UTH1 and the Genetic Control of Aging in the Yeast, *Saccharomyces*

by

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Submitted to the Department of Biology in partial fulfillment of the
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ABSTRACT

Individual yeast cells have a restricted division capacity called their lifespan after which they stop cycling and lyse. Furthermore, the mortality kinetics of a population of yeast cells can be described by the Gompertz equation, the classical definition for aging populations, which states that the mortality rate, the probability of dying in the next time interval or generation, increases exponentially with age. Aging cells increase in size, experience a lengthening of their cell cycle times, and accumulate lipid containing granules which are expelled into the media when the cells lyse. Intriguingly, in several wildtype backgrounds, the length of a strain's lifespan correlated with its ability to remain viable during starvation. To test the hypothesis that the same genes may regulate both the starvation response and the lifespan in yeast, a screen was performed to isolate stress-resistant mutants which were also long-lived.

The screen identified three recessive complementation groups and a dominant mutation with both increased viability after starvation and extended lifespan. These youth (*UTH*) mutants have a variety of phenotypes which suggest that the length of the lifespan of a strain is a reflection of its fitness and its ability to cope with stress.

Cloning of *UTH1* was done by complementation of the paraquat sensitivity of the mutant. Paraquat is a drug which is thought to generate superoxide radicals *in vivo*. *UTH1* is a novel gene located on chromosome XI. Disruption of the putative open reading frame extends longevity suggesting that *UTH1* is a yeast aging gene. Epistasis analysis demonstrated that *UTH1* is genetically upstream of the *SIR* genes which regulate transcriptional silencing in yeast. Furthermore, the analysis of *uth1* mutations in strains where telomeres or the HM loci are marked with a reporter sensitive to silencing levels suggested that *UTH1* encodes an "anti-silencer". We propose that loss of *UTH1* would lead to enhanced silencing throughout the genome and a subsequent increase in lifespan from the silencing of an aging gene(s).

UTH1 is a founding member of the *SUN* (*SIM1*, *UTH1*, *NCA3*) family of *Saccharomyces* genes which share a 215 amino acid domain at their C-termini. Two additional *SUN* genes have recently been identified including *SUN4*, another gene from *Saccharomyces*, and *bglA*, a putative beta-glucosidase from *Candida wickerhamii*. Null mutations in the other *Saccharomyces SUN* genes decreased the mean lifespan but not the maximum lifespan of the strain and it is still not clear if these genes directly affect the yeast aging process.

Characterization of the *UTH* genes provided the first links between transcriptional silencing and aging in the yeast cell and suggested that still unidentified aging genes (called AGE) were subject to gene silencing. Two other regions of the yeast genome, the telomeres and the HM loci, are known to be silenced. To examine the relationship between these known silenced regions and the hypothetical AGE locus, we determined the effects of changing telomere length on the lifespan of the yeast cell. We discovered an inverse correlation between telomere length and lifespan. Mutations which lengthen telomeres including *rap1s* and *Δrif1* lead to a shortening of lifespan.

The significant shortening of lifespan in the *Δrif1* strain was partially suppressed by alleles of *SIR4* which are thought to mislocalize the silencing complex to AGE. In contrast, overexpression of truncated *TLC1*, the gene encoding the yeast telomerase RNA, leads to a shortening of telomeres and a delay in senescence. Strikingly, this extension in lifespan requires *SIR3* which encodes an essential component of the silencing machinery. We propose that short telomeres increase the pool of available silencing complexes which enhances silencing at AGE delaying senescence. Our findings suggest that the telomere shortening seen in senescing mammalian cells may not cause senescence but may actually be a cellular response to an age-dependent loss of genomic silencing: telomeres shorten so that cells can continue proliferating.

In summary, the identification of single gene mutations which modulate longevity has opened up the field of aging to the power of yeast genetics. It remains to be seen, however, if any of these regulatory pathways is functionally conserved in models of replicative senescence in higher cells. What is clear is that yeast aging has become more than just a bud scar phenomenon. It is an area of research which is still young.

Thesis Supervisor: Leonard P. Guarente
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to my parents

My whole life I have known that I am loved.
Thank you for everything.

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Arnold Seto has continuously challenged my beliefs and in the process, made me even more of a believer. He's been a good friend. Arnold, I still think I will win our bet. You'll see...

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Finally, I thank GOD. My greatest discovery here at MIT was the joy and the peace that comes with the call to be a fool for Christ. He has made the good times great and the bad times better. I can ask for no more.

6

A TWO THOUSAND YEAR OLD SOLUTION TO THE AGING PROBLEM

พระผู้เป็นเจ้าทรงรักมนุษย์มาก
ถึงกับมอบพระบุตรองค์เดียวของพระองค์ให้
เพื่อว่าผู้ที่เชื่อในพระองค์จะไม่ตาย
แต่จะได้รับชีวิตนิรันดร

จอห์น 3:16

Gayon na lamang ang pag-ibig ng Diyos sa sanlibutan,
kaya ibinigay niya ang kanyang bugtong na Anak,
upang ang sumampalataya sa kanya ay hindi mapahamak,
kundi magkaroon ng buhay na walang hanggan.

Juan 3:16

For God so loved the world
that He sent His only begotten Son
So that whosoever believes in Him may not die
but have eternal life.

John 3:16

TABLE OF CONTENTS

CHAPTER ONE

Aging Genes, Longevity Genes, and the Quest for Immortality

INTRODUCTION

Are there aging and longevity genes? Species-specific lifespans and longevity differences among members of the same species have always been given as evidence that genetic factors influence aging. The nature of the genetic elements involved, however, has remained controversial. One possibility, that of aging genes which act solely to promote the deterioration and eventual death of the organism, has often been dismissed on evolutionary grounds. The primary argument is that there is no selection for genes which act during a period of life which most organisms in a natural population never reach because of predation or deleterious environmental conditions. In fact, natural selection would favor the selection of aging gene loss-of-function mutants which do not age and therefore continue to produce immortal progeny.

A more nonadaptive view looks at aging as the biological equivalent of wear and tear. The functional decline of the organism would arise from the accumulation of damage during its post-reproductive period of life which is not subject to the pressures of natural selection. The genetic elements which determine lifespan, according to this view, would not be aging genes but longevity genes which act to prevent damage and delay the organism's loss of viability.

A priori, loss of function mutations in aging and longevity genes should lengthen and shorten an organism's lifespan respectively. In recent years, candidate longevity and aging genes have been identified in different genetic model systems and the functional analysis of their gene products should answer some outstanding questions on the nature of the aging process. This chapter reviews the progress that has been made to identify genes which determine the lifespans of different organisms. I begin by describing both the aging process in the budding yeast, *Saccharomyces cerevisiae,* and the recent searches for the genes which regulate the yeast lifespan. Yeast, however, is only one of the genetic systems that has been exploited in aging research and the next section outlines the numerous insights into aging that have emerged from studies with other fungi, worms, fruit flies, and mice. Ultimately, though, the goal of all aging research is to understand the limits to the lifespan of the human species and most of the work with human aging,

11

described at the end of this chapter, has focused on unraveling the genetic mechanisms behind the aging of human cells with the hope that this would lead to an understanding of organismal aging.

THE GENETICS OF AGING IN THE YEAST, *SACCHAROMYCES*

The Aging Process in Budding Yeast

Defining aging in budding yeast is possible only because of the asymmetry of its cell division cycle. The mother cell is usually larger than its newly budded daughter and can therefore be identified at the end of each cell division. This allowed Mortimer and Johnston to show for the first time that an immortal yeast colony was in fact made up of mortal yeast cells (Mortimer and Johnston, 1959). Using a tetrad dissecting microscope, they were able to isolate virgin cells and determine that these cells only completed a certain number of cell cycles before arresting. They called this limited division potential the lifespan of the yeast cell. The colony, however, was immortal because each daughter cell reset its clock and had the potential for a full lifespan (Johnston, 1966).

In the past thirty five years, a few labs have characterized the yeast aging process and defined several biomarkers for this organism. Probably the most obvious feature of a senescent yeast cell is its large size. Several studies have shown that the volume of the yeast cell increases linearly with age and that the senescent cell can be four times as large as an exponentially growing cell (Mortimer and Johnston, 1959; Egilmez, et al., 1990). Another parameter that changes with age is the duration of the cell cycle which increases exponentially with increasing generations (Mortimer and Johnston, 1959; Egilmez and Jazwinski, 1989). This lengthening in the period of the cell cycle results from an increase in the time spent in the unbudded portion of the cell cycle and though it is not absolute, most old cells arrest during this G1 stage (Johnston, 1966). One thing that is clear is that aging in yeast is not dependent upon chronological time. Extending the cell cycle time either by decreasing the temperature or by changing the carbon source does not change the mean number of generations that a population of cells can complete even though it can double the calendar time required to age (Muller, et al., 1980; Kennedy, et al., 1994). In

12

fact, changing the carbon source from glucose to ethanol which nearly doubles the cell cycle time increases the lifespan (Muller, et al., 1980). Finally, aging yeast cells undergo morphological changes while they age. First, they accumulate bud scars, the chitinous remnants of the mother-bud neck formed at each cell division. They also acquire wrinkles from a loss in turgor pressure (Muller and Wolf, 1978) and, as described in chapter two of this thesis, accumulate refractile granules which stain with Sudan Black B, a lipid specific dye. These lipid granules are reminiscent of the lipid pigment, lipofuscin, which is known to accumulate in aged cells of a diverse range of organisms (Katz, et al., 1984).

At the molecular level, RNA and protein content are increased several fold with age (Motizuki and Tsurgugi, 1992) while specific transcripts have been shown to either increase or decrease with increasing generations (Egilmez, et al., 1989). In contrast, the protein synthetic rate measured by the incorporation of radioactive amino acids per unit amount of protein is reported to decrease linearly with age (Motizuki and Tsurgugi, 1992). It has been speculated that the decrease in the synthesis of critical limiting proteins may be responsible for the prolongation of the unbudded phase in older cells (Motizuki and Tsurgugi, 1992).

These phenotypic changes including the increase in cell size, the accumulation of lipid granules and the lengthening of the cell cycle are typical of all aging yeast cells. This recognition of universal similarities that define the aging process in yeast naturally led to attempts to explain the phenomenon.

Unlikely Explanations for Yeast Aging

Five major hypotheses have been proposed to explain the senescence of yeast cells. One of the earliest is that the yeast cell arrests when it has exceeded some critical cell size and a minimum surface to volume ratio (Mortimer and Johnston, 1959; Johnston, 1966). Experiments that have increased cell size and volume either by varying the ploidy of the cell (Muller, 1971) or by enlarging arrested cells with mating pheromone (Kennedy, et al., 1994), however, demonstrate that these do not decrease lifespan. Finally, experiments described in the Appendix with isogenic strains which differ in size because they have

different alleles of the G1 cyclin gene, *CLN3*, rule out a correlation between yeast size and lifespan.

Mortimer and Johnston also suggested that bud scars may limit the availability of surface area for budding or for nutrient exchange with the environment (Mortimer and Johnston, 1959). Recent studies of daughters of older mothers have obtained data inconsistent with this hypothesis (Kennedy, et al., 1994). As mentioned before, during each asymmetric division, the smaller bud has a full life expectancy. At a low probability, however, an aging cell produces a bud of similar size which has a lifespan identical to the mother's remaining lifespan. These symmetric divisions argue against a causal role for bud scars in determining longevity because mother and daughter cells do not have the same number of scars. In addition, increasing the deposition of chitin, the major component of bud scars, using a conditional *cdc24* mutant has little effect on longevity (Egilmez and Jazwinski, 1989).

Yeast senescence cannot be easily explained by theories which posit the accumulation of mutations or genetic errors because most daughters of old cells which inherit a copy of the old genome are young (Johnston, 1966) and because haploid and diploid cells have identical lifespans (Muller, 1971; Kennedy, et al., 1995). In addition, no increase in the numbers of auxotrophies or *petite* mutations have been observed in old cells (Muller, 1971). Finally, senescence has been shown to be dominant in the rare zygotes generated between a young and an old haploid (Muller, 1985). Thus, the young genome cannot replace any functions that may have been lost in the old cell.

Another popular hypothesis for the senescence of cell populations both yeast and mammalian is that division capacity is limited by the length of telomeres -- cells die when their telomeres get too short (Allsopp, et al., 1992; Wright and Shay, 1995). This can be directly tested in yeast by taking advantage of mutations which modulate telomere length. For example, a deletion of *SIR4* shortens telomeres (Palladino, et al., 1993) while a *rap1s* mutation lengthens them (Sussel and Shore, 1991). Lifespan analyses of strains bearing these mutations described in chapter four of this thesis show that there is no direct correlation between telomere length and longevity. Furthermore, populations of senescent

14

yeast do not show any evidence of telomere shortening suggesting that loss of telomeres does not play a major role in yeast longevity control (D'mello and Jazwinski, 1991; Smeal, et al., 1996).

Given these unlikely explanations, why do yeast cells age? One possibility, supported by several observations, is that yeast contain a senescence factor that accumulates in old mother cells and is inherited by their daughters (Egilmez and Jazwinski, 1989; Kennedy, et al., 1994). A primary goal for yeast aging research has become the isolation and characterization of genes which may encode this senescence factor or other regulatory elements of longevity.

The Search for Yeast Aging and Longevity Genes

There is a growing amount of support for the notion that genes exist in yeast which regulate its lifespan. Aging in yeast can be described by the Gompertz equation, the classical definition for aging populations, which states that the mortality rate, the probability of dying in the next time interval or generation, increases exponentially with age (Pohley, 1987). This is evident in the sigmoidal shape of the yeast mortality curve which depicts the percentage of a cell population able to complete a given number of cell cycles. Furthermore, different wildtype yeast strains have different mean and maximum lifespans suggesting that aging in yeast is not a purely stochastic process and that the genetic background can influence the longevity of the strain (Mortimer and Johnston, 1959; Kennedy, et al., 1994; Kennedy, et al., 1995). Tetrad analysis showed that longevity in yeast is not cytoplasmically inherited and that it is a polygenic trait influenced by a still undetermined number of genes (Kennedy, et al., 1995).

Though the genetics suggest that genes can regulate the yeast lifespan, mutations in many yeast genes have no effect on longevity. Auxotrophic mutations fall into this category as do mutations in the mating pheromone response pathway (Kennedy, et al., 1994; Kennedy, et al., 1995). Not surprisingly, loss-of-function changes in genes involved in the maintenance of crucial growth functions shorten lifespan. Included in this class are UV-sensitive mutants (Muller and Wolf, 1978). Though these genes fit the

15

definition for yeast longevity genes, loss of these genes also results in a slow growth phenotype. It is therefore difficult to interpret the role of these genes in the direct control of the aging process because the shortened lifespan could simply be a consequence of a decrease in the overall fitness of the mutant strain. Ideally, a candidate longevity gene should be defined by loss-of-function mutations which shorten lifespan without changing any cell cycle parameters or growth characteristics of the cell. In addition, overexpression of the gene might be expected to extend lifespan. The RAD9 gene involved in cell cycle checkpoint regulation is one gene which satisfies the first criterion (Kennedy, et al., 1994). The second criterion for RAD9 has not yet been tested.

Yeast homologs of genes involved in proliferation control of higher eukaryotic cells are prime candidates for genes which determine longevity in yeast. Jazwinski and colleagues have reported that a deletion in RAS2, one of the yeast homologs of the mammalian RAS oncogene, shortens lifespan while overexpression leads to a 30% increase in longevity (Sun, et al., 1994). The precise role of the RAS2 gene in longevity control is still unclear, however, because deletions of this gene have also been reported to lengthen lifespan (Pichova, et al., 1995). Finally, deletion of the yeast gene PHB1, the yeast prohibitin homolog, is reported to lengthen lifespan (Franklin and Jazwinski, 1993). Prohibitin is a candidate tumor suppressor gene which has been implicated in the biogenesis of breast cancer (McClung, et al., 1995). Because mammalian prohibitin is a mitochondrially localized protein (Ikonen, et al., 1995; K.McClung, personal communication), it will be interesting to see how the mitochondria influences cell cycle and proliferation control.

Two approaches, one biochemical and one genetic, have been taken to identify novel genes which regulate longevity. Jazwinski and coworkers used a differential hybridization screen to isolate cDNAs that are preferentially expressed either in young or in old cells (Egilmez, et al., 1989). One gene called LAG1 (longevity assurance gene) encodes a putative transmembrane protein (D'mello, et al., 1994). Transcript levels decreased with replicative age and deletion of the gene reportedly increased the yeast lifespan by 50%. Five more transcripts remain uncharacterized.

16

As described in this thesis, we have taken advantage of a correlation between stress resistance and longevity to isolate long-lived yeast mutants (Kennedy, et al., 1995). Aging cells, as described in chapter two, morphologically resemble haploid cells starving in sporulation media suggesting that starvation and aging may be similar processes regulated by the same genes. This was confirmed when we identified mutations in the *UTH* (youth) genes which increased both stress resistance and longevity.

UTH2 was the first gene to be cloned by complementation of its sterile phenotype (Kennedy, et al., 1995). Sequencing showed that it is allelic to *SIR4* (silent information regulation), a gene involved both in the repression of the silent mating type loci (Ivy, et al., 1986) and in the regulation of telomeric silencing and structure (Palladino, et al., 1993). *Uth2-42* is a gain-of-function mutation in the *SIR4* gene; a deletion of *SIR4* had varying effects depending upon the genetic background of the strain. The extension of lifespan, however, is dependent upon an intact *SIR3/4* complex suggesting that the silencing complex somehow regulates the expression of an aging gene(s), the putative AGE locus. Strikingly, recent work with populations of old cells isolated with a purification protocol involving sorting with magnetic beads demonstrated that derepression of the silent mating type information occurs with age (Smeal, et al., 1996). Furthermore, experiments with single *MATα* cells deleted of the *HMRa* information have demonstrated that derepression of the silent mating type information occurs in individual senescent cells (Smeal, et al., 1996).

Cloning of *UTH1* described in chapter three was done by complementation of the paraquat sensitivity of the mutant. It is a novel gene located on chromosome XI. Disruption of the open reading frame extends longevity suggesting that *UTH1* is a yeast aging gene. The gene is a founding member of the *SUN* (*SIM1, UTH1, NCA3*) family of yeast genes which share a 215 amino acid domain (the SUN domain) at their C-termini. *SIM1* (START independent of mitosis) was isolated in a screen for cell cycle mutants which undergo two rounds of DNA synthesis without an intervening mitosis (Dahmann, et al., 1995). *NCA3* (nuclear control of ATPase) has been implicated in the regulation of the

17

expression of the mitochondrial ATP synthetase (Pelissier, et al., 1995). Another yeast gene in this family tentatively called *SUN4* has been identified by the yeast sequencing effort (Pohlmann and Philippsen, 1996). Lifespan analysis of cells carrying disruptions in the other members of the *SUN* family revealed that *SIM1*, *NCA3*, and *SUN4* have characteristics of yeast longevity genes.

Finally, the dominant mutation in *UTH4* (B. Kennedy and L. Guarente, unpublished data) is a change in the previously isolated gene *HTR1* (Kikuchi, et al., 1994)/*MPT5* (Coglievena, et al., 1995) which was identified in screens for genes required either for high temperature growth and recovery from pheromone arrest or for suppression of a mutation in *POP2*, a gene involved in glucose repression. It is still unclear how these phenotypes are related to the yeast aging process.

Our experiments, some of which are described in chapter three, have placed the different *UTH* genes in a genetic pathway that may regulate the yeast aging process. Like the original *uth2-42* mutant, mutations in *UTH1* and *UTH4* which extend lifespan require the presence of an intact *SIR4* gene. This places these *UTH* genes upstream of the SIRs suggesting that they may affect the localization or activity of the silencing complex at the putative AGE locus. Attempts are underway to determine the molecular basis for *UTH* gene action and to identify downstream targets. One possibility is that aging in yeast may result from inappropriate gene expression due to defective silencing late in the lifespan causing death.

THE GENETICS OF AGING IN OTHER EXPERIMENTAL SYSTEMS

Many of the advances in our understanding of different biological processes have emerged from studies in model systems which share similar genes and genetic pathways. Though this has not yet proved true in aging research, this goal has fostered work to identify genes which promote longevity in other organisms. Increased longevity has now been obtained by artificial selection, loss-of-function mutations in candidate aging genes, and gain-of-function mutations in candidate longevity genes.

Longevity from Artificial Selection

In genetically tractable organisms including mice, flies, worms, and fungi, attempts have been made to selectively breed strains with different lifespans to obtain long-lived variants. These experiments have had two goals. The first is to determine the number and locations of the genetic loci which determine longevity and the second is to look for phenotypic differences between the original individuals and those selected for postponed senescence.

Several studies of inbred mice have estimated that a small number of genes contribute to the differences in their lifespans. For example, Goodrick indicated that as little as one gene might be responsible for the differences between the C57BL/6 and the AJ strains which have mean lifespans of 823 and 655 days respectively (Goodrick, 1975). In contrast, after analyzing 20 recombinant inbred hybrid lines derived from the mating of the C57BL/6 and the DBA/2 strains, Gelman et al. concluded that at least six regions on four different chromosomes are statistically associated with the increased lifespan of the longerlived inbred strain (Gelman, et al., 1988). Difficulties inherent to the analysis of complex phenotypic traits have prevented further characterization of these loci.

Two different laboratories have bred extraordinarily long-lived strains of the fruit fly, *Drosophila melanogaster*, by selectively breeding the progeny of the longest surviving 20-30% of the population (Rose, 1984; Luckinbill, et al., 1987). In one case, twenty-fifth generation flies were living about twice as long as the original parental strains (Luckinbill, et al., 1987). Mapping experiments using these strains show that the major longevity determining genes accounting for about 70% of the observed variation in longevity are located on chromosome 3 although all the chromosomes have some contribution (Luckinbill, et al., 1988). Furthermore, Tyler et al. have shown that these longlived flies have alleles coding for more active superoxide dismutase (SOD) protein, an enzyme which counteracts damage from oxygen radicals (Tyler, et al., 1993). Similar mapping experiments have recently been performed with a complex recombinant-inbred population of the nematode, *Caenorhabditis elegans* (Ebert, et al., 1993). Five regions of the worm genome were identified in which one parental allele was significantly enriched in

19

the long-lived variants within the population. This is probably a low estimate of the number of longevity determining genes in the worm given that the study could only detect those loci which were polymorphic between the two parental strains.

All laboratory strains of the red bread mold, *Neurospora crassa*, are capable of indefinite growth. However, this fungi produces postmitotic cells called conidia which are comparable to the spores of other fungi. Aging of these cells is defined as the time dependent loss of viability under laboratory conditions which resemble the organism's tropical habitat (Munkres and Furtek, 1984). Selective breeding of *Neurospora* led Munkres and colleagues to produce a conidial longevity variant termed *age+* which produced conidia with a viability of 60 days compared to the wildtype viability of 22 days (Munkres and Furtek, 1984). They traced this extension to significant increases in the activity of a gene complex of different enzymes, including superoxide dismutase and catalase, which combat the deleterious effects of oxygen radicals (Munkres, et al., 1984). Furthermore, when they obtained short-lived, *age-* strains, through mutagenesis, they discovered that the mutations mapped to the same gene complex of oxygen radical genes, and resulted in decreases in enzyme activity (Munkres, et al., 1984). It is striking that long-lived variants in organisms as divergent as flies, fungi, and as will be discussed below, worms, show similar increases in their ability to protect themselves against oxygen radicals.

Longevity from Loss-of-Function Alleles of Aging Genes

By definition, a loss-of-function mutation in an aging gene should result in the extension of longevity. Mutants which fit this category have been found in *Caenorhabditis elegans*, and in the filamentous fungi, *Podospora anserina*, *Neurospora crassa* and *Neurospora intermedia*.

Several mutations in potential aging genes have been found in *C. elegans*. The first in the gene *age-1* was found in a screen for long-lived worms (Klass, 1983). This recessive mutation extends the lifespan by up to 70% by decreasing the mortality rate at every point in the animal's lifespan (Johnson, 1990). Larsen has proposed that *age-1*

could be a negative regulator of SOD and catalase because mutant worms showed both an age-dependent increase in enzyme activities that was not seen in the parental strain and a hyperresistance to oxidative stress (Larsen, 1992). It is still not clear, however, if this is a direct effect of the mutation. An even more impressive increase in longevity was seen in a mutation in the genetic pathway which regulates the developmental switch that converts an L3 larva into a dauer larva. The dauer larva is an alternate developmental stage in the worm which is long-lived and resistant to stress. Kenyon and colleagues showed that a mutation in the *daf-2* gene doubled the worm lifespan and that this was dependent upon a functional *daf-16* gene product (Kenyon, et al., 1993). Larsen and coworkers extended this observation by showing that the *daf-23(m333)* mutation also confers life-span extension. In addition, *daf-12* mutants interact with both *daf-23* and *daf-2* to cause an lifespan extending enhancement four-fold over wildtype (Larsen, et al., 1995). These results suggest that this genetic pathway might regulate the mechanism that ensures the longevity of the dauer larva. Intriguingly, recent work indicates that *age-1* may be in the same dauer regulatory pathway because *age-1 daf-16* double mutants show no extension of longevity (Dorman, et al., 1995). This has been confirmed with the demonstration that *age-1* is allelic to *daf-23* encoding a putative P-I-3 kinase (H. Tissenbaum, personal communication). Van Voorhies has shown that sperm production decreases the longevity of male worms (Van Voorhies, 1992). A mutation in the *spe-26* gene made the worms sterile but increased their lifespan by 65%. Given that the dauer larva is sterile, it should be interesting to see if *spe-26* is also involved in the establishment of the dauer state. A recent report proposed that it is copulation and not egg or sperm production which limits the lifespan of *C. elegans* (Gems and Riddle, 1996). Though initial reports (Friedman and Johnson, 1988) suggested that the *age-1* mutant also decreased fecundity, recent linkage data show that this phenotype is unlinked to the original mutation which confers longevity (Johnson and Hutchinson, 1993). Finally, *rad-8* mutant worms exhibit 50% longer mean lifespans than wildtype worms at 16°C and normal oxygen tension levels (Ishii, et al., 1994).

In *Podospora* and *Neurospora*, senescence has been linked to the destruction of

21

their mitochondrial genomes. In *Podospora*, an intron (the alpha-senDNA element) in the mitochondrially encoded cytochrome c oxidase gene excises itself over time and then transposes itself to new locations in the mitochondrial genome beginning the destruction of the mtDNA (Jamet-Vierney, et al., 1980; Wright and Cummings, 1983; Osiewacz and Esser, 1984). Combinations of nuclear mutations in the *incoloris* (*i*), *vivax* (*viv*), and *gresea* (*gr*) loci inhibit the generation of this extragenomic plasmid preventing senescence (Tudzynski and Esser, 1979; Tudzynski, et al., 1980). The *i*, *viv*, and *gr* gene products have yet to be identified but are postulated to encode proteins required for the DNA excision step of the alpha-senDNA (Esser, et al., 1980) (Kuck, et al., 1985). It is important to note that it has been proposed that senescence in *Podospora* is an artifact of laboratory culture conditions because cultures serially passaged in liquid media do not senesce (Turker and Cummings, 1987). It is not clear how different growth conditions prevent mitochondrial destruction.

As mentioned earlier all laboratory strains of *Neurospora* like the standard Oak Ridge wildtype are immortal. However two isolates from natural populations, the Kalilo strains found in Hawaii and the Maranhar strains from Aarey, India, were found to undergo senescence (Griffiths and Bertrand, 1984; Court, et al., 1991). Extragenomic linear plasmids, the 9-kb kalDNA and the 7-kb marDNA, were associated with these senescent strains and were absent in the immortal races. As with *Podospora*, senescence in *Neurospora* can be linked to the destruction of the mtDNA by the amplification and eventual insertion of these extragenomic elements (Bertrand, et al., 1985).

Longevity from Gain-of-Function Alleles of Longevity Genes

Again by definition, longevity should be associated with gain-of-function mutations in longevity genes. Recent advances in recombinant DNA technology have allowed investigators to increase the expression levels of genes which might increase lifespan. As described above, studies with different longlived organisms lend support to the hypothesis that the levels of oxygen radical defenses correlate with extend longevity. To further test this hypothesis in *Drosophila*, Orr and Sohal overexpressed both the SOD

22

and catalase genes and showed that this resulted in a 6-33% increase in the lifespan of flies (Orr and Sohal, 1994). However, individual overexpression of these genes had no effect (Orr and Sohal, 1992; Orr and Sohal, 1993). They suggest that co-overexpression of the these genes might increase the activity of the pathway that converts the superoxide radical, known to be damaging to proteins and DNA, into water, something that cannot be accomplished with overexpression of either gene alone.

Finally, it has been shown that transgenic flies overexpressing the elongation factor EF-1α have an increased lifespan (Shepherd, et al., 1989). The interpretation of this result is complicated both by the pleiotrophic effects that come with overexpressing any component of the protein synthetic machinery and by the finding that the lifespan extending phenotype depends on the position of the transgene and the genetic background of the strain tested (Stearns and Kaiser, 1993).

Conclusions

In summary, the application of genetic principles to the study of lifespan determination has yielded a number of candidate longevity and aging genes. Some common themes emerge. One in particular is that oxygen radicals probably contribute to the aging process because long-lived fungi, flies, and worms, all have increased levels of antioxidant enzymes including superoxide dismutase and catalase. In the end, however, the ultimate goal of these studies is to reveal insights into human aging.

THE GENETICS OF AGING OF THE HUMAN SPECIES

Human Aging and Longevity Genes?

