surface of mother cells does not contribute to that of its subsequent daughters \cite{Linnemans77}\cite{Figure3}. Young cells were labeled on their cell surface with biotin, which covalently attaches to primary amine. These cells were allowed to divide for a fixed period of time and then were mixed with streptavidin-coated magnetic beads \cite{Smeal96}. Cells were then separated by placing a magnet next to the culture and selectively isolating the biotin-avidin coated cells. Cells recovered were those originally labeled, which had now divided many times. Northern analysis of these old cell populations revealed a five-fold decrease in the amount of RNA from \textit{STE12}, a haploid specific gene whose repression is dependent on simultaneous expression of both \textit{a} and \textit{\alpha} information \cite{Fields87}.

The observed decrease in expression of a haploid-specific gene suggested that \textit{HM} loci were being expressed in old cells \cite{Smeal96}. To directly establish this hypothesis, old mating type \textit{\alpha} cells were assayed for the appearance of \textit{a1} mRNA. Since \textit{a1} expression levels are not high, northern analysis required more old cells than could be effectively collected. To achieve a higher level of resolution, a RT-PCR based method was employed. PCR products derived from mRNA could be distinguished from those derived from genomic DNA since the \textit{a1} gene is one of the few yeast genes containing an intron \cite{Miller84}. The results demonstrated unambiguously that \textit{a1} was

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expressed in old α cells (Smeal et al., 1996). Expression of genes repressed through Sir-independent mechanisms was undetectable by RT-PCR in these old cells, demonstrating that the observed loss of repression was specific to HM loci.

These results suggest that derepression of silent mating type loci is the cause of sterility in old cells (Smeal, et al., 1996). To demonstrate this directly, two Mata strains were tested for their ability to respond to α factor during the aging process by micro-manipulation of individual cells. Cells of each strain were followed to different ages then moved near filters containing α factor. They were scored as responding to α factor only if they became arrested in the G1 phase of the cell cycle and if they formed characteristic projections or "schmoos" (Byers and Goetsch, 1975). A wild-type Mata strain began to lose α factor responsiveness after approximately 50% of its life span (Smeal, et al., 1996). Only a small fraction of very old cells of this strain retained their ability to respond to α factor. In contrast, Mata cells deleted for HMLα retain their ability to respond to α factor throughout their life span. These experiments corroborate the evidence that repression of HM loci is significantly diminished in old cells, and directly demonstrate that derepression of HM loci is the causal agent in an age-specific phenotype: sterility.

Experiments identifying and assessing the role of Sir proteins in the yeast aging process serve as a paradigm for yeast aging research (Kennedy, et al., 1995; Smeal, et al., 1996). That a mutation in SIR4 can alter the rate of aging in yeast does not on its own prove that this gene is important in the normal aging process. Linking sterility to the loss of function of the Sir proteins also provides evidence that the Sir protein complex may be a molecular target in the aging process. However, the fact that deleting SIR4 is not lethal suggests
that other events must occur in old mother that, together with the loss of silencing, cause aging. The unbiased screens described above may shed light on these events.

Uth Genes and Silencing

Two other genes identified in the screen for starvation resistance have been cloned and partially analyzed. *UTH1* encodes a protein with two putative domains separated by a linker of several serine residues (N.R. Austriaco and L. Guarente, unpublished). One of the domains has been termed the SUN domain and is conserved in several other yeast genes. The other genes have seemingly unrelated phenotypes.

*Δuth1* strains live approximately 25% longer than wild-type progenitors. Epistasis experiments place *UTH1* in a genetic pathway upstream of *SIR4*. Consistent with the hypothesis that *UTH1* inhibits silencing, *Δuth1* strains exhibit increased silencing at *HM* loci and telomeres. It remains to be determined in what manner Uth1 down-regulates Sir activity.

Uth4 also exerts its effect on yeast aging by regulating Sir activity (Chapter 4). Whereas *UTH1* seems to generally repress Sir activity, Uth4 regulates Sir activity in a locus specific manner. At telomeres and *HM* loci, *Δuth4* strains have increased levels of silencing. Life span, however, correlates directly with Uth4 levels suggesting that Uth4 increases silencing at the Aging locus. These results have led to a general model whereby Uth4 recruits silencing factors away from telomeres and *HM* loci to the Aging locus.

*UTH4* encodes an 834 amino acid protein. The central region of the protein contains eight 36 amino acid repeats. Several other proteins in a variety of organisms share this sequence motif, including one other yeast
protein, Ygl023 (Chen et al., 1991). Given the considerable homology between these proteins, the potential role of Ygl023 in transcriptional silencing was addressed. As in the case of Uth4, Ygl023 also recruits Sirs away from telomeres. However, there is no evidence that Ygl023 increases silencing at the Aging locus. Very likely, Ygl023 acts to increase silencing levels at yet another chromosomal locus.

Marker genes inserted at telomeres are transcriptionally silenced in a Sir-dependent manner, but there is no evidence that endogenous telomeric genes are subject to silencing. The Sir complex is required for localization of telomeres to nuclear periphery and contributes in a minor fashion to telomere length maintenance (Palladino, et al., 1993). Nevertheless, strains deleted for SIR genes maintain stable telomeres. Therefore the reason for the high concentration of Sirs at telomeres remains substantially unexplained. One emerging hypothesis is that telomeres serve as a reservoir for silencing factors so they can be quickly localized to other chromosomal loci as needed (Marcand, et al., in press). Uth4 and Ygl023 may be two of the conduits by which Sirs are re-directed.

Proteins with repeat regions similar to Uth4 and Ygl023 exist in a variety of organisms including humans, S. pombe, C. elegans and D melanogaster. With the exception of Drosophila pumilio (Barker et al., 1992; Macdonald, 1992), the role of these proteins remains unaddressed. pumilio functions to repress translation of the hunchback mRNA in a developmentally regulated manner. It directly binds the 3' untranslated region of hunchback (Murata and Wharton, 1995). Two obvious possibilities exist concerning the relationship between other repeat containing proteins and Uth4. Either the repeat domain behaves in a similar manner in proteins with different functions (i.e. as a conserved protein: protein interaction motif) or these
proteins function in a similar manner. The function of the Uth4 and Ygl023 repeats must be further addressed before the answer to this question can be determined. Nevertheless other proteins with these repeats are preliminary candidates for aging factors in their respective organisms.

Summary

The study of yeast aging has increased dramatically in recent years. In 1959, Mortimer and Johnston were the first researchers to question how many times one yeast cell can divide (Mortimer and Johnston, 1959). Their finding that the division potential of yeast cells is finite was not immediately pursued. Almost two decades later, the Jazwinski laboratory and the Müller laboratory undertook the task of determining the reasons for this limitation. Today several researchers have joined the field.

In the last few years, the aging phenomenon has finally been addressed from a genetic perspective. Several genes have been identified that either restrict or enhance life span potential. In some cases, the functions of the gene products are partially understood. UTH4 and SIR4 encode proteins which act as nuclear regulatory factors of the aging rate. Unfortunately, the gene(s) they help silence remain unidentified. Identification of these genes may finally lead to a molecular understanding of yeast aging.

A diverse range of organisms meet the standards of aging as defined by an exponential increase in the rate of mortality over time. This does not imply that the molecular determinants of aging in these organisms are identical or even related. It is presently impossible to address this question since aging is not well defined in any organism. However, two of the yeast aging genes identified (LAGI and UTH4) have potential homologues in a variety of
organisms including humans. Cloning these homologues and addressing their potential roles in aging may help determine the extent to which the aging process is conserved. In any case, yeast may well be one of the first organisms in which the aging process is defined.

References


Marcand, S., Buck, S.W., Moretti, P., Gilson, E., Shore, D. Nucleation of silent chromatin by Sir3p and Sir4p is controlled by sequestration of silencing factors at telomeres by Rap1 protein. Genes Dev. in press.


Table 1. Mutations that alter yeast life span potential.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of Mutation</th>
<th>Mean Life Span Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAG1</td>
<td>Loss-of-Function</td>
<td>47% Increase</td>
</tr>
<tr>
<td>RAS1</td>
<td>Loss-of-Function</td>
<td>25% Increase</td>
</tr>
<tr>
<td>RAS2</td>
<td>Loss-of-Function</td>
<td>23% Decrease</td>
</tr>
<tr>
<td>UTH1</td>
<td>Loss-of-Function</td>
<td>25% Increase</td>
</tr>
<tr>
<td>UTH2/SIR4</td>
<td>Gain-of-Function</td>
<td>50% Increase</td>
</tr>
<tr>
<td>UTH4</td>
<td>Loss-of-Function</td>
<td>50% Decrease</td>
</tr>
</tbody>
</table>
Figure 1. The genetic pathway of dauer larva formation

This figure has been redrawn from Larsen et al. (1995). The pathways are drawn to depict wild-type gene functions that regulate subsequent steps.
\( daf-1 \)
\( daf-4 \)
\( daf-7 \)
\( daf-8 \)
\( daf-14 \)

\( daf-2 \)
\( daf-12 \)
\( daf-23 \)

\( daf-3 \)
\( daf-5 \)
\( daf-16 \)

\( dauer \)

\( longevity \)
In wild-type young cells (a), the majority of SIR2,3,4 mediated transcriptional silencing occurs at HM loci, telomeres and the aging locus. As the cells age (b), silencing dissipates causing cells to lose division potential due to high level expression of the Aging locus. The cells also lose mating potential as transcription at HM loci is increased. Telomere silencing has not been directly analyzed in old cells. In SIR4-42 young cells (c), transcriptional silencing is re-directed from HM loci and telomeres to the Aging locus, strengthening silencing at that site. These cells are then sterile, but are presumably able to maintain silencing of the Aging locus through more cell divisions than SIR4 cells.

Figure 2. Model for life span extension by SIR4-42
(a) **SIR4 Young Cells**

- HM Loci
- Telomeres
- Aging Locus

(b) **SIR4 Old Cells**

- HM Loci
- Telomeres
- Aging Locus

(c) **SIR4-42 Young Cells**

- HM Loci
- Telomeres
- Aging Locus

- Sir 2, 3, 4 Protein Complexes
Preparing populations of old yeast cells

Cells are labeled on their surface with biotin and then allowed to divide for a period of time sufficient for them to undergo 10-15 cell divisions. The culture now contains the original cells, now old, and a large number of younger unlabeled cells. Avidin covered magnetic beads are then bound to biotin and the old cells are specifically sorted using a magnet. This process is feasible since the cell surface of emerging daughter cells is synthesized de novo at the budding site. Any buds attached to old cells will be retained in addition. Nevertheless, this process results in a substantial purification of old cells. The age of the cells can then be determined by staining the cell surface with Calcofluor and counting the number of bud scars present in a fluorescent microscope.
Label Cell Surface with Biotin

10-15 Generations

Bind Avidin-Magnetic Beads to Biotin

Separate Old Cells with a Magnet

Stain and Count Bud Scars
Chapter 2: Daughter cells of S. cerevisiae from old mothers display a reduced life span

This chapter was previously published in Journal of Cell Biology, Volume 127, pages 1985-1993 in 1994. The authors were Brian K. Kennedy, Nicanor R. Austriaco, Jr., and Leonard Guarente
Summary

The yeast *S. cerevisiae* typically divides asymmetrically to give a large mother cell and a smaller daughter cell. As mother cells become old, they enlarge and produce daughter cells that are larger than daughters derived from young mother cells. We found that occasional daughter cells were indistinguishable in size from their mothers, giving rise to a symmetric division. The frequency of symmetric divisions became greater as mother cells aged and reached a maximum occurrence of 30 percent in mothers undergoing their last cell division. Symmetric divisions occurred similarly in *RAD9* and *ste12* mutants. Strikingly, daughters from old mothers, whether they arose from symmetric divisions or not, displayed reduced life spans relative to daughters from young mothers. Because daughters from old mothers were larger than daughters from young mothers, we investigated whether an increased size *per se* shortened life span and found that it did not. These findings are consistent with a model for aging that invokes a senescence substance which accumulates in old mother cells and is inherited by their daughters.
Introduction

A hallmark of aging in an organism is that the probability of death increases exponentially with age (Gompertz, 1825). In Saccharomyces cerevisiae, cell division involves budding of daughter cells which are smaller than mother cells upon division (Hartwell and Unger, 1977). By micro-manipulating daughters away from mothers, the fate of mother cells can be followed during multiple rounds of cell division (Mortimer and Johnston, 1959). Through this kind of analysis, it was determined that mothers divide a relatively fixed number of times before stopping, and the probability of stopping increases exponentially as the number of prior divisions increases. These experiments therefore showed that yeast cells age and have a specific life span that varies around a given mean.

A number of phenotypes are manifest during the aging process in yeast. Cells enlarge as they age (Mortimer and Johnston, 1959). The increase in volume has been demonstrated to be linear with regard to the age of the cell (Egilmez et al., 1990). Aging cells divide more slowly: cell cycle time can increase as much as six-fold during the course of a cell’s life span (Mortimer and Johnston, 1959). Finally, a decrease in fertility has been observed in old cells (Müller, 1985).

When old cells are mated to young cells, the resultant diploids live for a number of generations most similar to the remaining life span of the older cells, suggesting that aging in yeast may be a dominant characteristic (Müller, 1985). This dominance may be due to some substance that is synthesized or accumulates in old cells. Consistent with the possibility of preferential synthesis in old cells, Egilmez et al. have demonstrated that certain mRNAs are preferentially found in old cells (1989).
Aging in yeast appears in many ways to be similar to senescence in mammalian fibroblasts. There appears to be an underlying genetic basis to both processes. Fibroblasts undergo an increase in cell size as they age (Sherwood, et al., 1988), and a correlation between cell size and senescence can be demonstrated by incubating young human diploid fibroblasts (HDFs) in low serum medium. These cells arrest in the G1 phase of the cell cycle, enlarge and display a decreased division potential when returned to normal serum containing medium (Angello et al., 1989). Also, cell fusion studies between old and young HDFs indicate that senescence is dominant (Norwood et al., 1974). Injection of polyA+ RNA from senescent HDFs into young cells was sufficient to inhibit DNA synthesis, further supporting the notion that senescence is dominant and suggesting that a gene or a number of genes encoding inhibitory proteins are expressed in senescent cells (Lumpkin et al., 1986). These apparent similarities between yeast and animal cells suggest yeast may be a fruitful model system in which to study aging. Indeed, the mechanisms of other cellular processes, such as that of transcription and cell cycle progression, appear to be conserved in these organisms.

Many theories have been put forth to explain yeast aging. The most prominent involves the accumulation of bud scars as the cell ages (Johnston, 1966). After each division, a mother cell accumulates a chitin-containing ring which covers approximately one percent of the surface area of the cell (Bartholomew and Mittwer, 1953). Since the cell wall of the daughter is newly synthesized in the process of division, old scars are not transferred from mother to bud (Farkas, 1979; Johnson and Gibson, 1966). In S. cerevisiae, these bud scars have never been observed to overlap on the surface of a cell (Bartholomew and Mittwer, 1953). If metabolic processes are altered at the site of a bud scar, the mother may continually lose active surface area until it
becomes unable to maintain division potential. Short-term exposure of \textit{cdc24} ts mutant cells to the restrictive temperature, conditions under which it accumulates chitin in the cell wall, did not affect their life span (Egilmez and Jazwinski, 1989). Therefore, artificially induced deposition of a major component of bud scars is not life-shortening.

The increase in size as a mother cell ages results in a decrease in the surface area-to-volume ratio. Researchers have speculated that a decrease in the ratio below a certain point may be detrimental to the cell (Mortimer and Johnston, 1959). Once the cell reaches a certain volume, transport of metabolites may no longer be sufficient to satisfy the growing need and thus the old cell may arrest division by a mechanism similar to starvation induced arrest.

In this report, we demonstrate that old mother cells are unable to produce daughters with a full life span potential, further indicating that bud scars are not the direct cause of aging. Instead these mothers produce larger than normal daughters with severely restricted life spans. Although these experiments show a direct correlation between size and life span of daughters cells, increasing the size of small daughters by a physiological regime did not shorten their life spans. Our findings are consistent with a model for aging in which a substance accumulates in old mother cells to cause senescence. When this substance is inherited by daughters, their life spans are shortened.

\textbf{Results}

The haploid strain PSY142 was used in all of the experiments presented. Figure 1 depicts the mortality curve for this strain. The mean life span for
this strain was 29.1 generations with a standard deviation of 10.9 generations. The aging characteristics of this strain follow a Gompertz distribution; the rate of death increases exponentially with age (see Materials and Methods). This is characteristic of many organisms including yeast (Pohley, 1987).

**Symmetric Divisions in Old Mother Cells**

During the life span determination of this strain, a number of observations were made concerning the size of the buds being removed. Mothers in the last third of their life span produced daughters that were significantly larger than normal and, in the extreme, were indistinguishable in size from the mother (a symmetric division). In no case was a daughter obviously larger than the mother at the time of division. Visual observation of divisions destined to be symmetric indicated that they occurred through the normal yeast budding process; the daughters were initially visible as small buds before enlarging to abnormal sizes. Also, the size of the mother cell did not decrease noticeably upon symmetric division. Instead, the daughter grew aberrantly large. These observations have also been made with a number of other unrelated strains in our laboratory (data not shown).

Larger and/or symmetric divisions occurred later in a mother's life span. Figure 2a shows a young mother undergoing a normal asymmetric division, while figure 2b depicts an old mother producing a bud larger than usual, yet asymmetric. Symmetric divisions are shown in figures 2c and 2d. In all non-symmetric cases the buds were micro-manipulated away from the mother prior to photography to demonstrate that the budding cycle was completed.
We sought to quantitate the frequency of appearance of symmetric cell divisions as a function of the relative age of the mother cell (figure 3). Since the life spans of the mother cells varied substantially, the best way to align the mothers for analysis was to consider not how many buds a mother had produced prior to a symmetric division, but what percentage of that mother's own life span had been completed. No symmetric buds were observed in mothers which had completed less than 50% of their life span. However, as the mothers aged further, symmetric budding began at a low frequency and increased exponentially to a maximum of 30% during the last five percent of the mothers' life span.

The daughters arising from symmetric divisions were not aberrant in that they gave rise to normal asymmetric divisions (8/11 divisions scored) at a frequency similar to daughters arising asymmetricly from old mothers (7/11 scored in the same experiment).

