Cell, Vol. 89, 381–391, May 2, 1997, Copyright ©1997 by Cell Press

Redistribution of Silencing Proteins from Telomeres to the Nucleolus Is Associated with Extension of Life Span in S. cerevisiae

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Summary

A prior genetic study indicated that activity of Sir silencing proteins at a hypothetical *AGE* locus is essential for long life span. In this model, the *SIR4-42* mutation would direct the Sir protein complex to the *AGE* locus, giving rise to a long life span. We show by indirect immunofluorescence that Sir3p and Sir4p are redirected to the nucleolus in the *SIR4-42* mutant. Furthermore, this relocalization is dependent on both *UTH4* a novel yeast gene that extends life span, and its homologue *YGL023*. Strikingly, the Sir complex is relocalized from telomeres to the nucleolus in old wild-type cells. We propose that the rDNA is the *AGE* locus and that nucleolar function is compromised in old yeast cells in a way that may be mitigated by targeting of Sir proteins to the nucleolus.

Introduction

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

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

The potential to isolate mutants with altered life spans makes yeast an attractive organism for aging research. Until recently, however, the yeast aging phenotype could only be followed in single cells, making a direct screen for life span mutants daunting. The problem was circumvented by the observation that life span correlates with stress resistance in some strain backgrounds (Kennedy et al., 1995). Stress-resistant mutant strains were isolated and then tested for an increased life span potential. Several aging mutants were recovered that defined four genes (*UTH1–4*).

The mutation in the *UTH2* gene caused an additional, unique phenotype: a severe reduction in mating potential (Kennedy et al., 1995). This gene was cloned and demonstrated to be identical to *SIR4*, a gene (along with *SIR2* and *SIR3*) previously shown to be required for transcriptional silencing of genes at *HM* loci and telomeres (Klar et al., 1981; Ivy et al., 1986; Rine and Herskowitz, 1987; Gottschling et al., 1990; Aparicio et al., 1991).

In addition, the *SIR2* gene product acts to limit mitotic recombination (Gottlieb and Esposito, 1989) and to repress transcription of a marker gene (Bryk et al., 1997; Smith and Boeke, 1997) at rDNA repeats. The rDNA in yeast consists of a tandem array of about 140 copies of a 9 kb repeat encoding 35S RNA and 5S RNA residing on chromosome 12. This rDNA array is assembled into a crescent-shaped, subnuclear structure termed the nucleolus, in which assembly of ribosomes occurs.

The allele of *UTH2*/*SIR4* isolated (*SIR4-42*) was unusual in that it behaved as a null allele for some phenotypes and as a gain-of-function allele for others (Kennedy et al., 1995). Silencing at *HM* loci and telomeres was abolished, but life span was extended. Deleting *SIR4* also abolished silencing at *HM* loci and telomeres but caused a decrease in life span. These findings suggested that the ability of the *SIR4-42* allele to extend life span was a gain of function and was due to an activity at a locus independent from telomeres and *HM* loci, termed *AGE*. The *SIR4-42* mutation deletes the C-terminal 121 amino acids of the 1358 residue protein (Kennedy et al., 1995), a region of *SIR4* shown to interact with *RAP1* (Moretti et al., 1994; Cockell et al., 1995), a protein found at *HM* loci and telomeres (Shore and Nasmyth, 1987; Longtine et al., 1989; Conrad et al., 1990; Klein et al., 1992). The loss of the Rap1p interaction domain presumably frees the Sir protein complex to relocate to *AGE*. Increased silencing or another activity of Sir proteins at *AGE* would delay the aging process.

Confocal microscopy using antibodies directed against Sir3p, Sir4p, or Rap1p detects a limited number of foci within the yeast nucleus of wild-type cells that correspond to the location of telomeric DNA (Palladino et al., 1993; Cockell et al., 1995; Gotta et al., 1997). Thus, a significant portion of these proteins appears to be localized to telomeres. Consistent with this observation, Rap1p has been shown to interact with yeast telomeres in vivo (Conrad et al., 1990), and reporter genes placed near telomeres are silenced (Gottschling et al., 1990). Despite the high concentration of these proteins, no clear physiological role has been established for silencing complexes at telomeres.

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Here, through immunostaining in the *SIR4-42* mutant, we find the majority of Sir3p and Sir4p, but not Rap1p, redirected from telomeres to the nucleolus. In addition, we describe two novel genes, *UTH4* and *YGL023*, that regulate yeast life span and are required for this redirection of Sir proteins. Finally, we show that the redistribution of the Sir complex actually occurs in wild-type cells when they are old. We propose a model whereby Sir protein recruitment to the nucleolus is mediated by *UTH4* and *YGL023* and is necessary for long life span. Thus, rDNA may be the *AGE* locus.