In humans, Cutler (Cutler, 1975) and Sacher (Sacher, 1975) independently concluded that the difference in lifespans between *Homo sapiens* which has a maximum attainable lifespan (MAL) of 115 years and our recent ancestor *Australapitechus africanus* which had a MAL of 51 years, must be due to relatively few changes in regulatory genes, to account for such an extension of lifespan occurring in only 250,000 years. If there are

CHAPTER ONE: AGING AND LONGEVITY GENES

indeed a few genes which extend the human lifespan, it would be reasonable to assume that mutations in any of these could be responsible for the rare premature aging syndromes including Progeria, where a dominant mutation seems to accelerate aging such that the median age is 12 years, and Werner's Syndrome, where an autosomal recessive mutation acts such that the median age at death is 47 years. The locus responsible for Werner's Syndrome was localized to chromosome 8 (Goto, et al., 1992)and the *WRN* gene has been cloned. The predicted protein is composed of 1432 amino acids and shows significant similarity to DNA helicases (Yu, et al., 1996) . The homology suggests that defective DNA metabolism is involved in the complex process of aging in Werner's Syndrome patients.

The Hayflick Limit and Cellular Senescence

The inability to manipulate the human lifespan has limited aging research in the human species to the study of the aging of human cells. The dogma that has governed this field for the past 30 years is known as the Hayflick hypothesis which has at its core the premise that an understanding of human cellular aging will also result in an understanding of the aging of the human organism.

In 1961, Hayflick and Moorhead demonstrated that human fibroblasts had a limited division potential *in vitro* at about 50 population doublings (Hayflick and Moorhead, 1961). Later, Hayflick proposed that organisms including humans age because their cells age (Hayflick, 1965). Two correlations support Hayflick's thesis. For the first albeit weaker correlation, it has been shown that cells taken from human embryos had a larger potential for division that those taken from a centenarian (Martin, et al., 1970; Schneider and Mitsui, 1976). For the second, it has been shown that there is a relationship between species-specific cell doubling potential and species-specific maximum lifespans: cells from short-lived species had a lower division capacity than those from longer-lived ones (Rohme, 1981). These findings have been extended to many other cell types but fibroblasts remain the system of choice.

According to this theory, aging genes would cause organismal aging by

24

shortening the lifespans of each of the animal's cells. In fact, recent work with fibroblasts obtained from patients with Werner's syndrome suggest that the potential aging gene mutated in this disease is responsible for counting the number of cell divisions completed by a clone (Faragher, et al., 1993; Kill, et al., 1994). The Werner's syndrome fibroblasts, like ones obtained from Progeria patients, not only had a lower division potential as predicted by the Hayflick hypothesis, but also seem to exit the cell cycle inappropriately as if they are unable to tell how many cell cycles they had completed.

Numerous studies of the Hayflick phenomenon (reviewed by Stanulis-Praeger, 1987) have shown that fibroblasts undergo reproducible changes as they senesce: they enlarge and their cell cycles lengthen until they arrest in late G1. The process is not time dependent but is governed by the number of divisions undergone by the cells. Genetic analyses using cell fusion/heterokaryon technology have shown that senescence is a dominant phenotype (Pereira-Smith and Smith, 1983). By fusing different immortal cell lines, Pereira-Smith and Smith concluded that senescence is determined by at least four complementation groups (Pereira-Smith and Smith, 1988). Using microcell fusion techniques, several labs have shown that genetic determinants of senescence lie on chromosome 1 ((Sugawara, et al., 1990; Hensler, et al., 1994)) , 4 (Ning, et al., 1991), 6 (Hubbard-Smith, et al., 1992), 7 (Ogata, et al., 1993) , 11 (Koi, et al., 1993) and 18 (Sasaki, et al., 1994). Work by Smith and colleagues (Lumpkin, et al., 1986) has shown that a species of mRNA found in senescent fibroblasts can prematurely arrest young cells. Intriguingly, a differential screen to identify this mRNA obtained SDI1 which is identical to CIP1 or p21, a tumor suppressor gene encoding an inhibitor of cyclin kinase activity (Noda, et al., 1994). This suggests that cellular senescence could have evolved as a defense mechanism against cancer. In fact, several cellular proteins that are important for the establishment and/or maintenance of the senescent phenotype are tumor suppressors including pRb, the retinoblastoma gene product, and p53, a component of the cell cycle checkpoint machinery (reviewed in Afshari and Barrett, 1996).

In recent years, the link between cellular senescence and cancer has been brought into focus by the telomere hypothesis for cell growth control (Harley, et al., 1990)

(Hastie, et al., 1990). This hypothesis mentioned earlier proposes that the proliferation of normal somatic tissue is limited by telomere length which shortens after every cell division. As telomeres shorten and approach a critical minimum length, cell division slows and the cells eventually senesce. At a low frequency, telomerase is activated in these cells which extends telomere length allowing them to proliferate indefinitely. Evidence for this premise has been accumulating. Nearly all somatic tissues in the human lack significant telomerase activity. In contrast, most immortal human cell lines have now been shown to contain telomerase activity (Kim, et al., 1994). Furthermore, the use of an antisense RNA to the telomerase RNA template significantly inhibits cell division (Feng, et al., 1995). Though this data show that a telomerase activity is required for continued proliferation, it is still not proof that senescence is caused by critically short telomeres. Work described in chapter four of this thesis proposes an alternative interpretation of this phenomenon based upon our work with yeast telomeres.

Conclusions

At present the outstanding question in the field of mammalian aging research, given the growing appreciation that senescence may be a tumor suppressor mechanism, is the nature of the link, if any exists, between cellular senescence as described by Hayflick, and organismal aging. It is clear that a correlation exists: shorter lived organisms and short lived mutants as is the case with the progeroid patients have fibroblasts with a smaller number of population doublings. What is not clear is whether cellular senescence is in fact the root cause of organismal aging as originally hypothesized by Hayflick. What is needed is a transgenic mouse which has been engineered so its fibroblasts and other cells have an extended reproductive capacity. Further work with both the oncogenes and the tumor suppressor genes involved in regulating cellular senescence could make this possible. The question would then be: does this animal now live longer?

OVERVIEW: COMMON AGING AND LONGEVITY GENES?

One of the most unexpected and exciting paradigms in modern biology is the sequence and often functional conservation of regulatory pathways and genes between organisms belonging to divergent genera. The field of aging research is still young and none of the regulatory pathways that determine the lifespan of any organism have been defined. One of my goals for the research effort which is described in this thesis was and still is to determine if any genes which regulate cellular senescence are functionally conserved between yeast cells and human fibroblasts. The two processes share some characteristics. Senescence in both systems is dominant, both cell types take longer to traverse the G1 portion of their cell cycles as they senesce and both arrest primarily in G1. One of the next avenues of my research, discussed in detail in chapter five, will be to bridge the two systems and to use the power of yeast genetics to possibly understand fibroblast senescence, and if the Hayflick hypothesis is true, even organismal aging. This search for synthesis and efforts like it will direct the aging field in the years to come.

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CHAPTER TWO

Stress-Resistant and Long-lived Mutations Define the
UTH Genes

INTRODUCTION

When we began our work in 1991, the field of yeast aging was already thirty years old (Mortimer and Johnston, 1959). The basic phenomenology had been described: yeast mother cells have a limited division potential and they enlarge and have longer cell cycles as they age. Several studies had also demonstrated that different strains had different lifespans suggesting that aging was under genetic regulation. Our primary goal at the outset was to identify the genes which regulate this process.

This chapter describes the initial characterization of the aging properties of several strains in our lab and our identification of genes which regulate aging in yeast. This mutant hunt was based upon a fortuitous finding that lifespan correlated with stress resistance in several yeast strains. We identified four complementation groups which define the *UTH* (youth) genes confirming our hypothesis that the same genes can regulate both stress-resistance and longevity in yeast.

RESULTS AND DISCUSSION

Initial Work

To establish the basis for our work in yeast aging, we determined the lifespans of two wildtype laboratory strains in the Guarente laboratory, BWG1-7a (Guarente and Mason, 1983) and PSY142 (Kennedy, et al., 1994), which have divergent genetic backgrounds. Exponentially growing cells of both strains were streaked on YEPD plates and allowed to grow overnight. Virgin cells which had not yet budded were then picked using a tetrad dissecting microscope, transferred to isolated locations on the plate, and allowed to divide. At every division, the new bud was separated and moved away from the mother cell until further division ceased. Cells were considered senescent when no division occurred within a 48 hour period. As shown in Figure 2.1, cells of strains BWG1-7A and PSY142 have mean lifespans of 18 and 27 generations and maximum lifespans of 35 and 70 generations respectively. This difference in lifespans confirmed earlier findings in the literature (Mortimer and Johnston, 1959; Johnston, 1966) that a wide range of mean lifespans existed in laboratory yeast strains suggesting that aging in yeast is not a purely stochastic process but is one that is influenced by the genetic background of the strain.

36

Programmed Cell Death in Yeast?

The key observation leading to the screen which yielded mutations in the *UTH* genes was fortuitously made during a random examination of cells taken from a prolonged incubation at $4^{\circ}C$. Microscopic analyses revealed that they contained bleb-like bodies which resembled those pictured in a then recent *Science* paper describing programmed cell death in insect cells (Clem, et al., 1991) (Figure 2.2). These bleb-like bodies in the dying insect cells were thought be lipid encapsulated remnants of the cellular degeneration accompanying programmed cell death or apoptosis. To determine if the blebs seen in the yeast cells exposed to long cold incubations were also lipid coated, these cells were immersed in a 1% solution of Sudan Black B, a lipid soluble dye, in 70% ethanol for 15 minutes, washed and examined. As seen in Figure 2.3, the bleb like structures stained positive with the dye.

Why would yeast cells undergo programmed cell death? One hypothesis was that the program would be initiated in starving cells some of which would actively die and lyse releasing nutrients into the surrounding environment allowing neighboring genetically identical clones to survive. To test this, exponentially growing haploid cells of strains BWG1-7A, PSY142, and BKy1-14c were resuspended in yeast sporulation medium and incubated at $30^{\circ}C$. Cells were examined microscopically and their viability was monitored by plating dilutions of cells to determine the plating efficiency. As pictured in Figure 2-4, after several days, starving cells did develop bleb-like structures resembling those seen in the cells removed from the cold.

A shorter experimental regimen which reproducibly produced "apoptotic-like" blebs allowed us to better characterize the phenomenon. A hallmark feature for programmed cell death in a variety of systems is the appearance of characteristic DNA ladders that arise from the active degradation of nuclear DNA into nucleosomal size fragments (Wyllie, 1980). Repeated attempts to find these ladders in starving cells containing the lipid blebs were unsuccessful. In addition, overexpression of BCL-2, a protein which is known to block programmed cell death in several systems (Hengartner and Horvitz, 1994), did not prevent the appearance of the blebs or change the yeast lifespan (data not shown). We did not pursue the hypothesis that programmed cell death

37

occurred in yeast.

What was striking, however, was the observation that the loss of viability of the cells in starvation medium over time correlated with their mean lifespans (Figure 2.5). These results were extended to four strains derived from a single tetrad with varying lifespans (Figure 2.6), suggesting that starvation and aging may be similar processes regulated by the same genes. To test this hypothesis, I initiated a screen to look for mutations which increased both the starvation resistance and the lifespan of the starvation-sensitive and short-lived strain, BKy1-14c. 39,000 cells were mutagenized with EMS with a viability of 40%. Colonies were allowed to grow on YEPD for two days then were replica plated onto starvation plates consisting of sporulation media lacking a rich carbon and nitrogen source. The starving cells were incubated at 30°C for 7 days before being replica plated back to YEPD plates to look for sustained viability. Thirty nine mutant colonies which had a significant and reproducible increase in viability over the wildtype were recovered and lifespan analysis was performed on 20 cells from each candidate. Of these mutants, eight had longer lifespans. Complementation analysis for the starvation resistance phenotype summarized in Table 2.1 placed the mutants into four classes (data not shown). We called these mutants *uth 1-4* (youth).

Phenotypic Characterization of the *UTH* mutants

To better understand the nature of the mutations we had obtained, the mutant strains were put through a battery of physiological tests. In addition to their starvation resistance, the mutants also exhibited several phenotypes which suggested that they were all stress resistant. Primarily, they were better growers both at 37°C (Figure 2.7) and on plates with ethanol as a carbon source and were also resistant to a 5 minute heat shock at 55°C (data not shown). Finally, the *uth* mutants also saturated in rich medium at a higher cell density than control wildtype cells. Surprisingly, they exhibited a slow-growth phenotype on plates containing 3mM paraquat, a drug that is thought to generate oxygen radicals *in-vivo* (Blaszczynski, et al., 1985). This phenotype, however, did not translate into a general sensitivity to other oxygen related stresses because the mutant strains had wildtype growth characteristics when exposed to hydrogen peroxide and to a

38

100% oxygen environment (data not shown). In general, however, these results suggest that the increase in lifespan is accompanied by a general increase in stress resistance.

MATERIALS AND METHODS

Strains, Media, and Lifespan Analysis

Yeast strains (see Table 2.2) were grown using standard media and conditions (Sherman, et al., 1979). To determine the lifespan of a strain, cells were taken from logarithmically growing liquid cultures and plated at low density on complete medium. The plates were incubated at $30^\circ C$ overnight. 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 lifespans of these cells were determined by noting and removing all subsequent daughters they generated. The plates were incubated at $30^\circ C$ during working hours and shifted to $4^\circ C$ overnight. On very rare occasions, an apparently small, young cell was observed to lyse immediately after micromanipulation and was excluded from the data set.

Mutagenesis

Growing BKy1-14c cells were mutagenized with ethyl methanesulfonate (approximately 60% cells killed) and allowed to form colonies on rich medium (yeast extract-peptone-dextrose, or 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 2 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. There were 39 mutants recovered that reproducibly tested starvation-resistant. Lifespans of all 39 mutants were determined for a minimum of 20 cells of each mutant. Eight mutants demonstrated significantly higher mean lifespans as determined by the Wilcoxen signed rank test. These eight mutants were used for subsequent analysis.

Determination of Stress Phenotypes

To determine starvation resistance, haploid cells were grown in rich medium to log phase, collected by centrifugation, and resuspended in minimal sporulation media for a period of 7-9 days. After starvation, cells were again collected by centrifugation and plated on YEPD to measure colony-forming units per milliliter. 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 3 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 5 days, with the number of cells per milliliter counted in a hemacytometer on a periodic basis. Control experiments indicated that the medium was completely saturated after this time period.

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

Statistical Analysis

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

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100
90
80
70
60
50
40
30
20
10
0

% CELLS ALIVE

PSY142
BWG1-7A

0   10   20   30   40   50   60

GENERATIONS
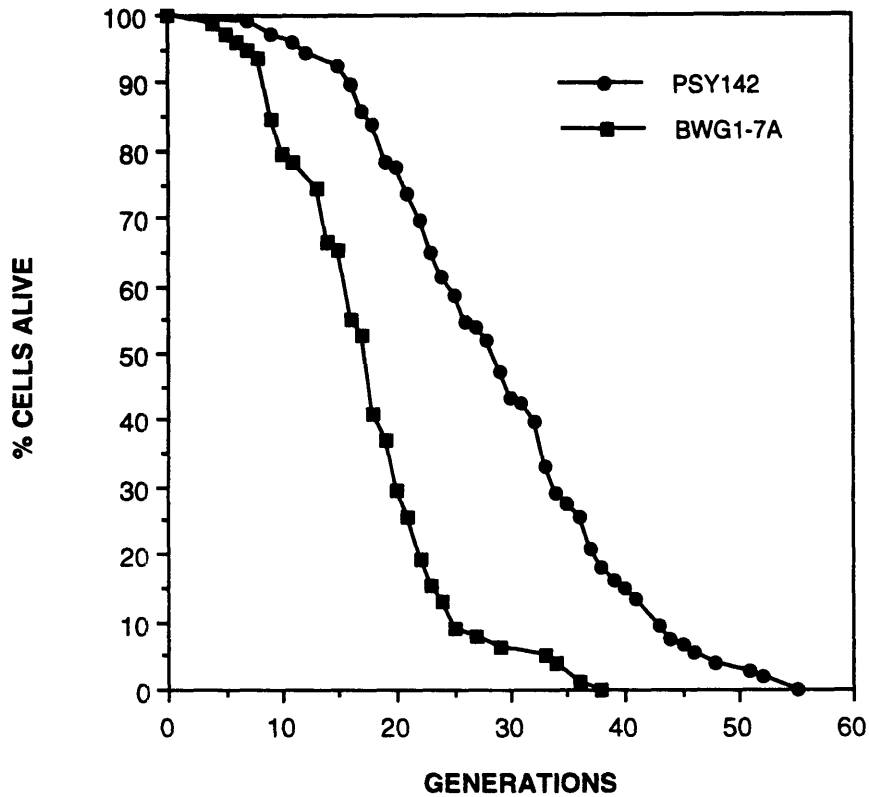
Figure 2.1: Mortality Curves for Strains PSY142 and BWG1-7a
Mortality curves are shown for two wildtype strains, PSY142 and BWG1-7a. All
curves are derived from at least two experiments. The difference in lifespans
between these two strains was shown to be statistically significant by the
Wilcoxen Ranked Signed Test. The mean lifespans for PSY142 and BWG1-7a
were 28 and 17 generations respectively. Sample sizes were 105 cells for PSY142
and 87 cells for BWG1-7a
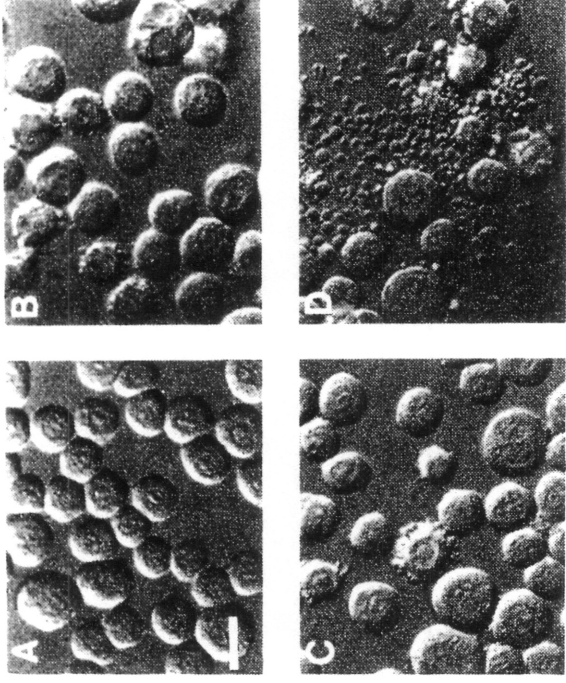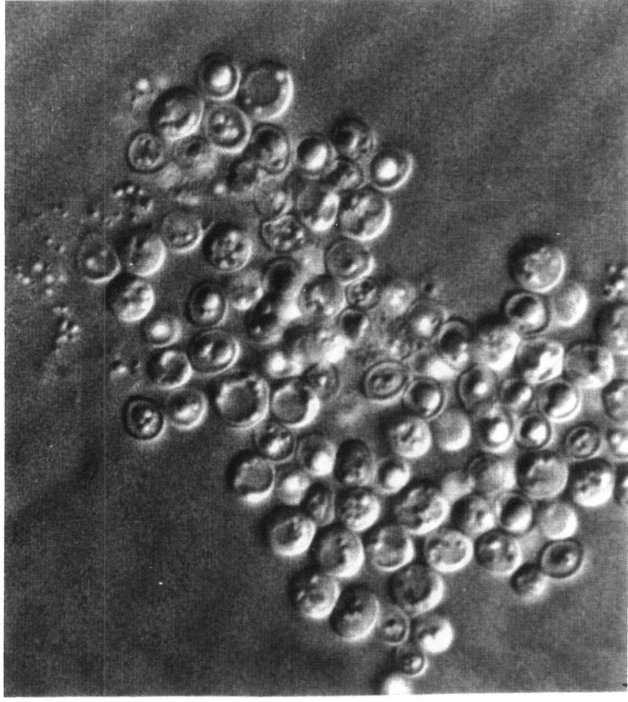
Figure 2.2: Starving Yeast Cells Accumulate and Release Bleb Like Structures Resembling
Apoptotic Bodies Found in Dying Insect Cells

Haploid yeast cells in minimal sporulation media accumulate bleb like structures which are
released into the media after lysis (Left Panel). Photograph was taken at 1000x magnification
using Nomarski optics. These "blebs" were similar to those seen in cells undergoing
programmed cell death after an infection with baculovirus lacking the p35
gene. (Right Panel reprinted with permission from Clem, R.J., Fechheimer, M., and Miller, L.K.
Prevention of apoptosis by a baculovirus gene during infection of insect cells.
Science 254: 1388-1390. Copyright 1991 American Association for the Advancement of Science)
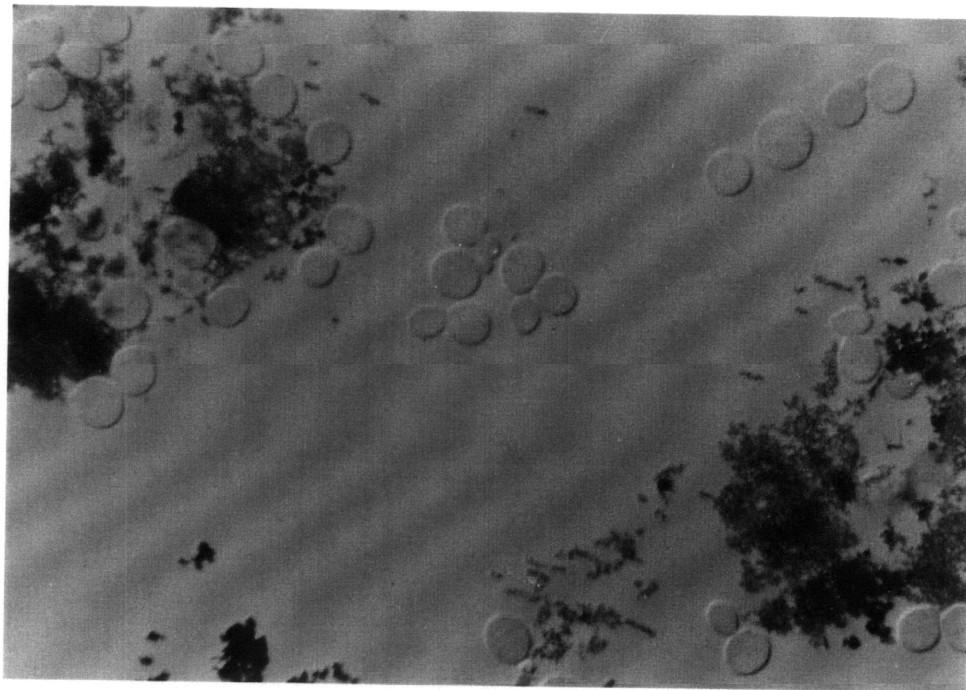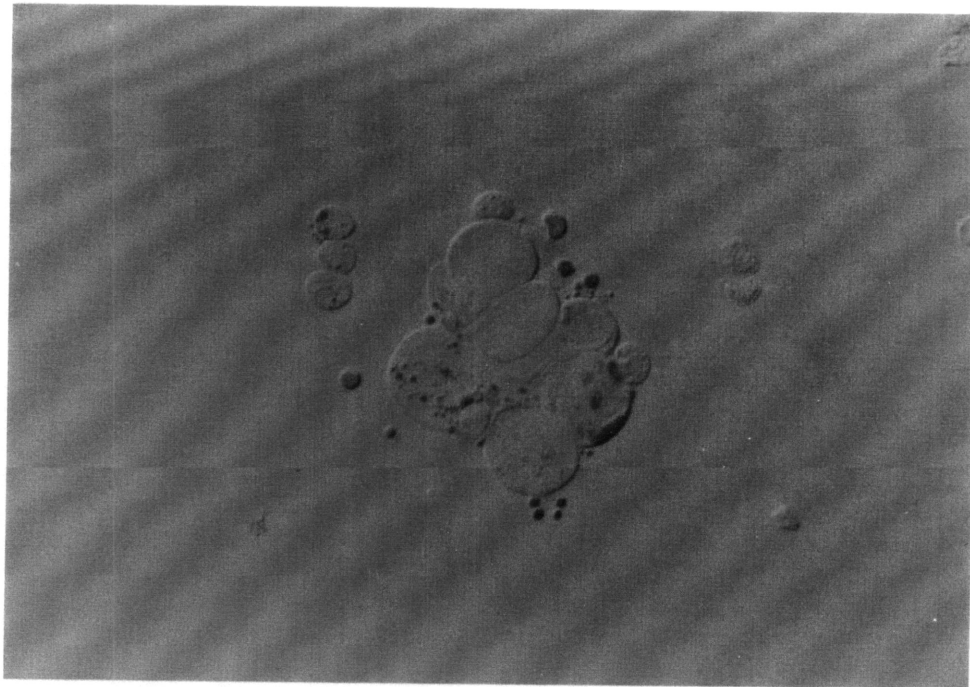
Figure 2.3: The Blebs Found in Starving Yeast Cells Stain with Sudan Black B
Haploid yeast cells in minimal sporulation media accumulate bleb like structures which are released into the media after lysis. Cells were harvested, immersed in a 1% solution of Sudan Black B, a lipid soluble dye, in 70% ethanol for 15 minutes, washed and examined. The blebs found in starving cells stained black. (Top panel) Exponentially growing cells lacked these blebs and did not stain with the dye. (Bottom panel) All photographs were taken at 1000x magnification using Nomarski optics.

Figure 2.4: Similarity Between Aging and Starving Yeast Cells
Exponentially growing haploid yeast cells in rich medium (upper panels). Haploid cells growing in sporulation medium (middle panels) increase in volume and accumulate granular particles. These starving cells resemble cells of the same strain which have been micromanipulated until they senesced (lower panels). All photographs were taken at 1000X magnification using Nomarski optics.
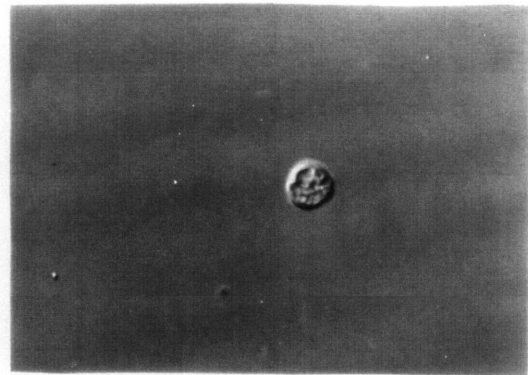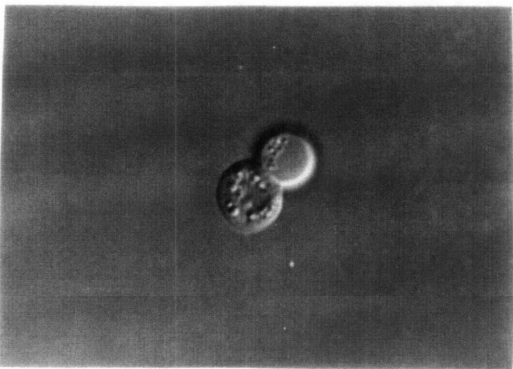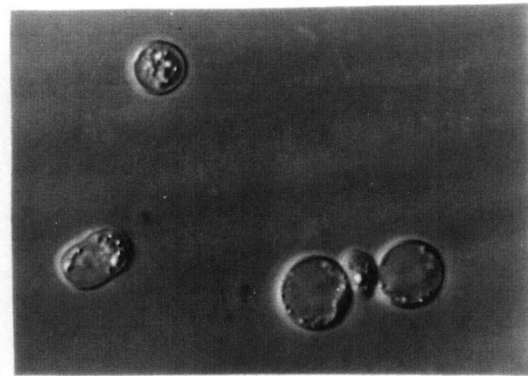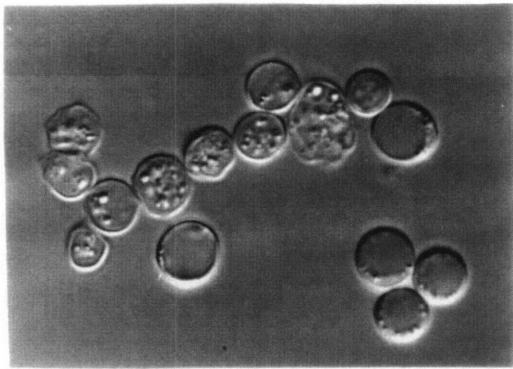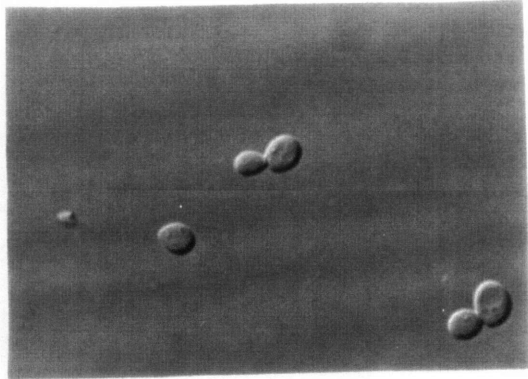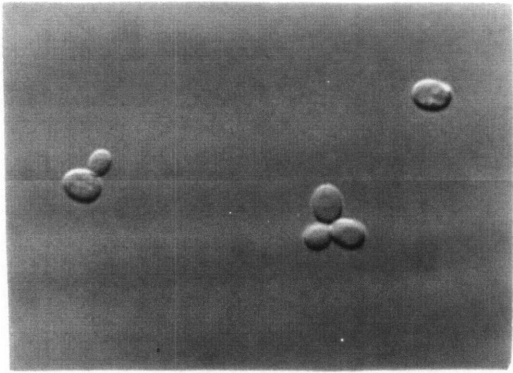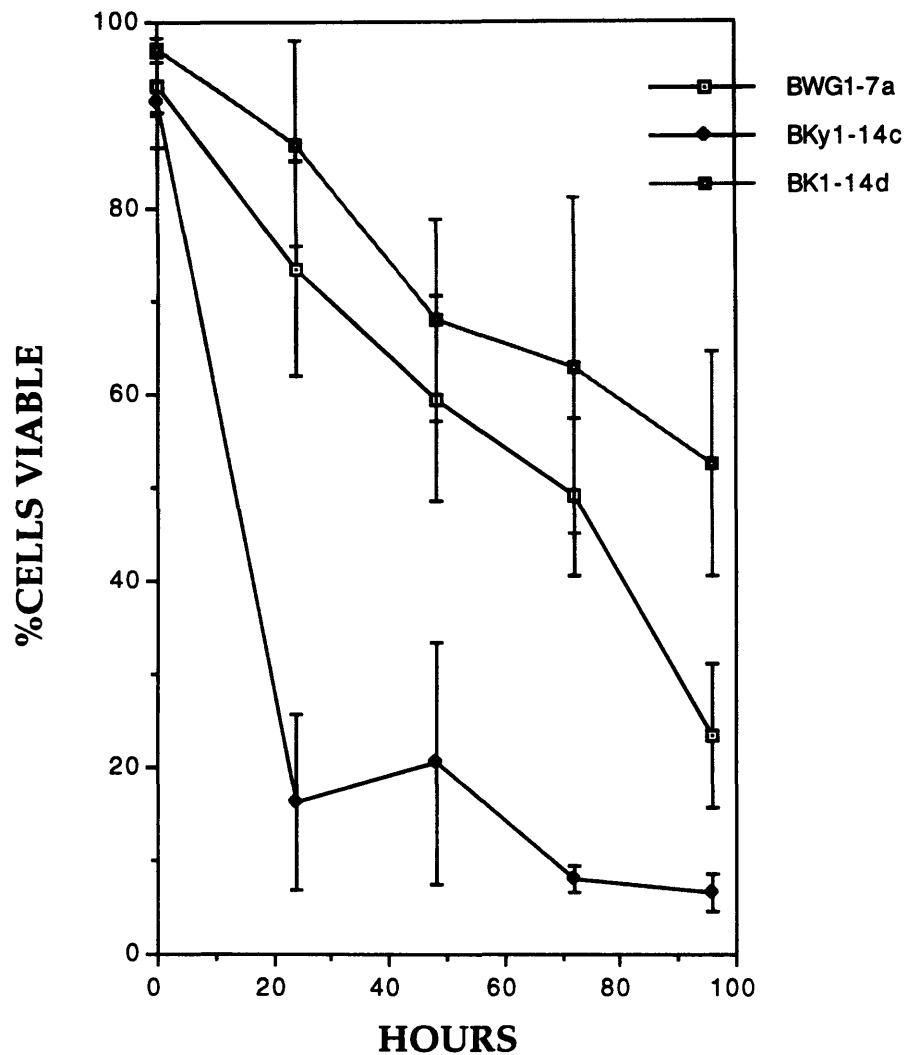
Figure 2.5: Loss-of-Viability Correlates with Lifespan for Three Yeast Strains
Loss of viability in sporulation media correlates with lifespan for three wildtype
yeast strains. Exponentially growing cells were resuspended in minimal sporulation
media at the beginning of the experiment at a concentration of 10^6 cells per ml.
At the indicated times, cell counts were taken with a hemocytometer and an aliquot
was plated on YEPD to determine cell viability. Standard deviations were derived
from three independent experiments. Typical mean lifespans for the strains tested
are 14, 18, and 38 generations for BKy1-14c, BWG1-7A, and BKy1-14d respectively.