**Symmetric Divisions Do Not Require Integrity of the \textit{RAD9} Checkpoint or the Pseudohyphal Growth Pathway**

We considered the possibility that symmetric divisions arose in old mother cells as a consequence of a pause in the cell cycle due to the accumulation of genetic damage. This pause could cause the bud to grow abnormally large. The one well characterized regulatory step that halts the cell cycle in response to DNA damage is the \textit{RAD9} checkpoint (Weinert and Hartwell, 1988). Thus, we examined whether symmetric divisions occurred in \textit{\textDelta RAD9} cells and their isogenic \textit{RAD9} parent at comparable frequencies. Initially, we found that the \textit{\textDelta rad9} mutant YJJ53 had a life span that was reduced about 30% compared to the isogenic \textit{RAD9} parent DBY747 (Figure 4a).
Nonetheless, symmetric divisions were observed, and, when plotted as a function of the life span of the \textit{RAD9} strain, these divisions occurred at a frequency indistinguishable from the \textit{RAD9} parent (Figure 4b).

We also considered whether other cases in which normal budding is altered might be relevant to symmetric divisions. One such case is pseudohyphal formation, which results in symmetric divisions and requires the integrity of the pheromone-response pathway (Liu et al., 1993). We determined whether this pathway was also required for symmetric divisions by constructing a deletion of \textit{STE12} in PSY142 using plasmid pNC163 (Company et al., 1988), and analyzing these cells microscopically. The deletion of \textit{STE12} did not shorten the life span of PSY142 (Figure 4c). Moreover, there was no substantial change in the frequency of symmetric divisions in the \textit{Astel2} strain (Figure 4d).

\textbf{Decreased Life Span in Daughters from Old Mother Cells}

The life spans of daughters derived from mothers of varying ages were determined by taking at random daughters produced during different points in mothers' life spans. In figure 5, the mean life span of daughters is plotted against the percent of the mothers' life span that had been completed at the time of the division. Daughters from mothers in the first 40% of their life span generally enjoyed full life spans themselves, while daughters from older mothers exhibited reduced life spans, the percent reduction increasing progressively as the mothers increased in age. At the extreme, daughters from mothers in the last ten percent of their life span lived only 25% as long as the mothers from which they were derived.
This decrease in life span in daughters of old mothers was not heritable. We analyzed the life spans of daughters of the daughters of mothers of varying ages (grand-daughters). Figure 6 shows that the reduced life spans of daughters of old mothers was restored back toward normal in grand-daughters. Further, great-grand-daughters, great-great-grand-daughters, and great-great-great-grand-daughters all displayed a similar normal life span, regardless of the age of the mother cells from which they descended.

To determine if the increased relative size of daughters from old mothers was correlated to the decreased life span, the life spans of symmetric buds were analyzed and compared to both the mean life span of the strain and the remaining life spans of the mothers after they produced the symmetric bud (Figure 7). An analysis of variance (ANOVA) was performed to compare the variance of each set of two data points relative to the variance of all points combined. The results demonstrate that a symmetric bud's life span was significantly more similar to the post-symmetric division life span of its mother than to the life spans of the other cells in the data set (p<0.05). In fact, the remaining life spans of the daughters and mothers in a symmetric division are not obviously different. The data in Figure 7 compares the life spans of symmetrically arising daughters with the life spans of cells randomly distributed with regard to age. However, their life span is also much shorter than asymmetrical daughters from older mothers (i.e. mothers of a similar age to those giving symmetrical daughters, not shown).

The substantial reduction in the mean life span of daughters derived from old mothers indicates that bud scars are not a necessary agent in yeast aging. The similarity in the remaining life spans of daughters and mothers from symmetric buddings is a further confirmation of this conclusion. The only way to resurrect the bud scar hypothesis is to assume that many of the
bud scars are somehow transferred to the bud in a symmetric division (an event which has been shown not occur in typical asymmetric divisions). If many of the bud scars were being transferred to the daughter in instances of symmetric divisions, not only would the daughter's life span be diminished, but the mother's life span would be substantially lengthened. To determine the effect on the mother cell, the life spans of 56 cells were compared to the number of symmetric buds they generated. Table 1 depicts both the number of cells having 0,1,2 etc symmetric buds and the mean life spans of those cells. Mothers which produced more symmetric buds had at most a slight increase in mean life span, though not the mean life span increase that would be expected if many of the bud scars had been transferred to the daughters. The slight observed increase in life span can be explained simply by the fact that mothers who live longer will have more chances to produce symmetric buds. It is thus likely that the production of symmetric buds does not affect the mother's life span.

Increased Size Does Not Shorten Life Span

Because old mother cells are larger than young mother cells, their daughters are larger than daughters from young mothers. A correlation between the size of mother cells and their age was noted previously (Mortimer and Johnston, 1959), and it was proposed that an increase in size might cause senescence. We thus wished to test whether the shortened life span in daughters from old mothers was due to their large size. Our approach was to cause the size of young cells to increase in the absence of cell division, and to measure whether this shortened their life span. We used α-factor to arrest cells from a random culture at start. After four hours of arrest, these treated
cells were much larger than cells of the same culture that were not treated (Figure 8A). The size of the treated cells remained large throughout their life spans. The arrows in the untreated control cells indicate cells that are in G1 and can be directly compared to the arrested cells. The α-factor was removed and mortality curves derived for the treated and control cells. Although we could not determine the age of cells at the start of the experiment, in a random population 1/2 are virgins, 1/4 are mothers that have divided once, 1/8 are mothers that have divided twice, etc. Thus these life spans will closely approximate those starting with only virgin daughters. Strikingly, there was no difference in the life spans of these two samples (Figure 8B), indicating that an increase in cell size does not necessarily lead to a shortening in life span.

Discussion

In this report we have microscopically followed mother cells in the budding yeast, *S. cerevisiae*, through many cell divisions to senescence. As previously noted, the number of cell divisions that mother cells undergo to give rise to daughters is finite and fixed around a distribution characteristic of aging in many organisms (Mortimer and Johnston, 1959; Pohley, 1987; Sacher, 1978). In this Gompertz distribution, the probability of cessation of life increases exponentially with age. Further, the size of mother cells, as well as their daughters, increases with age. We made three observations, discussed in greater detail below, that provide insight into the aging process. First, the fidelity with which cells divide asymmetrically to give a small daughter cell and a large mother cell, the normal mode of cell division in budding yeasts,
decreases with age. Older cells can display symmetric divisions, in which the bud grows to the same size as the mother at division. The oldest mother cells, which are in the last 10% of their life span, feature up to 30% symmetric divisions. Second, the daughter cells of the oldest mother cells have much shorter life spans than daughters of younger cells. This finding goes against the conventional view that the full life span is regenerated in all daughter cells (Johnston, 1966). Third, an increase in cell size does not shorten life span.

**Symmetric Divisions**

Why do symmetric divisions occur in old mother cells? One possible explanation is that an active mechanism is required to give rise to the normal asymmetric divisions in budding yeasts and this mechanism breaks down in older cells. Whether such a breakdown may be related to the events that cause senescence is not evident. It is clear that symmetric divisions are not related to aging in any obligatory way. A significant fraction of aging cells reach senescence without ever giving rise to a symmetric division. Intriguingly, after a symmetric division, the life spans of both the mother and daughter cells are short and approximate the remaining number of divisions that a mother cell of that age would be expected to possess. Thus, the mother cell apparently does not gain any division potential by giving rise to a daughter of equivalent size.

Several possible explanations for symmetric divisions have been eliminated. One is that old cells accumulate DNA damage which invokes the RAD9 checkpoint (Weinert and Hartwell, 1988) to slow the cell cycle in emerging buds. We report that deletion of RAD9 does not reduce the frequency
of symmetric divisions. We can not rule out the possibility, however, that other controls on the cell cycle slow progression in old cells and give rise to symmetric divisions. While our observations suggest that symmetric cycles occur over a longer period of time than asymmetric cycles, our attempts to quantitate these measurements were confounded by a highly variable time required for cytokinesis and daughter cell detachment in old cells.

While deletion of RAD9 does not eliminate symmetric divisions, it does result in a significant shortening of the life span (by about 30%). This could indicate that RAD9 serves a function in non-irradiated cells, perhaps to allow repair of DNA that is damaged in the absence of irradiation. When RAD9 is deleted, the accumulation of genetic damage may impose an artificial limit on the life span of cells.

A second explanation for symmetric divisions is that an altered program of budding is accessed in old cells, such as that used in pseudohyphal growth, which involves symmetric divisions and requires the pheromone-response pathway (Liu et al., 1993). Again, since deletion of STE12 does not alter symmetric divisions in old cells, we conclude that there must be some other underlying basis.

It would be of interest to inquire whether regulatory processes based upon the distinction between mother and daughter cells break down after a symmetric division. One such process is regulation of the HO endonuclease that initiates mating type switching (Strathern and Herskowitz, 1979). The HO gene is transcribed only in mother cells (Nasmyth, 1983). The basis for this regulation is not known but may relate to the increased time in G1 spent by daughter cells to increase their size (Nasmyth et al., 1990). After a symmetric division, because the mother and daughter are the same size, they appear to transit G1 with equivalent kinetics. If HO regulation were retained after
symmetric divisions, then some difference between mother and daughter cells that is not related to their difference in size would be implicated in the control.

**Decreased Life Span in Daughters of Old Mothers**

The decrease in the life spans in daughters of old mothers is substantial, 7.9 divisions in daughters from mothers in the last 10% of their life spans, as compared to 26.5 divisions for the daughters of mothers in the first 70% of their life span. Daughters from mothers in the last 70-80% and the last 80-90% of their life spans show reductions intermediate between daughters from young mothers and daughters of the oldest mothers. The reduction in life span applies to daughters arising from asymmetric and symmetric divisions alike.

What does this reduction in life span imply about the mechanism of senescence? We considered two models consistent with these findings. First, the increased size of daughters from old mothers *per se* shortens their life span. To address this possibility, we used α-factor to increase the sizes of G1-arrested cells in a random population. Mortality curves of the treated cells and untreated controls were indistinguishable. Since the treated cells were much larger than the control cells throughout their life spans, this experiment shows that an increase in cell size does not necessarily cause a shortening in life span.

A second model consistent with our findings is that the daughters of old mothers inherit a substance that shortens their division potential. Assuming the premature aging of these daughters is related to the normal senescence of mother cells, the substance would be the agent that accumulates in old mother cells to cause senescence. That substance may occur in the form of macromolecular damage that cannot be repaired rapidly enough to prevent
accumulation. The levels of this substance may be so high in old cells that daughters have a high probability to inherit a portion. Since this shortening of life span in daughters does not occur until mothers are very advanced in their aging program, it is likely that the substance does not accumulate until cells are fairly old. According to this model, the substance could kill cells directly or prevent growth by arresting the cell cycle.

Can the damage that accumulates in old cells be genetic damage? Several observations render this explanation unlikely. First, the daughters of the daughters of old mother cells displayed a life span that was corrected back toward normal. This finding is consistent with a senescence substance that is inherited in daughters of old mothers and is diluted in subsequent generations. It is not consistent with a theory of aging invoking damage to the DNA. Second, the cell cycling time of daughters from old mothers is increased in their first cell cycle (Egilmez and Jazwinski, 1989). This slowing of the cell cycle is alleviated in subsequent divisions. Third, yeast chromosomes are not inherited in a manner that is biased to confine the old DNA strand to the mother cell (Neff and Burke, 1991). However, none of these findings excludes the possibility that genetic damage might occur in a minority of old cells.

The reduction in life span in daughters of old mothers argues against bud scars as the immediate causative agent in yeast senescence. Bud growth in old cells is visually identical to growth in young cells. Thus, it is very likely that the physical parameters of bud growth, including confinement of the bud scars to the mother cells, does not change in old cells. Since the cell wall and membrane of the bud are derived from new synthesis, the substance inherited by the daughters of old mothers is probably an intracellular constituent. It is still possible, however, to retain the notion that bud scars cause senescence, but only if their effect is indirectly mediated by an intracellular component.
In summary, we have described several properties of aging in *S. cerevisiae* that help delimit possible mechanisms of senescence in that organism. Our findings argue against two proposals for aging in this organism: an accumulation of bud scars, or an enlarged cell size. Rather, we suggest that an intracellular substance accumulates in old mother cells that is generated by a failure to repair macromolecular damage. The identity of this substance and insights into its generation would shed light on the aging process in yeast cells, and perhaps other eukaryotic cells.

**Experimental Procedures**

**Strains and Media**

The haploid strain PSY142 (*α lys2-801, ura3-52, leu2-3, 112*) was used in most experiments reported. In the α-factor experiment, strain JFC17 (*MATα, his4, ura3-52, leu2-3, 2-112*) was used. All experiments were conducted on complete medium (YPD) prepared as described (Sherman et al. 1986). DBY747 and its ΔRAD9 derivative YJJ53 were a gift of L. Prakash (Schiestl et al. 1989). The *STE12* disruption was constructed using plasmid pNC163 as described (Company et al. 1988).

**Life Span Determinations**

To determine the life span of a strain, cells were taken from logarithmically growing liquid cultures and plated at low density on complete medium. The cells were incubated at 30°C for approximately 3 hours. At this
time daughter cells were isolated as buds that had emerged from mother cells and moved with a Zeiss Micro-manipulator to uninhabited parts of the plate. All future buds produced by these daughter cells were then micro-manipulated away. The plates were grown at 30°C during working hours and shifted to 4°C overnight. The life spans generated in this manner were compared to life spans from cells incubated at 30°C continuously and the means were not found to be statistically different (unpublished data). The positions of mother cells relative to partially formed buds were carefully noted to distinguish mothers from daughters during symmetric division. The daughters were then picked with the needle and moved to a different location on the plate for analysis of life span. On very rare occasions, a cell was observed to lyse immediately after micro-manipulation and was excluded from the data set. We observed that it was important that the initial daughter cells are isolated from a logarithmically growing culture prior to being micro-manipulated, otherwise they frequently do not begin to divide.

Photography

Photographs of cell divisions were taken with Nomarski Optics at 1000X magnification using a Zeiss Axioskop microscope with an accompanying Zeiss MC100 camera attachment.

Statistical Methods

To determine if the strain PSY142 had an exponential increase in the rate of senescence, the rate of death per generation was calculated in the
range where a sufficient amount of data was available and this rate was shown to increase exponentially by standard statistical methods.

The analysis of variance (ANOVA) was conducted according to standard statistical methods.

Acknowledgments

We would like to thank Chris Kaiser for use of his Zeiss microscope and camera, L. Prakash for the RAD9 strains, and B. Errede for permission to use the Δste12 vector. This work was supported by grants to L.G. from NIH (AG11119 and GM30454). NRA is a predoctoral fellow of the Howard Hughes Medical Institute.

References


### Table 1: Life Span of Mothers with Differing Numbers of Symmetric Buds

<table>
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<th>Number of Symmetric Buds</th>
<th>Number of Mothers</th>
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<td>21</td>
<td>2.7</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
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<td>2</td>
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<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
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<tr>
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<td>2</td>
<td>3.2</td>
</tr>
<tr>
<td>&gt;4</td>
<td>0</td>
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*Table 1.* The mean life spans of mothers producing differing numbers of symmetric buds. No mothers from this strain were observed to produce more than 4 symmetric buds. In other strains, as many as 6 symmetric buds have been recorded (data not shown).
Figure 1. The mortality curve for strain PSY142

The method used to determine the life span of this strain is described in the Materials and Methods.
PSY 142 (SAMPLE SIZE = 106)
Figure 2. Symmetric and asymmetric cell divisions

All photographs were taken at 1000x magnification using Nomarski optics. (A) A mother having completed one generation with her daughter. Mother cells are labeled with the letter "m" and daughters with the letter "d". (B) A mother cell and her 42nd daughter to the right. While this is clearly an asymmetric division, the daughter is noticeably larger than the daughter cell in (A). (C) A symmetric division. The mother has undergone 26 divisions prior to this one. (D) Another symmetric division. The mother has undergone 30 divisions prior to this one.
Figure 3. The exponential rate of increase of symmetric division with increasing age of the mother cell

The age of the mothers are given in terms of the percent of a mothers life span that had been completed when a symmetric bud occurred. The life spans of 38 mothers were followed in this experiment. The column labeled "overall" provides the percent likelihood per cell division that a mother would produce a symmetric bud regardless of its age. Symmetric buddings occur at a rate of 4.0% for the strain PSY142.
Figure 4. Life spans and symmetric bud formation in $\Delta RAD9$ and $\Delta ste12$ strains

Panels A and B depict the wild type DBY747 and its isogenic $RAD9$ derivative YJJ53 examined for life span (panel A) and symmetric bud formation (panel B). Panels C and D show analogous experiments for PSY142 and its isogenic $\Delta ste12$ derivative. In both experiments the sample consists of 40 cells of the wild type and 40 cells of the mutant, which were followed microscopically for life span and symmetric divisions.
Figure 5. The progressive decrease in the life span of daughters as a function of the mothers age at the time of division

The strain column reflects the mean life span for the strain. The sample sizes for each column are as follows: strain (106 cells), 0-10 (16), 10-20 (12), 20-30 (10), 30-40 (7), 40-50 (10), 50-60 (11), 60-70 (8), 70-80 (10), 80-90 (9), 90-100 (8).
MOTHERS' LIFE SPAN FROM WHICH DAUGHTER WAS DERIVED
Figure 6. The shortening in life span in daughters from old mothers declines in their descendents

Life spans of daughters from mothers of varying ages were determined microscopically. Daughters were from mothers that had passed through the first 60% of their life span (solid black, 11 cells), 60-80% of their life span (dark hatched, 6 cells), 80-90% of their life span (solid grey, 5 cells), or 90-100% of their life spans (light hatched, 21 cells). The life spans of the first daughters of each of these cells (grand-daughters) were also determined, and so on for great grand-daughters and great-great grand-daughters. The differences in life spans of daughters depending on the age of their mothers is evident in the first generation, and is reduced or eliminated in subsequent generations.
Figure 7. The life spans of symmetric buds compared to the post symmetric bud life spans of their mothers and the life span of the strain

The sample size is 19 sets of symmetric buds and their mothers. The strain sample size was 106 cells.
Figure 8. Enlargement of young cells does not shorten their life span

Cells from a growing culture of JFC17 (at a density of $10^7$ cells/ml) were treated with 20μg/ml α-factor for four hours. After washing away the α-factor, cells were spread on plates for life span analysis. Panel A shows untreated cells and panel B shows treated cells. The arrows point to cells in G1, the sizes of which can be compared to the G1-arrested cells in panel B. Panel C shows that the life spans of the arrested and control cells are very similar.
Chapter 3: Mutation in the silencing gene SIR4 can delay aging in Saccharomyces cerevisiae

This chapter was previously published in Cell, volume 80, pages 485-496 in 1995. The authors were Brian K. Kennedy, Nicanor R. Austriaco, Jr., Jisi Zhang, and Leonard Guarente. I would like to specifically recognize the contribution of Nick Austriaco to this paper. The screen and initial genetic characterization of aging mutants was a joint effort.
Summary

Aging in *Saccharomyces cerevisiae* is exemplified by the fixed number of cell divisions that mother cells can undergo (termed their life span). We have exploited a correlation between life span and stress-resistance to identify mutations that extend the life span. These mutations fall into four genes, one of which is *SIR4*, encoding a component of the silencing apparatus at *HM* loci and at telomeres. *sir4-42* is an amber mutation that removes 121 residues from the 1358 residue protein. This allele extends life span by more than 30%, and is semi-dominant for this property. The *sir4-42* mutant, like a *sir4* null mutant, exhibits shortened telomeres and is defective in silencing at the *HM* loci. However, a *sir4* null mutant does not extend life span. These findings suggest that *sir4-42* may extend life span by preventing recruitment of the SIR proteins to *HM* loci and telomeres, thereby increasing their concentration at one or more other chromosomal regions. Maintaining silencing at these other regions may be critical in preventing aging in this organism. Consistent with this view, expression of only the carboxyl-terminus of *SIR4* interferes with silencing at *HM* loci and telomeres and also extends the life span. Possible links between silencing, telomere maintenance, and aging in this and other organisms are discussed.
Introduction

Aging is a process in which all individuals of a species undergo a progressive decline in vitality leading to death. Many ideas on the causes of aging have been proposed, including the accumulation of damage to cellular constituents, and the implementation of a developmentally determined state (Finch, 1990). A classical definition of aging, which is based on the statistical analysis of individuals, states that the probability of death increases exponentially with age (Gompertz, 1825). This conclusion, originally deduced from studies of human actuarial tables, applies to many diverse organisms that also display morphological landmarks of aging (Finch, 1990).