Figure 2.6: Correlated Phenotypes for Tetrad BKy1-14

Mean lifespans of segregants of tetrad BKy1-14 were determined by mortality curves involving 20 cells of each strain except 14d (19 cells). (Top panel) The viability of BKy1-14 segregants stored for 4.5 months in the cold room. The percent viability is the number of colony-forming units per total microscopic cell count. For strain BKy1-14c, no viable cells were detected. (Middle panel) 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 YEPD medium to minimal sporulation medium lacking nitrogen and carbon sources and incubated for 7 days before plating on YEPD. The number of colony-forming units per milliliter is shown.

MEAN LIFE SPAN OF TETRAD 14
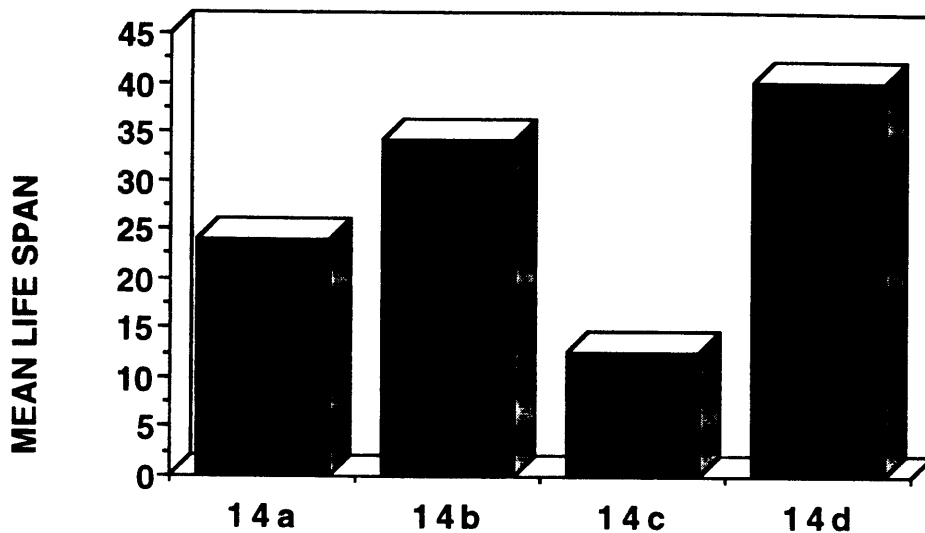
VIABILITY OF TETRAD 14 AFTER 4.5 MONTHS AT 4 DEGREES
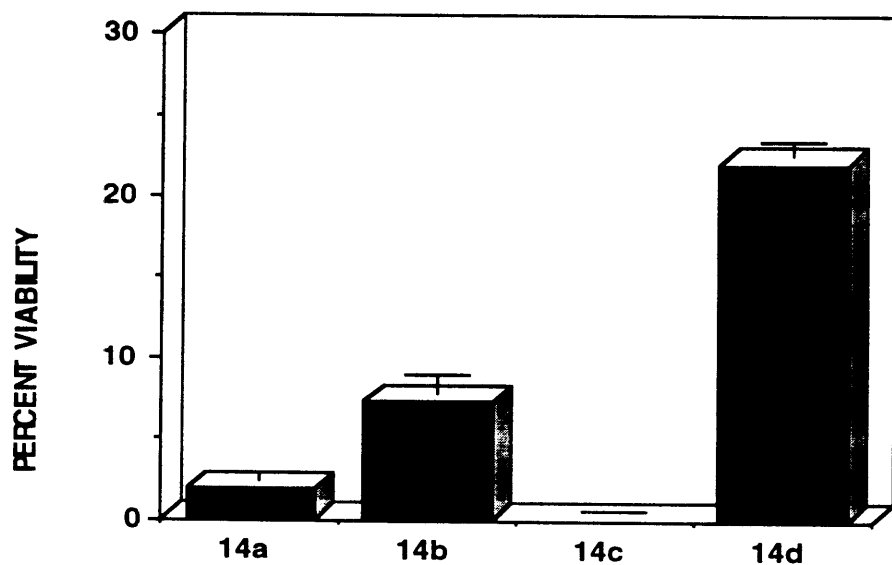
VIABILITY OF TETRAD 14 AFTER 7 DAYS STARVATION
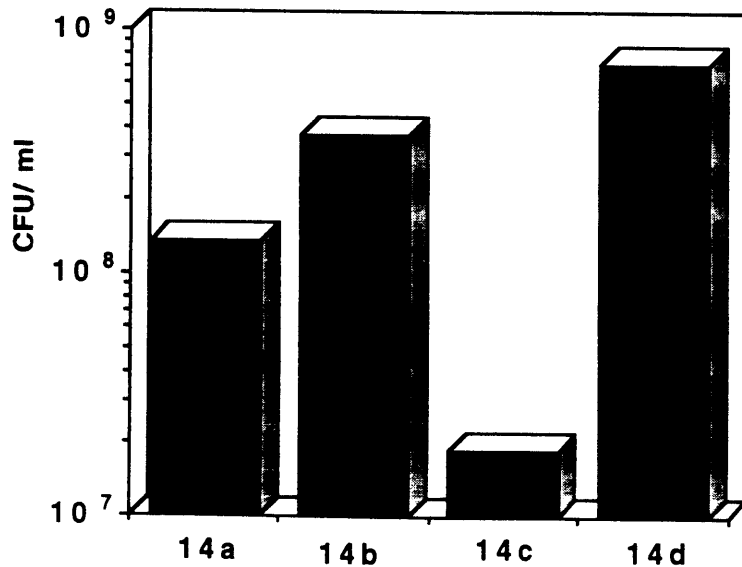
STRAIN	SAMPLE SIZE	MEAN LIFESPAN	%INCREASE
14C	137	14.1	–
UTH1 Complementation Group			
uth1-324	37	16.62	19.5
uth1-328	38	18.00	29.4
uth1-330	38	19.05	37.0
uth1-342	34	18.29	31.5
UTH2 Complementation Group			
uth2-42	19	20.95	50.6
UTH3 Complementation Group			
uth3-26	49	17.44	29.0
uth3-335	40	19.38	39.3
UTH4 Complementation Group			
uth4-326	59	21.68	55.9

Table 2.1: Complementation Data for the UTH Genes
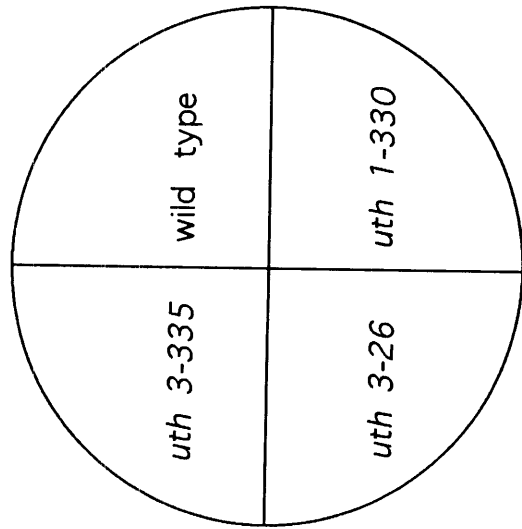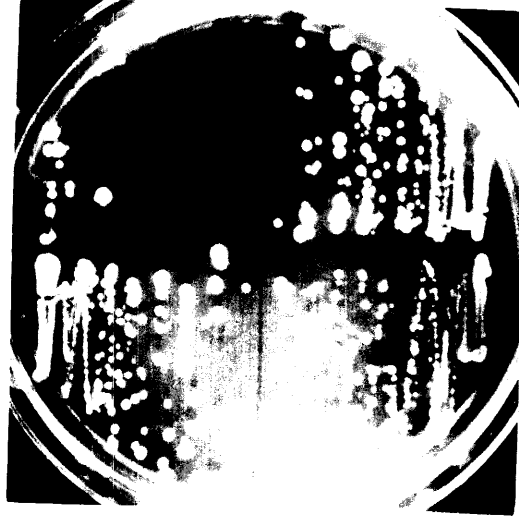
30°C   37°C

Figure 2.7: Stress Resistance of the *Uth* Mutants
Uth1 and *uth3* mutants have an enhanced growth phenotype
at elevated temperatures. Exponentially growing wildtype
and mutant strains from the 14C strain background were
streaked on YEPD plates and allowed to grow for two days
at the indicated temperatures.

Table 2.2: Yeast Strains Used in This Study

Strain	Genotype	Reference
BWG1-7A	MATa *ade1-100 his4-519 leu2-3,112 ura3-52*	Guarente and Mason, 1983
PSY142	MATα *leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1995
BKy1-14a	MATa *ade1-100 leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1995
BKy1-14b	MATα *leu2-3,112 ura3-52*	Kennedy, et al., 1995
BKy1-14c	MATα *ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1995
BKy1-14d	MATa *his4-519 leu2-3,112 ura3-52*	Kennedy, et al., 1995

CHAPTER THREE

The Yeast Aging Gene, *UTH1*, Encodes an "Anti-silencer"

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INTRODUCTION

As first described by Mortimer and Johnston, individual yeast cells are not immortal (Mortimer and Johnston, 1959). They have a limited division potential, called their lifespan, which ends in proliferation arrest and lysis. Several biomarkers have been defined for the aging process of this organism. Both the cell's size and its transit time through the cell cycle increase with age (Johnston, 1966; Egilmez, et al., 1990) and senescent cells accumulate wrinkles and lipid granules which are reminiscent of the aging pigment, lipofuscin (Katz, et al., 1984). The mortality kinetics of a yeast cell colony can be described by the Gompertz equation (Pohley, 1987), the classical definition for aging populations, and the differences in lifespans among different strains support the notion that aging in yeast is under genetic control (Johnston, 1966).

Several hypotheses have been proposed to explain aging in yeast. First, Mortimer and Johnson proposed that the cell arrests when it has exceeded some critical cell size and a minimum surface to volume ratio (Mortimer and Johnston, 1959; Johnston, 1966). Experiments which have increased cell size and volume either by varying the ploidy of the cell (Muller, 1971) or by enlarging arrested cells with mating pheromone (Kennedy, et al., 1994) demonstrate that these do not decrease lifespan. Second, it has also been suggested that bud scars, the chitinous deposits which remain on the mother cell after budding may limit the availability of surface area for further budding or for nutrient exchange with the environment (Mortimer and Johnston, 1959). Our work with aging cells, however, has demonstrated that at a low probability, old mother cells produce a bud of similar size which has a lifespan identical to the mother's remaining lifespan (Kennedy, et al., 1994). These "symmetric" divisions argue against a causal role for bud scars in determining longevity because mother and daughter cells do not have the same number of scars but have the same remaining lifespan potential. These studies suggest that yeast may contain a senescence factor that accumulates in old mothers and is inherited by their daughters.

To identify this senescence factor or other genetic elements which regulate the yeast lifespan, we carried out a screen, described in the previous chapter, for mutants which have enhanced stress resistance and delayed senescence. The first gene to be characterized in this screen (Kennedy, et al., 1995) was the regulator of transcriptional silencing, *SIR4* (silent information regulation), which represses gene activity at telomeres (Palladino, et al., 1993) and at *HMR* and

54

HML, the silent information cassettes for the yeast mating type locus (Ivy, et al., 1986). Characterization of *UTH2-42*, the hypermorphic allele of *SIR4* which resulted in extended longevity, led us to propose a model that posits the existence of an aging gene or genes which we call the AGE locus, which is silenced by a *SIR4* dependent mechanism. According to the model, this AGE locus would gradually be expressed as silencing is lost during yeast aging eventually resulting in cell death. One prediction of this model was confirmed when sensitive RT-PCR analysis demonstrated the loss of transcriptional silencing in old cells (Smeal, et al., 1996).

What is the AGE locus? By definition, the AGE locus is a yeast aging gene: loss-of-function mutations in AGE should result in delayed senescence. The same screen which yielded *SIR4* also identified two genes, defined by the recessive complementation groups, *UTH1* and *UTH3*, which were prime candidates for AGE. Several attempts to clone *UTH3* were not successful. Cloning of *UTH1* demonstrated that it encodes a novel protein belonging to a family of proteins that share a 215 amino acid domain which we call the SUN domain. Epistasis analysis placed *UTH1* genetically upstream of *SIR4* and it is now clear that the gene is not AGE but another aging gene which encodes an antagonist of transcriptional silencing.

RESULTS

Phenotypic Characterization of *uth1-330*

Four alleles of *UTH1* were isolated in the mutant hunt for stress-resistance and longevity which also identified *SIR4*. The mortality curves for these *uth* mutants are shown in Figure 3.1. *Uth1-330* had the largest lifespan extension of 37% over the wildtype and like the other *uth1* alleles was recessive both for its stress-resistant and longevity phenotypes. This suggested that *UTH1* unlike *SIR4* may be a yeast aging gene whose gene product is required for normal aging. To better understand the nature of the *uth1* mutations, the *uth1-330* strain was put through a battery of physiological tests which revealed that it grew better than wildtype at 37°C and was also resistant to a 5 minute heat shock at 55°C. Surprisingly, the strain exhibited a slow-growth phenotype on plates containing 3mM paraquat, a drug that is thought to generate oxygen radicals *in-vivo* (Blaszczynski, et al., 1985). This phenotype, however, did not translate into a general sensitivity to other oxygen related stresses because the mutant strains had wildtype growth

characteristics when exposed to hydrogen peroxide and to a 100% oxygen environment (data not shown). In general, however, these results suggest that the increase in lifespan is accompanied by a general increase in stress resistance.

Cloning of the *UTH1* Gene

To clone the *UTH1* gene, a yeast genomic library in the centromeric vector YCp50 (Pfeifer, et al., 1987) was screened for those plasmids that could complement the paraquat sensitivity and enhanced growth phenotypes of the *uth1-330* mutant strain. Approximately 1.5 million URA+ transformants were tested. They were plated on dropout plates containing 3mM paraquat with galactose as a carbon source. (During the course of our work, I had discovered that *petite* cells in the BKy1-14c strain background were also resistant to paraquat but grew slowly on galactose.) Colonies that were able to grow robustly on the paraquat plates were restreaked to selective plates at 37°C to look for complementation of the enhanced growth phenotype (Figure 3.2). Four different plasmids consistently complemented both phenotypes but only one designated pNRA10/1, was shown to be linked to the *uth1* mutation (See Materials and Methods). As shown in Figure 3.3, this plasmid was also able to complement the longevity phenotype of mutant *uth1-330*. One possible reason for the difficulty I faced in identifying *UTH1* became clear when I discovered that the plasmid containing the correct clone is toxic in all 12 strains of *Escherichia coli* tested to date causing a slow-growth phenotype.

Characterization of the Null Mutation: *UTH1* is a Yeast Aging Gene

Subcloning of plasmid pNRA10/1 established that a 2.5 kb HindIII fragment could complement the stress resistance phenotype and sequencing showed that the fragment contained the novel ORF YKR042w on chromosome XI (Dujon, et al., 1994). To determine the phenotype of a null mutation in *UTH1*, a gene disruption was constructed by inserting the 3.8 kb hisG-URA3 cassette into the unique AgeI site just downstream of the putative first ATG of the open reading frame. A 4.8 kb SpeI fragment containing the disrupted *UTH1* gene was then transformed into both the haploid BKy1-14c strain and an isogenic diploid. Haploid transformants were tested for stress resistance and those that had enhanced growth at 37°C (Figure 3.4) were confirmed to be

disrupted by Southern analysis demonstrating that *UTH1* is not an essential gene.

Lifespan analysis derived from two independent transformants (Figure 3.5) showed that the disrupted strain had phenotypes resembling those of the original *uth1-330* mutant. Further analysis confirmed that *uth1-330* has characteristics of a null allele of *UTH1*: a diploid *uth1-330/Δuth1* is phenotypically no different than a diploid homozygous for a disruption of *UTH1* (data not shown). In addition, we have demonstrated that the effects of disrupting *UTH1* are independent of the genetic background of the strain tested because enhanced stress and extended lifespan were also seen with the PSY316 strain (Table 3.1). No effect on lifespan or stress resistance was seen in the PSY142 strain background. It is still not clear why this strain is unique in its behavior with respect to mutations in *UTH1*.

UTH1 is a Founding Member of the Novel *SUN* Family of Yeast Genes

The predicted protein sequence of *UTH1* shown in Figure 3.6 was compared with all the sequences in the GenBank. As shown in Figure 3.7, the gene is a founding member of the novel *SUN* (*SIM1*, *UTH1*, *NCA3*) family of yeast genes which share a 215 amino acid domain (the *SUN* domain) at their C-termini. *SIM1* (START independent of mitosis) was isolated in a screen for cell cycle mutants which undergo two rounds of DNA synthesis without an intervening mitosis (Dahmann, et al., 1995). *NCA3* (nuclear control of ATPase) has been implicated in the regulation of the expression of the mitochondrial ATP synthetase (Pelissier, et al., 1995). Another yeast gene in this family tentatively called *SUN4* has been identified by the yeast sequencing effort (Pohlmann and Philippsen, 1996). A recent search has also put *bglA*, a putative beta-glucosidase from *Candida wickerhamii*, into this gene family (Skory and Freer, 1995).

To see if the other *SUN* genes from *S. cerevisiae* also played a role in regulating the yeast lifespan, null mutations of all four genes were constructed in the wildtype strain, BKy1-14c, and their lifespans were determined. Null mutations in the other *SUN* genes decreased the mean lifespan but not the maximum lifespan of the strain (Figure 3.8). Simultaneous disruptions of both *UTH1* and *SIM1* resulted in wildtype lifespan (Table 3.2).

57

UTH1 Requires *SIR4* for its Longevity Enhancing But Not Stress Related Function

The lifespan extending mutation *UTH2-42* in *SIR4* requires an intact SIR complex in order to function implicating silencing in the yeast aging process. To explain this, we proposed that an aging gene, the putative AGE locus, is subject to transcriptional silencing and is gradually expressed when silencing is lost during aging. To determine if *UTH1* was this AGE locus a double mutant of *SIR4* and *UTH1* was constructed. Lifespan analysis showed that *SIR4* was epistatic to *UTH1*: the lifespan of the *Δsir4Δuth1* double mutant was indistinguishable from the lifespan of the *Δsir4* mutant (Figure 3.9). This suggested that *UTH1* was genetically upstream of *SIR4* and that Uth1p may be involved in either the localization of the SIR complex or its activity at the AGE locus.

In contrast to this, the mechanism which increased the stress resistance of the *uth1* mutant did not require Sir4p. As shown in Figure 3.10, a triple mutant *Δuth1Δsir4ΔHMRa*, was as stress resistant as the original *Δuth1* mutant. The deletion of the *HMRa* information was necessary because the deletion of *SIR4* alone enhances stress resistance but this stress resistance is dependent upon an intact *HMRa* cassette in a *MATα* strain (data not shown).

UTH1 is Involved in Alleviating Transcriptional Silencing

Though epistasis analysis suggested that *UTH1* is involved in transcriptional silencing, no significant defects were detected in a *uth1* mutant's strain ability to mate (data not shown). Furthermore, as shown in Figure 3.11, no changes in telomere length are seen with the *uth1-330* null allele of *UTH1*. In contrast, many mutations in genes involved in silencing in yeast are associated with defects in mating ability or changes in telomere length (Ivy, et al., 1986; Sussel and Shore, 1991; Palladino, et al., 1993).

To detect more subtle changes in silencing, *UTH1* was disrupted in a strain where the *ADE2* gene is at one of its telomeres (Gottschling, et al., 1990). Wildtype cells with *ADE2* at a telomere produce sectored red and white colonies because of the metastable transcriptionally repressed state of genes at this location. This is known as telomere position effect or TPE. As

58

shown in Figure 3.12, loss of *UTH1* resulted in enhanced telomeric silencing and a greater number of sectored red colonies as compared to the number in the wildtype strain. In addition the introduction of an additional copy of *UTH1* decreased telomeric silencing (Figure 3.13). A similar result was seen in a strain where *ADE2* lies within an HM silencer (Sussel, et al., 1993). HM silencing is inherently more stable than telomeric silencing and the colonies are homogeneously red or pink (Sussel, et al., 1993). Disruption of *UTH1* resulted in colonies which were redder than the control (Figure 3.12). To further show that the *UTH1* had an effect of transcriptional silencing, the gene was disrupted in strains where either *TRP1* is at a crippled HM silencer (Sussel and Shore, 1991) or *URA3* is at a telomere (Gottschling, et al., 1990) and viability studies showed that the mutants had a small but reproducible increase in levels of repression at both loci (Figure 3.14). These result suggest that the *uth1* effect on silencing is not gene specific. The effect on *TRP1* or *URA3* gene expression is not a general effect on transcription because no significant effects were seen in *uth1* strains where either gene was at its normal genomic locus (Figure 3.14).

Finally, to check if any of the other *SUN* genes had an effect on silencing *SIM1* was deleted in the strain with *ADE2* at an HM silencer. No effects on silencing were seen in the *sim1* mutant though the stress resistance of the mutant was enhanced (data not shown).

DISCUSSION

The yeast colony is immortal but the yeast cell is not. To better understand the genes which regulate the yeast lifespan, we performed a screen to isolate mutants which were both stress resistant and long-lived. The first gene identified in this screen was the silencing regulator, *SIR4*, and the characterization of the extended lifespan mutant provided the first link between gene silencing and the the aging process in budding yeast (Kennedy, et al., 1995) .

This chapter describes the cloning and characterization of *UTH1*, another gene identified in the same screen that yielded *SIR4*. *UTH1* is different from *SIR4* because all the alleles of this gene identified in the mutant hunt were recessive suggesting that it is a yeast aging gene which normally functions to limit the yeast lifespan. Cloning of *UTH1* revealed that it is a novel gene on chromosome XI which encodes a protein belonging to a novel family of yeast proteins which

share a 215 amino acid domain at their carboxyl termini which we have called the *SUN* domain.

Disruption of the *UTH1* open reading frame extended the yeast lifespan confirming the hypothesis that the gene is a yeast aging gene. Epistasis analysis for extension of lifespan placed the gene genetically upstream of *UTH2 /SIR4* suggesting that Uth1p may be involved in the activity or localization of the SIR complex. To determine if Uth1p may directly affect gene silencing, the gene was disrupted in several reporter strains bearing an *ADE2* marker either at crippled HM locus or at a telomere. Wildtype cells with *ADE2* at one of their telomeres produce variegated red and white colonies because of the metastable transcriptionally repressed state of genes at this location, a phenomenon known as telomere position effect or TPE. Disruption of *UTH1* resulted in colonies which were redder and more variegated suggesting that telomeric silencing is enhanced in these cells. A similar result was noted in strains with *ADE2* at an HM silencer. These cells were redder than wildtype controls. Furthermore, this effect is not gene specific because two other genes, *TRP1* and *URA3*, behave in a similar fashion though the effects are small. The small effects seen with *TRP1* and *URA3* despite clear differences in the red color of strains marked with *ADE2* has been noted before (Sussel, et al., 1993). In a study with the *HMR* silencer, cells containing an *ADE2* marked silencer displayed clear color changes and variegation when the silencer was crippled (Sussel, et al., 1993). This exact strain, however, did not exhibit any differences in growth on plates lacking tryptophan when *ADE2* was replaced by *TRP1* (Sussel and Shore, 1991). Furthermore, the subtle changes in silencing seen with the *ADE2* marked strain were not detected in studies with the same strain which looked at the steady-state levels of *a1*, the gene normally present at *HMR* (Brand, et al., 1985). *ADE2* appears to be more sensitive to small changes in silencing than either *TRP1* or *a1*. In conclusion, these results suggest that *UTH1* encodes an "anti-silencer" which acts to limit silencing at the HM loci, at telomeres, and at AGE. According to this model, loss of *UTH1* would lead to enhanced silencing of AGE and a subsequent increase in lifespan.

Many genes have been identified which affect gene silencing in yeast. The central players are the *SIR2/3/4* genes which are absolutely required for silencing (Ivy, et al., 1986). Deletion of any one of these genes results in complete derepression of gene transcription at HM (Ivy, et al., 1986) or at telomeres (Aparicio, et al., 1991). Though the precise function of the Sir proteins is

still not known, they have been shown to form a protein complex (Moretti, et al., 1994; Cockell, et al., 1995) which interacts with histone H4 (Hecht, et al., 1995). The Sir complex is therefore thought to be involved in heterochromatin reorganization which results in the repression of gene transcription.

Mutations in a number of additional genes result in the loss of transcriptional silencing at both the HM loci and at telomeres. These include the genes encoding histone H4, *HHF1* and *HHF2* (Johnson, et al., 1992), the DNA binding regulatory protein, *RAP1* (Sussel and Shore, 1991; Kurtz and Shore, 1991), the components of the N-terminal acetyltransferase, *NAT1/AAA1* and *ARD1* (Mullen, et al., 1989), the transcriptional coactivator, *GAL11* (Suzuki and Nishizawa, 1994), and the Rap1p interacting factor, *RIF1* (Hardy, et al., 1992). The proteins encoded by these genes are thought to affect silencing either by affecting localization of the Sir complex as in the case of Rap1p and Rif1p, or by modulating the chromatin structure at silencers as in the case with histone H4, Gal11p, Ard1p, and Nat1p.

UTH1 is unique among these genes found to affect silencing because loss of gene function results in enhanced rather than decreased silencing at both HM loci and at telomeres. Furthermore, this increase in TPE is not accompanied by any significant change in telomere length. The only other genetic modification known to enhance silencing at telomeres without affecting telomere length is the overexpression of Sir3p (Renauld, et al., 1993) and one possibility is that Uth1p regulates Sir3p levels. Nevertheless, this cannot be the only function for Uth1p because stress resistance in the *UTH1* loss-of-function mutants does not require an intact silencing complex. *UTH1* has several effects related to stress response which are not likely to be related to silencing including paraquat sensitivity, heat shock resistance, and enhanced growth on ethanol and at elevated temperatures. In this manner, *UTH1* resembles *ARD1* which encodes one of the subunits of N-terminal acetyltransferase (Whiteway and Szostak, 1985; Mullen, et al., 1989). Loss-of-function mutations in *ARD1* give rise to phenotypes of slow growth, inability to sporulate, and failure to enter G0 (Whiteway and Szostak, 1985). Based on other studies with mutants which cannot enter G0, loss-of-function mutations in *ARD1* should result in heat shock sensitivity and stress resistance (see Werner-Washburne, et. al., 1993). Ard1p is required for N-terminal acetylation of histone H2B and the proper assembly of chromatin (Mullen, et al., 1989).

However it is also required for the modification of many other yeast proteins including iso-1-cytochrome c (Mullen, et al., 1989) and the absence of these other modifications could result in the pleiotrophic defects seen in the *ard1* mutants. Loss-of-function mutations in *UTH1* result in phenotypes opposite to those predicted with *ARD1* and it would be intriguing to see if Uth1p is also involved in some post-translational modification of the histones and other yeast proteins.

UTH1 is a founding member of a growing gene family which includes the *Saccharomyces* genes, *SIM1*, *NCA3*, and *SUN4* (Figure 3.7A). Deletions of these other genes show that they too affect yeast aging but, in contrast to *UTH1*, result in a shortened lifespan. It is still unclear if these genes directly affect the yeast aging process because a shortening of lifespan can easily result from any change which decreases the viability of the cells. *SIM1* was identified in a screen for mutants which undergo two rounds of DNA replication without an intervening mitosis (Dahmann, et al., 1995). The precise function of Sim1p is unclear but disruption of the gene promotes the destabilization of the *CLB5*, a B-type cyclin involved in progression through the S phase of the cell cycle. *Uth1* mutants do not have significant cell cycle defects. Furthermore unlike *UTH1*, *SIM1* does not seem to be involved in silencing regulation. *NCA3* has been implicated in the regulation of the expression of the mitochondrial ATP synthetase (Pelissier, et al., 1995). *Nca3* mutants have decreased levels of the mitochondrially encoded transcripts for the Atp6p and Atp8p subunits of the synthetase. Mitochondria isolated from *uth1* cells do not show any significant changes in ATP synthetase function. What could the *SUN* proteins be doing? One possibility is suggested by their homology to *BglA*, a putative beta-glucosidase identified in the fungi, *Candida wickerhamii* (Figure 3.7B). *BglA* was identified in an immunological screen of a *C. wickerhami* library with polyclonal antibodies against the organism's cell-bound, extracellular beta-glucosidase (Skory and Freer, 1995). As shown in Figure 3.15, the *SUN* genes contain a conserved region which resembles the catalytic site found in class I beta-glucosidases. However, we have not been able to reproducibly detect any enzyme activity in *Escherichia coli* cells overexpressing Uth1p or BglA.

What does *UTH1* tell us about aging in yeast? *UTH1* strengthens the link between gene silencing and the regulation of the yeast lifespan. The epistasis analysis suggests that Uth1p enhances lifespan by enhancing silencing at a putative AGE locus. The general effects seen with

the *uth1* mutant at both the HM loci and at telomeres argue that AGE is probably a locus which is normally silenced in wildtype cells and not one which is silenced by a neomorphic gain-of-function mutation in *SIR4* which extends lifespan. However, only the identification of AGE will test this prediction.

Our work with *UTH1* has also separated the stress-resistance and longevity enhancement phenotypes in yeast. It has been proposed that aged animals may be compromised during environmental stress and accumulate misfolded or aggregated proteins at a higher rate than young animals (Johnson, et al., 1996). *Caenorhabditis elegans*, for example, shows increased sensitivity to thermal stress as it ages (Lithgow, et al., 1994) and several mutant worm strains which are long-lived have been shown to have increased thermotolerance (Lithgow, et al., 1995). These observations, along with the finding that several dauer mutants which are inherently stress-resistant are also long-lived suggested that the extended lifespan was a consequence of an enhanced stress response. Though the *uth1-330* mutant was originally identified because of its stress-resistance, the *Δuth1Δsir4ΔHMRa* triple mutant which is stress-resistant but not long-lived suggests that the mechanisms responsible for these phenotypes are distinct in yeast.

As discussed in chapter one, evolutionary arguments have been used to argue against the presence of aging genes since loss-of-function mutants would be expected to produce more progeny than wildtype short-lived cells. Aging genes could only exist if they benefit young cells. Mutant *uth1* cells which are able to live longer are sensitive to paraquat. Though the mechanism of paraquat action is unclear, there may have been evolutionary pressure to retain *UTH1* because paraquat mimics some stress normally experienced by yeast in the wild.

In summary, we have cloned and characterized the yeast aging gene, *UTH1*. Loss of function mutations in *UTH1* increase both the lifespan and stress-resistance in yeast. Epistasis analysis shows that *UTH1* lies upstream of the *SIR* complex for its longevity enhancing function and the analysis of *uth1* mutations in strains where telomeres or the HM loci are marked, suggest that *UTH1* encodes an "anti-silencer".

MATERIALS AND METHODS
<u>Strains, Plasmids, and Media</u>

63

Yeast strains (see Table 3.3) were grown using standard media and conditions.The *UTH1* disruption construct was made by digesting the original library plasmid, pNRA10/1, with SpeI and subcloning the 1 kb which contains the putative 5' end of the ORF into the SpeI site in pRS305 (Sikorski and Hieter, 1989). Next, a 3.8 kb fragment containing the hisG-URA3 fragment from pJLZ25 (generously provided by J. Zamanian) was blunt-ended with Klenow and ligated into a blunt-ended AgeI site just downstream of the *UTH1* ATG. Digestion of the final plasmid, pNRA113.17, with SpeI results in a 4.8 kb fragment that can used to disrupt *UTH1* in yeast by transformation and gene replacement. The *SIM1* disruption was constructed using a *sim1::URA3* plasmid as described (Dahmann, et al., 1995). Disruption of *NCA3* was constructed using a *nca3::URA3* plasmid as described (Pelissier, et al., 1995). To construct the *SUN4* disruption construct, PCR was used to clone 1 kb of the upstream region of *SUN4* (position -538 to +616 relative to the ATG) from yeast genomic DNA obtained from the wildtype strain, Bky1-14c. The primers were as follows:

delSUN45'Bam: CCC GGG ATC CTA TAA TCG CCG TAT TAT TCA CTA

delSUN43'H3: CCC CGG GAA GCT TTA CTA GAG CCA CCC GTA GAA

The PCR product was purified, digested with BamHI and HindIII, and inserted into pUC18 (Yanisch-Perron, et al., 1985) digested with same enzymes. A hisG-URA3 fragment was obtained by digesting pJLZ25 with SacII and the 3.8 kb fragment was inserted into the pUC18 plasmid containing the 5' region of *SUN4* at the SacII site 27 base pairs downstream from the putative first ATG of ORF. Digestion of the final plasmid with BglI and EcoRI results in a 4.9 kb fragment which can be used to disrupt *SUN4* in yeast by transformation and gene replacement. Disruption of *SIR4* was constructed with the *sir4::URA3* plasmid as described (Marshall, et al., 1987). Disruption of *HMRa* was constructed with plasmid pXW102 as described (Wu, et al., 1996). All disruptions were verified by Southern analysis or PCR.

To determine the lifespan 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 overnight. 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 lifespans 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. On very rare occasions, an apparently small, young cell was observed to lyse immediately after micromanipulation and was excluded from the data set.

Determination of Stress Phenotypes

To determine starvation resistance, haploid cells were grown in rich media to log phase, collected by centrifugation, and resuspended in minimal sporulation media for a period of 7-9 days. After starvation, cells were again collected by centrifugation and plated on YEPD to measure colony-forming units per milliliter. 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 know 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 3 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 5 days, with the number of cells per milliliter 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.

Cloning of *UTH1*

A genomic library in the low-copy centromeric vector, YCp50 (Pfeifer, et al., 1987), was transformed into the *uth1-330* mutant by standard methods and plated on dropout plates containing 3mM paraquat with galactose as a carbon source. Colonies that were able to grow robustly on the paraquat plates were restreaked to selective plates at 37°C to look for complementation of the enhanced growth phenotype. Plasmids were recovered from these colonies by standard methods

and retransformed into the *uth1-330* mutant. Four plasmids which consistently complemented both phenotypes were identified. Tetrad analysis showed that three were unlinked to the mutation. The last plasmid to be characterized, pNRA10/1, also complemented the extended longevity phenotype of the *uth1-330* mutant. This plasmid was toxic in several strains of *Escherichia coli* which complicated further molecular analysis of the clone. However, restriction analysis and subcloning into plasmid pRS316 (Sikorski and Hieter, 1989), identified a 2.5 kb HindIII fragment which could complement the *uth1-330* mutant. Sequencing of this fragment using the universal primers complementary to the Bluescript polylinker of pRS316 and the single-strand approach (Sequenase version 2; United States Biochemical Corporation) showed that the fragment contained the novel ORF YKR042w on chromosome XI. Disruption of YKR042w resulted in stress resistant phenotypes identical to those seen with the original *uth1-330* mutant. Finally, 32 spores generated from a diploid of the original *uth1-330* strain and a strain disrupted of YKR042w were all stress-resistant demonstrating that *UTH1* and YKR042w are tightly linked.

Determination of Telomere Length and Telomeric and HM Silencing

Total genomic DNA was isolated from an overnight culture of the appropriate yeast strain, digested with XhoI, and separated on a 0.7% agarose gel and transferred to a GeneScreen Plus hybridization transfer membrane (New England Nuclear Research Products). Hybridization and wash conditions were as suggested by the manufacturer of the membrane. A plasmid containing 600bp located within the conserved Y' region of yeast telomeres, supplied by V. Zakian, was used to generate a random primed probe which 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.

To test telomeric position effect, strains with with either *ADE2* or *URA3* located in the telomeric region of chromosomes V-R or VII-L respectively were constructed as described (Gottschling, et al., 1990). With the strain marked with *ADE2*, wildtype and *uth1* cells were streaked on fresh YEPD and allowed to grow at 30°C for 2-3 days before being photographed. Strains containing *UTH1* on a low-copy vector or a control were streaked on low adenine (one-third standard supplementation) plates which select for the presence of the plasmid and allowed to

grow at 30°C for up to a week for color development.   With the strain marked with *URA3*, wildtype and *uth1* cells were grown overnight in YEPD and then plated either on 5-fluoro-orotic acid (5-FOA) or YEPD plates and allowed to grow at 30°C for three days.   Levels of gene silencing are expressed as the ratio of the number of colonies which grew on FOA over the number which grew on YEPD. To control for transcription effects with the *URA3* gene, the experiment was repeated with wildtype and *uth1* cells with *URA3* at its own locus. All values were obtained from three independent transformants of the indicated genotype.

To test HM silencing, *UTH1* was disrupted in strains which contain either *ADE2* or *TRP1* at an HM silencer. With the strain marked with *ADE2*, wildtype and *uth1* cells were streaked on fresh YEPD and allowed to grow at 30°C for three days before being photographed. With the strain marked with *TRP1*, wildtype and *uth1* cells were grown overnight in YEPD and then plated either on dropout plates lacking tryptophan or YEPD plates and allowed to grow at 30°C for three days. Levels of gene silencing are expressed as the ratio of the number of colonies which grew on the plates lacking tryptophan over the number which grew on YEPD. To control for transcription effects with the *TRP1* gene, the experiment was repeated with wildtype and *uth1* cells with *TRP1* at its own locus. All values were obtained from three independent transformants of the indicated genotype.

Statistical Analysis

Determination of the significance of differences in mean lifespans between two strains was performed using the nonparametric Wilcoxen signed rank test (Systat5 Statiscal Software, Systat, Incorporated). Whenever the mean lifespans of two strains are said to be statistically significant, the analysis showed a confidence level greater than 99%. Determination of significance of differences between levels of silencing was performed using the chi-square test at a confidence level greater than 95%.

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100
80
60
40
20
0

% CELLS VIABLE

GENERATIONS

0   10   20   30   40

— □ — BKy1-14c
— ◆ — uth1-324
— ■ — uth1-328
— ◇ — uth1-330
— ■ — uth1-342
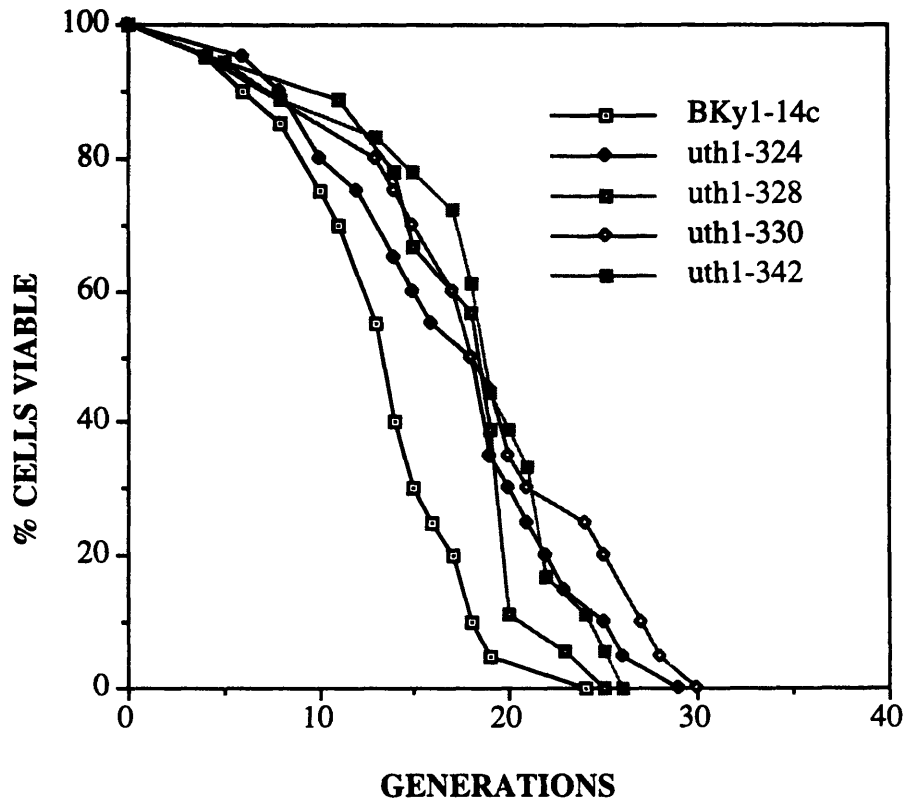
Figure 3.1: Mortality Curves for the *UTH1* Mutants

Mortality curves are shown for all the *uth1* strains obtained in the screen. Curves were derived from at least two independent experiments and the differences in mean lifespans between the mutants and the wildtype strain were shown to be statistically significant by the Wilcoxen Signed Ranked Test. Sample sizes were *uth1-324*, 37 cells; *uth1-328*, 38 cells; *uth1-330*, 38 cells; *uth1-342*, 34 cells; wildtype, 40 cells.
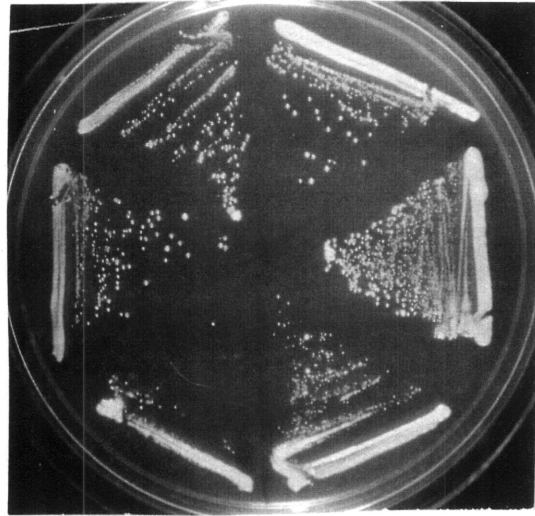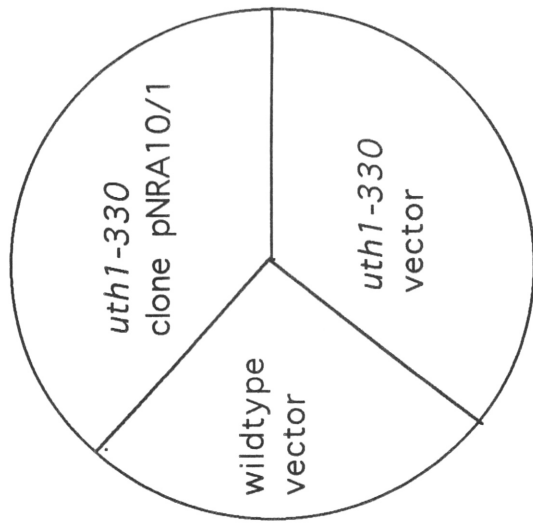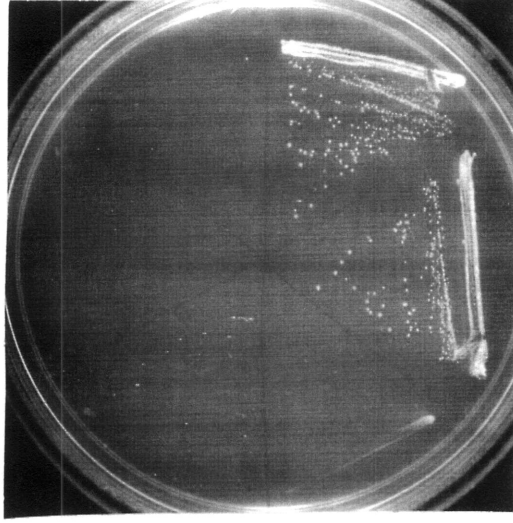
37°C   30°C

Figure 3.2: Complementation of the Stress Resistance of *uth1-330*
Clone pNRA10/1 complements the stress resistance of *uth1-330*. Exponentially
growing cells of the designated genotype were streaked on dropout plates
and allowed to grow for three days at the indicated temperatures.
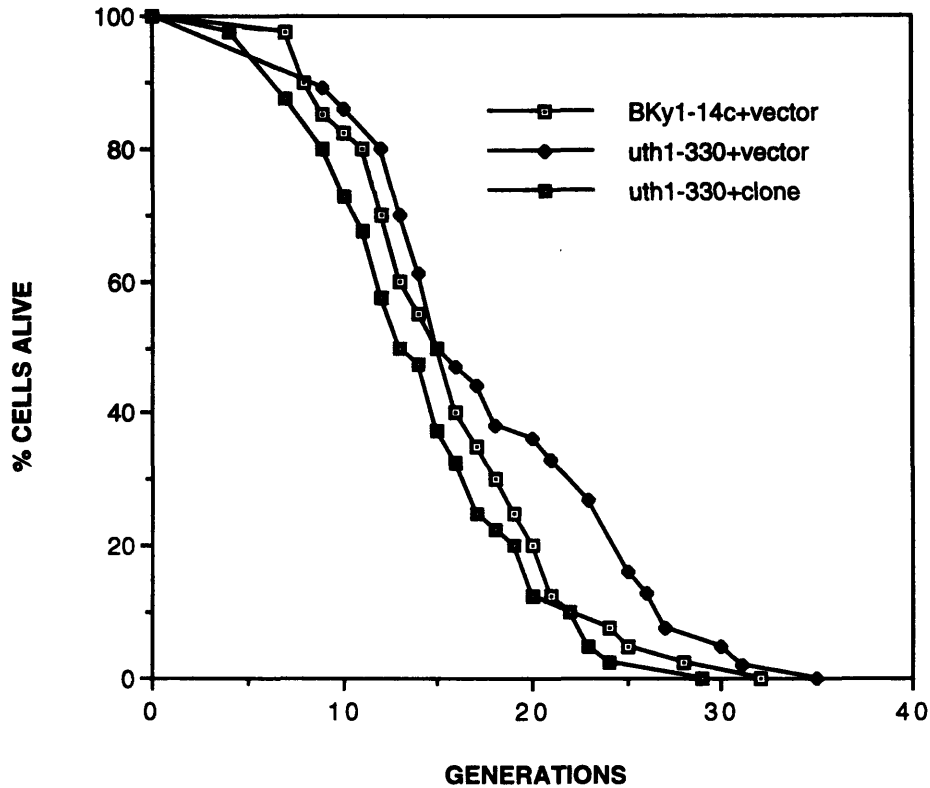
Figure 3.3: Complementation of the *uth1-330* Mutant by Clone, pNRA10/1
Mortality curves are shown for the *uth1-330* mutant transformed with the plasmid,
pNRA10/1, which carries the *UTH1* clone, or a control vector. In addition,
lifespan analysis was also done with wildtype 14C cells transformed with the control
vector. Curves were derived from two experiments with mean lifespans of 15.8, 18.2,
and 14.3 generations for wildtype cells with vector, mutant cells with vector, and
mutant cells with clone, respectively. Sample sizes were as follows: BKy1-14c vector,
40 cells; *uth1-330* vector, 36 cells; *uth1-330* clone, 40 cells.

37°C   30°C
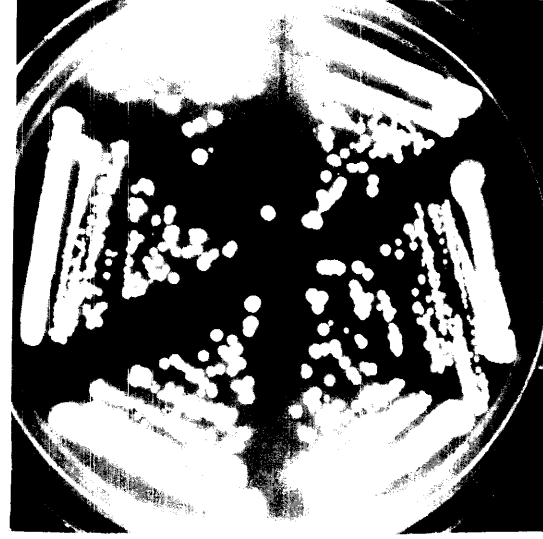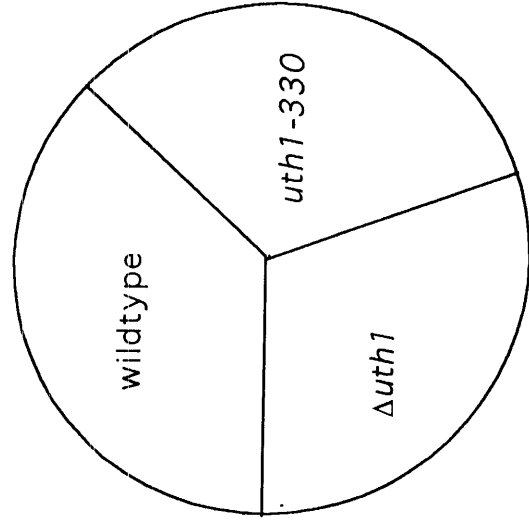
Figure 3.4: Disruption of *UTH1* Enhances Growth at Elevated Temperatures
A strain carrying a disruption of *UTH1* has an enhanced growth phenotype at elevated temperatures which is indistinguishable from the growth phenotype of the original *uth1-330* mutant. Exponentially growing cells of the designated genotype were streaked on YEPD plates and allowed to grow for two days at the indicated temperatures.
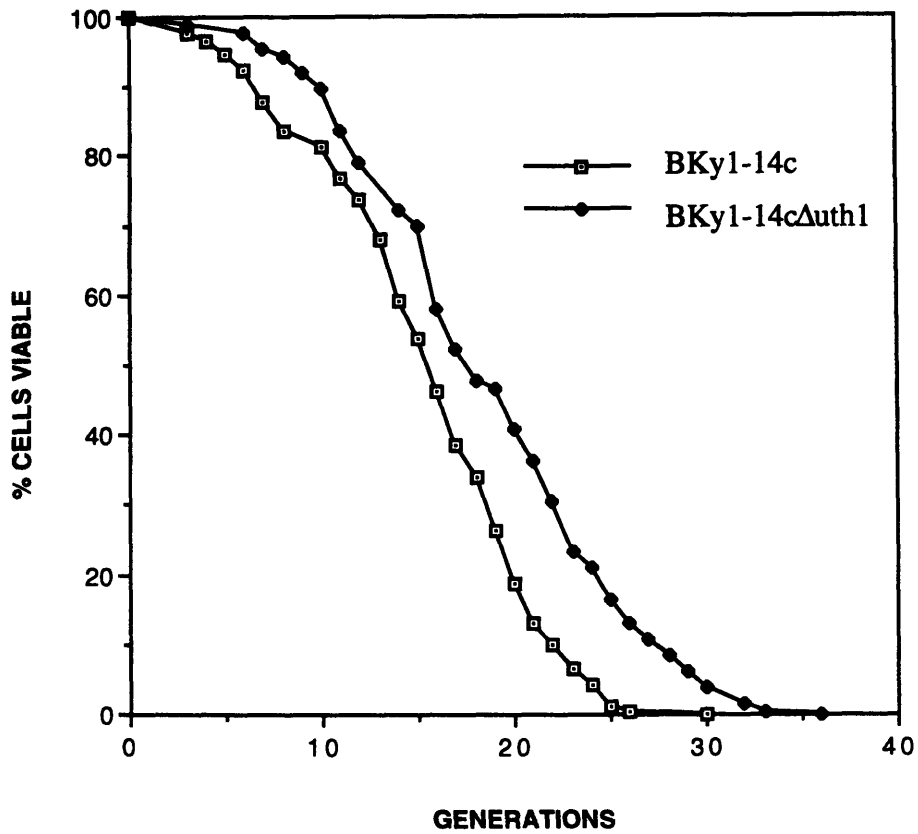
Figure 3-5: Disrupting *UTH1* Extends the Yeast Lifespan
The mortality curve for a strain with a disruption of *UTH1* is shown. The
curves were derived from four experiments with mean lifespans of
16 and 19 generations for wildtype and disruption strain respectively.
Sample sizes were wildtype, 94 cells, and mutant, 86 cells.