Aging has also been studied in the budding yeast, *S. cerevisiae*. This yeast divides asymmetrically to give rise to a larger mother cell and a smaller daughter cell. In this organism life span is defined by the number of cell divisions undergone by mother cells before they stop dividing (Müller, et al., 1980). Mortimer and Johnston (1959) followed the fate of mother cells through many rounds of cell division by manipulating the daughter cells away microscopically and showed that mothers had a relatively fixed life span. The probability of these mothers dying increased exponentially with the number of cell divisions that they had undergone (Jazwinski et al., 1989; Pohley, 1987). Thus aging is thought to occur in yeast, with the metric of age equaling the number of divisions undergone by mother cells.

An early hypothesis proposed to explain yeast aging was that cell death is due to the accumulation of bud scars, which are chitinous rings deposited on the surface of the mother cells to mark each cell division (Johnston, 1966). However, several experiments indicate that bud scars do not cause cell death. First, shifting a *cdc24* temperature-sensitive mutant to the restrictive
temperature will cause an accumulation of chitin on the cell surface, but does not limit life span when cells are returned to the permissive temperature (Egilmez and Jazwinski, 1989). Second, daughters arising from old mother cells have a longer cell cycling time than daughters arising from young mothers (Egilmez and Jazwinski, 1989). Third, daughters from old mothers have a reduced life span compared to daughters from young mothers (Kennedy et al., 1994). Since daughters arising from old mothers do not contain bud scars, these latter two observations indicate that bud scars are not a direct cause of aging. These experiments, rather, suggest the possibility of a senescence factor that accumulates in old mother cells and which can be inherited by their daughters.

It has been suggested that the senescence of human diploid fibroblasts (HDFs) in culture is a model for aging (Goldstein, 1990; Hayflick, 1965). This claim is bolstered by the finding that fibroblasts from patients with the premature aging syndrome, Werner's syndrome, divide fewer times in culture before senescing (Salk et al., 1981). Two features of senescent HDFs bear similarity to aging yeast cells. First, in both cases cells undergo enlargement (Egilmez, et al., 1990; Mortimer and Johnston, 1959; Sherwood, et al., 1988) and a slowing in the rate of cell division as they age (Grove and Cristofalo, 1977; Mortimer and Johnston, 1959). In the case of yeast, the correlation between cell size and aging is not an obligate one (Kennedy et al., 1994). Second, in both cases, senescence is dominant. This was demonstrated by cell fusions between old and young HDFs (Norwood et al., 1974) or by mating between old and young yeast mother cells (Müller, 1985).

A limitation to studying aging in any of these systems is the difficulty in identifying genes that could be important in the process. In mammalian cells, cell fusion experiments have identified four chromosomal loci that
appear to be important in promoting senescence (Pereira-Smith and Smith, 1988). While chromosomal locations of these loci have been narrowed (Hensler, et al., 1994; Ning, et al., 1991; Sugawara, et al., 1990), they have not yet been cloned. In *C. elegans* a single mutation, *age-1*, extends the life span of the worm by 50% (Friedman and Johnson, 1988; Johnson, 1990). Both *age-1* and the gene for Werner's syndrome have been mapped (Goto, et al., 1992) but not yet cloned.

The potential for isolating mutants with altered life spans makes the yeast system attractive for studying aging. However, a barrier to attaining this goal is the difficulty in applying genetic strategies to a phenomenon that can only be measured at the level of individual cells. Here, we describe an approach that circumvents this difficulty, resting on the observation that certain yeast strains with longer life spans are more stress-resistant than strains with shorter life spans. We describe mutations in four genetic loci which increase the life span of the parental strain 20-55%. We did not seek mutants with shorter life spans because we imagined that their effects could be less specific. One of the loci that promoted longevity corresponds to a known gene involved in silencing of chromosomal domains. The nature of this mutation and its interaction with other genes involved in silencing provides a framework upon which to build models of aging in yeast.

**Results**

**Strategy for the isolation of longer-lived mutants.**

The strategy we adopted to isolate mutants with longer life spans results from two observations. First, we determined the life spans of several unrelated
lab strains and found that they were all different. A typical mortality curve is shown if FIGURE 1, in which the life spans of two strains are determined by plotting the percentage of cells from the initial population (chosen to be generation 1 daughter cells) that remain viable after an increasing number of divisions. The average number of divisions of the initial population is the mean life span, which is 18 and 29 for these two strains, BWG1-7A and PSY142. Similar life spans were obtained in assays in which cells were kept at 30°C, or in assays in which cells were refrigerated overnight. For most of the life span assays described in the paper, cells were stored at 4°C overnight for convenience. When the above strains were crossed, segregants displayed a spectrum of mean life spans that varied over a three-fold range. The variation was due to the segregation of more than one gene. A typical tetrad is shown in FIGURE 2a in which life spans vary from 13 (14c) to 37 (14d).

A second important observation for our approach was a correlation between life span and stress resistance in segregants of the BWG1-7A X PSY142 cross. This observation was initially made when we tried to recover cells from plates that had resided at 4°C for an extended period of time. Strains with longer life spans contained a greater percentage of viable cells after this period. Thus, the ability of these strains to remain viable upon storage mimicked their life spans. FIGURE 2b shows the viable count of each segregant of tetrad 14 after storage at 4°C for 4.5 months. Next, we tested whether a simple starvation protocol would elicit similar differences in survival. Cells were starved for nitrogen and then plated on complete media after intervals of time. FIGURE 2c shows that the ability of these strains to withstand seven days of starvation again correlates with the length of their life spans. Other assays of stress tolerance, such as heat shock resistance, also showed this same pattern (data not shown). This finding suggested that genes affecting life span
could be identified by isolating mutations of segregant 14c that caused an increase in resistance to starvation.

**Isolation and characterization of longer-lived mutants.**

BKx1-14c was mutagenized and grown into colonies, which were then replica-plated to media without nitrogen or carbon sources. After incubating under these starvation conditions for eight days, colonies were replica-plated back to rich plates. 39 mutants were recovered that survived this treatment, thereby displaying an increased stress-resistance. Of these, 8 exhibited life spans which were elongated compared to the parent (by 20-55%). The mutants all exhibited other phenotypes consistent with an increase in stress tolerance. They were more resistant to heat shock and showed an enhanced ability to grow on ethanol (a carbon source that induces the heat shock response in *S. cerevisiae* (Plesset, et al., 1982)). The link between starvation and heat shock was not surprising because starvation is known to induce heat shock proteins (Kurtz, et al., 1986). Further, the mutants all grew to a higher saturation density than the parent, probably because of their enhanced ability to utilize the ethanol in the media after the diauxic shift. One mutant, designated uth2-42, displayed two additional phenotypes: it mated poorly, and exhibited a bipolar budding pattern characteristic of diploids.

Eight mutants were crossed to BKy5 (isogenic to 14c, but with the opposite mating type), sporulated, and seven were shown to segregate 2:2 for stress-related phenotypes in more than 10 tetrads each. The uth3-335 mutant may have an additional mutation that contributes to stress-resistance. Genetic analysis indicated that seven mutants were recessive and one was dominant for stress phenotypes. The recessive mutations fell into three
complementation groups. The dominant mutation was not linked to representatives of any of these groups, and representatives of each group were not linked to each other. These genes were designated \textit{UTH1-4} (youth) and mortality curves for each complementation group are shown in FIGURE 3 a-d. The differences in life span in this and subsequent experiments presented in this paper were deemed significant by a Wilcoxon signed rank test (Experimental Procedures).

Because of its additional phenotypes, we focused on \textit{uth2-42}, and showed that sterility and bipolar budding pattern both cosegregated with stress tolerance. Moreover, in three complete tetrads we found that a lengthened life span also cosegregated with the other mutant phenotypes. Thus, a single mutation is likely to cause all of these phenotypes. Finally, we found that the extension in life span caused by \textit{uth2-42} was observed in a continuous assay in which cells were kept at 30° C for the entire course of the experiment (data not shown).

\textbf{Cloning \textit{UTH2} and its identity to \textit{SIR4}.}

\textit{UTH2} was cloned by its ability to restore mating to the \textit{uth2-42} strain, assayed by replica-plating transformants to a lawn of a tester strain of opposite mating type (CKy21). Positive clones were recovered in E. coli, and one, pBK40, was able to confer efficient mating ability when retransformed back into yeast. This clone also restored starvation and heat shock sensitivity to the \textit{uth2} mutant. pBK40 contained an insert of approximately 8kb. An internal fragment was labeled and used to probe a panel of \textit{\lambda} clones containing yeast DNA (Riles, et al., 1993). The \textit{\lambda} clone that hybridized contained \textit{SIR4}, a component of the yeast silencing complex that represses copies of \textit{MAT}α and
MATa information at HML and HMR (Hartwell, 1980; Laurenson and Rine, 1992; Rine and Herskowitz, 1987). Restriction enzyme mapping of pBK40 indicated that it contained SIR4 and at least 1kb of flanking DNA to either side. A loss of function mutation in SIR4 would explain the sterile and bipolar budding phenotypes of the uth2 mutant. To determine linkage, the insert was transferred to a LEU2-containing integrating vector and targeted to the SIR4 locus in BKy5. This integrant (BKy30) was mated with uth2-42 (containing pBK40 to allow mating) and, after eviction of pBK40, the diploid sporulated. 13/13 tetrads contained 2 Leu+, fertile : 2 Leu-, sterile segregants, showing that SIR4 is tightly linked to the uth2-42 mutation. We concluded that uth2-42 was probably an allele of SIR4 (now designated as sir4-42).

Specificity of elongation of life span.

The SIR4 mutant is sterile because it expresses a and α information simultaneously. We carried out two experiments to show that the lengthening of life span was not because of the a/α cell type. First, we determined the life span of the isogenic BKx1-14c/BKy5 diploid, designated BKy6, (Experimental Procedures) and found that, as expected (Müller, 1971), it was not significantly different from the haploid parents (Table 2). Second, we introduced into BKy5 a plasmid which expressed MATα, and found that this transformant also did not have a lengthened life span (Table 2).

In C. elegans, it has been shown that certain mutations that cause sterility also confer a lengthened life span (Van Voorhies, 1992). To determine whether sterility, more generally, could be related to life span in S. cerevisiae, we disrupted STE4 or STE12 (Experimental Procedures), genes involved in the
mating pheromone-response pathway. Again, life span was not affected in either of these sterile strains (Table 2).

Finally, because the stress and mating phenotypes of \textit{sir4-42} were recessive, we surmised that the phenotype of a \textit{SIR4} null mutation would mimic that of \textit{sir4-42}. The entire \textit{SIR4} gene was deleted in BKx1-14c (Experimental Procedures), and the strain with the deletion (BKy104), indeed, was stress tolerant, sterile, and exhibited bipolar budding (data not shown). Importantly, however, it did not have a lengthened life span (FIGURE 4A). In fact, because of the large number of cells studied in this experiment, we can conclude that the deletion shortened life span by a small, but statistically significant, degree.

The above finding suggested that the effect of \textit{sir4-42} on life span, unlike its effects on stress and mating, might be due to a gain of function. To test this, we determined whether the \textit{sir4-42} allele was dominant to \textit{SIR4} for the phenotype of lengthened life span. The wild type \textit{SIR4} was transferred to an integrating vector and targeted to \textit{URA3} in the \textit{sir4-42} mutant. The resulting \textit{SIR4/sir4-42} haploid (BKy109) was stress sensitive and mated efficiently, as expected. However, the life span of this strain was intermediate between the \textit{SIR4} parent, BKx1-14c, and the \textit{sir4-42} mutant (FIGURE 4B), indicating that the \textit{sir4-42} mutation is semi-dominant with respect to life span. As a second test for dominance, we used mating to construct isogenic diploids, \textit{SIR4/SIR4} (BKy6), \textit{SIR4/sir4-42} (BKy17), and \textit{sir4-42/sir4-42} (BKy28) (using the \textit{SIR4} plasmid to permit mating in \textit{sir4-42} mutants as described in Experimental Procedures). The homozygous diploids had life spans similar to their haploid parents, and the heterozygous diploid displayed a life span intermediate between the homozygotes (data not shown). These findings show
that the extended life span in the sir4-42 mutant is semi-dominant and, therefore, due to a gain of function mutation.

The SIR4-42 mutation was cloned by gap repair and mapped to a SmaI fragment spanning codons 743 to the UAA stop at the end of the 1358 residue SIR4 open reading frame (Experimental Procedures). The clone was shown to contain the mutation by a functional test in which it was transferred to an integrating vector, and targeted to LEU2 in strain BKy104 (Δsir4). The resulting strain had an extended life span indicating that the integrating vector contained the sir4-42 allele (data not shown). The SmaI fragments from the mutant or wild type SIR4 gene were incorporated into Bluescript and sequenced across the entire SIR4 coding sequence. A single difference was found in the mutant which generated a stop at codon 1237, removing 121 residues from SIR4.

The lengthening of life span by sir4-42 requires SIR3.

How does sir4-42 extend life span? We initially began by asking whether sir4-42 acted alone or in concert with other members of the SIR complex. The activities of SIR2, SIR3, and SIR4 are closely coupled in that all are required for silencing at the HM loci and at telomeres (Aparicio, et al., 1991; Rine and Herskowitz, 1987). The function of SIR1 is different in that it is only required at the HM loci (Aparicio, et al., 1991), and even there, its requirement is not absolute (Pillus and Rine, 1989). To determine whether SIR3 and SIR1 were required for the extention of life span, the genes were disrupted in the sir4-42 mutant and, as a control, in BKx1-14c (Experimental Procedures). The sirl disruptions did not exert any effect on the sir4-42 mutant or its SIR4 parent (FIGURE 5A). In contrast, the sir3 disruption
abolished the extension in life span conferred by *sir4-42* (FIGURE 5B). This shortening of life span in the *sir4-42* strain was specific because disruption of *SIR3* did not greatly alter the life span of the *SIR4* parent (FIGURE 5B). The number of cells examined in this experiment was not sufficiently large to reveal any small reduction in the life span of the Δ*sir3* strain. Similarly, we found that deleting *SIR2* abolished the extension in life span conferred by *sir4-42* (not shown). Thus, the gain of function caused by *sir4-42* is probably an activity of the entire SIR complex, and not *SIR4* alone.

**Effects of the *sir4-42* mutation on telomeres.**

Because the *sir4-42* mutation results in a loss of activity at HM loci, we considered the possibility that it redirects the SIR complex to another chromosomal location, resulting in the observed extension in life span. One obvious possible location was telomeres, because loss of function mutations in *SIR2, SIR3,* or *SIR4* relieve silencing at telomeres and also result in shorter telomeres (Aparicio et al., 1991; Palladino et al., 1993). In mammalian cells, telomeres have been shown to shorten with age (Harley et al., 1990), and this shortening has been proposed as a causative agent of aging (Allsopp et al., 1992; Olovnikov, 1973). If telomere shortening imposed a limit to life span in yeast, then excessive recruitment of the SIR complex might counter aging by lengthening telomeres.

Thus, we determined the length of telomeres and the degree of silencing at telomeres in Δ*sir4* and *sir4-42* mutants. The 1.2 kb band in the *SIR4* parent represents the G1-3T repeats and Y' region distal to the Xho1 sites at most yeast chromosomes (FIGURE 6A) (Chan and Tye, 1983; Walmsley et al., 1984). As previously reported, deletion of *SIR4* in BKx1-14c resulted in a shortening of
telomeres of approximately 50-100 bases (Palladino, et al., 1993). Surprisingly, the length of telomeres in the sir4-42 mutant was indistinguishable from the Δsir4 mutant, indicating that the mutant behaved like the deletion with respect to activity at telomeres (FIGURE 6B). Next, we determined the degree of silencing of URA3 positioned at telomere VIIL in strain UCC1001 and derivatives in which SIR4 was deleted and sir4-42 (or SIR4) were reintroduced. Deleting SIR4 greatly relieved silencing, as expected, resulting in sensitivity to 5-flouroorotic acid (5-FOA) (FIGURE 6C). Introduction of SIR4 but not sir4-42 restored silencing to the Δsir4 strain. There are two important conclusions from these findings. First, the sir4-42 mutant behaves like the SIR4 deletion at telomeres, i.e. it exhibits a loss of function phenotype. Second, since sir4-42 extends life span and Δsir4 does not, the lengthened life span is probably unrelated to telomere length or telomere silencing.

Expression of the carboxyl-terminus of SIR4 also extends life span.

Our results indicate that the sir4-42 mutation abolishes activity at the HM loci and at telomeres, and endows the SIR complex with the ability to extend life span. This extension in life span could be because the sir4-42 complex is more available to function at yet another chromosomal location, which is crucial in the determination of life span. By this logic, a different means of preventing recruitment of the SIR complex to the the HM loci and to telomeres should also extend life span.

Since the sir4-42 mutation removes the carboxyl-terminus of the protein, it seemed possible that this fragment of SIR4 localized the complex to HM loci and telomeres. Thus, over-expression of a carboxyl-terminal fragment of SIR4 might compete with the wild type protein for recruitment to HM loci.
and telomeres. Indeed, a construct expressing only the carboxyl 154 residues of SIR4 has been shown to behave as an anti-SIR4 dominant negative mutant with respect to silencing at HM loci (Ivy, et al., 1986; Marshall, et al., 1987). We introduced this construct into BKx1-14c and confirmed that it functioned as a dominant negative inhibitor of mating. The transformant was also stress resistant, as expected. Strikingly, the construct extended the life span of BKx1-14c by about 30% (FIGURE 7). To verify that this extension in life span was due to the wild type SIR complex, we expressed the construct in the BKy 1-14c Δsir4 strain and observed no extension (FIGURE 7).

Discussion

In S. cerevisiae, the number of cell divisions that mother cells undergo is relatively fixed and termed their life span (Mortimer and Johnston, 1959). We have exploited a correlation between stress resistance and life span within a set of yeast strains to isolate mutations that increase the life span of mother cells. Mutations were isolated by an increased tolerance to stress and some extended the life span by 20-55%. This screen resulted in mutations in four genes termed UTH1-4. One of these genes UTH2 is identical to SIR4, known to be required for silencing regions of yeast chromosomes (Rine and Herskowitz, 1987).

A correlation between stress tolerance and life span may be more general than what we have observed here. In Drosophila, selective breeding of flies at an advanced age results in strains with an elongated life span (Luckinbill, et al., 1984; Rose and Charlesworth, 1981). These flies are also more resistant to stress treatments, such as dessication and starvation (Arking, et al.,
1991). In *C. elegans*, first stage larvae can enter a dormant state termed dauer larvae and remain viable for prolonged periods in the absence of nutrients. While this pathway is normally activated by starvation, it can be induced inappropriately when *daf-2* temperature sensitive mutants are shifted to the restrictive temperature (Riddle et al., 1981). Such induction of Dauer gene expression does not enable post larval worms to enter the dormant state. However, adults display a life span that is elongated 2-fold compared to the uninduced control (Kenyon, et al., 1993). These findings indicate that a pathway which functions to promote stress resistance in starved larvae can extend the life span of adults.

**A framework for relating silencing to aging and stress.**

We consider a speculative framework for interpreting the effects of the various alleles of *SIR4* on life span, stress resistance, and telomere silencing. Table 3 summarizes the effects of three mutant alleles of *SIR4*; Δsir4, sir4-42, and the anti-sir4 in the presence of the wild type gene. These alleles all alleviate silencing at HM loci and promote stress-resistance. Δsir4 and sir4-42 also alleviate silencing at telomeres and reduce telomere length. However, these alleles exert very different effects on life span. sir4-42 and the anti-sir4 extend life span, while the Δsir4 shortens life span.

To explain these findings, we first propose that a locus that is repressed by the SIR complex can promote resistance to stress when repression is eliminated (FIGURE 8). In principle, this locus could be linked to *HML, HMR*, a telomere, or reside at some other location. Linkage to HM loci is not likely, however, because deletion of *SIR1*, which weakens repression at the HM loci,
does not promote stress resistance. For simplicity, we show the case of a
telomere-linked, stress-resistant locus under SIR control.

We further suggest that life span is controlled by a different locus
(termed "AGE", FIGURE 8) that is unlinked to HM loci or telomeres, but
regulated by the SIRs. The repression of the "AGE" locus by SIR4 is essential to
longevity, according to this view, and aging may result from a breakdown in
the silencing of that locus. Thus, the Δsir4 exhibits a shortened life span. It is,
of course, possible that silencing at more than one chromosomal region
governs aging. In any case, the "AGE" locus is shown as unlinked to telomeres
or HM loci because the sir4-42 mutation and the Δsir4 exhibit similar effects at
HM loci and at telomeres, but have opposite effects on life span. Further, the
extension of life span by sir4-42 is semi-dominant in a strain also containing
SIR4, indicating that it is a gain of function mutation with regard to life span.
The function gained in the mutant must relate to the normal silencing activity
of the SIR complex because the ability of sir4-42 to promote longevity requires
the integrity of SIR3 and SIR2.

Why is the sir4-42 mutant more able to repress a hypothetical "AGE"
locus? We suggest that the mutation prevents recruitment of the SIR complex
to HML, HMR, and telomeres, rendering the complex more available for any
other site of action in the cell. The carboxyl 121 residues that are missing in
the sir4-42 mutant may be important in the recruitment of the SIR complex to
these chromosomal sites. Consistent with the view that the carboxyl terminus
of SIR4 helps localize the SIRs to HM loci and telomeres, over-expression of the
carboxyl 163 residues of SIR4 is known to exert a dominant negative effect on
repression at HM loci (Ivy et al., 1986; Marshall et al., 1987). We expressed this
SIR4 fragment and found that in addition to blocking repression at HML and
HMR, it promoted longevity in a SIR4 strain but not in a Δsir4 strain. This
finding in consistent with the above recruitment model, and suggests that the
sir4-42 mutation does not function by creating some novel activity of the SIR
complex. Determining the identity of new sites of repression by the SIR
complex will be an important goal for future studies.

Aging and chromosome silencing.

Our findings lead to the suggestion that a breakdown in silencing by the
SIR complex may be causally related to aging in S. cerevisiae. Is there any
indication that a breakdown in chromosomal silencing is related to aging in
any other system? X-chromosome inactivation is perhaps the most extensive
eexample of heterochromatic gene inactivation in mammals (Lyon, 1961). By
employing strains with X-autosomal translocations, it was shown that X-linked
markers, normally silenced in embryos and young adults, became reactivated
as a function of age in the mouse (Wareham, et al., 1987). It is intriguing that
the inactivated X chromosome displays a hypoacetylation of histone H4
(Jeppesen and Turner, 1993), as do the silenced HM loci (Braunstein, et al.,
1993). In the latter case, hypoacetylation is abolished by mutations in SIR2, 3,
or 4.

Further, in cultured human diploid fibroblasts, CpG methylation, which
correlates very well with gene inactivation, is lost as the cells progress
towards senescence (Wilson and Jones, 1983). The rate of loss is greater in
fibroblasts derived from shorter-lived mammals, such as mice, than in cells
from humans. While these examples only demonstrate correlations between
aging and a loss of silencing, they are consistent with the possibility that a
loss of silencing may be a primary cause of aging in a wide range of biological
systems.
Aging and telomeres.

An interesting current hypothesis proposes that a progressive loss of telomeres causes senescence in cultured animal cells and aging in the intact animal (Allsopp, et al., 1992; Olovnikov, 1973). This view is based on several observations on cell cultures and intact animals. In cell cultures, there is a good correlation between the length of telomeres and the replicative capacity of primary human fibroblasts taken from a range of different individuals (Allsopp, et al., 1992; Harley, et al., 1990). Cells from individuals with the premature aging syndrome, Huntington's-Gilford progeria, display the shortest telomeres and weakest replicative capacity. In rare instances, cells emerge from crisis and become immortalized in culture. In such cells, the telomeres lengthen and telomerase activity increases (Allsopp, et al., 1992). In humans, telomeres from several human organs, such as lymphocytes or peripheral blood leukocytes, have been shown to shorten with age (Schwartz, et al., 1993; Vaziri, et al., 1993).

The identification of *SIR4* as a gene that affects life span in yeast appears to relate telomeres and aging in that organism. However, our findings show that telomeres in the *sir4-42* strain, just as in the *Δsir4* null mutant, are shorter than wild type. This suggests that telomere length is not causally related to aging. However, this conclusion must be qualified because we have not examined the effect of the *sir4-42* mutation on the state of telomeres specifically in old cells. It is theoretically possible that the mutation counters telomere shortening selectively in old cells. Due to the technical difficulties in obtaining a large population of old cells, we have not yet analyzed the effects of aging on telomere length in wild type or mutant senescent cells. D'Mello
and Jazwinski have followed cells out to 80% of their life spans and found that telomeres have not shortened (D'Mello and Jazwinski, 1991). We are currently developing methods to obtain large numbers of cells at the ends of their life spans that will make these important experiments possible.

**Overview.**

We describe the start of a genetic analysis on aging in *S. cerevisiae*. More broadly, genetics is likely to be a useful approach, whatever the underlying mechanism of aging. If aging is based on a developmental program, the identification of important genes will be a crucial first step toward an understanding of the process, just as in embryonic development. If, rather, aging is a response to accumulated damage, mutations that extend life span will shed light on which processes are most damage-sensitive.

**Experimental Procedures**

**Strains, Plasmids, and Media**

Yeast strains (Table 1) were grown using standard media and conditions (Sherman et al., 1979). The *STE4* disruption was constructed using a *ste4::URA3* plasmid as described (Whiteway, et al., 1989). Disruption of *STE12* was constructed using plasmid pNC163 as described (Company et al. 1988). The plasmid pKC2 containing the *Mata* locus was kindly provided by G. Fink. The region from 153 base pairs 5' to *SIR4* through the entire open reading frame was deleted and replaced with the *URA3* gene using the plasmid, pAR59.
provided by J. Broach (Marshall et al. 1987). The sir4Δ was confirmed by Southern analysis. The sir1Δ was generated using plasmid pJI23.2 which removes the C-terminal 335 amino acids from the 648 amino acid protein (Ivy et al. 1986). The sir3Δ was constructed with plasmid pDM42 which deletes 123 amino acids at the C-terminus of SIR3 (Mahoney and Broach, 1989). pJH3a was originally described in Ivy et al. 1986 and later characterized in Hicks et al. 1988.

Integration plasmids were generated by subcloning the entire library insert containing SIR4 from pBK40 into pRS305 or pRS306 by a NotI SalI double digest (Sikorski and Hieter, 1989). Integration was directed to the URA3 locus by a StuI digest. Integration was directed to the LEU2 locus by a XcmI digest. All integration events were verified by Southern analysis.

To generate BKy5, strain BK1-14c was transformed with a (GAL-HO) plasmid and plated on galactose medium to induce mating type switching (Herskowitz and Jensen, 1991). Colonies were tested by mating to CKy20 or CKy21 to determine their mating type. A Mata colony was picked and the GAL-HO plasmid was segregated using 5-FOA (Boeke, et al., 1987). This strain, BKy5 was mated to BKx1-14c and zygotes were isolated by micromanipulation to generate BKy6. To verify that BKy6 is a diploid, the strain was shown to be sporulation-competent. The SIR4/sir4-42 heterozygote (BKy17) was generated by mating the sir4-42 mutant containing pBK40 to BKx1-14c and subsequently losing the plasmid with 5-FOA. BKy17 was sporulated and a Mata sir4-42 segregant (BKy21) was chosen to generate the homozygous sir4-42 diploid (BKy28). BKy21 carrying pBK40 was mated to the sir4-42 mutant also carrying pBK40 and diploids were isolated.
**Life Span Analysis**

To determine the life span of a strain, cells were taken from logarithmically growing liquid cultures and plated at low density on complete medium. The plates were incubated at 30°C for approximately 3 hours. At this time, daughter cells were isolated as buds that had emerged from mother cells and moved with a Zeiss Micromanipulator to uninhabited regions of the plate. The life spans of these cells were determined by noting and removing all subsequent daughters they generated. The plates were incubated at 30°C during working hours and shifted to 4°C overnight. Life spans generated by this incubation schedule do not differ significantly from those generated by incubating cells continuously at 30°C (data not shown). On very rare occasions an apparently young cell was observed to lyse immediately after micromanipulation and was excluded from the data set.

**Mutagenesis**

Growing BKx1-14c cells were mutagenized with ethyl methanesulfonate (approximately 60% of cells killed) and allowed to form colonies on rich media (YEPD). These colonies were then replica-plated to sporulation plates and allowed to starve for 7-9 days. At this point the cells were returned to YEPD and incubated for two days. While the majority of colonies were unable to grow appreciably, a small percentage were able to resume growth. These colonies were isolated and their starvation-resistant phenotype confirmed. 39 mutants were recovered that reproducibly tested starvation-resistant. Life spans of all 39 mutants were determined for a minimum of 20 cells of each mutant. 8 mutants demonstrated significantly higher mean life spans as
determined by the Wilcoxon signed rank test. These eight mutants were used for subsequent analysis.

**Cloning of UTH2**

A genomic library, CT3, supplied by R. Young (Thompson, et al., 1993) was transformed into the \textit{uth2-42} mutant by standard methods. Transformed colonies were tested for their ability to complement the mating defect in the \textit{uth2-42} mutant. Plates containing library-transformed colonies were replica-plated onto permissive plates containing a lawn of strain CKy21. Cells were incubated at room temperature for 1 day to allow mating and then replica-plated to plates selective for diploid growth. Colonies were picked which clearly grew on the selective plates. Plasmids were recovered from these colonies by standard methods and re-transformed into \textit{uth2-42} mutant cells. One plasmid was isolated which restored mating efficiency in the \textit{uth2-42} mutant. This plasmid, pBK40, also conferred heat shock sensitivity and starvation sensitivity to \textit{uth2-42} making it a good candidate for the \textit{UTH2} gene.

A 1.6 kb fragment located entirely within the pBK40 library insert was random primed by manufacturer's protocol (U. S. Biochemical), and used to probe a \lambda clone library (Riles, et al., 1993). Only one clone, which contained the \textit{SIR4} gene, showed a distinguishable signal.

**Determination of Stress Phenotypes**

To determine starvation resistance, haploid cells were grown in rich media to log phase, collected by centrifugation, and re-suspended in minimal sporulation media for a period of seven to nine days. After starvation, cells
were again collected by centrifugation and plated on rich media (YEPD) to measure colony forming units/ml. Colonies could be assayed for their ability to withstand starvation by utilizing sporulation plates instead of liquid culture.

Heat shock resistance was determined by collecting logarithmically growing cells and plating them at a known concentration on YEPD plates. The cells were heat-shocked at 55°C for periods varying from 5 minutes to 1 hour. Plates were then incubated at 30°C for three days and the number of colonies was noted.

Saturation density was measured by suspending logarithmically growing cells in YEPD liquid culture at a density of 10^6 cells/ml. Cultures were incubated for a period of five days with the number of cells/ml counted in a hemacytometer on a periodic basis. Control experiments indicated that the media was completely saturated after this time period.

Growth on ethanol was measured by directly streaking a strain on either rich media containing ethanol or synthetic media supplemented with necessary nutrients and containing ethanol as the sole carbon source.

**DNA Sequencing**

Gap repair was utilized to clone both the wild-type \textit{SIR4} allele from BKx1-14c and the \textit{sir4-42} allele from the \textit{SIR4} mutant strain. A SmaI, AatII double digest was performed to remove the coding region of \textit{SIR4} from pBK40. The linear plasmid was gel purified and transformed into either BKx1-14c or the \textit{sir4-42} mutant. Ura+ colonies were picked and the plasmids were recovered by standard methods. Restriction digests were conducted to determine if the gap repair event was successful. To localize the mutation within \textit{SIR4}, digests were conducted with AatII, SmaI, and SphI, all of which have one site in the
SIR4 gene and another within the pBK40 insert either 5' or 3' to SIR4. These linearized plasmids were transformed into sir4-42 and transformants were tested for their ability to complement the sir4-42 associated mating defect. This analysis localized the mutation to the region from amino acid 743 to the 3' end of the gene.

A SmaI fragment containing this region was gel purified from both the BKx1-14c SIR4 allele and the mutant allele from sir4-42 and subcloned into Bluescript (Stratagene). Sequencing primers were made approximately 200 base pairs apart for this entire region and it was sequenced by the single-strand approach (Sequenase version 2, U.S. Biochemical). Only one base pair change was discovered generating a stop at codon 1237.

**Determination of Telomere Length and Silencing**

Total genomic DNA was isolated from BKx1-14c, 14c SIR4Δ and sir4-42, digested with XhoI and separated on a 0.7% agarose gel and transferred to a GeneScreen Plus Hybridization Transfer Membrane (NEN Research Products). Hybridization and wash conditions were as suggested by manufacturer of the membrane. A plasmid containing 600 base pairs located within the conserved Y' region of yeast telomeres, kindly supplied by V. Zakian, was nick translated (GIBCO BRL) and used as a probe (Chan and Tye, 1983). This probe overlaps the XhoI site and thus hybridizes to fragments both telomere-proximal and telomere-distal to the restriction site. Most yeast telomeres contain the Y' region (Walmsley, et al., 1984).

A strain (UCC1001) with the URA3 gene located in the telomeric region of the left arm of chromosome VII was provided by D. Gottschling (Renauld, et al., 1993). For this experiment, SIR4 had to be disrupted with LEU2 (Ivy et al.,
1986) to generate BKy109. Either the wild-type \textit{SIR4} gene or \textit{sir4-42} was re-introduced into BKy109 on a \textit{TRP1} marked ARS-CEN vector (pRS314) (Sikorski and Hieter, 1989). UCC1001 and BKy109 were transformed with pRS314 as a control. All media used in the experiment lacked tryptophan to maintain selection for the plasmids.

**Statistical Analysis**

Determination of the significance of differences in mean life span between two strains was performed using the non-parametric 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%.