Strain Background	UTH1 allele	Growth			Lifespan		n*
		30°C	37°C	39°C	mean	maximum	
BKy1-14c	wildtype	++	+/-	-	16	30	94
	Δuth1	++	++	+	19	36	86
PSY316	wildtype	++	++	+/-	15.8	24	25
	Δuth1	++	++	+	18.1	35	26
PSY142	wildtype	++	++	++	28.7	52	30
	Δuth1	++	++	++	27.2	56	30
W303	wildtype	++	++	+/-	ND		
	Δuth1	++	++	++			
YPHDF-1A	wildtype	++	++	+/-	ND		
	Δuth1	++	++	++			

Table 3.1: Effects of *UTH1* in Different Genetic Backgrounds

* n: sample size

Figure 3.6: Sequence of *UTH1*
The nucleotide and amino acid sequences for *UTH1* are listed in the following pages as they are entered in Genbank in the entry for the gene YKR042 on chromosome XI. The figure was generated using DNA Strider

```
  1 TGAAAAAGTGGAACTAGACCCCACGTCAGCGGGCCCTAGGCCCTTCA ATG TGT TAG AATACACAGCGTGCCTAGTTC 76
 77 CTGGTGCCTGGATCTCGAGGCCGCGGCACTGGAAAAGCCCTTTCTTTTCCAGATCGGGAAACCTA ATG AGT CCA   150
151 TAA AAAGAA ATG TAG AGGTGGTGTTGACGTTTTGCCGCTTTTGGGCAAGTAGGTCTTTCTGCACGGCCCGGCCCGG 226
227 GTCGTGCGGAAAAAAGAAAAAAGCAGACAAAACAAAATTTTTCCTTTTTTTTCGCCTTTGTTTCTCCTGATTCGGGTATATA 306


307 AGTGAATACCATCTA ATG TGT TTC CTT CTC GAG ACC TCG GCG TCT CCC AGA TCA AAG CTC AGC 369
  1                 M   C   F   L   L   E   T   S   A   S   P   R   S   K   L   S   16


370 AAA GAT TTT AAA CCG CAA TTT ACG CTC CTT TCA TCG GTA ACT AAG AAG AAA AAA AAA AAA 429
 17 K   D   F   K   P   Q   F   T   L   L   S   S   V   T   K   K   K   K   K   K   36


430 GTA CGA CCA CAC AAT TTC CAG TGT ATT CAT TCC TTA AAC TTC GTT TAT TTT TTA TTC ATT 489
 37 V   R   P   H   N   F   Q   C   I   H   S   L   N   F   V   Y   F   L   F   I   56


490 CAT TCA TTT TTA TTT GAA TAT AAC CAA CTA CTA GTC CTT CCT TTA AAC AAA AAT TTA CCC 549
 57 H   S   F   L   F   E   Y   N   Q   L   L   V   L   P   L   N   K   N   L   P   76


550 TCC CTT AAT TTT TCA AGA AAT TCC AGT ATG AAA TTA TCC GCT CTA TTA GCT TTA TCA GCC 609
 77 S   L   N   F   S   R   N   S   S   M   K   L   S   A   L   L   A   L   S   A   96


610 TCC ACC GCC GTC TTG GCC GCT CCA GCT GTC CAC CAT AGT GAC AAC CAC CAC CAC AAC GAC 669
 97 S   T   A   V   L   A   A   P   A   V   H   H   S   D   N   H   H   H   N   D   116


670 AAG CGT GCC GTT GTC ACC GTT ACT CAG TAC GTC AAC GCA GAC GGC GCT GTT GTT ATT CCA 729
117 K   R   A   V   V   T   V   T   Q   Y   V   N   A   D   G   A   V   V   I   P   136


730 GCT GCC ACC ACC GCT ACC TCG GCG GCT GCT GAT GGA AAG GTC GAG TCT GTT GCT GCT GCC 789
137 A   A   T   T   A   T   S   A   A   A   D   G   K   V   E   S   V   A   A   A   156


790 ACC ACT ACT TTG TCC TCG ACT GCC GCC GCC GCT ACT ACC TCT GCC GCC GCC TCT TCT TCC 849
157 T   T   T   L   S   S   T   A   A   A   A   T   T   S   A   A   A   S   S   S   176


850 TCC TCT TCC TCT TCC TCC TCT TCC TCT TCT TCC TCT GTT GGT TCT GGA GAT TTT GAA GAT 909
177 S   S   S   S   S   S   S   S   S   S   S   S   V   G   S   G   D   F   E   D   196


910 GGT ACC ATT TCC TGT TCT GAT TTC CCA TCC GGA CAA GGT GCT GTC TCC TTG GAC TGG TTA 969
197 G   T   I   S   C   S   D   F   P   S   G   Q   G   A   V   S   L   D   W   L   216


970 GGT CTA GGC GGC TGG GCT TCC ATC ATG GAC ATG AAC GGT AAC ACC GCC ACC TCT TGT CAA 1029
217 G   L   G   G   W   A   S   I   M   D   M   N   G   N   T   A   T   S   C   Q   236


1030 GAC GGA TAC TAC TGT TCT TAC GCT TGT TCT CCA GGT TAC GCT AAG ACC CAA TGG CCT TCT 1089
237  D   G   Y   Y   C   S   Y   A   C   S   P   G   Y   A   K   T   Q   W   P   S   256


1090 GAA CAA CCT TCC GAT GGT AGA TCC GTT GGT GGT TTA TAC TGT AAG AAC GGT AAA TTA TAC 1149
257  E   Q   P   S   D   G   R   S   V   G   G   L   Y   C   K   N   G   K   L   Y   276


1150 CGT TCC AAC ACC GAC ACT AAC AGT TTG TGT GTA GAA GGT CAA GGC TCT GCT CAA GCT GTT 1209
277  R   S   N   T   D   T   N   S   L   C   V   E   G   Q   G   S   A   Q   A   V   296


1210 AAC AAG GTC TCC GGC TCC ATT GCT ATC TGT GGT ACC GAT TAT CCA GGT TCT GAA AAC ATG 1269
297  N   K   V   S   G   S   I   A   I   C   G   T   D   Y   P   G   S   E   N   M   316


1270 GTC GTT CCT ACC GTA GTT GGC GCT GGT TCC TCC CAA CCA ATC AAC GTC ATC AAG GAG GAC 1329
317  V   V   P   T   V   V   G   A   G   S   S   Q   P   I   N   V   I   K   E   D   336
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337  S   Y   Y   Q   W   Q   G   K   K   T   S   A   Q   Y   Y   V   N   N   A   G   356

1390 GTC TCT GTG GAA GAT GGT TGT ATC TGG GGT ACT GAG GGT TCC GGT GTC GGT AAC TGG GCC 1449
357  V   S   V   E   D   G   C   I   W   G   T   E   G   S   G   V   G   N   W   A   376

1450 CCA GTT GTC TTG GGT GCT GGT TAC ACT GAT GGT ATC ACT TAC TTG TCC ATC ATT CCA AAC 1509
377  P   V   V   L   G   A   G   Y   T   D   G   I   T   Y   L   S   I   I   P   N   396

1510 CCA AAC AAC AAA GAA GCA CCA AAC TTT AAC ATC AAG ATC GTT GCC ACC GAT GGC TCT ACC 1569
397  P   N   N   K   E   A   P   N   F   N   I   K   I   V   A   T   D   G   S   T   416

1570 GTC AAT GGT GCT TGC TCT TAC GAA AAT GGT GTC TAC TCT GGC TCT GGC TCT GAC GGT TGT 1629
417  V   N   G   A   C   S   Y   E   N   G   V   Y   S   G   S   G   S   D   G   C   436

1630 ACT GTT TCA GTT ACT TCT GGT TCT GCT AAC TTT GTC TTC TAC TAG GCCTTTTTTCCTTGAATATT 1694
437  T   V   S   V   T   S   G   S   A   N   F   V   F   Y   *                     451

1695 GCAAATAAGCTTTTCCTAGTACTTTTTTTTACTCCGTTCATTTT ATG GTT TAT TTT TCA ATT AGT TCG TTT 1764
```

THE *SUN* GENE FAMILY

UTH1
1   236   451

NCA3
1   123   338

SIM1
1   211   426
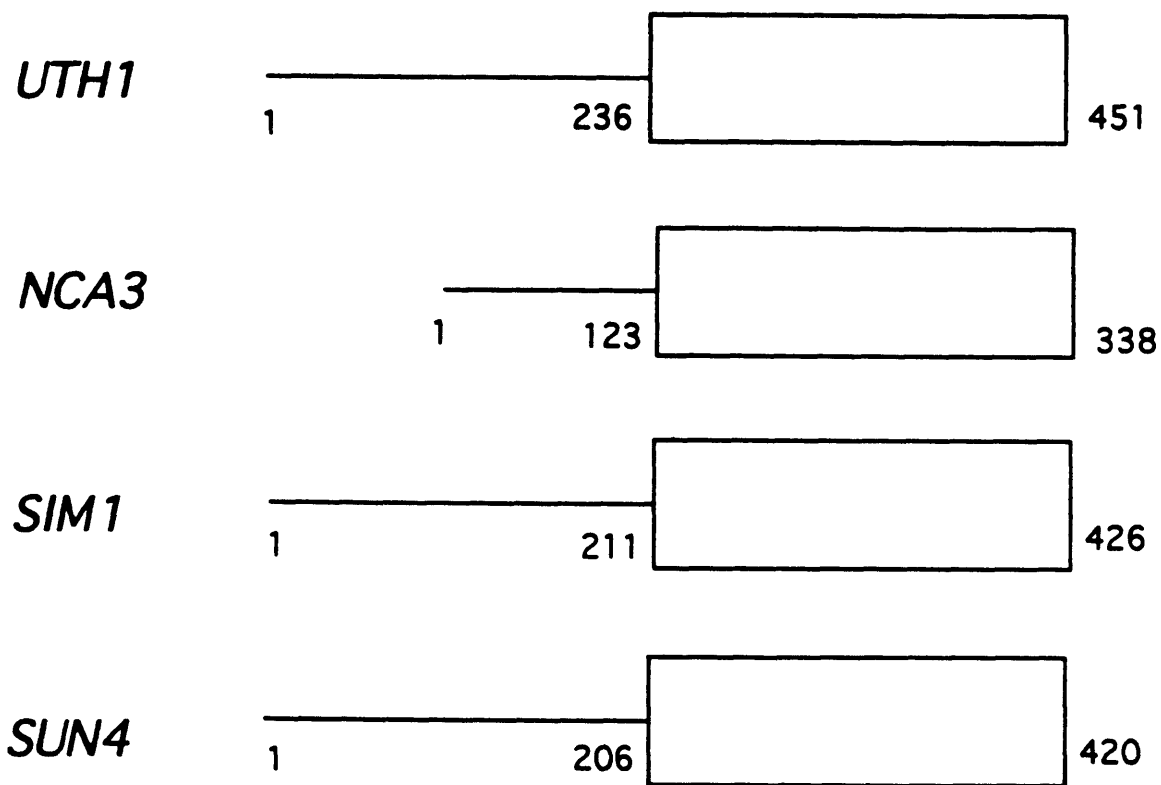
SUN4
1   206   420

Figure 3.7A: The *Saccharomyces SUN* Genes

NCA3	GYYCSYACEPGMSKTQWPSDQPSDGKSVGGLYCKNGYLYRTNTDTSDLCS
UTH1	GYYCSYACSPGYAKTQWPSEQPSDGRSVGGLYCKNGKLYRSNTDTNSLCV
SIM1	GSYCSYSCQPGMSKTQWPSDQPSDGRSVGGLLCKNGYLYRSNTDADYLCE
SUN4	GSYCSYACQPGMSKTQWPSDQPSDGRSIGGLLCKDGYLYRSNTDTDYLCE
bglA	GYYCSYACQPGMSKTQWPSDQPANGVSVGGLLCKGGKLYKSSS-SDYLCE

NCA3	TDETSAKAINKKSDSIALCRTDYPGSENMVIPTVVDGGDSQPISVVDEDT
UTH1	EGQGSAQAVNKVSGSIAICGTDYPGSENMVVPTVVGAGSSQPINVIKEDS
SIM1	WGVEAAYVVSKLSKGVAICRTDYPGTENMVIPTYVEGGSSLPLTVVDQDT
SUN4	WGVDAAYVVSELSWDVAICRTDYPGTENMVIPTYVQAGDSLPLTVVDQDT
bglA	WGVDAARVVSEISDVVSICRTDYPGTENMVIPTAVEGGDEAILTVVNGET

NCA3	YYQWQGKKTSAQYYINNAGVSAEDGCIWGTSGSDVGNWAPLVLGAGSTNG
UTH1	YYQWQGKKTSAQYYVNNAGVSVEDGCIWGTEGSGVGNWAPVVLGAGYTDG
SIM1	YFTWEGKKTSAQYYVNNPGVSVEDGCIWGTSGSGIGNWAPLNFGAASTGG
SUN4	YYTWQGLKTSAQYYVNNAGISVEDACVWGSSSSGVGNWAPLNFGAGSSDG
bglA	YYQWEGMTTSAQYYVNNAGVSYEDGCIWGDSNGDIGNWAPLNFGAGYDGG

NCA3	ETYLSLIPNPNSNQAANFNVKIVASDG.ANVQGSCAYEDGSFTGDGSDGC
UTH1	ITYLSIIPNPNNKEAPNFNIKIVATDG.STVNGACSYENGVYSGSGSDGC
SIM1	VTYLSLIPNPNNSDALNYNVKIVAADDSSNVIGECVYENGEFSG.GADGC
SUN4	VAYLSLIPNPNNGNALNFNVKIVAADDSSTVNGECIYENGSFSG.GSDGC
bglA	ISYLSLIPNPNNKNALNFNVKIIAYDDKSNVNGECVYENGSYNGDGTDGC

		Identity to UTH1	
NCA3	TVSVLSGSAEFVFY	NCA3	73% identity
UTH1	TVSVTSGSANFVFY	SIM1	68% identity
SIM1	TVSVTSGKAHFVLYN	SUN4	58% identity
SUN4	TVSVTAGKAKFVLYN	bglA	60% identity
bglA	TVAVTSGKAKYVLY		

Figure 3.7B: The *Saccharomyces cerevisiae* *SUN* genes share a 215 amino acid domain with the *bglA* gene from *Candida wickerhamii*
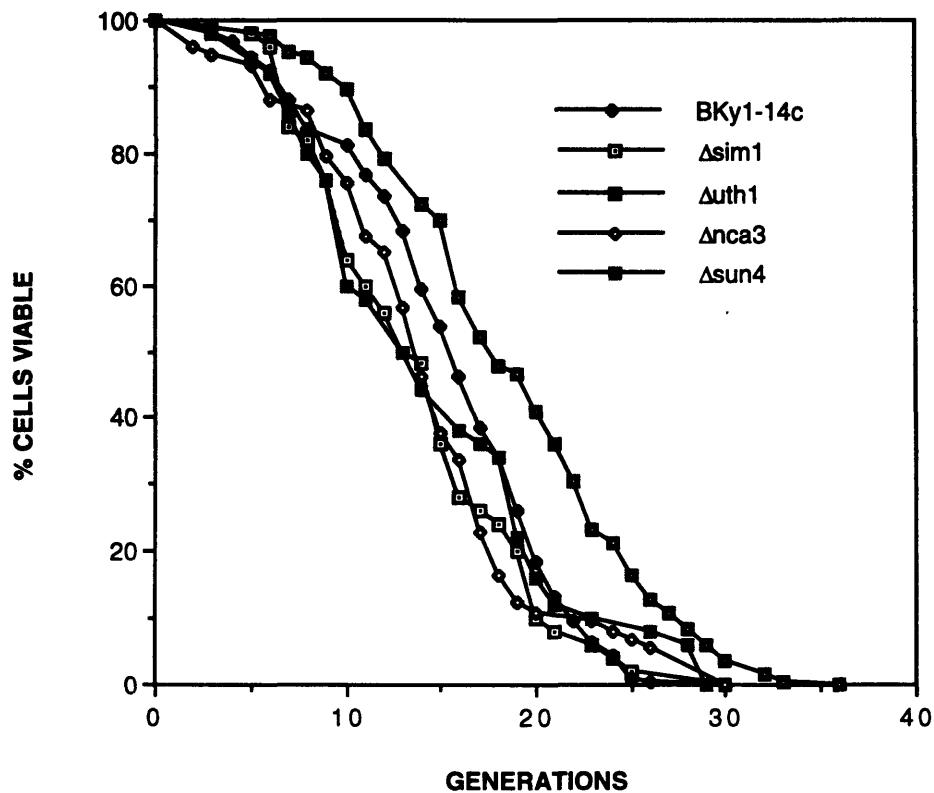
Figure 3.8: Mortality Curves for Mutants Carrying Null Mutations in the*SUN* Genes
Mortality curves are shown for strains in the BKy1-14c background with null alleles in
the different *SUN* genes. All curves are derived from at least two independent experiments.
The difference in lifespans between these the mutants and the wildtype strains was
shown to be statistically significant by the Wilcoxen Ranked Signed Test. Mean
lifespans and sample sizes are listed in Table 3.2

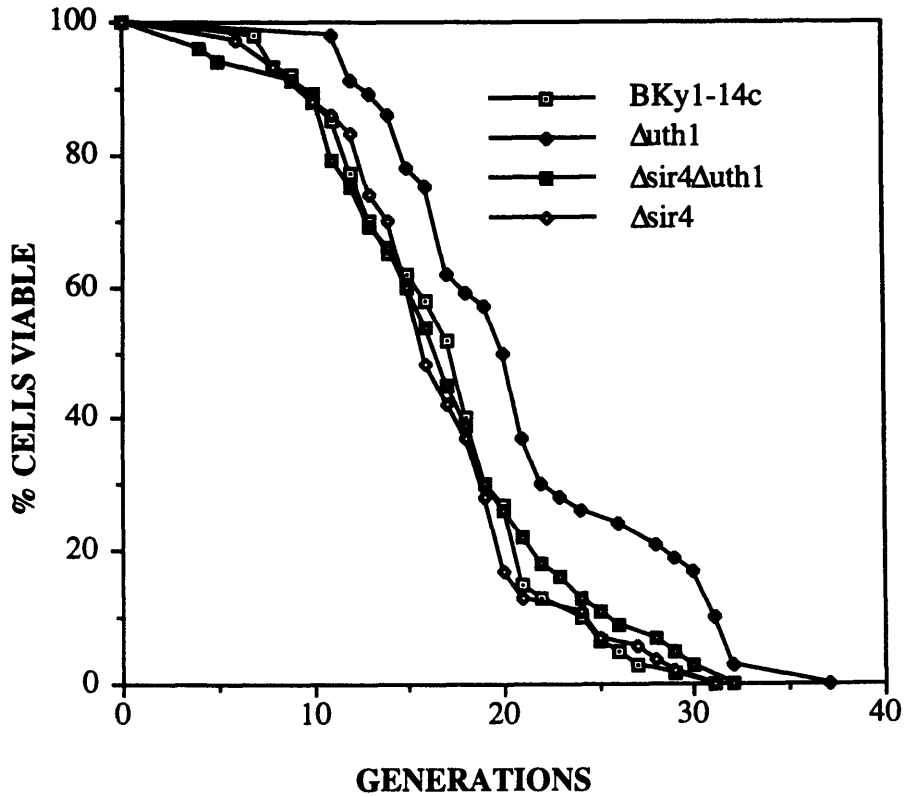| STRAIN	MEAN LIFESPAN	MAXIMUM	SAMPLE SIZE
wildtype	16	30	94
Δuth1	19	36	86
Δnca3	14	31	74
Δsim1	14	30	50
Δsun4	14	30	49
Δsim1Δuth1	16	34	40

Table 3.2: Lifespan Data for the SUN Genes

Figure 3.9: *SIR4* is Epistatic to *UTH1* for the Regulation of the Yeast Lifespan
Mortality curves are shown for the wildtype, *SIR4* and *UTH1* null mutants, and the
double mutant. All curves are derived from at least two experiments. The
difference in lifespans between the *uth1* mutant and the other strains was shown to
be statistically significant by the Wilcoxen Ranked Signed Test. The mean lifespans
were 17.8, 21.1, 16.8, and 18.2 generations for the wildtype, *uth1* , *sir4* and *uth1sir4*
strains. Sample sizes were as follows: wildtype, 60 cells; *uth1*, 55 cells; *sir4*; 57;
uth1sir4, 52 cells.

37°C   30°C

wildtype
MAT α

Δuth1
MAT α

Δuth1Δsir4
MAT αΔHMLa
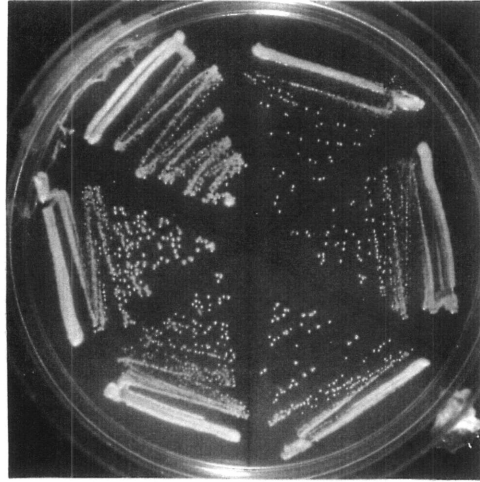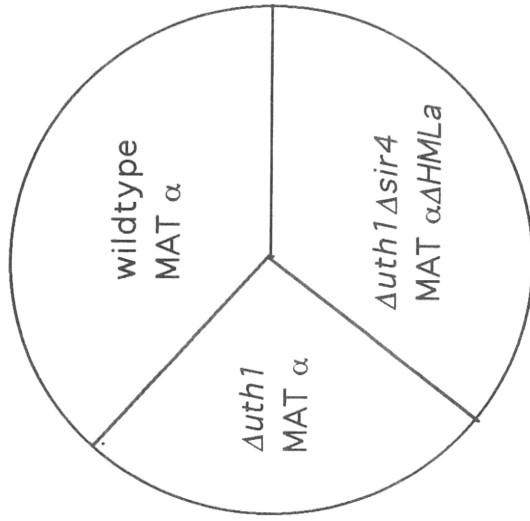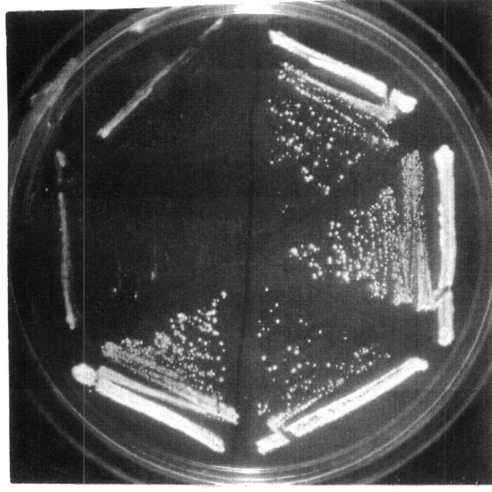
Figure 3.10: Stress Resistance Phenotype of *UTH1* is Independent of *SIR4*
Unlike the mutant *Uth2-42*, the stress resistant phenotype of a *uth1* mutant is independent of *SIR4* and the mating type of the strain. Exponentially growing cells of the designated genotype were streaked on dropout plates and allowed to grow for three days at the indicated temperatures.
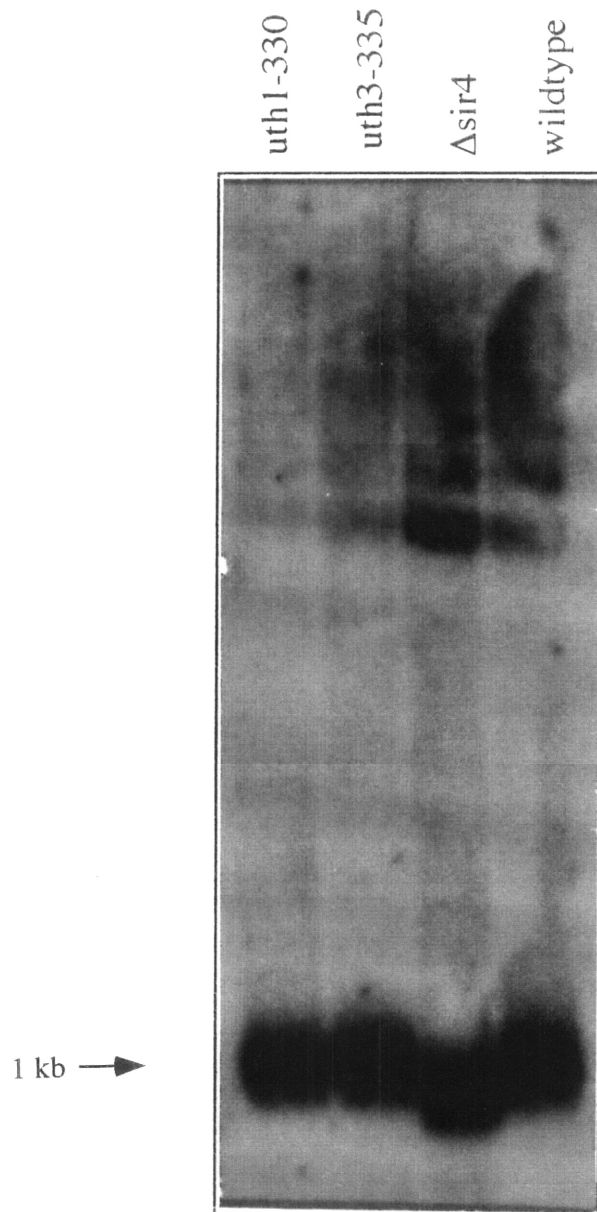
Figure 3.11 Telomere Length is Unchanged in the *uth1-330* Mutant
XhoI digested DNA is probed with a labeled 600 bp fragment that hybridizes
to Y' DNA that is telomere distal to the XhoI site. The broad smear at the
bottom of the gel consists of yeast telomeres. Sources of DNA were strains
bearing the indicated genotype in the BKy1-14c background.
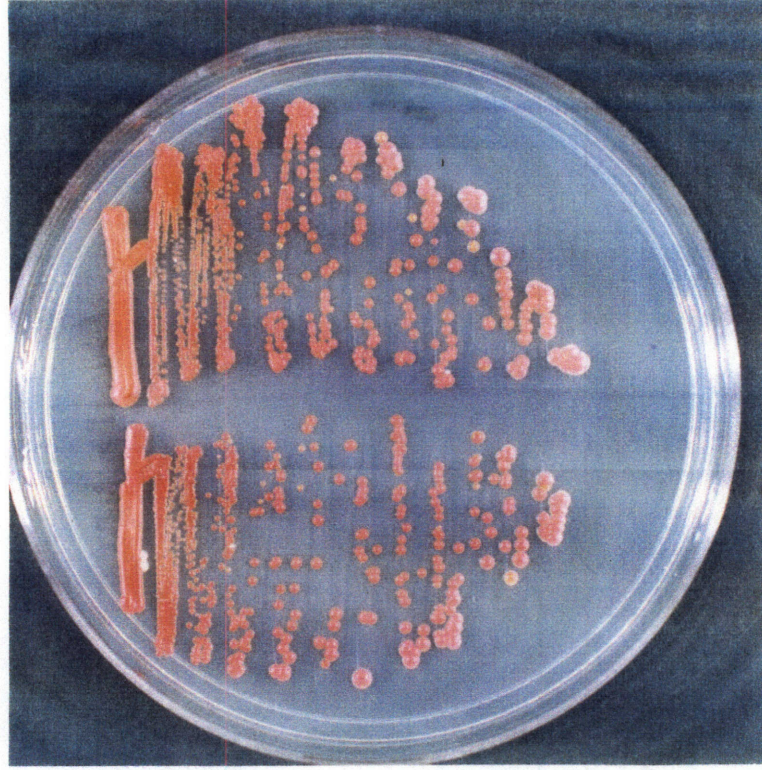
Figure 3.12: *UTH1* is Involved in Alleviating Silencing at Telomeres and at the HM Loci
A. *UTH1* was disrupted in PSY316AT, a strain which has *ADE2* at the telomeric region of chromosome V-R. The extent of silencing is depicted by the red color of the colonies: red indicates silencing, white indicates gene expression. A YEPD plate showing the Δ*uth1* (right) and the wildtype control (left) was incubated at 30°C for 2-3 days for color development.
B. *UTH1* was disrupted in OAy23, a strain which has *ADE2* at an HM silencer. As above, silencing is depicted by the red color of the colonies. A YEPD plate showing the Δ*uth1* (left) and the wildtype control (right) was incubated at 30°C for 2-3 days for color development.
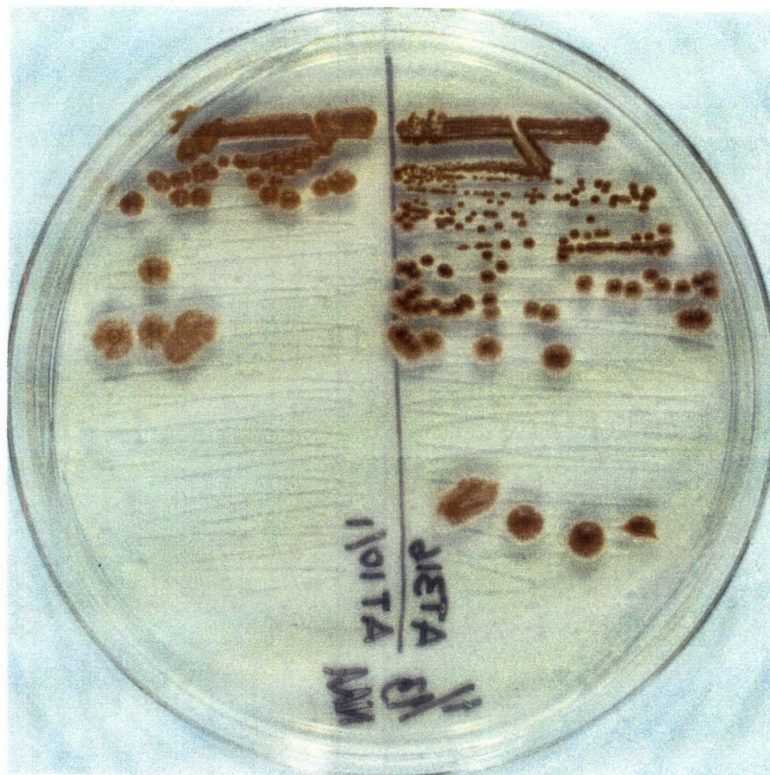
Figure 3.13: Additional Copies of *UTH1* Result in Loss of Telomeric Silencing
A low-copy vector carrying *UTH1* was introduced into the PSY316AT strain which has *ADE2* at the telomeric region of chromosome V-R. A plate selecting for the *UTH1* plasmid (left) or vector alone (right) was incubated at 30°C for 2-3 days and then left in the cold room for color development. To enhance color differences, the plate was supplemented with only one third of the standard amount of adenine.
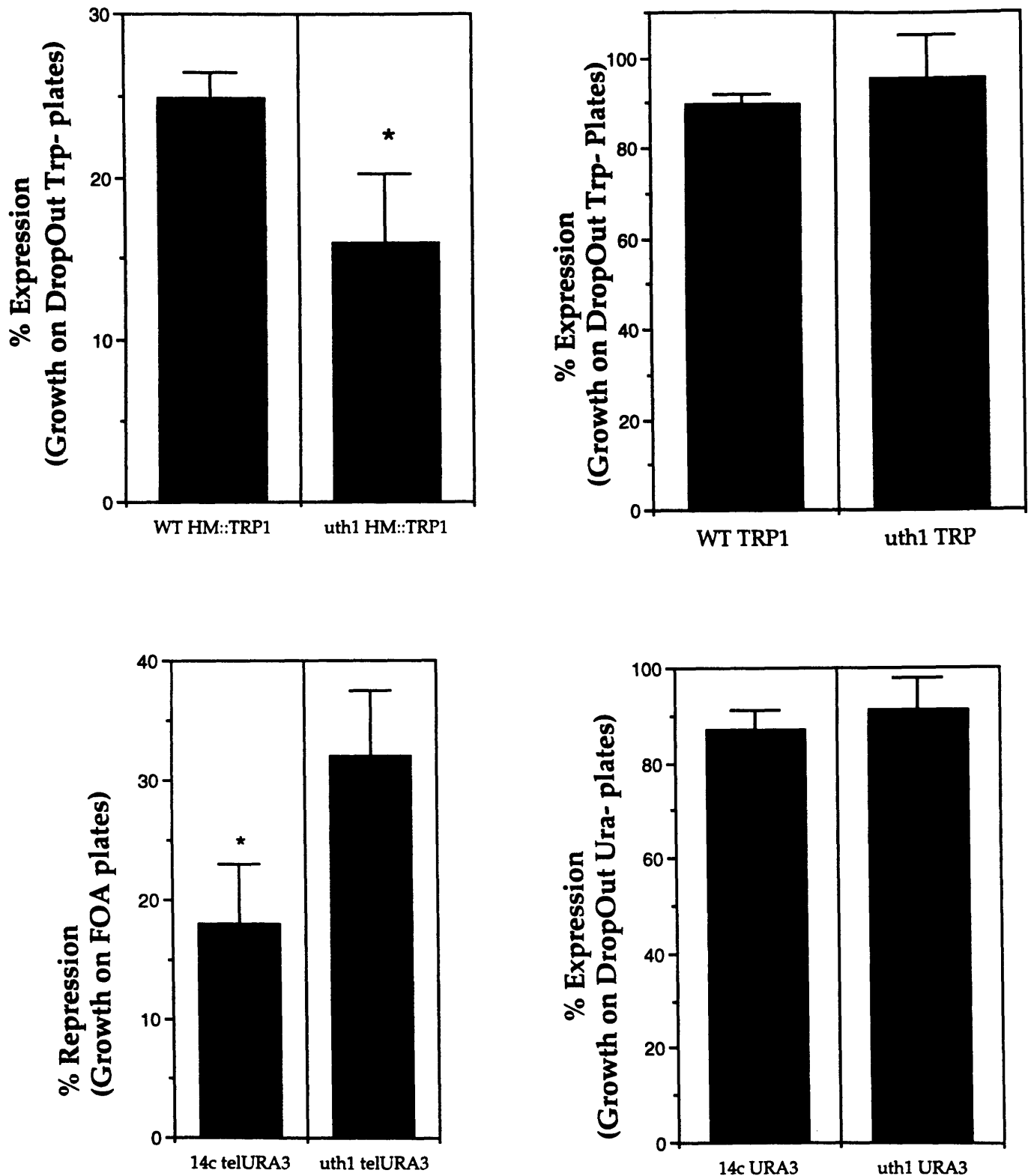
Figure 3.14: *UTH1* Decreases Silencing at Both Telomeres and HM Loci
Disruption of *UTH1* results in a small but significant increase in silencing both at HM loci and at telomeres. Cells of the designated genotype were grown overnight on YEPD and plated on either the indicated plates or YEPD. Standard deviations were derived from three independent transformants and statistical significance indicated by the asterisk was determined using the chi square test at a $p < 0.05$. Each experiment was repeated at least twice and representative values are shown. Strains backgrounds were W303a for TRP1 experiments and BKy1-14c for URA3.

```
S. solfataricus      LYMYVTENGIA---------------DDADYQRPYY    400
S. solfataricus      LPLYVMENGIA---------------DDADYQRPYY    402
E. coli              -PLFIVENGLGAKDSVEADGS-----IQDDYRIAY     383
S. aureus            HKIYITENGLGYKDEFIES---EKTVHDDARIDY      398
S. lactis            KKIYITENGLGYKDEFVD------NTVYDDGRIDY     405
L. casei             PVTYVTENGIGLKESLPENATPDTVIEDPKRIDY      401
C. thermocellum      PNIVISENGAAFKDEIGSNGK-----IEDTKRIQY     376
C. saccharolyticum   IPIYITENGAAYNDIVTEDGK-----VHDSKRIEY     385
A. faecalis          -ECYITENGACYNMGV-NGQ-----VNDQPRLDY      380
B. polymyxa          IDIYITENGACINDEVV-NGK-----VQDDRRISY     373
B. polymyxa          LPLILTENGAAMRDELV-NGQ-----IEDTGRHGY     377
H. sapiens           PPIYVTENGVSQREE-----------TDL-NDTARIYY  391
rabbit               PPIYVTENGVSHRGD-----------SYL-NDTTRIYY  392
H. sapiens           IPIYITENGVGL-TN-----------PNT-EDTDRIFY  389
rabbit               IPIYITENGVGL-TN-----------PRL-EDIDRIPY  389
UTH1                 NGACSYENGVYSGS-----GS-----DGCTVSV

NCA3                 QGSCAYEDGSFTGD-----GS-----DGCTVSV
SIM1                 IGECVYENGEFSG------GA-----DGCTVSV
SUN4                 NGECIYENGSFSG------GS-----DGCTVSV
BglA                 NGECVYENGSYNGD-----GT-----DGCTVAV
                     -----ENG--------------D------
```