**Acknowledgments**

We would like to thank K. Cunningham for the MAT \( \alpha \) clone, J. Broach for the \textit{SIR4} disruption construct, D. Mahoney for the \textit{SIR3} disruption construct and the \textit{Anti-SIR4} construct, D. Shore for the \textit{SIR1} and \textit{SIR4} disruption constructs, C. Styles for the \textit{STE4} and \textit{STE12} disruption constructs, L. Riles for information on the map position of \textit{UTH2}, D. Gottschling for a strain with \textit{URA3} at a telomere, and V. Zakian for the telomere probe. We would also like to thank G. Fink and P. Sorger for comments on the manuscript. This work was supported by NIH grants GM3054 and AG11119 to LG, a HHMI predoctoral grant to NA, and an NIH training grant for BK.
References


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<td><em>Mata adel-100 leu2-3,2-112 lys2-801 ura3-52</em></td>
</tr>
<tr>
<td>BKy1-14b</td>
<td><em>Mata leu2-3,2-112 ura3-52</em></td>
</tr>
<tr>
<td>BKy1-14c</td>
<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52</em></td>
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<tr>
<td>BKy1-14d</td>
<td><em>Mata his4-519 leu2-3,2-112 ura3-52</em></td>
</tr>
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</tr>
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</tr>
<tr>
<td>BKy17</td>
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<td></td>
<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42</em></td>
</tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>BKy30</td>
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</tr>
<tr>
<td>BKy100</td>
<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 ste4::URA3</em></td>
</tr>
<tr>
<td>BKy101</td>
<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 ste12::URA3</em></td>
</tr>
<tr>
<td>BKy102</td>
<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir1::LEU2</em></td>
</tr>
<tr>
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<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir3::URA3</em></td>
</tr>
<tr>
<td>BKy104</td>
<td><em>Mata adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4::URA3</em></td>
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</tbody>
</table>
BKy105  \textit{Mat\textalpha} ade\textit{1-1} 1 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42
\textit{sir1::LEU2} \\
BKY106  \textit{Mat\textalpha} ade\textit{1-100} his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42
\textit{sir3::URA3} \\
BKy107  \textit{Mat\textalpha} ade\textit{1-100} his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4::URA3
\textit{LEU2/sir4-42} \\
BKy108  \textit{Mat\textalpha} ade\textit{1-100} his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42
\textit{URA3/SIR4} \\
BKy109  \textit{Mat\textalpha} ade\textit{2-101} his3-\Delta200 leu2-\Delta1 lys2-801 trp1-\Delta1 ura3-52
\textit{adh4::URA3-TEL sir4::LEU2} \\

All strains were generated in this study except BWG1-7A which is described in Guarente and Mason, 1983, UCC1001 which was a gift of D. Gottschling and the mating testers CKy20 and CKy21 which were gifts of C. Kaiser. The terminology \textit{LEU2/sir4-42} in strain BKy107 indicates that the \textit{sir4-42} allele has been integrated at the \textit{LEU2} locus, for example.
Table 2. The Effects of Sterility on Mean Life Span

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample Size</th>
<th>Mean Life Span</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKx1-14c</td>
<td>20</td>
<td>15.6</td>
<td>25</td>
</tr>
<tr>
<td>BKy5</td>
<td>20</td>
<td>14.5</td>
<td>20</td>
</tr>
<tr>
<td>BKy6</td>
<td>20</td>
<td>15.3</td>
<td>27</td>
</tr>
<tr>
<td>BKy100 (Δste4)</td>
<td>20</td>
<td>15.9</td>
<td>24</td>
</tr>
<tr>
<td>BKy101 (Δste12)</td>
<td>20</td>
<td>16.5</td>
<td>24</td>
</tr>
<tr>
<td>BKy5 + MATα</td>
<td>20</td>
<td>14.6</td>
<td>26</td>
</tr>
</tbody>
</table>

The column labeled maximum indicates the number of daughters produced by the oldest mother cell. The data presented derives from one independent experiment. Life span analysis has been repeated at least three times for all of these strains and similar results have been observed.
<table>
<thead>
<tr>
<th>Allele</th>
<th>Amino Acids</th>
<th>Mating</th>
<th>Stress Response</th>
<th>Life Span Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIR4</td>
<td>1-1358</td>
<td>+</td>
<td>Sensitive</td>
<td>---------------</td>
</tr>
<tr>
<td>sir4-42</td>
<td>1-1237</td>
<td>-</td>
<td>Resistant</td>
<td>increase</td>
</tr>
<tr>
<td>Δsir4</td>
<td>-----</td>
<td>-</td>
<td>Resistant</td>
<td>decrease</td>
</tr>
<tr>
<td>SIR4 +</td>
<td>1-1358</td>
<td>+</td>
<td>Resistant</td>
<td>increase</td>
</tr>
<tr>
<td>Anti-SIR4</td>
<td>1205-1358</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mortality curves are shown for BWG1-7A and PSY142 from several experiments totaling 78 cells for BWG1-7A and 106 cells for PSY142. The relative difference between the two strains was consistently observed. The mean life spans for these two strains are 18 generations for BWG1-7A and 29 generations for PSY142.
Figure 2. Correlated Phenotypes for Tetrad BKy1-14

(A) Mean life spans of segregants of tetrad BKy1-14 (resulting from BWG1-7A X PSY142) were determined by mortality curves involving 20 cells of each strain except 14d (19 cells). (B) The viability of BKy1-14 segregants stored for 4.5 months at 4°C. The percent viability is the number of colony-forming units per total microscopic cell count. For strain BKx1-14c, no viable cells were detected. (C) The ability of BKy1-14 segregants to maintain colony forming potential during starvation. An equal number of cells of each strain was inoculated from rich YPD media to minimal sporulation media lacking nitrogen and carbon sources, and incubated for seven days before plating on YPD. The number of colony-forming units per ml is shown.
Figure 3. The Mortality Curves of *uth* mutants

Mortality curves are shown for BKx1-14c and uth1-4 mutants in panels 3A-D, respectively. All curves are derived from two experiments. The data presented in Figure 2B was generated in two experiments unrelated to the experiments performed to generate Figure 2A, C, and D. The differences in the life spans between each uth mutant and BKx1-14c were shown to be statistically significant (see Experimental Procedures). The sample size for strain BK1-14c was 40 cells. (A) The sample sizes for *UTH1* mutants were: *uth1*-324 - 37 cells, *uth1*-328 - 38 cells, *uth1*-330 - 38 cells, *uth1*-342 -34 cells. (B) The sample size for uth2-42 was 40 cells. (C) The sample sizes for *UTH3* mutants were: *uth3*-26 - 49 cells, *uth3*-335 - 40 cells. (D) The sample size for *UTH4*-326 was 40 cells.
Figure 4. Deletion of the *SIR4* Gene does Not Extend Life Span; the *sir4-42* Mutation is Co-dominant for Increased Longevity

(A) Mortality curves are shown for BKx1-14c (*SIR4*), *sir4-42* (i.e. BK1-14c *sir4-42*) and BKy104 (*Δsir4*) and are derived from seven experiments. Statistical analysis showed that the mean life span difference between *sir4-42* and *SIR4* was significant as was the smaller difference between *SIR4* and *Δsir4* (see Experimental Procedures). The sample sizes for the strains in this experiment were as follows: BKx1-14c - 139 cells, *sir4-42* - 139 cells, BKy104 - 136 cells.

(B) In strain BKy108 (*sir4-42 + SIR4*), a wild-type *SIR4* gene has been integrated into the *URA3* locus of *sir4-42* giving the strain one mutant and one wild-type copy of *SIR4*. Mortality curves for BKx1-14c (*SIR4*), *sir4-42*, and BKy108 (*sir4-42 + SIR4*) are derived from one experiment. Similar results have been obtained in other experiments. Statistical analysis determined that the mean life span of BKy109 was significantly different from the means of both *sir4-42* and BKx1-14c (see Experimental Procedures). The sample sizes for the strains in this experiment were as follows: BKx1-14c - 20 cells, *sir4-42* - 20 cells, BKy108 - 20 cells.
Figure 5. The Increased Longevity Conferred by sir4-42 Requires SIR3 but Not SIR1.

Mortality curves are shown for BKx1-14c (SIR4), sir4-42, and their isogenic Δsir1 and Δsir3 derivatives. The sample sizes were as follows: (A) BKx1-14c (SIR4) - 20 cells, BKy102 (Δsir1) - 19 cells, sir4-42 - 18 cells, and Bky105 (sir4-42 Δsir1) - 19 cells (B) BKx1-14c (SIR4) - 60 cells, BKy103 (Δsir3) - 20 cells, sir4-42 - 19 cells, and BKy106 (sir4-42 Δsir3) - 30 cells.
Figure 6. Telomere Length Shortens and Telomere Silencing is Eliminated in the \textit{sir4-42} Mutant

(A) The Structure of most yeast telomeres. The variable G\textsubscript{(1-3)}T Region at the chromosmome end is flanked by the conserved Y' Region which contains a XhoI site (Walmsley et al., 1984). The average length from the XhoI site to the terminus of the chromosome is approximately 1.2kb. (B) XhoI-digested DNA is probed with a labeled 600 base pair fragment which hybridizes to Y' DNA that is telomere-distal to the XhoI site. The broad smear just above a 1 kb size marker consists of yeast telomeres. (C) Viability of strains having URA3 at a chromosome VIIL telomeric locus on complete synthetic media with or without 5-fluoro-orotic acid (5-FOA). Strains used were UCC1001 \textit{(SIR4)} or BKy109 \textit{(\textit{Asir4})} containing the ARS-CEN vector pRS314, or BKy109 \textit{(\textit{Asir4})} with \textit{SIR4 or \textit{sir4-42}} carried on pRS314. Cells were pre-grown in synthetic liquid media and then plated at ten-fold serial dilutions from right to left on either complete media or media containing 5-FOA. Silencing of \textit{URA3} gives rise to a \textit{Ura\textsuperscript{-}} phenotype and growth on 5-FOA.
Figure 7. The Dominant Negative Anti-\textit{SIR4} Construct Extends Yeast Life Span

The strain labeled \textit{SIR4} + Anti-\textit{SIR4} is BKx1-14c transformed with the plasmid pJH3A, a 2 \( \mu \) plasmid containing the C-terminal 154 amino acids of the \textit{SIR4} gene (Ivy, et al., 1986). The strain labeled \textit{Asir4} + Anti-\textit{SIR4} is BKy104 transformed with plasmid pJH3A. The control strains (\textit{SIR4} or \textit{Asir4}) contain a 2 \( \mu \) LEU2 vector plasmid. Mortality curves for all four strains are from two experiments. The mean life span of the strain labeled \textit{SIR4} + Anti-\textit{SIR4} was shown to be statistically different from that of the \textit{SIR4} strain (see Experimental Procedures). The sample sizes were as follows: \textit{SIR4} - 56 cells, \textit{SIR4} + Anti-\textit{SIR4} - 48 cells, \textit{Asir4} - 54 cells and \textit{Asir4} + Anti-\textit{SIR4} - 48 cells.
Figure 8. A Model for the Effects of Silencing on Yeast Life Span

A locus that confers stress-resistance is labeled STRESS R and is shown located near a telomere. A non-telomeric locus that causes aging when expressed in old cells is labeled "AGE". The arrows indicate transcription. Silencing by SIR2, SIR3 and SIR4 is indicated. In a SIR4+ strain, the majority of the SIR complex is localized at telomeres, at the expense of the "AGE" locus. The sir4-42 mutation prevents recruitment of the SIR complex to telomeres (and HM loci) resulting in increased occupancy at "AGE" and a concomitant slowing of the aging process.
**SIR4+**
STRESS SENSITIVE
SHORT-LIVED

**sir4-42**
STRESS RESISTANT
LONG-LIVED

**Δsir4**
STRESS RESISTANT
SHORT-LIVED
Chapter 4: The Uth4 protein extends life span in *S. cerevisiae* by regulating transcriptional silencing factors

This chapter will be submitted for publication. While I was the principal researcher on this work, several members of the Guarente laboratory contributed to this work, including David McNabb, Mala Murthy, Sally Pak, and Leonard Guarente. Confocal microscopy experiments were performed by Thierry Laroche and Susan Gasser at the Swiss Institute for Experimental Cancer Research in collaboration.
Summary

By screening for mutations which increased stress resistance in a strain background where stress resistance and life span were shown to correlate, mutations were isolated in four genes that extended life span (UTH1-4) (Kennedy et al., 1995). In this report, UTH4, a gene encoding a protein with an eight 36 amino acid repeat domain homologous to the D. melanogaster pumilio protein and others, has been cloned and its role in yeast aging characterized. The UTH4 gene is required for strains to achieve full life span potential. Life span extension by UTH4 is completely dependent on an intact UTH2/SIR4 gene. The Uth4 protein reduces SIR4-mediated silencing at both telomeres and, to a small extent, at silent mating type loci. In addition, over-expressing UTH4 increases life span of all strains tested while further reducing telomere and HML silencing. YGL023, another yeast gene with a similar pumilio-like domain, also regulates telomere silencing. These findings have led to a model whereby Uth4 acts to re-direct silencing factors away from telomeres and HML loci to an Aging locus, thereby extending yeast life span.
Introduction

Aging is characterized by an exponential increase in the rate of mortality over time (Gompertz, 1825). This fundamental property of aging is manifest in organisms as complex as humans (Gompertz, 1825) to those as simple as the single-celled yeast, *Saccharomyces cerevisiae* (Pohley, 1987).

Yeast aging is measured by determining the number of daughter cells that a mother cell can produce (Mortimer and Johnston, 1959). The two cell types can be differentiated on the basis of size (Hartwell and Unger, 1977). Mean and maximum life spans vary broadly among yeast strains with the means ranging from 13-30 divisions or generations (Kennedy et al., 1995). As mother cells become older they undergo a number of accompanying phenotypic changes. These include an increase in cell size and slowing of the cell cycle (Mortimer and Johnston, 1959), loss of mating potential (Müller, 1985; Smeal et al., 1996), and a decrease in the ability of old mother cells to produce small daughter cells with full life span potential (Kennedy et al., 1994).

The potential to isolate mutants with altered life spans make yeast an attractive organism for aging research. Until recently however, the yeast aging phenotype could only be followed in single cells, making a direct screen for life span mutants impossible. The problem of isolating yeast aging mutants was circumvented by utilizing an observation that life span correlated with stress resistance in tetrads resulting from a cross of two unrelated yeast strains (Kennedy, et al., 1995). Stress resistant mutant strains were isolated and then tested for an increased life span potential. Several aging mutants were recovered which defined four genes (*UTH1*-4).

The mutation defining the *UTH2* gene had an additional, unique phenotype: a severe reduction in mating potential (Kennedy, et al., 1995). This
gene was cloned and demonstrated to be identical to SIR4, a gene, along with SIR2 and SIR3, that has been previously shown to be required for transcriptional silencing of genes at HM loci and telomeres (Aparicio et al., 1991; Gottschling et al., 1990; Rine and Herskowitz, 1987). While silencing is absolute at HM loci, silencing at telomeres is variable; some cells in a given strain silence a marker gene placed at telomeres and others do not (Gottschling et al., 1990). The nature of this heterogeneity has not been explained.

The allele of SIR4 isolated (SIR4-42) was unusual in that it behaved as a null allele for some phenotypes and as a dominant allele for others (Kennedy et al., 1995). Silencing at HM loci and telomeres was abolished, but life span was extended even though Δsir4 strains displayed shorter life spans. These findings suggested that the ability of the SIR4-42 allele to extend life span was independent of its role at telomeres and HM loci. The SIR4-42 mutation results in a truncation of the C-terminal 121 amino acids of the 1358 residue protein (Kennedy et al., 1995). This C-terminal region of Sir4 has been shown to interact with Rap1 (Moretti et al., 1994; Shore and Nasmyth, 1987) (Cockell et al., 1995), a protein found at HM loci and telomeres (Conrad et al., 1990; Klein et al., 1992; Longtine et al., 1989). Rap1 plays an important role in silencing at these loci. In addition, Rap1 serves as a transcriptional activator for several other genes. The loss of the Rap1 interaction domain presumably frees the Sir complex to re-locate to the Aging locus. Increased silencing of this Aging locus would delay the aging process. This model was further supported by the observation that over-expressing the C-terminal 154 amino acids of Sir4, previously shown to interfere with telomere and HM silencing (Ivy et al., 1986), increased life span (Kennedy et al., 1995). Moreover, this increase in
life span was dependent on the presence of a wild-type \textit{SIR4} gene, indicating that life span extension was mediated by intact Sir-complexes.

If silencing of the Aging locus were to become defective in old cells, Sir-complexes with a greater relative affinity for the Aging locus would be likely to silence the Aging locus for a longer period of time and thus delay aging. To address this issue, the aforementioned age-specific sterility phenotype was carefully analyzed. In all strains tested, yeast cells lose the ability to mate as they age (Müller, 1985). Loss of mating ability results from an inability of cells to respond to pheromones (Smeal, et al., 1996). An old \textit{Mata} cell does not arrest division and form schmoos in the presence of α factor. Deleting \textit{HMRa} completely restores the ability of these old cells to respond to α factor, directly linking mating in old age to Sir-mediated silencing at \textit{HM} loci. These experiments provide the first evidence that a loss of Sir-mediated silencing occurs in old cells. They were also performed in long-lived stress resistant strains, suggesting that Sirs are general aging factors.

Here, we report the cloning and characterization of \textit{UTH4}, a gene previously identified as \textit{MPT5}, a multi-copy suppresser of a glucose derepression defect (Coglievina et al., 1995; Sakai et al., 1992), and as \textit{HTR1}, a gene required for growth at high temperature in some strain backgrounds (Kikuchi et al., 1994). Little is known about the molecular nature of these phenotypes. \textit{UTH4} has eight 36 amino acids repeats which are highly homologous to the eight repeats found in the \textit{Drosophila melanogaster pumilio} protein (Barker et al., 1992; Macdonald, 1992). \textit{Pumilio} and its cohort, Nanos, act as a repressor of hunchback translation (Murata and Wharton, 1995). Several other genes in the database also have this repeat region including two human genes, two \textit{C. elegans} genes, and another yeast gene, \textit{YGL023} (Chen et al., 1991).
We have studied the role of the *UTH4* and *YGL023* gene products in transcriptional silencing and aging. These proteins cause a re-direction of silencing factors away from *HM* loci and telomeres. Uth4 presumably recruits Sir complexes to the Aging locus and thereby extends yeast life span. Life span directly correlates with *UTH4* expression levels in all strains tested, demonstrating that like *SIR4*, *UTH4* is a general yeast aging factor.

To fortify the model that life span extension in Uth mutants correlates with a re-direction of silencing components, we have undertaken experiments to localize silencing factors in the yeast nucleus. In wild-type yeast cells, Sir3, Sir4 and Rap1 all co-localize with yeast telomeres at the nuclear periphery (Cockell, et al., 1995; Palladino et al., 1993). These results are consistent with the observed silencing of genes placed near telomeres (Gottschling, et al., 1990). Sir4-42, however, localizes predominantly to one nuclear location and does not co-localize with telomeres. Sir3 displays an identical staining pattern in strains containing *SIR4-42*. We propose that this novel nuclear location may contain the Aging locus.

**Results**

**Cloning *UTH4***

The *UTH4-326* allele was identified as a dominant mutant which conferred stress resistance and increased life span potential (Kennedy, et al., 1995). This gene was cloned by constructing an ARS-CEN genomic library from a strain containing the *UTH4-326* allele (see Experimental Procedures). Clones were isolated that conferred resistance to one of the stress resistant phenotypes (see Experimental Procedures). One clone, pBK4b3, was isolated.
that conferred resistance to stress and extended life span when re-integrated into the yeast genome (data not shown). A LEU2 gene was integrated at the genomic locus corresponding to the pBK4b3 clone. This strain was crossed to the original UTH4-326, leu2 strain and tetrads were dissected. In all 12 complete tetrads analyzed, two spores were stress resistant, Leu- and two spores were stress sensitive, Leu+ (data not shown). This demonstration of tight linkage indicated that the genomic DNA present on pBK4b3 contained the UTH4 gene.

**Sequence Analysis of UTH4**

Sequence analysis of the 4kb genomic insert of pBK4b3 revealed that the clone contained one gene which is identical to the previously isolated MPT5/HTR1 gene. This gene contains a putative open reading frame of 834 amino acids that contains a region of eight 36 amino acid repeats in the middle of the protein that is highly similar to those of the *Drosophila pumilio* protein (Figure 1a-b)(Barker, et al., 1992; Macdonald, 1992). A search of the sequence database revealed another yeast gene, YGL023, that contained a similar repeat structure (Figure 1c)(Chen, et al., 1991). A molecular function has not been attributed to these repeats in either protein. The N and C-termini of Uth4 display no significant homology to proteins in the database.

To sequence the dominant allele, UTH4-326, the presumed wild-type UTH4 allele from strain BKx1-14c was cloned by gap repair (see Experimental Procedures) (Kennedy, et al., 1995). The region of UTH4 containing the mutation was determined to be in the region of the open reading frame between the BglIII restriction site (+557 relative to the putative start of translation) and the Bsg1 restriction site (+2155) by gap repairing the wild-
type plasmid in the UTH4-326 mutant strain (data not shown). This entire region was sequenced. Surprisingly, when the sequences of the two alleles of UTH4 were compared to the published sequence, the presumed wild-type allele had a one base pair insertion in repetitive stretch of adenosines relative to the published sequence. This frameshift led to a stop codon after 206 amino acids and an additional 5 out-of-frame amino acids (Figure 1d). To confirm that this sequence alteration in the BKx1-14c allele was not a gap repair artifact, the allele was cloned three additional times independently and sequenced. The same 1bp insertion was present in all clones. Since BKx1-14c is the product of a cross between BWG1-7A (Guarente and Mason, 1983) and PSY142 (Kennedy, et al., 1994; Kennedy, et al., 1995), the UTH4 alleles from these parent strains were cloned and their sequence analyzed. The BWG1-7A, but not the PSY142, UTH4 allele contained the 1bp insertion. This truncated UTH4 allele will be hereafter referred to as uth4-14c.

The UTH4-326 allele contains a one base pair deletion in the same multi-adenosine stretch that restores the frame of the Uth4-14c allele creating an allele with a sequence identical to the published sequence of UTH4 (Figure 1d). This allele will hereafter be referred to as UTH4-WT. To reiterate, the dominant mutant allele of UTH4 originally isolated reverts a frameshift mutation in the Uth4-14c allele and restores the function of UTH4 in that strain.

Effects of UTH4 and YGL023 on Aging

To analyze the role of UTH4 and YGL023 in yeast aging, both genes were disrupted individually and in combination (see Experimental Procedures). Life spans were determined in two strain backgrounds, BKx1-14c and PSY316AT (Table 1). BKx1-14c contains the truncated uth4-14c allele described above.
Consistent with the notion that the uth4-14c allele contains a loss of function mutation, deleting the remaining portion of UTH4 (BKyl13) does not alter the life span potential of the strain (Figure 2a). The presence of UTH4-WT (BKyl3.26) causes a 50% extension in both the mean and maximum life span. This life span difference was determined to be statistically significant (see Experimental Procedures). Deleting YGL023 has no effect on life span in strains containing the uth4-14c allele (BKyl12) or the UTH4-WT allele (BKyl14). A similar pattern of life span phenotypes is observed in the genetically unrelated PSY316AT strain (Figure 2b). PSY316AT normally contains a functional UTH4 gene. A Δuth4 strain (BKyl20) has greatly reduced life span potential. Again, Δygl023 strains (BKyl121, BKyl122) do not display altered life span potential. None of the mutant strains presented above has altered growth relative to the wild-type parent strains.

Relationship between UTH4 and SIR4

UTH4 was isolated in the same screen for stress resistance as UTH2/SIR4. To determine if the ability of Uth4 to extend life span was related to role of SIR4 in aging, SIR3 and SIR4 were disrupted a UTH4-WT strain (BKyl15, BKyl16). Δsir3 and Δsir4 strains cause only a 5-10% reduction in mean life span in a strain bearing the uth4-14c allele (Kennedy et al., 1995). The ability of UTH4-WT to extend life span is completely abolished in the absence of either SIR3 or SIR4 (Figure 3a). This result suggests that Uth4 may increase life span potential by regulating Sir activity.

The SIR4-42 allele that extends life span was also isolated in the BKx1-14c strain. A UTH4-WT allele was integrated into a SIR4-42 strain (BKyl117) to determine the combined effect of the two life span extending alleles.
Individually, either allele extends mean life span approximately 50% relative to BKx1-14c. Life span is not further increased in BKy117 (Figure 3b). This result further strengthens the theory that \textit{UTH4} and \textit{SIR4} act in a common pathway regulating yeast aging and suggest that strains containing a \textit{UTH4-WT} allele may experience increased silencing at the Aging locus.

If \textit{SIR4} was downstream of \textit{UTH4} in a genetic pathway regulating yeast aging then the \textit{SIR4-42} mutant strains should retain long life span potential in the absence of \textit{UTH4}. This result was expected since the \textit{SIR4-42} allele was isolated in a strain containing \textit{uth4-14c}. Surprisingly, we find that life span extension by \textit{SIR4-42} is completely abrogated when the \textit{uth4-14c} allele is deleted (Figure 4). This finding, together with the observation that \textit{SIR4-42}, \textit{UTH4} and \textit{SIR4}, \textit{UTH4} strains have the same life span potential demonstrate that \textit{SIR4-42} is an allele specific suppressor of \textit{uth4-14c}. Further, \textit{uth4-14c} is not a null allele.

\textbf{Role of \textit{UTH4} in Telomere and \textit{HM} Silencing}

Since the ability of \textit{UTH4} to extend life span was clearly Sir dependent, a series of experiments were undertaken to determine whether \textit{UTH4} played a regulatory role in transcriptional silencing at other loci. To measure the levels of transcriptional silencing at telomeres, the \textit{ADE2} gene was integrated near chromosome VR telomere, generating strain PSY316AT (Table 1). Genes located near yeast telomeres exist in two states: transcriptionally active and transcriptionally silent (Gottschling et al., 1990). Both of these states are heritable, with switching occurring from one state to another at a low but observable rate. Cells in which the \textit{ADE2} gene is silenced behave as adenine auxotrophs; the colonies that they produce turn a red-orange color (Roman,
Colonies arising from cells in which *ADE2* is transcribed remain white. The wild-type strain clearly contains a mixture of red and white colonies (Figure 4a).

**UTH4** and **YGL023** deletions were made alone and in combination in the PSY316AT strain background. BKy122, deleted for **UTH4** and **YGL023**, was almost uniformly red, indicating that transcriptional silencing is nearly universal in the absence of these proteins (Figure 5a). Strains containing either **UTH4** or **YGL023** (BKy120, BKy121) have a higher frequency of white colonies, although to different extents. Remarkably, BKy121, containing only **UTH4**, was almost entirely white. In the absence of **YGL023**, **UTH4** virtually abolishes telomere silencing. BKy120, containing only Ygl023, exhibited a much more modest reduction in telomere silencing. As previously stated, a wild-type (**UTH4**, **YGL023**) strain shows an intermediate amount of silencing with more white colonies than the **Auth4**, **Ygl023** strain, but less than the **UTH4**, **Aygl023** strain. This surprising result suggests that Uth4 and Ygl023 may be competing with each other for the ability to interfere with telomere silencing. Ygl023 seems to counteract the ability of Uth4 to influence telomere silencing. It should be noted that the intensity of silencing, reflected in the deepness of the red color of a strain is also altered in these mutants. Silenced colonies from the **Auth4**, **Aygl023** strain are deep red, while the few predominantly silenced colonies (1/130) from the **UTH4**, **Aygl023** strain are lightly pink, barely distinguishable from the white background (Figure 5a).

In addition to telomere silencing, Sir proteins influence telomere length in yeast. Strains containing disruptions of **SIR3** or **SIR4** display a reduced telomere length relative to wild-type strains (Palladino et al., 1993). Telomere length was assayed in the PSY316AT strain background containing **Auth4**, **Aygl023**, or both and compared to that of the wild-type strain (Figure 6).
In this Southern to assay telomere length, the broad, variable band at approximately 1.2 kb reflects the variance in length of the G(1-3)T repeats at the telomere termini. BKy122 (Δuth4, Δygl023) has the longest telomeres. Adding either of these proteins back individually reduces telomere lengths. Just as for telomere silencing, Uth4 is much more effective at reducing telomere length than Ygl023. Both the results from the telomere silencing assays and the telomere length studies indicate that the role of both UTH4 and YGL023 is to remove the Sir complex from telomeres. The ability of UTH4 to extend life span suggests that it may be re-directing the Sir complex away from telomeres to the Aging locus.

To determine whether UTH4 had any effect on transcriptional silencing at the silent mating type loci, two strains (YLS56 and YLS227) with TRP1 integrated in place of HMRa were used (Table 1). YLS56, which contains a wild-type silencer, completely silences TRP1 gene expression and consequently is unable to grow on media lacking tryptophan. In strain YLS227, the Rap1 (E) binding site (Buchmann et al., 1988; Shore and Nasmyth, 1987) in the HMR E silencer has been deleted to weaken silencing (Brand et al., 1987; Kimmerly et al., 1988; Sussel and Shore, 1991). This strain grows slowly on media without tryptophan. UTH4 was deleted in both strains (BKy129, BKy130) and the resultant strains were also tested for their ability to grow in the absence of tryptophan (Figure 5b). As in the case of YLS56, BKy129 cannot form colonies. Unlike the YLS227 (ΔE) strain, the BKy130 (ΔE Δuth4) strain also cannot form colonies on tryptophan-lacking media. Deleting UTH4 increases the ability of the crippled silencer to silence TRP1 expression. As with telomere-mediated silencing, Uth4 can interfere with HM silencing. Other experiments were conducted to determine whether deleting Uth4 could increase the level of silencing in strains with wild-type silencers, but no
effect was observed. The Sir1 protein specifically acts to recruit Sir2-4 to $HM$ loci (Aparicio et al., 1991; Pillus and Rine, 1989). Thus silencing at $HM$ loci may be inherently stronger than that at telomeres. This high affinity of the Sir complex for $HM$ loci could prevent Uth4 from interfering with silencing. The role of $YGL023$ in $HM$ silencing has not been determined.

**Overexpression of $UTH4$ and $YGL023$**

Life span is extended 50% in strains containing $UTH4$ compared to $\Deltauth4$ strains. To determine if overexpressing $UTH4$ caused a further increase in yeast life span the $UTH4$ coding region was placed under the control of the $ADH1$ promoter in a 2 micron vector. The $ADH1$ promoter confers high level constitutive expression of genes under its control. $YGL023$ was cloned in a similar manner. These plasmids were introduced into strain PSY316AT. Transformants containing either plasmid divided at a much slower rate than wild-type cells. Most mutations that confer slow growth rates have a reduced life span. Reasoning that reducing the copy number of plasmids may abrogate growth inhibition, both the $ADHI-UTH4$ and $ADHI-YGL023$ plasmids were transferred to integrating vectors and integrated into PSY316AT at the $URA3$ locus (BKy124, BKy125). The strain containing $ADHI-UTH4$ divide at a rate indistinguishable from the wild-type strain. The $ADHI-YGL023$ strain still divided at a slower rate than the wild-type strain.

Life span analysis was performed on two independent isolates overexpressing either $UTH4$ or $YGL023$, as well as two control strains containing the $ADH1$ promoter alone integrated at the $URA3$ locus (BKy123). The two strains overexpressing $UTH4$ extend life span an additional 25% beyond that of the wild-type $UTH4$ isolates (Figure 7a). This life span
difference was determined to be statistically significant (see Experimental Procedures). Overexpressing *YGL023* caused a severe reduction in life span. Given the slow growth of these strains, it is impossible to determine if the short life span observed is a result of an enhanced aging rate, a general reduction in the vitality of the strains, or both.

*UTH4* and *YGL023* were also overexpressed in PSY142 (BKy127, BKy128), which has the longest life span of any laboratory strain we have tested. Again two strains which overexpress *UTH4* have statistically significant, extended life spans (Figure 7b). In all strain backgrounds tested, life span correlates directly with Uth4 levels demonstrating that *UTH4* is fundamental to aging. Overexpressing *YGL023* does not reduce the growth rate of PSY142. Two isolates containing *ADH1-YGL023* display a 40% reduction in mean life span. Deleting *SIR4* or *UTH4* results in the same reduction in life span, suggesting that overexpressing *YGL023* inhibits Uth4 from recruiting Sirts to the Aging locus.

Telomere silencing was also determined in strains overexpressing either *UTH4* or *YGL023* in PSY316AT. Two isolates containing the *ADH1* promoter alone integrated at *URA3* (BKy123) display intermediate levels of silencing (Figure 7c). Overexpressing *UTH4* decreases silencing considerably; whereas overexpressing *YGL023* increases observed silencing levels. In general telomere silencing depends on the relative levels of Uth4 and Ygl023. The phenotype of a Δ*uth4* strain is similar to that of a *UTH4* strain with increased levels of Ygl023. Likewise the phenotype of a Δ*ygl023* strain is similar to that of a *YGL023* strain with increased levels of Uth4.

**Localization of Silencing Factors**
A large body of data has been collected which suggests that the \textit{SIR4-42} mutant allele causes the Sir complex to be re-localized away from telomeres and \textit{HM} loci to an Aging locus. In order to directly determine the \textit{in vivo} localization pattern of the \textit{SIR4-42} gene product, confocal microscopy was performed with antibodies directed to \textit{SIR3}, \textit{SIR4}, or \textit{RAP1} in homozygous \textit{SIR4} and \textit{SIR4-42} diploid (BKy28). In wild-type strains, all three of these proteins co-localize with yeast telomeres at the nuclear periphery (Palladino et al., 1993). In \textit{asir4} strains, Sir3 and Rap1 stain in a diffuse nuclear pattern demonstrating that \textit{SIR4} is required for appropriate Sir3 and Rap1 localization. In the following experiments, the nucleus is stained red with ethidium bromide and the proteins of interest are stained green with fluorescent secondary antibodies. The observed staining pattern of Sir3, Sir4, and Rap1 in the \textit{SIR4} strain are consistent with the previously published results (Figure 8 left hand panels). In the \textit{SIR4-42} strain, the staining pattern of these proteins is altered. Sir3 and Sir4-42 are relocalized primarily to one specific location in the nucleus (Figure 8 upper and middle right panel). Sir4-42 also displays a low background of diffuse nuclear staining in some cells. Rap1 is found in a diffuse nuclear staining pattern (Figure 8 lower right panel). The region of the nucleus not stained by Rap1 in the \textit{SIR4-42} strain corresponds to the nucleolus (data not shown). Rap1 is similarly de-localized in \textit{asir4} strains (Palladino et al., 1993).

We cannot rule out that this new location of Sir3 and Sir4-42 staining in a \textit{SIR4-42} strain corresponds to one telomere or one set of telomeres. This is unlikely since the single spot is often not peripheral. These experiments clearly demonstrate that the \textit{SIR4-42} mutation causes a re-localization of Sir proteins and suggest that they are specifically targeted to one nuclear location. This new location may contain the proposed Aging locus.
potential. The identification of one of those mutations in *UTH4* was initially troubling. It could be argued that the short life span observed in BKx1-14c was the result of a mutation which reduced the general health of the strain (i.e. altered its efficiency of cell cycle progression, interfered with damage-repair mechanisms etc.). Therefore, short life span potential in this strain may be unrelated to the normal aging process and the Uth mutants may not directly regulate aging. Since *SIR4-42* is an allele-specific suppresser of *uth4-14c*, the generality its affect on life span was also called into question. Three findings together eliminate this possibility. First and foremost, life span is directly proportional to Uth4 levels. Importantly, increasing Uth4 levels beyond that of wild-type strains increases life span potential in all strains tested. This includes PSY142, the longest-lived strain we have studied. Second, all strains that we have tested are dependent on a functional *SIR4* gene to achieve full longevity. Finally, we have demonstrated that Sir-dependent transcriptional silencing is abrogated as cells become old (Smeal et al., 1996). This phenotype is also observed in strains of different genetic backgrounds, including long-lived strains. Together these findings demonstrate that *UTH4* and *SIR4* encode factors that likely regulate the yeast aging process.

**Model for Telomere Silencing**

While gene silencing occurs when marker genes are placed near telomeres, there is no known evidence that any endogenous telomeric genes are subject to transcriptional silencing. *SIR3* and *SIR4* also help localize telomeres to the nuclear periphery and participate in telomere length maintenance, but strains deleted for either of these genes have stable telomeres and no growth defects (Palladino et al., 1993). In fact, disrupting
these genes confers increased stress resistance in many strain backgrounds (Kennedy et al., 1995)(Austriaco et al., unpublished). Therefore, it is not clear why silencing factors are localized specifically to telomeres.

One hypothesis recently presented suggests that telomeres serve as a reservoir for silencing factors (Marcand et al., in press). These factors could then be re-directed to other important loci quickly when needed. One example of such a factor is Sir1, which specifically increases silencing at $HM$ loci. Sir1 presumably serves as an intermediate between Sir2-4 and DNA binding proteins at silent mating type loci which facilitates the establishment of silencing at these two locations. We propose that Uth4 and Ygl023 act in a similar manner (Figure 9). Uth4 would re-direct Sirs to an Aging locus. Ygl023 may re-direct Sirs to yet another unknown location. At this point, we cannot, however, rule out the possibility Uth4 and Ygl023 are influencing silencing indirectly.