Figure 3.15: Alignment of Residues Around the Nucleophile of the Beta-glucosidases of family BGA

Mutational analysis of the beta-glucosidase from *Agrobacterium* identified Glu358 as the nucleophile in this enzyme. Similar results have been obtained with related enzymes from *Cellulomonas fimi* and *Eshcerichia coli*. Other critical residues in this family of enzymes include Gly 360 which when mutated reduces enzyme activity and Asp374 which may serve as the acid-base catalyst. These residues have been highlighted in bold. Intriguingly, the SUN family of genes contain a conserved region which may correspond to the catalytic site of this family of beta-glucosidases (adapted from Trimbur, et al., 1993).

Table 3.3 : Yeast Strains Used in This Study

Strain	Genotype	Reference
BKy1-14a	*MATa ade1-100 leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1995
BKy1-14b	*MATα leu2-3,112 ura3-52*	Kennedy, et al., 1995
BKy1-14c	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1995
BKy1-14d	*MATa his4-519 leu2-3,112 ura3-52*	Kennedy, et al., 1995
BKy110	BKy1-14c *sir4::LEU2*	Gift of B. Kennedy
BKy111	BKy1-14c *sir4:: LEU2 hmra::URA3*	Gift of B. Kennedy
BWG1-7A	MATa *ade1-100 his4-519 leu2-3,112 ura3-52*	Guarente andMason, 1983
LS59	MATα *ade2-101 his3-Δ200 trp1-1 leu2-3,112 can1-100 hmrΔA::TRP1*	Gift of D. Shore
PSY142	MATα *leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1994
PSY316	MATα *ade2-101 his3-Δ200 leu2,112 lys2 ura3-53*	Pina et al., 1993
PSY316AT	PSY316 *ADE2-TEL V-R*	Gift of B. Kennedy
OAy23	MATa *ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100 HIS3::HMR-ADE2-1*	Gift of O. Aparicio
W303a	MATa *ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	
YPHDF-1A	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	D'mello, et al., 1994
NRAy100	BKy1-14c *uth1::hisG*	
NRAy101	BKy1-14c *sim1::URA3*	
NRAy102	BKy1-14c *nca3::URA3*	
NRAy103	BKy1-14c *sun4::URA3*	
NRAy104	BKy1-14c *uth1::hisG sun4::hisG-URA3-hisG*	
NRAy105	BKy1-14c *uth1::hisG sir4::LEU2*	
NRAy106	BKy111 *uth1::hisG-URA3-hisG*	
NRAy107	BKy1-14c *adh::URA3-TEL*	
NRAy108	BKy1-14c *uth1::hisG adh::URA3-TEL*	
NRAy200	PSY316 *uth1::hisG-URA3-hisG*	
NRAy201	PSY316AT *uth1::hisG-URA3-hisG*	
NRAy300	PSY142 *uth1::hisG-URA3-hisG*	
NRAy400	W303a *trp1-1::TRP1*	
NRAy401	W303a *trp1-1::TRP1 uth1::hisG-URA3-hisG*	
NRAy402	OAy23 *uth1::hisG-URA3-hisG*	
NRAy403	OAy23 *sim1::URA3*	
NRAy404	LS 59 *uth1::hisG-URA3-hisG*	
NRAy500	YPHDF-1A *uth1::hisG-URA3-hisG*	

CHAPTER FOUR

Short Telomeres Delay Senescence in Yeast Via a Mechanism Requiring *SIR3*

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INTRODUCTION

Individual cells of the budding yeast, *Saccharomyces cerevisiae*, are mortal. As first described by Mortimer and Johnston (Mortimer and Johnston, 1959), yeast cells complete a strain-specific number of cell cycles called their lifespan before arresting. Aging yeast cells share several universal characteristics. They increase in size and senescent cells four times as large as an exponentially growing cell are not uncommon (Mortimer and Johnston, 1959; Egilmez, et al., 1990). Furthermore, their cell cycle times increase with age and final cell cycle periods of twenty-four hours have been observed (Mortimer and Johnston, 1959; Egilmez, et al., 1989). In contrast, young cells cycle about once every ninety minutes. The mortality kinetics of a yeast colony can be described by the Gompertz equation (Pohley, 1987), the classical definition of an aging population, and the differences in lifespans among different strains support the hypothesis that aging in yeast is under genetic control.

To identify the genes that regulate the yeast lifespan, we carried out a screen for stress-resistant yeast mutants which were also long lived. Mutations which fell into four complementation groups were identified. *UTH2* (youth) was the first gene to be cloned by complementation of its sterile phenotype (Kennedy, et al., 1995) and sequencing revealed that it was allelic to *SIR4* (silent information regulation) which encodes a component of a protein complex which is required for silencing at *HMLα* and *HMRa* (Ivy, et al., 1986), the silent information cassettes of the yeast mating type locus, and at yeast telomeres (Gottschling, et al., 1990). The identification of the longevity enhancing mutation in *SIR4* provided the first link between transcriptional silencing and aging in yeast. Additional support has also been provided by the cloning and characterization of the yeast aging gene, *UTH1*, which encodes an "anti-silencer" (Chapter 3).

SIR4-42, the mutation which enhances yeast longevity, is a gain-of-function mutation in the *SIR4* gene that removes about 200 amino acids of its carboxyl terminus (Kennedy, et al., 1995). However, the *SIR4-42* mutation is probably not a neomorph: the mutation is only able to enhance yeast longevity if *SIR2* and *SIR3* which encode the other essential components of the silencing complex are intact. This along with the observation that the deletion of *SIR4* shortens

lifespan in several yeast backgrounds suggest that the mutant is still involved in silencing. Strikingly, overexpression of only the carboxyl terminus of Sir4p which is missing in the *SIR4-42* mutant (the dominant negative anti-*SIR4* allele) resulted in an extension of lifespan similar to that seen with *SIR4-42* (Kennedy, et al., 1995). Again, this extension of lifespan from the overexpressed C-terminus of Sir4p required *SIR2* and *SIR3*. To explain these results, we proposed that the lifespan of the yeast cell is determined by the expression levels of an aging gene called AGE which is subject to transcriptional silencing requiring the gene products of the *SIR* genes. According to this model, this AGE locus would gradually be expressed as silencing is lost during yeast aging resulting in cell death. Both the *SIR4-42* mutation and the overexpression of the carboxyl terminus of Sir4p increase the affinity of the silencing complex for AGE enhancing the silencing of this aging gene. Enhanced silencing of AGE would then delay senescence. One prediction of this model has been confirmed with sensitive RT-PCR analysis which demonstrated that transcriptional silencing is lost in old yeast cells (Smeal, et al., 1996).

Recent work has revealed that a delicate equilibrium of limited silencing complexes exists between the HM silent information cassettes and the telomeres of the cell (Buck and Shore, 1995; Marcand, et al., 1996; Lustig et al., 1996). Though HM and telomeric silencing are similar, both requiring the Sir2, Sir3, and Sir4 proteins, it is clear that HM silencing is more stable than the silencing at telomeres which undergoes variegation (Gottschling, et al., 1990; Aparicio, et al., 1991). This difference is probably because these locations have different affinities for the Sir complex: HM silencing is more stable than telomeric silencing because the HM loci have a higher affinity for Sir2p, Sir3p, and Sir4p due to the presence of Sir1p (Chen, et al., 1993). The analysis of the *rap1s* mutation in Rap1p, the DNA binding protein which is thought to tether the Sir proteins to DNA (Moretti et al., 1996), supports this hypothesis. Buck and Shore have demonstrated that this allele of *RAP1* increases telomeric silencing by preferentially recruiting the Sir proteins to telomeres (Buck and Shore, 1995). As predicted, this increase in telomeric silencing (also known as telomere position effect or TPE) is accompanied by a decrease in HM silencing.

In the last several years, one model for cellular aging that has become popular is that division capacity is limited by the length of telomeres (reviewed in Greider and Harley, 1996). Two studies have shown that the telomeres of senescing fibroblasts gradually shorten as the cells

age (Harley, et al., 1990; Hastie, et al., 1990). According to this hypothesis, cells die when their telomeres get too short. This hypothesis can be directly tested in yeast by taking advantage of mutations which modulate telomere length. Though the analysis of a population of senescent yeast cells demonstrated that gradual telomere loss does not occur with aging in yeast (D'mello and Jazwinski, 1991; Smeal, et al., 1996), it still did not rule out the possibility that telomere length played any role in the regulation of yeast senescence. We therefore wanted to determine the effects of changing telomere length on the lifespan of the yeast cell. In this chapter we show that telomere length, *per se*, does not regulate yeast longevity: shortening of telomeres from the deletion of a *SIR* gene or from the overexpression of truncated *TLC1*, the gene encoding the telomerase RNA (Singer and Gottschling, 1994), have opposite effects on lifespan. However, the length of a cell's telomeres can determine the yeast lifespan by possibly altering the delicate balance of silencing complexes in the cell. In fact, shorter telomeres can delay senescence via a mechanism which requires *SIR3*. We propose that the overexpression of truncated *TLC1* shortens telomeres and increases the pool of available silencing complexes which enhances silencing of AGE. Our findings suggest that the telomere shortening in mammalian cells may not cause senescence but may actually be a cellular response to an age-dependent loss of genomic silencing: telomeres shorten so that cells can continue proliferating.

RESULTS

Telomere Length Alone Does Not Limit the Yeast Lifespan

Previous work with different alleles of *SIR4* suggested that telomere length itself does not determine the yeast lifespan. Both the deletion of *SIR4* and the *SIR4-42* mutation in *SIR4* shorten yeast telomeres but have opposite effects on the reproductive capacity of the yeast cell (Kennedy, et al., 1995). In the short-lived strain BKy1-14c, a deletion of *SIR4* results in a small decrease in the yeast lifespan while the allele *SIR4-42* increases the lifespan by about 30%. However, given the small effect on telomere length from the *SIR4* mutations, we wanted to see if a more significant change of telomere length would affect lifespan. Therefore, the *rap1s* mutation was introduced into the short-lived strain, BKy1-14c, which increased mean telomere length (Figure 4.1). Lifespan analysis of this mutant showed that the yeast lifespan is not significantly

altered from this increase in telomere length (Table 4.1). This suggested that telomere length, *per se*, does not limit the yeast lifespan.

Deleting *SIR4* Removes the Differences in Lifespan Among Different Strains

In the course of our work, we discovered that deleting *SIR4* in different strains has different effects on lifespan (Table 4.1). As discussed above, the deletion of *SIR4* in a short-lived strain has a minimal effect on the yeast lifespan. However, the deletion of *SIR4* in the long-lived strain, PSY142, decreased mean lifespan from 27 to 18 generations. We therefore hypothesized that one reason why different strains have different lifespans may be because the levels of silencing of AGE varies among different strains. BKy1-14c, is short-lived because silencing at AGE is minimal, while PSY142 is long-lived because silencing at AGE effectively lowers gene expression. Loss of *SIR4* removes this difference in levels of silencing between the strains, PSY142 and BKy1-14c, and the mean lifespans of the strains are now more comparable.

Mutations Which Elongate Telomeres Shorten the Yeast Lifespan

The work of Buck and Shore suggested that the *rap1s* mutation increases both telomere length and telomere position effect by preferentially recruiting silencing factors to telomeres (Buck and Shore, 1995). To determine if increasing telomere length in a strain where AGE is subject to silencing would have an effect on lifespan, the *rap1s* mutation was introduced into the long-lived PSY142 strain. As predicted if long telomeres recruit silencing factors away from AGE, the mean lifespan of the PSY142 *rap1s* strain decreased to 19 generations, a number comparable to that of the strain with a deletion of *SIR4* which has a mean lifespan of 18 generations (Table 4.1). Surprisingly, this had little effect on telomere length (Figure 4.2). It is still not clear why *rap1s* does not produce a large change in telomere length in this strain background.

Given the lack of significant change in telomere length in PSY142, we first chose to repeat the experiments in another long-lived strain background where *rap1s* has been shown to increase telomere length and then to look at a mutation in the non-essential gene, *RIF1* (RAP1 interacting factor), which also increases telomere length (Hardy, et al., 1992). Strains in the W303 genetic background containing either the *rap1s* or the *Δrif1* mutation which are both known to increase

telomere length (Figure 4.3) were obtained and their lifespans were compared to a wildtype control. Both mutants had shortened lifespans and again, the mean lifespans of 24 generations for the *rap1s* strain and 20 generations for the *Δrif1* strain were no shorter than the 20 generation lifespan of a W303*Δsir3* mutant which completely lacks silencing (Table 4.1).

The Shortening of Lifespan in the *Δrif1* Strain is Suppressed by Mutations in *SIR4* Which Localize the Silencing Complex to AGE

In yeast, telomeres contain multiple *RAP1* binding sites which probably mediate telomeric silencing by serving as tethering sites for the Sir complex (Longtine et al., 1989; Wang and Zakian, 1990; Moretti, et al., 1994). In fact, immunolocalization of Rap1p, Sir3p and Sir4p demonstrate that they are concentrated in regions which are associated with telomeric DNA (Klein, et al., 1992; Palladino, et al., 1993). We propose that the *rap1s* and *Δrif1* mutations result in a decrease in lifespan because the longer telomeres shift the balance of silencing factors in favor of the telomeres by increasing the number of *RAP1* binding sites at this location. Loss of silencing factors at AGE would shorten the yeast lifespan. This explanation predicts that mutations in *SIR4* which relocalize the silencing complex to AGE away from telomeres should suppress the decrease in lifespan seen with *rap1s* or *Δrif1*. To test this prediction, we introduced the *SIR4-42* and the dominant negative *SIR4* C-terminus into the *Δrif1* strain which had the longest telomeres and shortest lifespan and repeated the lifespan analysis. Our previous work had suggested that both of these mutations relocalize the silencing complex to AGE increasing the yeast lifespan (Kennedy, et al., 1995). As expected, both mutations were able to partially reverse the shortening of lifespan seen with *Δrif*, increasing the mean lifespan of the strain by 2-3 generations (Figure 4.4). Neither mutation completely suppressed the lengthened telomere phenotype associated with the *Δrif1* mutation though there were different effects seen with the *SIR4-42* and dominant negative anti-*SIR4* alleles (Figure 4.3).

Shortening Telomeres Can Delay Senescence in Yeast Via a Mechanism Which Requires *SIR3*

Our results with mutations that both increase telomere length and decrease lifespan suggest that telomere length can regulate yeast longevity by modulating the balance of silencing factors at AGE. A decrease in lifespan, however, can always be attributed to subtle changes in cell viability hence the need for suppression analysis. It is therefore more convincing if a change in the cell leads to an increase rather than to a decrease in lifespan. According to our model, shortening telomere length should increase genomic silencing at AGE and delay senescence. To test this prediction, a truncated form of *TLC1*, the gene encoding the yeast telomerase RNA, was overexpressed in a wildtype strain and a strain deleted of *SIR3*. Overexpression of truncated *TLC1* has previously been shown to result in decreased levels of TPE (Figure 4.5) and shortening of telomeres (Figure 4.6; Singer and Gottschling, 1994). Lifespan analysis showed that overexpressed *TLC1* increased the mean lifespan of the strain by 4 generations (Figure 4.7). This extension, however, required *SIR3* suggesting that the increase is not simply from a shortening of telomere length or a loss of TPE but rather from some change in the state of silencing in the cell. We propose that the shortening of telomeres liberates silencing factors which increases genomic silencing at AGE.

DISCUSSION

Changes in Telomere Length Can Regulate the Yeast Lifespan by Modulating Silencing at AGE

Individual yeast cells have a limited division potential called their lifespan which varies among different strains and is determined by their genotype (Mortimer and Johnston, 1959). A screen for long-lived stress-resistant mutants yielded four complementation groups which define the *UTH* (youth) genes (Kennedy, et al., 1995). The characterization of three of these genes, *UTH1*, *UTH2* and *UTH4*, has provided evidence for a link between gene silencing and aging in yeast. *UTH1* is a novel yeast aging gene on chromosome XI encoding an "anti-silencer" which limits silencing throughout the cell. Deletion of *UTH1* increases the yeast lifespan by 20% and enhances both TPE and HM silencing (Chapter 3). *UTH2* is allelic to the previously identified gene *SIR4* which encodes an essential component of a protein complex that mediates silencing both at the HM loci which contain the silent information cassettes of the yeast mating type gene, *MAT*,

and at yeast telomeres (Kennedy, et al., 1995). *SIR4-42*, the mutation which enhances longevity, is a gain-of-function mutation in the gene which is believed to enhance silencing at a hypothetical aging gene called AGE. An increase in silencing at AGE then delays senescence. *UTH4* encodes a protein which is thought to recruit the silencing complex to AGE (B. Kennedy and L. Guarente, unpublished data).

Silencing at the HM loci and at telomeres in *Saccharomyces* is mediated by a protein complex composed of Sir2p, Sir3p, and Sir4p (Moretti, et al., 1994; Cockell, et al., 1995). Though the precise function of the Sir proteins is still not known, they have been shown to form a protein complex which interacts with histone H4 and are therefore thought to be involved in heterochromatin reorganization (Hecht, et al., 1995). None of the Sirs is known to bind DNA and it is believed that they are tethered to silenced regions of the genome by DNA binding proteins including Rap1p which binds to sites at both HM loci and at telomeres (Moretti, et al., 1994). Recent work has revealed that a limited pool of silencing complexes exists in the cell which must be partitioned between different regions of the genome (Buck and Shore, 1995; Lustig et al., 1996; Marcand et al., 1996). Mutations have been found in *RAP1* which shift the equilibrium of silencing factors either towards (Buck and Shore, 1995) or away (Marcand, et al., 1996) from telomeres. This results in either decreased or increased levels of silencing in other regions of the genome respectively suggesting that telomeres act as a "sink" for silencing factors.

In this chapter, we develop the link between gene silencing and aging in yeast by describing experiments which relate telomere length, silencing, and lifespan in yeast. Though previous studies had shown that senescent yeast cells do not gradually lose their telomeres as they age (D'mello and Jazwinski, 1991; Smeal, et al., 1996), they did not rule out the possibility that telomere length could play a role in regulating yeast longevity. We now show that telomere length, can influence the lifespan via a mechanism requiring the silencing complex. We suggest that shortening telomeres releases silencing factors increasing genomic silencing at AGE.

In the course of our work, we discovered that deleting *SIR4* has different effects in different yeast strains suggesting that variations in silencing at AGE can exist among strains. In the short-lived strain, BKy1-14c, a deletion of *SIR4* had little effect on lifespan. In contrast, deletions of a *SIR* gene in the long-lived strains, PSY142 and W303, resulted in significant

shortening in lifespan. The apparent differences in silencing at AGE in different strains is reminiscent of differences in the magnitude of TPE seen in different strains (compare strain YDS631, 54% FOA resistant, (Chen, et al., 1993) and strain DG28, 33% FOA resistant, (Gottschling, et al., 1990)). In the strains where loss of silencing lead to large decreases in lifespan, we demonstrate that telomere length can influence the lifespan of the strain via a mechanism involving silencing complexes in the cell. Mutations in *RAP1* and *RIF1* which elongate telomeres lead to a shortening of lifespan. This short lifespan phenotype is partially suppressed in the *Δrif1* strain by mutations in *SIR4* which are thought to enhance localization of the Sirs to AGE suggesting that the silencing complex is playing some role in decreasing longevity. In contrast, the overexpression of truncated *TLC1*, the gene encoding the telomerase RNA, which had previously been shown to result in shortening of telomeres (Singer and Gottschling, 1994) was able to delay senescence in yeast via a mechanism which required *SIR3*. We propose that telomere length may shorten or lengthen the yeast lifespan by trapping or liberating silencing factors which relieve or enhance silencing throughout the genome including AGE.

Telomere Length and Aging in Yeast

What is the relationship between telomeres and aging in yeast? A popular hypothesis to explain cellular senescence is that division capacity is limited by the length of a cell's telomeres -- the telomeres of proliferating cells gradually shorten with continued division and the cells die when their telomeres get too short (Harley, et al., 1990; Hastie, et al., 1990). Though it is clear that telomere shortening occurs in senescing mammalian cells, two studies with populations of senescent yeast cells have demonstrated that no significant telomere shortening occurs with age (D'mello and Jazwinski, 1991; Smeal, et al., 1996). The experiments described above, however, demonstrate that the steady-state length of yeast telomeres can influence the lifespan of the strain. One possibility is that telomere length sets the limits to the yeast lifespan but is not involved in the process of yeast aging. Walmsley and Petes have shown that telomere length varies greatly among different wildtype yeast strains and is under genetic control (Walmsley and Petes, 1985). Assuming that the expression levels of the Sir proteins is constant among strains, these differences in telomere length would result in differences in the pool of available silencing factors within the

cell and therefore in the levels of silencing throughout the yeast genome. This may be one reason why deleting *SIR4* decreases lifespan by different magnitudes in different strains. A consequence of this difference in levels of genomic silencing is that it would take different strains a different number of generations to completely derepress AGE. In sum, the differences in the steady-state length of a cell's telomeres may therefore account for some of the differences in mean lifespan seen among different strains of *Saccharomyces cerevisiae* and provide one explanation for the variation in longevity that is seen within the species.

Telomere Length and Aging in Mammalian Cells

Primary human fibroblasts have a limited division potential, *in vitro*, which is known as the Hayflick Limit (Hayflick and Moorhead, 1961). Cells that have exhausted this finite division potential become senescent and lose the ability to both synthesize DNA and divide despite the maintenance of basic metabolic processes (reviewed by Afshari and Barrett, 1996) . Many experiments suggest that senescence in human cells is composed of two independent mechanisms which block further cell division. The first block can be overcome with the expression of SV40 large T antigen. The second requires an additional immortalization event.

The observation that telomeres shorten in primary human cells and tissues, *in vivo* and *in vitro*, prompted many to speculate that telomeres make up the mitotic clock which limits the replicative capacity of these cells (Vaziri, et al., 1994; reviewed in Greider and Harley, 1996). Further work demonstrating that human somatic cells lack any detectable telomerase activity which is aberrantly reactivated when these cells became immortalized (Kim, et al, 1994) was consistent with the proposal that critical telomere loss signals irreversible cell cycle arrest and activation of the Hayflick Limit. Now known as the telomere hypothesis of cell aging and immortalization (Greider and Harley, 1996), the model suggests that the first block is a result of short telomeres which signal a cell cycle checkpoint and lead to arrest. Transformation events including the expression of SV40 large T antigen would allow cells to bypass this first block to further cell proliferation, often called M1, without activating telomerase. Continued proliferation then promotes further telomere shortening. When telomeres become critically short on an increasing number of chromosomes, the cells enter crisis, also known as the M2 block. Rare clones which activate

telomerase, stabilize their chromosomes, escape M2, and acquire an indefinite growth capacity.

In light of our results with telomere length changes in yeast, we propose that the critical changes in mammalian cellular aging may, in fact, not be the gradual decrease in telomere length, but be a gradual loss of silencing factors and transcriptional silencing paralleling the loss of *SIR*-dependent silencing demonstrated in yeast. Indeed, it has been shown that in cultured human diploid fibroblasts, CpG methylation, which correlates well with gene inactivation, is lost as the cells progress towards senescence (Wilson and Jones, 1983). In addition, though no *SIR* dependent silencing mechanism has been identified in human cells, a human *SIR2* homolog has recently been cloned (Brachmann, et al., 1995). The shortening of telomeres may then reflect a cellular response to this loss of silencing: the cell attempts to compensate for the decrease in genomic silencing by liberating silencing factors which are normally sequestered at its telomeres (Figure 4.8). The M1 block would correspond to a point in the life history of the cell when no further shortening of telomeres can liberate additional silencing factors and the loss of genomic silencing leads to irreversible cell cycle arrest via expression of an antiproliferative gene analogous to AGE. Transformation would be an event which abrogates AGE function. Continued proliferation after transformation would lead to loss of telomeres and crisis. Telomerase would then have to be reactivated for further cell division.

Our modification of the telomere model for aging might explain why murine fibroblasts which have telomere restriction fragment lengths several hundred kilobase pairs longer than their human counterparts (Kipling and Cooke, 1990) have a lower Hayflick Limit and can only complete half as many population doublings (Rohme, 1981). We propose that their longer telomeres act as a trap for silencing factors thus lowering the steady-state levels of genome wide silencing. Because of the smaller pool of silencing factors, it would therefore take a shorter duration and a lower number of population doublings for genomic silencing to be completely lost. Furthermore, murine cells would not be able to take full advantage of the gradual shortening of their telomeres to liberate silencing factors because of the constitutive activation of telomerase in these cells (Proswe and Greider, 1995; Blasco, et al., 1995). In sum, their replicative capacity would be restricted. This interpretation predicts that murine cells lacking telomerase and presumably have shorter telomeres would have an *increased* rather than a decreased replicative

capacity, a prediction that should be testable with the generation of transgenic mice lacking the telomerase RNA.

Finally, we suggest that the mechanism of telomere shortening may have evolved in multi-cellular organisms as their individual cells had to undergo numerous cell divisions and actually had to face the prospects of silencing loss and cellular senescence. Senescent fibroblasts, for example, have been detected *in vivo* using a senescence-specific galactosidase activity (Dimri, et al., 1995). In contrast, senescent yeast cells are extremely rare: in a colony of 2^{15} cells, only one cell, if it had not earlier already entered quiescence due to crowding and nutrient-limitation, would be 15 generations old. Yeast would, therefore, be under no selective pressure to escape senescence.

SUMMARY

In this chapter, we demonstrate that changes in the steady-state length of yeast telomeres can affect the yeast lifespan via a mechanism which involves the silencing complex. We propose that short telomeres can increase the pool of available silencing complexes within the cell enhancing silencing at AGE delaying senescence. Genetically determined differences in telomere length would then be one explanation for the variation in lifespans that is seen within the species, *Saccharomyces cerevisiae*. Furthermore, these results suggest that the telomere shortening in human cells may not cause senescence but may actually be a cellular response to an age-dependent loss of genomic silencing. Cells shorten their telomeres so they can continue dividing.

MATERIALS AND METHODS

Strains, Plasmids, and Media

Yeast strains (see Table 4.2) were grown using standard media and conditions. The construct used to replace the wildtype copy of *RAP1* with the *rap1s* (*rap1-12*) allele was made as follows: a 1400 bp BamHI-XbaI fragment from the *RAP1* gene containing the *rap1-12* double point mutations was isolated from plasmid, pS101, a gift of D. Shore, and ligated into the BamHI-XbaI sites of pRS305 (Sikorski and Hieter, 1989). Digestion of the final plasmid, pNRA305rap1s, with StuI results in a linearized fragment which, when transformed into yeast, can integrate at the *RAP1* locus to produce a *rap1s* allele and a null allele of *RAP1* lacking the 5'

end of the open reading frame. The plasmid used to overexpress truncated *TLC1* (a gift of D. McNabb) was constructed as follows with PCR using the following primers:

5-TLC1: GGG AAG CTT GAG CTC AAT AAA ACT AGA GAG GAA GAT AGG TAC CC

3-TTLC1: GGG AAG CTT TCT AAA TGC ATC GAA GGC ATT AGG AGA AGT AGC TGT G

A truncated clone of TLC1 was obtained from genomic yeast DNA, digested with HindIII and cloned into the HindIII site of plasmid pDB20 (Becker, et al., 1991) and checked for proper orientation with respect to the ADH promoter. The anti-SIR4 plasmid (pJH3A) was originally described by Ivy, et al. (1986). Integration of the *SIR4-42* was done as previously described (Kennedy, et al., 1995). All integration and disruption events were checked by Southern analysis or PCR.

To determine the lifespan 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 overnight. 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 lifespans 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. On very rare occasions, an apparently small, young cell was observed to lyse immediately after micromanipulation and was excluded from the data set. When lifespan analysis was being performed with cells containing a plasmid, daughter cells from the old mother cells were checked for the presence of the plasmid. Mother cells which had lost the plasmid were excluded from the data set.

Determination of Telomere Length and Telomeric Silencing

Total genomic DNA was isolated from an overnight culture of the appropriate yeast strain, digested with XhoI, and separated on either a 0.7% or a 1.5% agarose gel and transferred to a GeneScreen Plus hybridization transfer membrane (New England Nuclear Research Products). Hybridization and wash conditions were as suggested by the manufacturer of the membrane. A plasmid containing 600bp located within the conserved Y' region of yeast telomeres, supplied by V. Zakian, was used to generate a random primed probe which 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.

To test telomeric position effect, a strain with *ADE2* located in the telomeric region of chromosomes V-R was constructed as described (Gottschling, et al., 1990). Strains containing a vector overexpressing truncated *TLC1* or a control were streaked on low adenine (one-third standard supplementation) plates which select for the presence of the plasmid and allowed to grow at 30°C for up to a week for color development.

Statistical Analysis

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

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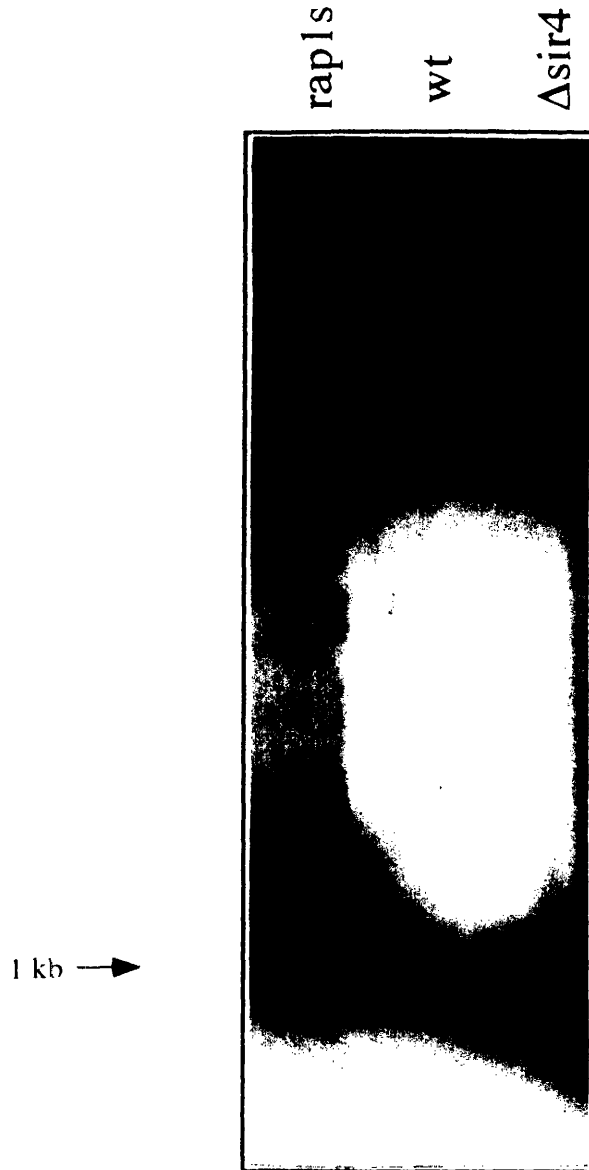
Figure 4.1: The *rap1s* Mutation Lengthens Telomeres in the BKy1-14c Strain XhoI digested DNA is probed with a labeled 600 bp fragment that hybridizes to Y' DNA that is telomere distal to the XhoI site. The broad smear at the bottom of the gel consists of yeast telomeres. Sources of DNA were strains bearing the indicated genotype in the BKy1-14c background.

Strain Background	Genotype	Lifespan		sample size
		mean	maximum	
BKy1-14c	wildtype	16	37	45
	rap1s	17	35	45
	Δsir4	16	31	57
PSY142	wildtype	32	50	20
	rap1s	18	46	19
	Δsir4	18	34	22
W303	wildtype	27	53	71
	rap1s	24	41	30
	Δrif1	20	29	47
	Δsir3	20	32	48

Table 4.1: Effects of Telomere Length Mutations on Lifespan

Figure 4.2: The *rapls* Mutation Has a Minimal Effect on Telomere Length in the PSY142 Background

XhoI digested DNA is probed with a labeled 600 bp fragment that hybridizes to Y' DNA that is telomere distal to the XhoI site. The broad smear at the bottom of the gel consists of yeast telomeres. The genotype *rap1t* indicates that the DNA was obtained from a strain containing a plasmid with the RAP1-17 allele which lengthens telomeres (Kyrion et al., 1993). Sources of DNA were strains bearing the indicated genotype in the PSY142 background.

Figure 4.3: Different Mutations Lengthen Telomeres in the W303 Background
XhoI digested DNA electrophoresed on a 0.7% agarose gel is probed with a
labelled 600 bp fragment that hybridizes to Y' DNA that is telomere distal to the
XhoI site. The broad smear at the bottom of the gel consists of yeast telomeres.

The genotypes Δrif1 anti-SIR4 or SIR4-42 indicate that DNA was obtained from

a Δrif1 strain containing either allele in addition to a wildtype SIR4 allele.
All strains were in the W303 background.
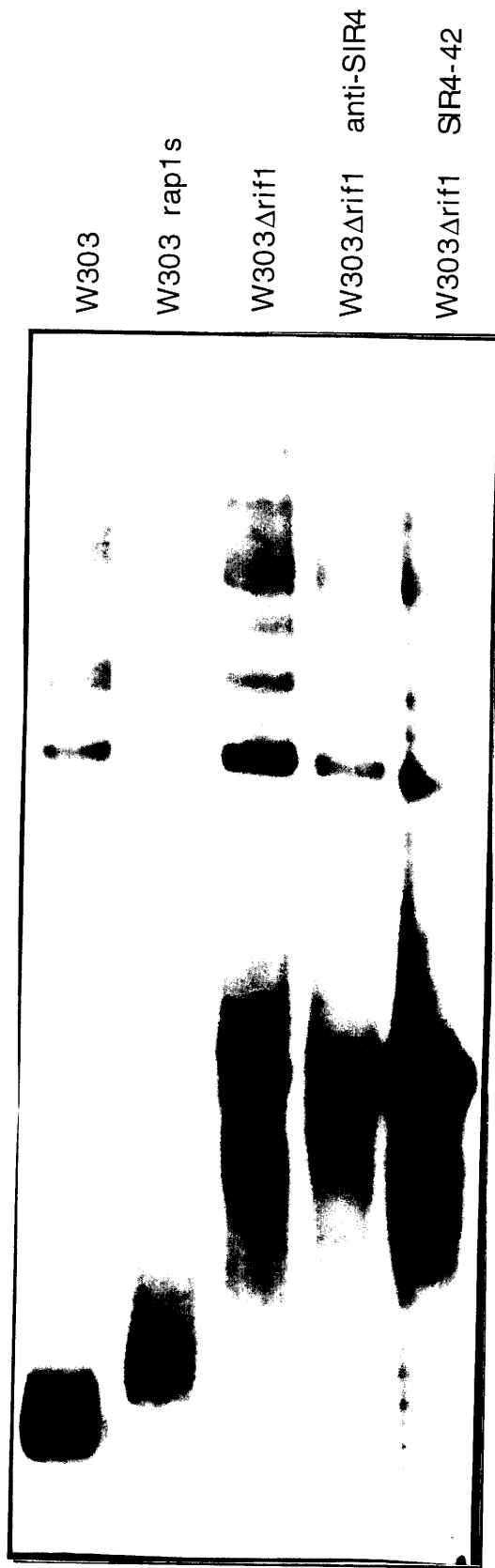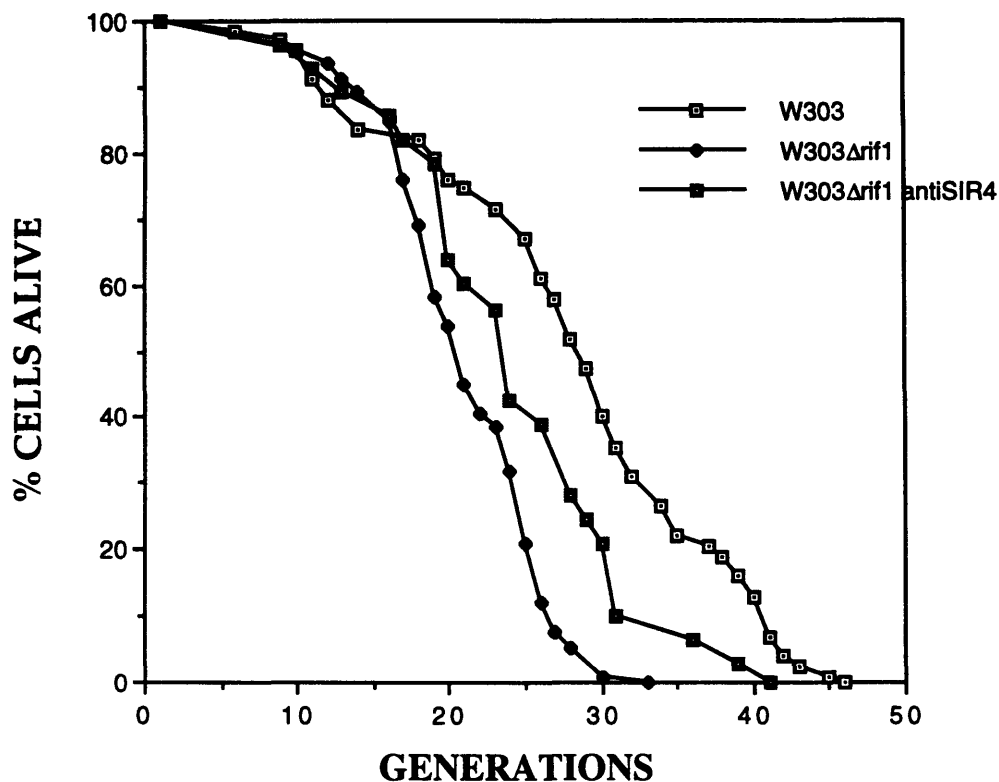
Figure 4.4A: Mortality Curves for Δrif1 and Suppression with Anti-SIR4
Mortality curves from two independent experiments are shown for the wildtype
and Δrif1 strains in the W303 background. Also shown is the mortality curve
for the Δrif1 strain containing the anti-SIR4 plasmid which overexpresses the
C-terminus of SIR4. Controls contained an empty vector. The mean lifespans
were 27.1, 20.2, and 23.4 generations for the W303, Δrif1, and Δrif1 anti-SIR4
strains respectively and were shown to be statistically significant by the Wilcoxen
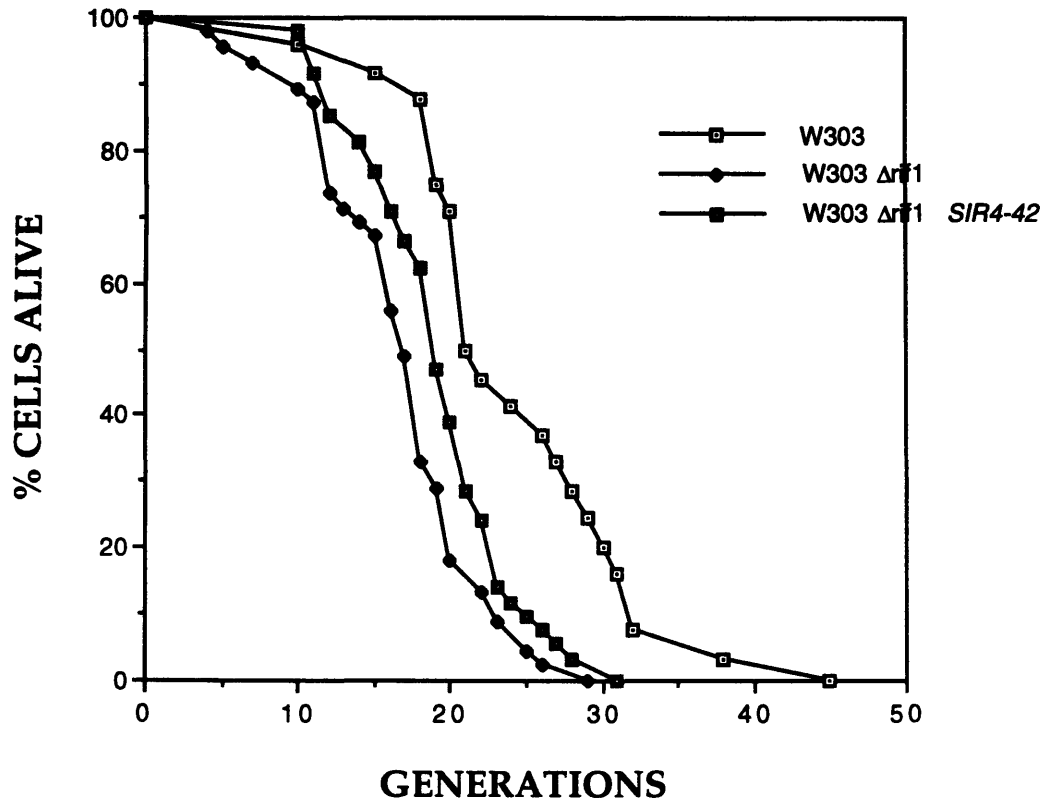Ranked Signed Test. Sample sizes were 67, 46, and 28 cells respectively.

Figure 4.4B: Mortality Curves for Δrif1 and Suppression with SIR4-42
Mortality curves derived from two independent experiments are shown for the
wildtype and Δrif1 strains in the W303 background. Also shown is the mortality
curve for the Δrif1 strain containing both the wildtype SIR4 the SIR4-42 alleles.
The mean lifespans were 24.5, 16.8, and 19.3 generations for the W303, Δrif1, and
Δrif1 SIR4-42 strains respectively and were shown to be statistically different by
the Wilcoxen Signed Rank Test . Sample sizes were 24, 45, and 48 cells
respectively.
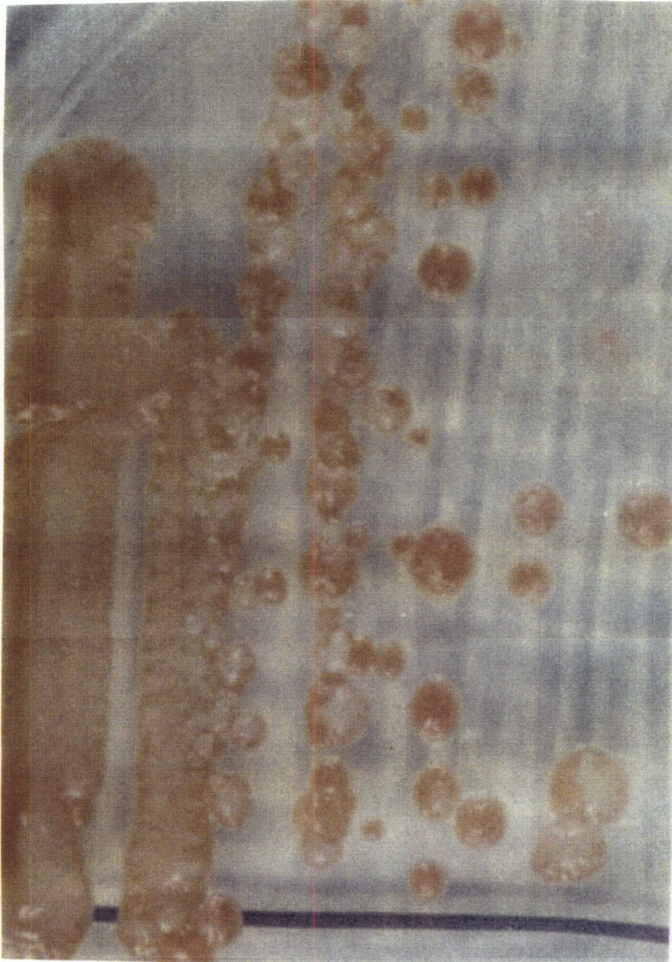
**ADE2-TEL vector**   **ADE2-TEL ADH-tTLC1**

Figure 4.5: Overexpression of Truncated *TLC1* Results in Loss of Telomeric Silencing
The vector overexpressing truncated *TLC1* was transformed into strain PSY316AT which
has *ADE2* at the telomeric region of chromosome V-R. The extent of silencing is
depicted by the red color of the colonies: red indicates silencing, white indicates gene
expression.

Figure 4.6: Overexpression of Truncated *TLC1* Shortens Telomeres
XhoI digested DNA electrophoresed on a 1.5% agarose gel is probed with a labelled
600 bp fragment that hybridizes to Y' DNA that is telomere distal to the XhoI site.
The broad smear at the bottom of the gel consists of yeast telomeres. The width
of the telomere band is indicative of the range of telomere lengths in each strain.
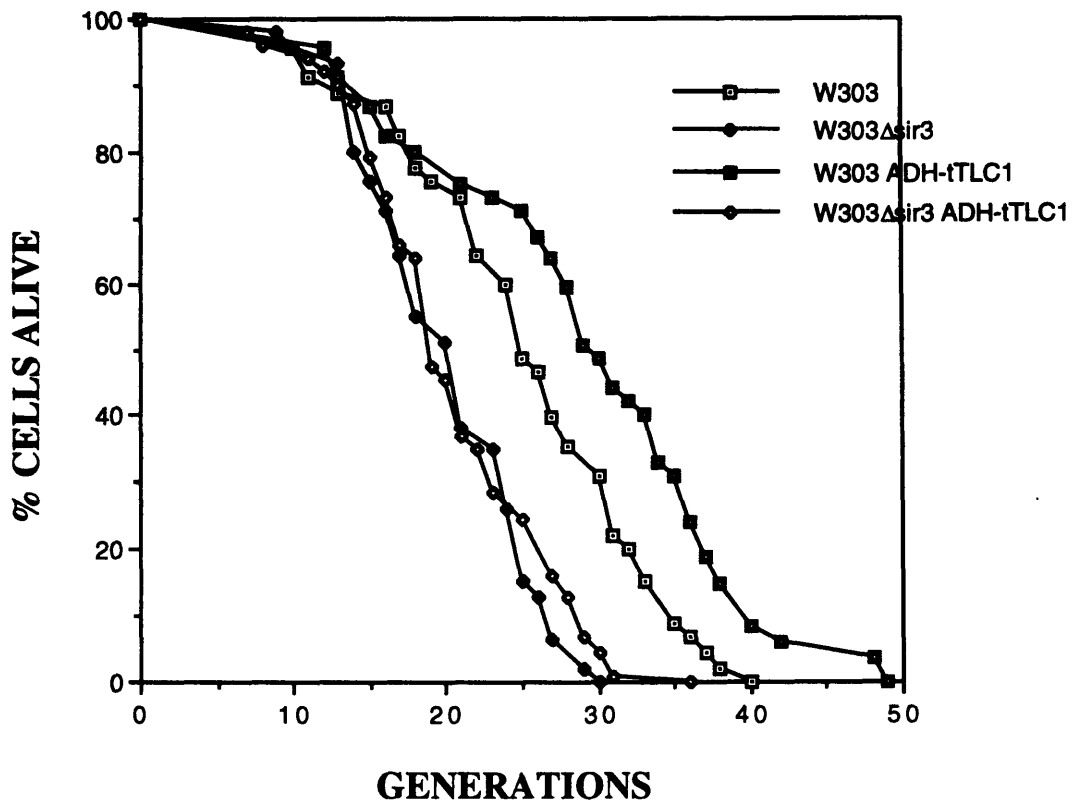All strains were in the W303 background.
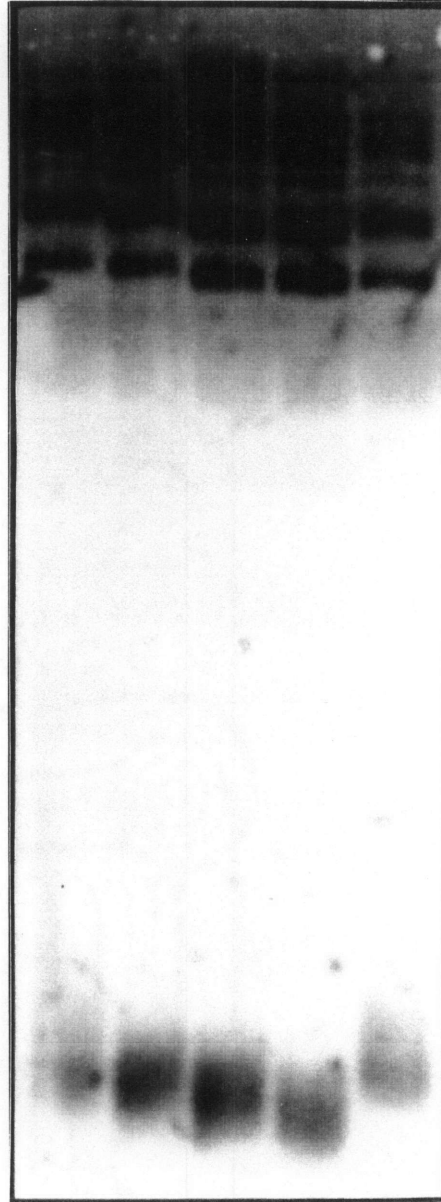
Figure 4.7: Mortality Curves for Strains Overexpressing Truncated *TLC1*
Mortality curves obtained from two independent experiments are shown for the
W303 and *Δsir3* strains overexpressing a truncated *TLC1*. Controls contain an
empty vector. The mean lifespans were 25.4, 20.3, 29.2, and 20.6 generations for
W303, W303*Δsir3*, W303 ADH-tTLC1, and W303*Δsir3* ADH-tTLC1, respectively.
The difference between the W303 strains with and without the ADH-tTLC1 plasmid
were statistically significant as determined by the Wilcoxen Signed Rank Test.
Sample sizes were 45 cells, 45 cells, 45 cells, and 48 cells repectively.

Figure 4.8: A Model for Sustaining Proliferation with Telomere Shortening
(A) In young cells, genomic silencing is intact and telomeres remain long. (B) As cells senesce, silencing factors are slowly lost. (C). To prevent expression of an anti-proliferative gene, here called AGE, the cell shortens its telomeres releasing a pool of silencing factors which repress AGE. (D) Telomeres shorten below a critical length, no silencing factors remain in the cell, and AGE is expressed. Cells arrest at M1. (E) A transformation event results in loss of AGE function and continued proliferation. Telomere shortening proceeds. Critical telomere length is reached and essential genes are lost. Cell arrests at M2. (F) Telomerase is activated stabilizing telomere length. Cells continue dividing indefinitely.
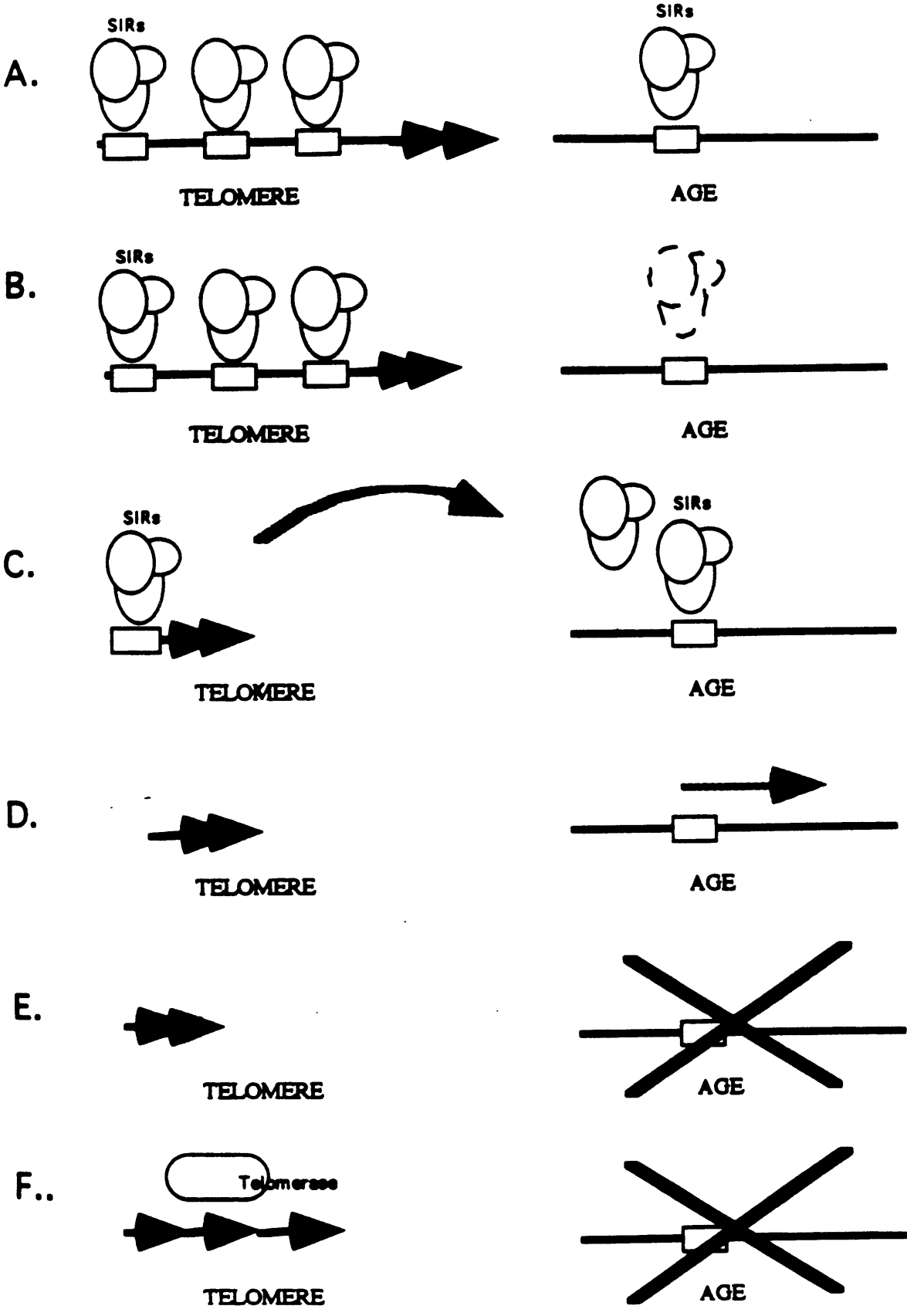
A.

SIRs

TELOMERE

SIRs

AGE

B.

SIRs

TELOMERE

AGE

C.

SIRs

TELOMERE

SIRs

AGE

D.

TELOMERE

AGE

E.

TELOMERE

AGE

F..

Telomerase

TELOMERE

AGE

Table 4.2: Yeast Strains Used in This Study

Strain	Genotype	Reference
BKy1-14c	MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52	Kennedy, et al., 1995
LS59	MATα ade2-101 his3-Δ200 trp1-1 leu2-3,112 can1-100 hmrΔA::TRP1	Gift of D. Shore
LS486	MATa ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100 hmrΔE::TRP1 rap1-12::LEU2	Gift of D. Shore
LS531	MAT α ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100 HMR::TRP1 rif1::URA3	Gift of D. Shore
PSY142	MATα leu2-3,112 lys2-801 ura3-52	Kennedy, et al., 1994
PSY316	MATα ade2-101 his3-Δ200 leu2,112 lys2 ura3-53	Pina et al., 1993
PSY316AT	PSY316 ADE2-TEL V-R	Gift of B. Kennedy
RS862	W303a sir3::TRP1	Rolf Sternglanz
W303a	MATa ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100	Gift of S. Bell
W303b	MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100	
NRAy109	BKy1-14c rap1-12::LEU2	
NRAy301	PSY142 rap1-12::LEU2	
NRAy405	LS531 LEU2::SIR4-42	

CHAPTER FIVE

The Future of the End: Yeast Aging in the Next Millenium

INTRODUCTION

Yeast aging research is still young. This chapter discusses the three areas in the field which I believe are promising avenues for discovery. First, the links between silencing and aging have to be strengthened. Second, the prospects of obtaining immortal yeast mutants should be considered. Finally, the possibility that the mechanisms behind yeast and human cellular senescence are conserved have to be explored.