Uth4 may be acting in much the same way as Sir1, except at the aging locus. While Sir1 has no known DNA binding activity, this is not clear with Uth4. There are no obvious regions of sequence homology between $UTH4$ and other DNA binding proteins. One future direction of research will entail determining whether Uth4 interacts with one of the Sir proteins and how Uth4 associates with the Aging locus. It should be noted that Ygl023 has a putative zinc finger (Chen et al., 1991).

$UTH4$ has been previously isolated in two different screens. In one screen, it was isolated as a gene required for growth at high temperature in some strain backgrounds (Kikuchi et al., 1994). We find $UTH4$ increases life span potential in all strains tested including those in which $\Delta a u t h4$ confers a ts phenotype and those in which $\Delta a u t h4$ has no effect on growth at high temperature (Kennedy and Guarente, unpublished). Therefore these two
phenotypes of $UTH4$ may not be directly related. The authors also demonstrate that $\Delta auth4$ strains are defective in recovery from pheromone arrest (Kikuchi et al., 1994). In another screen, $UTH4$ was isolated as a high copy suppressor of defects associated with a $pop2$ mutation, which include resistance to glucose derepression and sensitivity to caffeine (Sakai et al., 1992). It is unclear if any of these phenotypes of $UTH4$ are related to its role in silencing.

**Telomere Silencing Levels and Life Span**

Telomere silencing levels inversely correlate with life span potential. The $SIR4$-$42$ mutant allele was thought to increase silencing at the aging locus by reducing the affinity of the Sir complex for $RAP1$ and telomeres. Therefore the relative affinity for proteins at the aging locus would be increased. Several findings suggest that this theory may be too naive. Foremost among these is the result that Sir4-$42$ does not further extend life span compared to Sir4 in a $UTH4$ strain. Silencing at telomeres and $HM$ loci, however, are still reduced in $UTH4, SIR4$-$42$ strains. Therefore increasing the relative concentration of non-telomeric Sirs is not sufficient to extend life span in this strain.

Another finding suggests that the Sir complex may not be the limiting component for silencing at the Aging locus. In a $UTH4$ strain, telomere silencing can be reduced to a similar extent by overexpressing $UTH4$ or by deleting $YGL023$, but only overexpressing $UTH4$ significantly extends life span. Releasing Sirs from telomeres may have no effect on silencing of the Aging locus unless there is a sufficient amount of $UTH4$ to direct the silencing factors properly. Given that the $SIR4$-$42$ mutant is an allele-specific suppressor of the
\textit{uth4-14c} mutant, it is likely that these two proteins interact. Experiments are currently underway to address this possibility.

**Heterogeneity of Telomere Silencing**

Silencing of genes placed at telomeres is epigenetic; the gene is silenced in some cells and transcribed in others (Gottschling et al., 1990). Silencing at \textit{HM} loci occurs in the same manner in a \textit{Δsir1} strain (Pillus and Rine, 1989). The silencing state of the cell is generally conferred to its daughters, making silencing heritable. At a low but observable rate switching occurs resulting in red and white sectoring colonies when \textit{ADE2} is used as the marker gene. The molecular events that cause the cell to silence or transcribe these genes are not understood. \textit{UTH4} and \textit{YGL023} may be the conduits through which the cell decides the fate of telomeric genes. We find that telomere silencing varies dramatically in strains deleted for one of these genes. In a strain containing only \textit{YGL023}, a telomeric \textit{ADE2} gene is silenced more than normal, whereas a strain containing only \textit{UTH4} is virtually unable to silence \textit{ADE2}. Strains containing both genes display intermediate levels of silencing suggesting that these two proteins compete with one another for silencing components. While the mechanism of heritability in silencing remains poorly understood, it is possible that part of the mechanism could be to regulate whether Uth4 or Ygl023 is the determinant of silencing levels in a particular cell. When Uth4 is in control, most cells would transcribe telomeric genes and when Ygl023 is in control, most cells would silence telomeric genes. The reason for such a mechanism could relate to the relative needs of the cell to transcribe or silence the loci controlled by these two proteins. Alternatively, silencing at
telomeres could be stochastic. When these unknown silenced loci are discovered it should be possible to test these models.

Relationship between $UTH4$ and $YGL023$

$UTH4$ and $YGL023$ have considerable sequence homology in the eight repeat region. Aside from a long stretch of basic residues, they have little similarity outside the repeats. The model presented for silencing states that Uth4 and Ygl023 compete with each other for silencing components. This prediction suggests that the proteins may interact with silencing factors through the repeat region. This issue is presently being addressed. The best evidence suggests that Uth4 and Ygl023 recruit silencing factors to different loci. While the $UTH4$ sequence does not reveal any obvious DNA binding regions, $YGL023$ has sequence corresponding to a putative zinc finger. Alternatively, these proteins may interact with other proteins at the relevant loci.

$pumilio$ is a well characterized protein which acts as a translational repressor of $hunchback$ (Murata and Wharton, 1995). It directly binds to the 3' untranslated region of $hunchback$ mRNA. Therefore the function of pumilio seems unrelated to that of Uth4 and Ygl023. Published evidence indicates that the repeat region of pumilio is not required for RNA binding (Murata and Wharton, 1995). The most likely explanation for the strong sequence conservation between these proteins is that the repeat region serves as a protein: protein interaction domain that is used in different organisms for different purposes. Alternatively, Uth4 could be a multi-functional protein and also plays a role in translation. In support of this theory, there are several phenotypes ascribed to $UTH4$ that cannot be related to transcriptional
silencing as yet (Kikuchi et al., 1994; Sakai et al., 1992). The sequence database contains several other proteins with pumilio repeats in a variety of organisms including humans. It remains to be determined what the function of the proteins may be.

Silencing in Old Cells

Sir-mediated transcriptional silencing of silent mating type loci is compromised in old cells (Smeal et al., 1996). One model for this observation is that silencing factors become de-stabilized as cells age. This would lead to an increased expression of the aging locus causing the cells to age and die. Another possibility is that the aging cell has a greater need to silence internal loci such as the aging locus and silencing factors are recruited away from HM loci and telomeres to meet this increased need. This would also result in the sterility observed in old cells. Without cloning the Aging locus, it is impossible to determine which of these models may indeed be correct. Nevertheless, it is probable that UTH4 and SIR2-4 are critical determinants of aging in yeast. Further analysis of both Uth4 and Ygl023 function may also be instrumental in determining additional cellular roles for yeast silencing factors.

Experimental Procedures

Strains, Plasmids, and Media

Yeast strains (Table 1) were propagated using standard media and conditions (Sherman et al., 1986). The sir3Δ was constructed with URA3 using
plasmid pDM42 (Mahoney and Broach, 1989). SIR4 was disrupted and replaced with LEU2 (Ivy et al., 1986). The UTH4 disruption plasmid was constructed by replacing the region from 159 bp upstream of the putative translational start site through +166 with the LEU2 gene. The YGL023 disruption plasmid was constructed by replacing the region from base pair +925 to base pair +1386 of coding region with hisG-URA3. Disruptions were subsequently passed over 5-FOA to eliminate the URA3 gene (Boeke et al., 1987). All gene disruptions were confirmed by Southern analysis.

Gap repair analysis was conducted to map the mutation in UTH4-326. Digestions of pBK4-3 were performed with a variety of restriction enzymes which recognize sites in the UTH4 coding region. The digested ARS-CEN plasmid was then transformed into BKx1-14c. After repair of the plasmid, strains were scored for stress resistance. The mutation was clearly mapped to the region of UTH4 between base pair +557 and +2155.

ADE2 was integrated at the chromosome VIIL telomere using plasmid pHR10-6 kindly provided by D. Gottschling (Gottschling et al., 1990). ADH1-UTH4 and ADH1-YGL023 constructs were made by PCR cloning UTH4 or YGL023 and inserting them into pDB20 at the NotI site (Becker et al., 1991). To integrate these overexpression constructs, the entire promoter and terminator of ADH1 including either UTH4 or YGL023 was moved into pRS306 (Sikorski and Hieter, 1989) and integrated at URA3 by StuI digest. HA-UTH4 was cloned and integrated in a similar manner except the HA epitope was included in the 5' primer used to clone UTH4 by PCR. All integrations were confirmed by Southern analysis.

Strains BKyl9, Bky131, BKy132, and Bky133 were constructed in the following manner. SIR4 was disrupted with LEU2 in all strains as described (Kennedy et al., 1995). LEU2 was then disrupted with hisG-URA3 and
subsequently grown on media containing 5-FOA to recover the *URA3* marker. *UTH4* was then disrupted with *LEU2* as described above in strains Bky119 and Bky131. *SIR4-42* was integrated at the *URA3* locus by StuI digest in BKy131 and BKy133. *SIR4* was integrated at the *URA3* locus by StuI digest in BKy 119 and BKy132.

**Cloning of UTH4**

Overexpressing human cdk2 from a *GAL1* promoter causes toxicity in some yeast strains including BKxl-14c. The *GAL1*-cdk2 plasmid was kindly provided by M. Meyerson (Meyerson et al., 1992). The *UTH4-326* mutant allele confers resistance to this toxicity. The *UTH4* gene was cloned by selecting plasmids from the genomic pJZ1 library, constructed from the *UTH4-326* strain, which conferred resistance to cdk2 mediated toxicity. Clones were then tested for their ability to extend life span.

The cdk2 toxicity most likely does not relate to the aging phenotypes. Both Δ*asir4* and *SIR4-42* strains are also resistant to cdk2 toxicity. This finding suggests that the cdk2 phenotype more closely resembles the stress resistant phenotypes previously described because Δ*asir4* and *SIR4-42* strains behave differently with regard to life span (Kennedy et al., 1995).

**Construction of the genomic library pJZ1**

The pJZ1 library was constructed by partially digesting genomic DNA from BKy3.26 with Sau3A and cloning fragments into the partially filled XhoI site of pRS315 (Sikorski and Hieter, 1989). Average insert size was approximately 4 kb.
Immunofluorescence

Immunofluorescence experiments were performed as described (Palladino et al., 1993). Antibodies directed to Rap1 were generated as described (Klein et al., 1992). Antibodies were made that recognized Sir3 and Sir4 as described (Palladino et al., 1993).

Life Span Procedure

Life span analysis was performed as previously described (Kennedy et al., 1994).

Statistical Analysis

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, Incorporated). Whenever the mean life spans of two strains are said to be statistically significant, the analysis showed a confidence level greater than 99%.

Telomere Length Assays

Telomere lengths were determined as previously described (Kennedy et al., 1995).
Acknowledgments

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References


Marcand, S., Buck, S.W., Moretti, P., Gilson, E. and Shore, D. Nucleation of silent chromatin by Sir3p and Sir4p is controlled by sequestration of silencing factors at telomeres by Rap1 protein. Genes Dev. in press.


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The first listing for each strain background shows the full genotype of the strain. In subsequent listings, only the genetic alterations are displayed. The following strains were previously described: BWG1-7A (Guarente and Mason, 1983), PSY142 (Kennedy, et al. 1994), PSY316 (Berger et al., 1992), BKy4.2, BKy6 and BKy28 (Kennedy et al., 1995). Strains YLS56 and YLS227 were a gift of D. Shore (Brand et al., 1987).
Figure 1. Sequence comparison of \textit{UTH4, YGL023, and pumilio}

(A) Sequence of the eight 36 amino acid repeats of UTH4. The repeated regions of YGL023 (B) and \textit{pumilio} (C) were re-drawn from Barker \textit{et al.} (1992). The frameshift mutation of BKx1-14c is shown in (D). This allele of \textit{UTH4} has an extra adenosine within a repetitive stretch of adenosines. The \textit{UTH4-326} mutant allele has a loss of one adenosine in the same stretch restoring the wild-type, published sequence of \textit{UTH4}.
Mortality curves are shown for combinations of \textit{UTH4} and \textit{YGL023} alleles in either the BKx1-14c (A) or the PSY316AT (B) strain background. All curves were derived from two independent experiments. The relative differences between strains was conserved in both experiments. (A) The sample sizes are as follows: BKx1-14c, 40 cells; \textit{uth4} \textit{ygl023}, 39 cells; \textit{uth4-14c ygl023}, 40 cells; \textit{UTH4-WT ygl023}, 40 cells, \textit{UTH4-WT ygl023}, 39 cells. (B) The sample size for all four strains was 40 cells.
A. 

B. 

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**Figure A:** Graph showing % Cells Viable over Generations for different strains. 
- BKx1-14c (uth4-14c YGLO23)
- Δuth4 YGLO23
- uth4-14c Δygl023
- UTH4-WT YGLO23
- UTH4-WT Δygl023

**Figure B:** Graph showing % Cells Viable over Generations for different strains. 
- PSY316AT (UTH4-WT YGLO23)
- Δuth4 YGLO23
- UTH4-WT Δygl023
- Δuth4 Δygl023
Figure 3. Relationship between *UTH4* and the silencing genes *SIR3* and *SIR4*

(A) Mortality curves for *UTH4-WT* strains with or without either *SIR3* or *SIR4*. All curves are derived from two independent experiments. The sample sizes for each strain are as follows: *uth4-14c*, 40 cells; *UTH4-WT*, 39 cells; *UTH4-WT Δsir3*, 39 cells; *UTH4-WT Δsir4*, 38 cells. (B) Mortality curves for combinations of *UTH4* and *SIR4* alleles. All curves are derived from two independent experiments. The sample sizes for each strain are as follows: *SIR4 uth4-14c*, 40 cells; *SIR4-42 uth4-14c*, 34 cells; *SIR4 UTH4-WT*, 37 cells; *SIR4-42 UTH4-WT*, 34 cells.
A. 

Graph showing the percentage of cells viable over generations for different strains: 
- uth4-14c
- UTH4-WT
- UTH4-WT Δsir3
- UTH4-WT Δsir4

B. 

Graph showing the percentage of cells viable over generations for different strains: 
- SIR4 uth4-14c
- SIR4 Δ2 uth4-14c
- SIR4 UTH4-WT
- SIR4 UTH4-WT Δsir4
- SIR4 Δ2 UTH4-WT
**Figure 4: SIR4-42 does not extend life span in the absence of uth4-14c**

Mortality curves for the following strains are derived from two independent experiments. The sample sizes for each strain are as follows: *SIR4 uth4-14c*, 40 cells; *SIR4-42 uth4-14c*, 38 cells; *SIR4 Δuth4*, 39 cells; *SIR4-42 Δuth4*, 40 cells.
The graph shows the percentage of cells viable over generations for different strains:

- **SIR4 uth4-14c**
- **SIR4-42 uth4-14c**
- **SIR4 Δuth4**
- **SIR4-42 Δuth4**

The x-axis represents generations, and the y-axis represents the percentage of cells viable. The curves indicate the decline in cell viability over time for each strain.
Figure 5. The effect of Uth4 and Ygl023 on transcriptional silencing

(A) Transcriptional silencing at telomeres varies dramatically if Uth4 or Ygl023 is not present. Strains depicted have the following genotypes: lower left, Δuth4 Δygl023; lower right, Δuth4 YGL023; upper right, UTH4 Δygl023; upper left, UTH4 YGL023. Strains were grown for 4 days on SC + glucose media supplemented for all requirements. The concentration of adenine was reduced to 6μg/ml to induce color formation. (B) Transcriptional silencing at HM loci is enhanced in Δuth4 strains. Strains in the left portion were grown for 4 days on SC + glucose media supplemented for all strain requirements. The plate on the right is identical except that it lacks tryptophan. Strains were plated on the -Trp plate in the same order as the complete plate. In AEB TRP1 strains, the TRP1 gene has been integrated at HMR and is transcriptionally silenced. AEB TRP1 are similar except that the cis-acting E site has been deleted, resulting in a partially crippled silencer. The E site is a binding site for Rap1. Both strains were provided by D. Shore (Brand, et al., 1987).
The **UTH4** and **YGL023** gene products act to decrease telomere length.

Telomere length is reflected in the broad bands slightly larger than 1kb. A region of the telomeric Y' region was used as a probe (Kennedy, et al., 1995). Genomic DNA was digested with XhoI, which cuts in the Y' element of most telomeres and run on a 0.85% agarose gel. All strains shown are derived from the PSY316AT strain background (Table 1).
Figure 7 The effect of overexpressing *UTH4* and *YGL023* on aging and telomere silencing.

(A) Mortality curves of strains overexpressing either *UTH4*, *YGL023*, or nothing in the PSY316AT strain background. BKy123 is strain PSY316AT with a *URA3* gene integrated at the *ura3*-52 locus to make all strains isogenic (Table 1). Two isogenic strains of each genetic background are depicted. Curves are derived from two independent experiments. The sample sizes for each strain are as follows: BKy123 (1), 46 cells; BKy123 (2), 50 cells; *ADH1-UTH4* (1), 66 cells; *ADH1-UTH4* (2), 36 cells, *ADH1-YGL023* (1), 40 cells, *ADH1-YGL023* (2), 39 cells.

(B) Mortality curves of strains overexpressing *UTH4* or *YGL023* or nothing in the long-lived PSY142 strain background. BKy126 is strain PSY142 with a *URA3* gene integrated at the *ura3*-52 locus to make all strains isogenic (Table 1). Two isogenic strains of each genetic background are depicted. Curves are derived from two independent experiments. The sample sizes for each strain are as follows: BKy126 (1), 38 cells; BKy126 (2), 38 cells; *ADH1-UTH4* (1), 39 cells; *ADH1-UTH4* (2), 38 cells, *ADH1-YGL023* (1), 40 cells, *ADH1-YGL023* (2), 39 cells.

(C) Telomere silencing in overexpression strains. Strains used are identical to those used in (A). The strains were grown for 3 days on rich (YPD) media.
Figure 8. Immunolocalization of silencing factors in \textit{SIR4} and \textit{SIR4-42} strains.

Immunolocalization was performed using confocal microscopy (see Experimental Procedures). DNA is stained red with ethidium bromide. Proteins of interest appear green. The strain used in (A), (C), and (E) is BKy6. The strain used in (B), (D), and (F) is BKy28 (Table 1). Cells were stained with Anti-Sir4 antibodies in (A) and (B), Anti-Sir3 antibodies in (C) and (D), and Anti-Rap1 antibodies in (E) and (F).
Figure 9. A model for the regulation of silencing components in yeast

In the proposed model, proteins act to pull silencing factors away from telomeres to other chromosomal locations. Uth4 would recruit silencers to the Aging locus. Sir1 acts to recruit silencers to *HML* and *HMR*. Ygl023 would also recruit silencing factors to an unknown chromosomal location.
Appendix 1: Loss of transcriptional silencing causes sterility in old mother cells of *S. cerevisiae*

This appendix was previously published in Cell, Volume 84, pages 633-642. The authors were Tod Smeal, James Claus, Brian Kennedy, Francesca Cole, and Leonard Guarente. My contribution to this work was the analysis of factor responsiveness in old cells presented in Table 2.
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 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, 1984) or at the level of individual cells (Hayflick, 1965). In this 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; Alsopp et al., 1992; Vaziri et al., 1993; Schwartz et al., 1993). In paramecium (Gilley and Blackburn) and in yeast (D'Mello and Jazwinski, 1991), telomere shortening has not been observed in old cells.

In *C. 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 in adults of genes in this pathway by use of conditional mutations extends their life span substantially (Kenyon et al., 1993).

In the case of the yeast *S. 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 et al., 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, 1984). 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 crosslinking 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 which had increased mean and maximum life spans. One mutation to emerge was an unusual allele of \textit{SIR4}. This gene, along with \textit{SIR2} and \textit{SIR3}, silences the unexpressed copies of \(\alpha\) and \(\alpha\) 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 \textit{SIR4-42} mutation behaved like a null allele with respect to silencing at \(HM\) loci and telomeres; silencing was abolished. However, only the \textit{SIR4-42} allele extended life span, and was dominant to \textit{SIR4} for this effect. A null mutation in \textit{SIR4} actually shortened life span. These findings indicated that extension in life span by \textit{SIR4-42} was not due to phenomena occurring at \(HM\) loci or telomeres, but involved some other \textit{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 silencing in old cells which causes the aging-specific phenotype of sterility. Our findings provide the first molecular explanation for any 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 which 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 one in $2^{20}$ or about one in a million. 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 which have all divided a predetermined number of times.

Thus, we conjugated the surfaces of cells (strains PSY142 and BKx1-14c) with biotin (Experimental procedures), which covalently attaches to primary amines, such as the epsilon-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 unlabelled cells by FACS analysis. Starting with a mixture containing 20% fluorescent PSY142 cells and 80% non-fluorescent cells, a single sort produced a fluorescent population that was $>99\%$ pure. When cells which had been quantitatively conjugated with biotin were incubated in rich media at 30°C for six hours, populations of unlabeled daughters and also unlabeled buds still attached to fluorescent
mothers were now evident (data not shown), indicating that the label was not transferred to daughter cells.

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 a fraction of cells (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 the 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) 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 BKx1-14c 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 vortexed, and the magnet 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, grown for a period of time to allow 12-14 divisions, and cells magnetically sorted. The sorted cells were grown in fresh media for an additional 12-14 generations and again sorted. In parallel, BKx1-14c cells were sorted. Because of 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 counting the number of scars per cell in the fluorescence microscope. Bud scars consist of chitin rings which 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 0 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 BKx1-14c sort had a peak of bud scars around 11 (Figure 3C). There were a few cells from the first sort of PSY142 and from the sort of BKx1-14c 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 BKx1-14c sort were senescent was their very low
efficiency of plating (<1%) compared to 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 5, telomeres of cells obtained from BKx1-14c, which had an average number of bud scars of 12, were unaltered (Figure 5). The shortening seen in cycling cells of Δsir4 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 BKx1-14c cells. Note that this strain is MATα. If silencing were abridged in old cells, expression of HMRα would result, causing repression of haploid-specific genes, such as STE12. As expected, Northern blotting showed that the levels of STE12 compared to total RNA were reduced 5-fold in a/α diploids, or haploids that expressed HMRα due to mutation of SIR4 (Figure 6). 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 *HMRα* itself is
expressed in old cells. We developed a 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 which promote
reverse transcription in the presence of a dT primer followed by PCR
amplification. Two primers were used which bracket an intron in *a1* coding
sequences (Miller, 1984) (Figure 7A). Thus, the PCR product resulting from
reverse transcription of the RNA will be 33 bases smaller than the product
derived from genomic DNA.

A single fragment was observed using nucleic acid from cycling cells of
BKx1-14c roughly the expected size of the product amplified from *a1* genomic
DNA (Figure 7B, lane 1). When we tested nucleic acid from BKy5 (isogenic to
14c, but *MATα*) (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 BKx1-14c *sir4* mutants, which derepress *HMRα* (lanes 4 and 5). We
confirmed that this product was derived from *a1* RNA by demonstrating its
sensitivity to RNAse (lane 8) and its requirement for inclusion of the dT
primer (lane 10). When nucleic acid from old BKx1-14c cells was examined,
the RNA-derived product was clearly evident (lane 6). There is somewhat less
RNA-derived product in this sample than observed is 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 mis-priming by the dT primer on the *a1* cDNA).
Additional control experiments were carried out. First, a similar PCR analysis revealed no trace of α1 RNA in cells that were biotinylated and immediately sorted (not shown). Thus, its presence in the old cell preparation must due to the age of the cells and not the sorting method. Second, structural analysis of the MAT locus in DNA from old cells revealed no trace of α information (not shown). Thus, the α1 RNA observed above can not be due to rare, HO-independent switching between HMR and MAT, or to contaminating MATa cells. In sum, these experiments show that HMRα1 is transcribed in old cells.

Transcription of HMRα1 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 nucleotides for MEI4 and 91 nucleotides for DCM1), rendering them amenable to PCR analysis, in a manner analogous to HMRα1 above. Using primers that bracket the introns in MEI4 and DCM1 (Figure 8A), we observed that the RNA products of MEI4 (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 (lanes 8 and 13). In the same experiment, the HMRα1 transcript was observed in old cells, as expected (lane 3) (as well as in MATα, Δsir4, and sporulating a/α diploid cells). Thus, we conclude that old cells do not transcribe genes promiscuously, and the derepression of HMR is likely due to a loss in silencing by the SIR complex.
Old cells become sterile due to derepression of $H M$ loci

These findings suggested that old cells might become sterile due to derepression of $HML$ and $HMR$. To first quantitate the degree of sterility in old cells sterile, mother cells of DLy1 (isogenic to PSY142, but $MATa$) that had divided various numbers of times were prepared by microscopic manipulation and then exposed to $\alpha$-factor for four hours (Experimental procedures). 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 non-responders) were then moved away from the pheromone and allowed to complete their life spans. Note that exposure to $\alpha$-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 $\alpha$-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, 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 were due to derepression of the silent mating type loci in old cells, we deleted $HMLa$ 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 $\alpha$-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 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 
lifespan, and, second, an alteration in the phenotype of the organism with age.
In neither case is the molecular cause of aging known in any organism. 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 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; Alsopp 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 (D'Mello and Jazwinski, 1991). Second, our genetic studies indicated that while the SIR2/3/4 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, \textit{STE12}, was repressed in old cells of a haploid strain. This was consistent with the possibility that silencing at \textit{HMLα} and \textit{HMRα} had been lost in these cells. Second, and more directly, we demonstrated that silencing at \textit{HMRα} was lost in old cells of this \textit{MATα} strain. Other transcriptionally repressed genes, such as the sporulation-specific genes, \textit{MEI4} and \textit{DMC1}, remained silent in old cells. It is noteworthy that the appearance of transcription at \textit{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 \textit{HML} and \textit{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 \textit{HML} and \textit{HMR} is strengthened by SIR1 (Pilus and Rine, 1989). Indeed, assays for defects in trans-acting mediators of silencing frequently employ \textit{HM} loci that have been weakened by removal of one of the cis-acting elements (Sussel and Shore, 1991).

The loss of silencing at \textit{HMR} in old cells could be due to inactivation of the SIR2/3/4 complex or, perhaps, SIR1. In \textit{sir1} mutants, a fraction of cells transcribe \textit{HM} loci (Pilus and Rine, 1989), which could account for the \textit{HMRα} transcript observed in old cells. However, previous experiments showed that while deletion of \textit{SIR4} shortened life span, deletion of \textit{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 \textit{Δsir1} strain to exhibit a shortened life span similar to the \textit{Δsir4} strain.
Sterility in old cells due to loss of silencing

The simultaneous expression of $\alpha$ and $a$ information in growing cells results in sterility. We therefore surmised that old cells might have an aging-associated phenotype of sterility because of a loss of silencing at $HML\alpha$ and $HMR\alpha$. This would be consistent with earlier findings that the efficiency of zygote formation is reduced when old yeast cells are mated to young cells (Muller et al., 1985). Cells of various ages of a $MATa$ strain were thus exposed to $\alpha$-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 $HML\alpha$ 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? One possible answer 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 would still be fertile. In this case, 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-aza-cytosine, which inhibits DNA-methylase 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, or 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 invariant 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 BKx1-14c (MATα adel-100 his4-519 leu2-3,2-112 lys2-801 ura3-52), the isogenic MATα derivative, BKy5, the isogenic a/α diploid, BKy6, and PSY142 (MATα 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 OD600 of 0.5. Between 10^7 and 10^8 cells were spun, washed and resuspended in cold 1XPBS. Cells were then spun and resuspended in 0.33 ml of room temperature 10XPBS. Separately, 3.5g of NHSLC biotin (Pierce) was dissolved in 0.67 ml 10X PBS and immediately added to the cells. The mixture was shaken 15 minutes by vortexing at a slow setting at room temperature. Cells were then spun and washed eight times in 1 ml cold 1X 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 cold 1X PBS. Cells were sonicated 30 seconds at 20% power and streptavidin paramagnetic beads 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 minutes for 2 hours. The suspension was then placed in a test tube in a magnetic sorter (Perseptive Diagnostics/Advanced Magnetics) in the 4°C room. After 15 minutes the supernatent was carefully aspirated, 10 ml cold YPD added, and the mixture vortexed. The cells were again placed in the sorter for 10 minutes and the process repeated 10 times.

For early experiments involving the fluorescence activated cell sorter (FACS) up to 10^8 biotinylated cells were suspended in 1 ml of 1X PBS and 50μl of
fluorescein-conjugated avidin (Pierce, 5mg/ml) was added. The mixture was vortexed, incubated 10 minutes on ice covered with foil to avoid bleaching, and washed 2X with 1ml of 1X 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 α using a GAL-HO expression plasmid. HMLα was deleted with a LEU2 marker as described (White and Haber, 1990) and confirmed by Southern. 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 ΔHMLα. Cells were taken from YPD (rich) agar grown 24 hours and re-plated at low density on YPD agar. After 3 hours, 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 microns from filters with 0.02µg/µu α-factor (Sigma). This relatively low concentration of pheromone afforded the most sensitive identification of sterile cells. Cells were observed hourly for 4 hours and schmooing scored (Byers and Goetsch, 1975). This phenotype was unambiguous. Cells were then moved away from the pheromone and the filters 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 hrs at 30°C, spun down, washed 1x with SPM media (.3% potassium acetate, .02% raffinose) and incubated under well aerated conditions in SPM media for 37 hrs at 30°C and then harvested for RNA analysis.

**Northern analysis**

Total RNA was fractionated on 1% agarose-formaldehyde gels and transferred to Gene Screen Plus membranes (NEN Research). Blots were
prehybridized in 6X SSC, 5X Denhardt's, 20mM NaH2PO4, 100ug/ml salmon sperm DNA for 2 hours at 65°C. Hybridization to random primed STE12 or SPT15 (TBP) DNA probes was in 6X SSC, 0.4% SDS, 20mM NaH2PO4, 100ug/ml salmon sperm DNA for 20 hours at 65°C. Filters were washed with 6X SSC, 0.1% SDS for 2 hours at 65°C.

**Rt-PCR analysis of RNA**

Total RNA was reverse transcribed using the Perkin Elmer Gene Amp system as per manufacturer's instructions. 50 pmoles of oligo dT 12-18 and total RNA were incubated at room temperature for 10 minutes, the reverse transcription components added, and samples incubated for 10 minutes at room temperature followed by 10 minutes at 42°C and then 10 minutes 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 minutes at 50°C and then 15 minutes at 70°C). The cDNAs were amplified with a thermal cycler set for 35 step cycles of 94°C for 1 minute, 60°C for 1 minute, followed by a final extension step of 7 minutes at 60°C (except for MEI4 and DMC1 for which the thermal cycler was set for 40 step cycles of 94°C for 1 minute, 50°C for 30 seconds, 60°C for 1 minute, followed by a final extension step for 7 minutes at 60°C. The 5' primer (5' GGCGGAAACATAACAGAAGACTCTG 3') and 3' primer (5' CCGTGCTTGGGGTGTATTGTAG 3') were used to amplify all. MEI4 was amplified with the 5' primer(5' AGATATGGGAACAAAAGGAAACATCGG 3') and the 3' primer (5' AAATGATTGGCGCTCCCTITAAAACCAACTGCATCTTC 3'). DMC1 was amplified with the 5' primer (5' TCTGTTACAGGAAGTAAGTCCATAGTGCATCCTTC 3') and the 3' primer (5' CGCTGCTTTATATCATTTCAGTAGCAGGGGG 3'). Products were resolved
on 1.5% agarose/1X TAE gel stained with EtBr and photographed under short-wave UV light.

**Acknowledgements**

We wish to thank R. Isberg and R. Axel for helpful suggestions. This work was supported by a research grant from the N.I.H. to L.G (AG11119), a postdoctoral grant from the American Cancer Society to T.S, a predoctoral grant from the N.S.F. to J.C., and a predoctoral grant from the H.H.M.I. to F.C.

**References**


**TABLE 1 - Enrichment of biotinylated cells by magnetic sorting.**

<table>
<thead>
<tr>
<th>Cells</th>
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<th>Percentage of biotinylated cells</th>
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Biotinylated BKx1-14c 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 BKx1-14c, which is auxotrophic for these requirements. The number of BKx1-14c (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.
Divided at least one time after being removed from the presence of a factor. All cells labeled (non-responders). The relative ages of cells at the time of exposure to a factor were determined by removing them from the presence of a factor and determining the remaining number of divisions they underwent. Numbers in parentheses reflect the percentage of the cells that were relatively low (see experimental methods). Numbers in parentheses reflect the percentage of the cells that were relatively low (see experimental methods).

This is probably because the concentration of factor used passed through G1 in wild-type and + in AHMLA. A minority of cells in the K cells responded in their second round of complete passage through the cell cycle. A minority of cells in the K cells responded in their second round of complete passage through the cell cycle.

Indicates that the cells responded by forming colonies and indicates that the cells did not respond to factor. Table I (MALT1 AHMLA or DLY1 AHMLA) shows that different ages were examined for their ability to respond to factor.

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TABLE 2 - Alpha factor responsiveness in old cells
Figure 1. Sorting of biotinylated cells by the fluorescence-activated cell sorter (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 non-fluorescent cells (bottom panel) was subjected to sorting by FACS, and a fluorescent population of cells (middle panel) and non-fluorescent population (top panel) were obtained. The purity of the fluorescent population was estimated to be >99%.
Figure 2. Sorting old cells by biotinylation and avidin-conjugated magnetic beads

This details of the biotinylation and sorting are detailed 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 1 biotinylated cell -- the mother originally labelled. Avidin-magnetic beads (small black 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.
1. Label surfaces of growing cells with biotin

2. Grow labelled cells 10-15 generations

3. Isolate biotinylated cells with avidin-magnetic beads

4. Stain and count bud scars
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 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. Panels A and B show a histogram of bud scar number in cells from the first and second sort of the long-lived strain, PSY142, and panel C shows the same for a single sort of the short-lived strain, BKx1-14c.
A First Sort

Number of Bud Scars

B Second Sort

Number of Bud Scars
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, 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.
DNA was extracted from approximately $10^7$ old cells of BKx1-14c and telomeres analyzed as described (Kennedy et al., 1995). 14c and 14c Δsir4 DNA were extracted from exponentially 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 kilobase 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 Δsir4 strain. Note that there is no shortening of telomeres in DNA from old cells.
Figure 6. Northern blot of $STE12$, an a/α repressed gene, in DNA from old cells of BKx1-14c ($MAT\alpha$)

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 to the $MAT\alpha$ haploid, and about 3-fold in the old cell RNA, as determined by quantitation with the phosphorimager.
Figure 7. Sequential reverse-transcription/PCR amplification (rtPCR) analysis of a1 RNA from old BKx1-14c (MATα) cells

Panel A shows the a1 coding sequence present at HMR in BKx1-14c 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 nucleotides. Panel B shows an experiment using these primers in a sequential reverse transcription-PCR amplification assay (Experimental procedures) with nucleic acid extracted from the indicated strains. Lane 1: BKx1-14c. This strain does not express a1 RNA and the amplification product of a1 genomic DNA is evident. Lanes 2 and 3: BKy5 (MATa) and BKy6 (a/α diploid). These strains express a1, and the RNA-derived product predominates. Lanes 4 and 5: BKx1-14c 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 BKx1-14c 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 rtPCR showing only the DNA-derived product. Lanes 9 and 10: controls in which the dT primer for reverse transcription was omitted prior to rtPCR.
A

MATa1

P1

P2

1500
1554
1631
1684
1832

B

MATα
MATα
SIR4
OLD
RNase
dT primer

+ + + + + + + + + +
+ + + + + + + + + +
+ + + + + + + + + +
+ + + + + + + + + +
+ + + + + + + + + +
+ + + + + + + + + +

BP

500
300
150

1 2 3 4 5 6 7 8 9 10 11
Figure 8. rtPCR analysis of $MEI4$ and $DCMI$ RNA from old cells

Panel A shows the $MEI4$ and $DCMI$ 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. Panel B shows RT-PCR analysis of nucleic acid from the indicated strains. Lanes 1-5 use the $MATa1$ primers, as in the previous Figure, lanes 6-10 use $MEI4$ primers, and lanes 11-15 use $DCMI$ primers. The strains are as in Figure 7, OLD is nucleic acid from old BKx1-14c cells, and SPO is nucleic acid from a/a diploid cells sporulated for 20 hours. $a1$ RNA is present in cells expressing $MATa1$ (lanes 2,4,5) and in old cells (lane 3). $MEI4$ and $DCMI$ RNA (lower bands) is present in sporulating cells (lanes 10 and 15) but not in old cells (lanes 8 and 13) or in vegetatively grown cells (lanes 6,7,9,11,12,14). The upper bands represent $MEI4$ and $DCMI$ DNA.
### A.

<table>
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### B.

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[Image of gel electrophoresis results]