SILENCING AND AGING IN YEAST

Overview

There were two broad questions in the yeast aging field when we began our work five years ago. First, why did yeast cells age and what was the nature of the genes which regulate this aging process? Second, why do different yeast strains have different lifespans? To address these questions, we performed a mutant hunt to look for long-lived yeast mutants based on a fortuitous observation that stress-resistance and lifespan were correlated in several yeast strains. Our mutant hunt, described in this thesis, gave us several *UTH* genes which provided the first link between silencing and aging in yeast. The characterization and cloning of these genes has provided some answers to the two questions we had asked at the outset of our work. First, yeast age because, at some level, silencing is lost with age, resulting in inappropriate gene expression which kills the cell. It is still not clear how this actually occurs. Second, different yeast strains have different lifespans, in part, because the levels of silencing in a cell are regulated by a number of factors including telomere length which can vary among strains of different genotype. These two ideas form the basis for a model linking silencing and aging.

The AGE Model

To explain all the results obtained from the characterization of the *UTH* genes, we propose that a hypothetical aging gene, called AGE, is normally silenced in yeast (Figure 5.1). According to the model, this AGE locus would gradually be expressed as silencing is lost during yeast aging resulting in cell death. One prediction of this model was confirmed when sensitive RT-PCR analysis demonstrated the loss of transcriptional silencing at HM loci in old cells (Smeal, et al.,

1996). It is not clear how silencing is lost. Two possibilities include either loss of the silencing complex itself or relocalization of the Sirs away from the HM loci. Recently, Kim, et al. reported that TPE at telomere VII-L is not lost in old cells (Kim, et al., 1996) suggesting that the silencing complex remains intact during aging. Relocalization could result if the expression level or activity of a localization factor analogous to Sir1p or Uth4p changes with age. Western analysis of different proteins obtained from populations of old cells should address this issue.

What is the nature AGE locus? The AGE locus is a hypothetical locus which is distinct from the other two regions which are known to be silenced in yeast. It is distinct from the HM loci because *sir1* mutations which decrease silencing at HM have no effect on lifespan in either a short-lived or a long-lived strain (see the Appendix). Furthermore, AGE is distinct from telomeres because both the *SIR4-42* mutation and the overexpression of truncated *TLC* which abolish TPE enhance longevity via a mechanism requiring *SIR3*. Though, no other regions in the yeast genome are known to be silenced, candidate regions could include loci which contain *RAP1* binding sites. Multiple *RAP1* binding sites exist at the ribosomal DNA cluster, for example (Buchman et al., 1988). Also, *RAP1* binding sites exist upstream of many genes which encode components of the machinery for protein synthesis and glycolytic enzymes (reviewed in Shore, 1994). In this context, Rap1p acts as a transcriptional activator. Is it possible that the function of Rap1p in these promoters would switch from an activator to a silencer when the level of free Sir complexes rises above a certain threshold? Several efforts are underway in the lab to identify AGE.

Our work in the the short-lived strain, BKy1-14c, supports the notion that it is the gradual accumulation rather than the expression of the AGE product which kills cells. In this strain background, the wildtype and Δ*sir* strains have similar lifespans. This suggests that AGE is not silenced in any appreciable fashion in this strain. Why then is this strain still alive? There are two possible explanations. First, as suggested above, it is the accumulation of the AGE product which kills. In the absence of silencing, AGE is constitutively expressed but levels of protein still need to build up. Second, the possibility exists that a *SIR*-independent mechanism operates which gradually increases the expression of AGE with age. Cells would die when the level of gene expression rises above a certain threshold. Silencing would just be a second level of regulation for

the AGE gene. Work which suggests that a cytoplasmic senescence factor accumulates with age in yeast that can be inherited by daughter cell (Egilmez and Jazwinski, 1989; Kennedy, et al., 1994) better supports the first scenario. In addition, it might explain why senescence is dominant in those rare zygotes generated between an old and young haploid cell (Muller, 1985). The young cell could replenish "young" silencing factors but would not be able to decrease levels of AGE protein.

The AGE model predicts that lifespan can be extended via two mechanisms. The first mechanism is by enhancing levels of silencing at AGE so that its age-dependent expression is delayed. We have shown with *UTH1* that this can be accomplished by increasing the overall levels of silencing within the cell. AGE expression could also be repressed by shifting the balance of silencing factors within the cell so that AGE is preferentially silenced to the detriment of other silenced regions of the genome. *UTH2-42* accomplishes this, we believe, by increasing the affinity of the Sir complex at AGE. In addition, shortening of yeast telomeres can shift the balance of silencing to AGE by liberating silencing factors which are normally sequestered at telomeres. Several other mutations are expected to have similar effects on lifespan by changing the equilibrium of silencing factors within the cell. Both the *RAP1* mutation which increases genomic silencing (Marcand, et al., 1996) and the overexpression of *SIR4* which is reported to increase internal genomic silencing (S. Gasser, personal communication) should delay senescence . The second mechanism is by deleting AGE itself. Testing this prediction awaits the cloning and characterization of the locus.

Finally, silencing cannot be the only cause for aging in yeast because a Δsir mutant still ages and ages with Gompertz kinetics. Other genes including *RAS2* (Sun, et al., 1994) (Pichova, et al., 1995) and *LAG1* (D'mello, et al., 1994) have been identified which are reported to affect yeast longevity and a priority in the field should be to tighten the genetic and ideally the mechanistic relationships between these determinants of yeast longevity. In *Caenorhabditis elegans*, for example, most of the genes which affect lifespan isolated in different ways by different labs now seem to belong to the dauer pathway (Kenyon, et al., 1993; Larsen, et al., 1995; Dorman, et al., 1995). It will therefore be interesting to see if links between the *UTH* genes and *LAG1* or *RAS2* exist. Do these other genes affect silencing?

126

Lifespan Variations Within the Species, *Saccharomyces*

Two scenarios can be proposed to explain the differences in lifespan which exist among wildtype yeast strains. First, different lifespans may reflect the different levels of silencing which exist in different strains. Silencing can be influenced by a multitude of factors including the levels of the Sir proteins, the activities of many genes which regulate chromatin function, as well as factors which influence telomere length. There are at least 40 genes known which affect one of these processes. Many are not essential and there would not be any selection for the wildtype alleles of these genes in the laboratory environment. The allelic variation which exists with *UTH4*, for example, accounts for some of the differences in lifespan among strains studied in the lab (B. Kennedy and L. Guarente, unpublished data).

A second scenario to explain strain differences is that mutations exist in genes which, though not directly involved in silencing, affect the lifespan of the strain. Given the number and non-essential nature of the *SUN* genes, for example, it is not difficult to imagine that different combinations of alleles could exist in different strains accounting for the variation in lifespan. As shown in chapter three, the $\Delta sim1$ and $\Delta uth1$ combination actually results in wildtype lifespan.

The contrasting effects of *RAS2* deletions on lifespan (Sun, et al., 1994; Pichova, et al., 1995) mentioned in chapter one may be one manifestation of polymorphism. On the other hand, there is no evidence that different strains age in the same way. All yeast aging may result from inappropriate gene expression but different genes may become deregulated in different strains. Only a study of the effects of the same combinations of mutations in different strains will resolve this issue.

YEAST IMMORTALITY

One can argue that we will never really understand the yeast aging process until we can completely manipulate it at will. What are the prospects for an immortal yeast cell? Three possibilities should be explored. The first two depend on the number and nature of the pathways which regulate lifespan and the third is simply a matter of symmetry.

Simplistically, one may postulate that there is a single pathway regulating aging as there is

only one biosynthetic pathway in the cell for methionine production. The mutations in the known longevity determining genes lie upstream of a common pathway. A mutation downstream of the junction point like the elimination of the putative senescence factor would remove aging and the cell would be immortal. (Figure 5.2a) On the other extreme, one can envision a situation where there are several parallel and independent blocks to immortality. To achieve perpetual youth, the cell must acquire the correct combination of mutations which maximizes longevity and minimizes aging. This might involve deletions of all aging genes with concurrent high overexpression of several longevity gene products. (Figure 5.2b) This second scenario predicts that no single gene mutation can ever result in immortality.

The third route to the fountain of youth for yeast may be easier to achieve. The key to immortality may already exist given that the yeast colony is already immortal. This immortality results from the asymmetric resetting of the clock which determines the reproductive potential of the cell. Only one other instance exists where the mother/daughter asymmetry is clear and that is in the asymmetric regulation of *HO* expression; mothers switch, daughters do not (Nasymth, 1983). Even so, in some instances, daughters have been found to switch at a low frequency (Strathern and Herskowitz, 1979). Is this break in asymmetry analogous to the symmetric buds seen in old cells where the daughter fails to be rejuvenated (Kennedy, et al., 1994)? If asymmetry is the key to immortality for the colony, then a mutant which partitions the clock resetting mechanism to increase the chances that the mother's clock is reset at some frequency, making her "young", would be immortal. (Figure 5.2c) Some preliminary data with a *she1* mutant which breaks the asymmetry of *HO* switching (Jansen, et al., 1996) suggests that lifespan may be extended in this mutant (data not shown). One must keep in mind, however, that a symmetry mutant could also result in clonal senescence and a mortal colony. Nevertheless, the question of immortality is reduced then to a question of symmetry.

CELLULAR SENESCENCE: YEAST AND HUMAN LINKS?

As mentioned earlier, the preeminent goal in aging research is to reveal insights into human aging. Senescence in yeast and human cells share some characteristics. Senescence in both systems is dominant, both cell types take longer to traverse the G1 portion of their cell cycles as

they senesce and both arrest primarily in G1. In the human fibroblast system, senescence is mediated by numerous components including p53, p21, and pRB which function in cell cycle control (reviewed by Afshari and Barrett, 1996). Overexpression of the gene encoding p21 which acts as an inhibitor of the cyclin dependent kinase complex, for example, inhibits cell proliferation and senescent cells are now known to have extremely high levels of this protein (Noda, et al., 1994). Though none of these human proteins have homologs in *Saccharomyces*, the mechanism behind cell cycle and proliferation control are remarkably conserved. Inhibitors of cyclin dependent kinases (CDKs) exist in yeast and it is still possible that downstream events in yeast senescence eventually lead to cell cycle arrest via activation of a CDK. Furthermore, a human homolog exists for *LAG1* (GenBank Accession # S30034) suggesting that some aspects of senescence control may be conserved. Human *LAG1* function has not yet been tested in yeast. Finally, the recently identified gene mutated in the progeroid syndrome, Werner's Syndrome, is homologous to the yeast helicase, Sgs1p (Yu, et al., 1996). It is still not clear if this sequence homology can be extended to functional conservation.

In conclusion, studies in the yeast system with its powerful genetics and completely sequenced genome may enable us to answer long-standing questions on how proliferation, senescence, and cancer, are linked. The coming years promise to be an exciting time for yeast aging research and I look forward to being a part of it.

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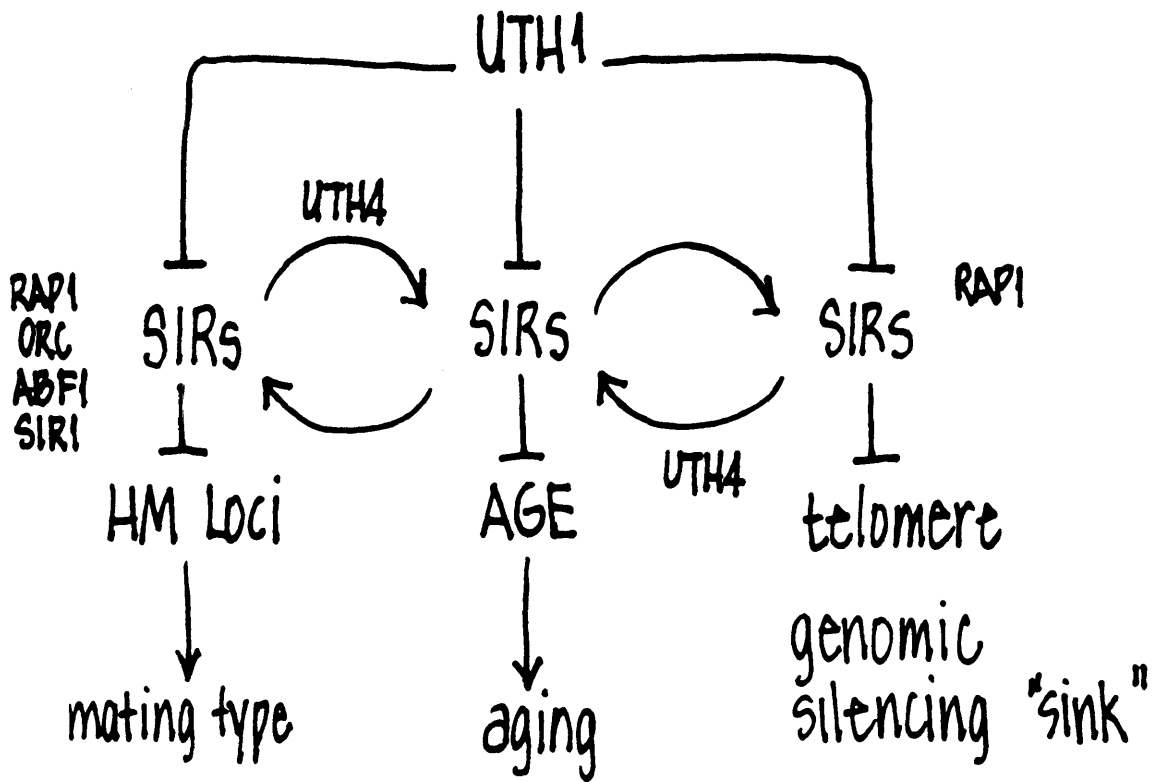
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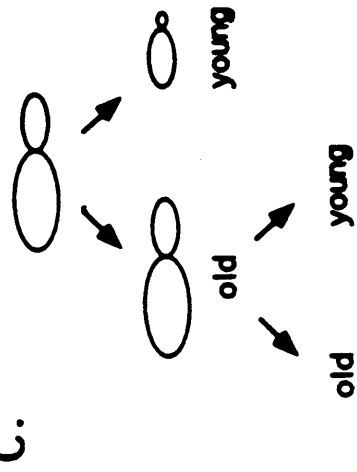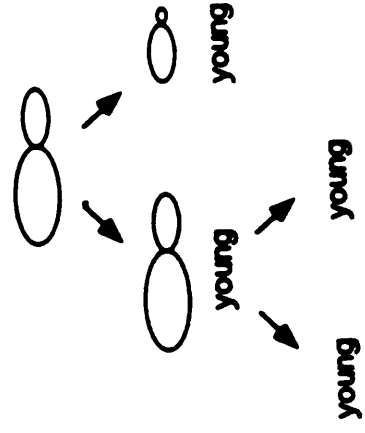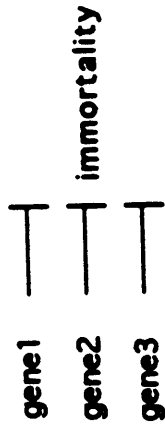
UTH1

UTH4

RAPI
ORC
ABFI
SIRI

SIRS   SIRS   SIRS   RAPI

UTH4

HM Loci   AGE   telomere

mating type   aging   genomic
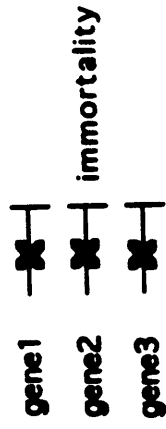   silencing "sink"
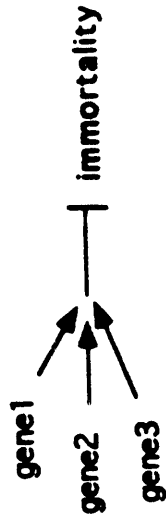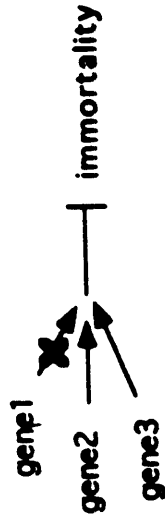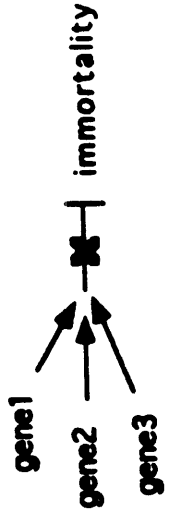
The AGE Model
FIGURE 5.1

FIGURE 5.2: Three scenarios for Yeast Immortality.
(A.) If one genetic pathway regulates longevity, then a single gene mutation can result in immortality. (B.) Several independent genetic mechanisms preventing immortality would mean that multiple genetic events would be required for perpetual youth. (C.) A mutation which breaks the asymmetry of the clock resetting mechanism would make the mother cell immortal without affecting the basic yeast aging mechanism.

A. WILDTYPE | LONGLIVED MUTANT | IMMORTAL MUTANT

gene1, gene2, gene3 → immortality

B.

gene1, gene2, gene3 — immortality

C.

young, old
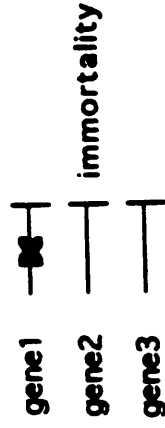
APPENDIX

SIR1 and *CLN3* Function in Yeast Aging

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My contribution to the work described in this appendix includes the lifespan analysis. Strain construction and verification were done by Andrew Holz.

SIR1 IS NOT INVOLVED IN LIFESPAN REGULATION IN YEAST

As discussed in chapter four, yeast cells have a delicate equilibrium of silencing factors which are distributed among the different regions of the yeast genome which are silenced. Of the three regions believed to be silenced, the HM loci, telomeres, and AGE, the HM loci appear to be the most stably repressed and cells carrying *ADE2* at an HM silencer give rise to colonies which are homogeneously red or pink (Sussel, et al., 1993). Telomeric silencing is inherently unstable and cells containing *ADE2* marked telomeres produce sectored colonies (Gottschling, et al., 1990). It is still not known if silencing at AGE is stable. It is believed that the HM silencer is stable because accessory factors, including Sir1p, increase the affinity of the locus for the Sir proteins. A null mutation in *SIR1* results in destabilization of silencing at the HM silencer: in *sir1* cells, the *HML* silent locus switches back and forth between repressed and derepressed states (Pillus and Rine, 1989). Pillus and Rine explained these results by proposing that *SIR1* is important in the establishment but not maintenance of silencing. Mutations in *SIR1* have no effect on telomeric silencing, however, suggesting that Sir1p does not function at that location (Aparicio, et al., 1991). Recent work, however, elegantly demonstrated that targeting Sir1p to a telomere enhances telomere position effect (Chen, et al., 1993). All of these results support a model where silencing at telomeres is inherently unstable because they lack a Sir1p-like protein to effectively compete for limiting silencing complexes.

Does *SIR1* play any role in regulating the yeast lifespan? Previous work had demonstrated that the *SIR4-42* mutant which extended the yeast lifespan in the BKy1-14c strain background did not require *SIR1* (Kennedy, et al., 1995). However, it was not clear if *SIR1* would have any effect on lifespan on its own. In addition, a deletion of *SIR3* or *SIR4* did not have any significant effects on lifespan in the short-lived strain, BKy1-14c, but had large effects in the PSY142 and W303 strain backgrounds. To clarify the role of *SIR1* in regulating the yeast lifespan, the lifespans of a wildtype and Δ*sir1* strain in the W303 strain background were compared. No significant difference in the mean lifespan was detected between these strains (Figure A.1) confirming our earlier work which had failed to detect a role for *SIR1* in lifespan control. This finding suggests that Sir1p does not function at AGE.

CLN3 IS NOT LIMITING IN THE CELL-CYCLE ARREST SEEN DURING YEAST SENESCENCE

The cell cycle of *Saccharomyces cerevisiae* is relatively well understood primarily because of the powerful genetics of the yeast system. It is made up of two phases, S (DNA synthesis) and M (mitosis), which are separated by two gaps, G1 and G2. The critical control point in the budding yeast cell cycle is called START, the point in late G1 after which a cell is committed to the completion of the next cell cycle. It is becoming clear that passage through START is dictated by the levels of the *CLN1*, *CLN2* and *CLN3* gene products, the predominant G1 cyclins in budding yeast (Richardson, et al., 1989). The dominant mutation *CLN3-1* which stabilizes the Cln3p protein shortens the G1 phase as cells progress through START at an accelerated pace (Cross, 1988). Because of this abbreviated G1 phase, cells do not have sufficient time to grow before each mitosis and are small (Cross, 1988; Nash, et al., 1988). In contrast, cells deleted of *CLN3* are large because they experience a significant delay in G1 (Cross, 1988; Nash, et al., 1988). In sum, Cln3p levels contribute to the cell's ability to progress through START.

The majority of senescent cells arrest as large unbudded cells (Table A.1) which is reminiscent of the cell cycle arrest seen with mutants which are unable to progress through START (Hartwell, et al., 1974; Reed, 1980; Richardson, et al., 1988). To determine if this senescence associated cell cycle arrest is mediated by a limiting amount of Cln3p, the lifespan of a wildtype strain was compared to strains carrying the dominant and null alleles of *CLN3* described above. If *CLN3* was limiting in aging cells, then the *CLN3-1* allele might be expected to ignore the signals to senesce, prevent arrest and possibly increase lifespan. For example, *CLN3-1* cells are unable to arrest in response to mating pheromone or nutrient starvation and continue proliferating (Cross, 1988). Lifespan analysis, however, showed that the dominant mutation did not extend the yeast lifespan (Figure A.2). In fact, there was a small but significant decrease in both the mean and maximum lifespan of the mutant strain. A similar decrease in lifespan was seen with the strain carrying a null allele of *CLN3*. These results, like all other decreases in lifespan, are difficult to interpret because they may result from a lowering of the viability of the strain. However, they suggest that the senescence associated cell cycle arrest differs from the arrest seen with mating pheromone exposure and nutrient limitation, both of which are delayed by *CLN3-1*. Also, this

experiment provides clear evidence that cell volume is not the key determinant of lifespan. As previously reported, these three strains vary in size: *CLN3-1* cells are smaller and Δ*cln3* cells are larger than wildtype (Cross, 1988; Nash, et al., 1988). Both of these mutant strains, however, had shorter lifespans than the wildtype control suggesting that size is not the primary determinant of the yeast lifespan as had been previously suggested (Mortimer and Johnston, 1959).

MATERIALS AND METHODS

Yeast strains (see Table A.2) were grown using standard conditions. *CLN3-1* and Δ*cln3* strains were constructed using the plasmids, YRpDAF1-1 and cln3::URA3, generously provided by F. Cross, as described (Cross, 1988) and the null mutation was verified by southern analysis. Lifespan analysis was performed as previously described (Kennedy, et al., 1994)

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Strain Background	% Unbudded senescent cells	% Budded senescent cells	Sample Size
BK1-14c	71%	29%	831 cells
W303	62%	38%	541 cells

Table A.1: Cell Cycle Characterization of Senescent Yeast Cells
Terminal phenotype of the senescent cell is noted above and derived from a compilation
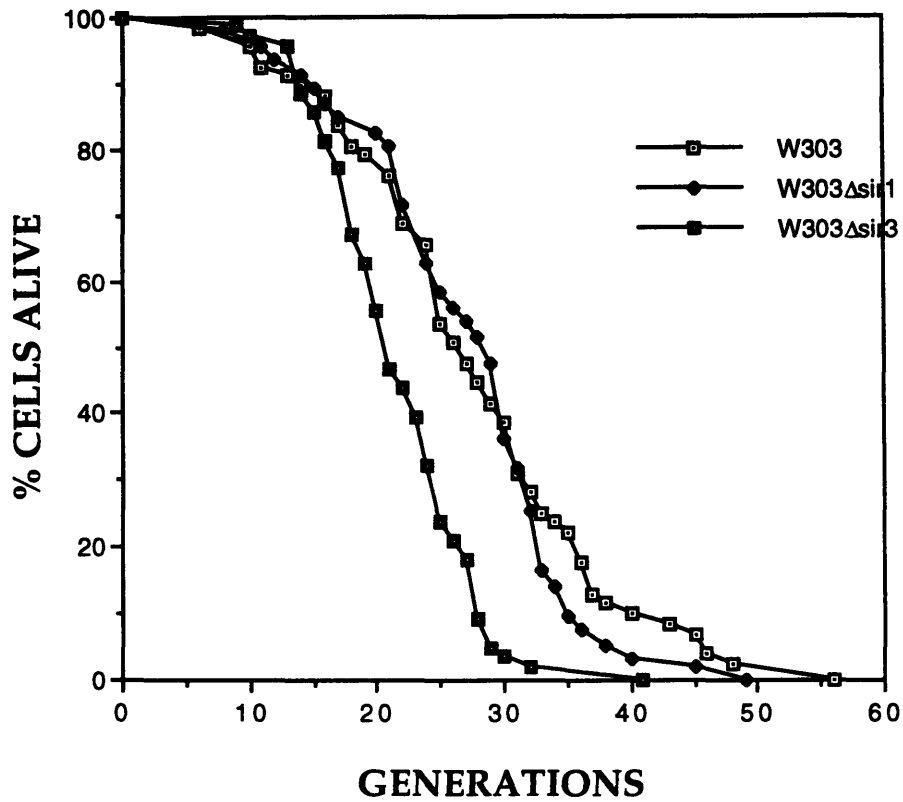of several data sets.

Figure A.1: Mortality Curves for the W303Δ*sir1* Strain

Mortality curves from two independent experiments are shown for the wildtype, Δ*sir1*, and Δ*sir3* strains in the W303 strain background. Mean lifespans were 27.5, 27.4, and 21.5 generations with sample sizes of 66, 46, and 69 cells respectively.
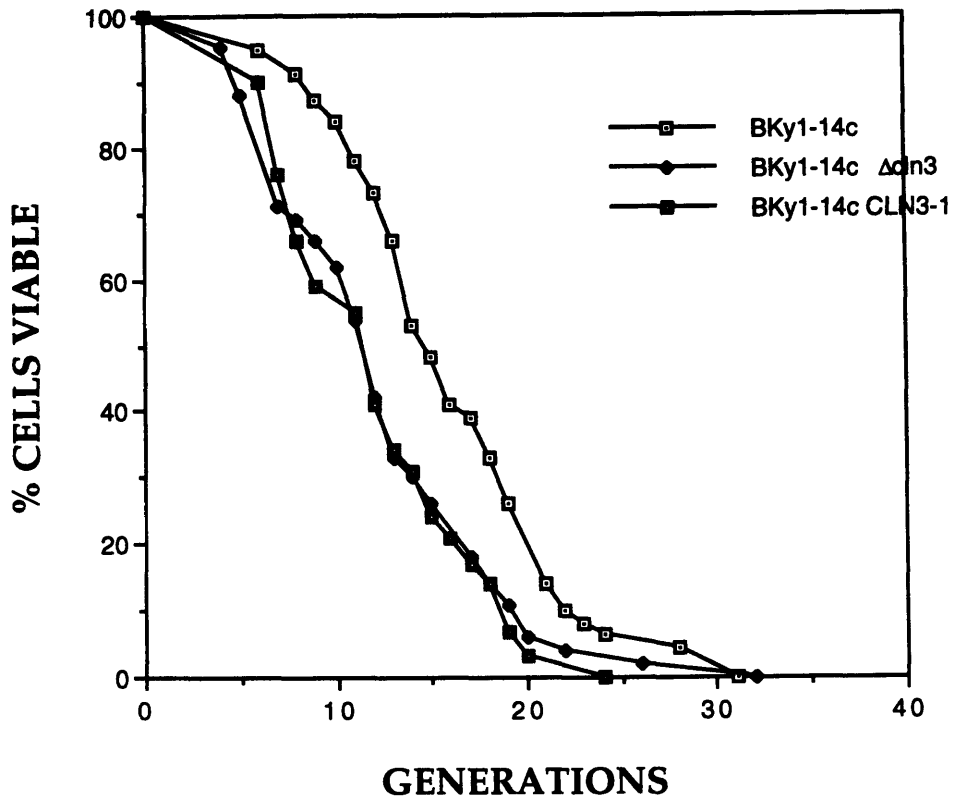
Figure A.2: Mortality Curves for Strains with Different *CLN3* Alleles
Mortality curves from two independent experiments are shown for isogenic strains in the BKy1-14c with different *CLN3* alleles. Mean lifespans were 15.6, 12.5, and 12.1 generations for the wildtype, Δ*cln3*, and *CLN3-1* strains. Differences between the mutant strains and the wildtype control were statistically significant by the Wilcoxen Signed Rank Test. Sample sizes were 56, 42, and 29 cells respectively.

Table A.2: Yeast Strains Used in This Study

Strain	Genotype	Reference
BKy1-14c	MATα *ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52*	Kennedy, et al., 1995
W303a	MATa *ade2-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	
yAL011	W303a *sir1::hisG*	Gift of A. Lau
ALHy1	BKy1-14c *cln3::URA3*	
ALHy3	BKy1-14c *TRP1::CLN3-1*	

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