Results

Localization of Sir Proteins to the Nucleolus in the *SIR4-42* Mutant

Genetic data suggests that the *SIR4-42* mutant allele extends life span by causing the Sir complex to be redirected away from telomeres and *HM* loci to another locus termed *AGE* (Kennedy et al., 1995). Confocal microscopy was performed with antibodies directed to *SIR3*, *SIR4*, or *RAP1* in order to determine the in vivo localization pattern of these factors in the *SIR4-42* background. In the following experiments, the nucleus of fixed spheroplasts is visualized by red fluorescent staining of DNA and RNA with ethidium bromide, and the proteins of interest are detected with fluorescein-conjugated secondary antibodies (green). In wild-type strains, all three of these proteins colocalize with yeast subtelomeric DNA clustered near the nuclear periphery (Palladino et al., 1993; Gotta et al., 1997) (Figures 1d–1f). The staining pattern of these proteins in the *SIR4-42* mutant, however, was altered such that Sir3p and Sir4-42p are relocalized primarily to one specific location in the nucleus (Figures 1a and 1b). Sir4-42p also displayed a low background of diffuse nuclear staining in some cells. Rap1p reveals a diffuse nuclear staining pattern (Figure 1c) similar to the delocalized patterns observed in Δ*sir4* strains (Palladino et al., 1993).

The only site at which Sir proteins are known to function other than telomeres and *HM* loci is the rDNA, where Sir2p was shown to limit rDNA recombination (Gottlieb and Esposito, 1989) and to mediate silencing (Bryk et al., 1997; Smith and Boeke, 1997). We therefore tested the possibility that the primary position of Sir complex localization in the *SIR4-42* mutant background was the nucleolus. Confocal microscopy was performed in the *SIR4* wild type and the *SIR4-42* strain with antibodies directed against Sir4p in combination with affinity-purified antibodies that recognize the abundant nucleolar protein Nop1p, a homolog of fibrillarin (Henriquez et al., 1990; Tollervey et al., 1993). Figures 1g and 1k show localization in strains that carry *SIR4-42* or *SIR4* alleles, respectively. Counterstaining Nop1p (Figures 1h and 1l) identifies the nucleolus as a crescent-shaped region of the nucleus. Strikingly, mergence of the two staining patterns with the ethidium bromide nuclear stain reveals colocalization of Sir4p and Nop1p in the *SIR4-42* mutant (Figure 1i; colocalization appears white). In the *SIR4* wild type, the typical perinuclear spots of Sir4p and the nucleolus were clearly distinct (Figure 1m).

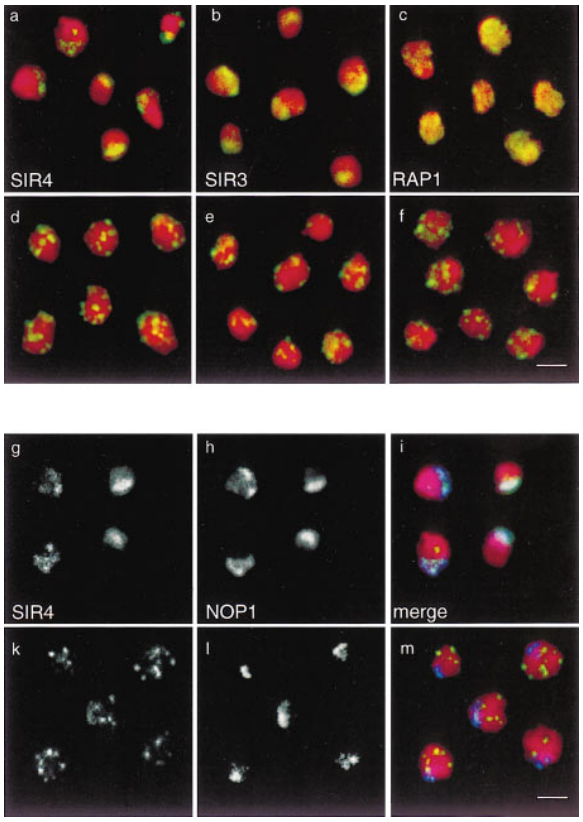

Figure 1. Sir Protein Redistribution Occurs in a *SIR4-42* Strain

(A) Confocal microscopic images of indirect immunofluorescence are shown for the indicated proteins (*SIR4* [a] and [d]; *SIR3*, [b] and [e]; and *RAP1*, [e] and [f]) performed on fixed yeast spheroplasts from the homozygous diploid yeast strain BKy28 (homozygous for *SIR4-42*) and BKy6, which carries *SIR4* alleles. Immunofluorescence, image capture, and analysis was done on a Zeiss Axiovert 100 microscope (Zeiss Laser Scanning Microscope 410) with a 100× Plan-Apochromat objective (1.4 oil) and appropriately filtered helium and argon lasers. Under standard imaging conditions, no signal from one fluorochrome could be detected on the other filter set. The immune reaction is visualized by a DTAF-conjugated secondary antibody (green fluorescence) that is superimposed on the red fluorescent signal of the ethidium bromide–stained nuclei. Coincidence of red and green fluorescent signals produces a yellow color.

(B) Shown is the result of double labeling by indirect immunofluorescence against *SIR4* ([g] and [k]) and the nucleolar antigen *NOP1* ([h] and [l]) on the *SIR4-42* ([g]–[i]) and wild-type ([k]–[m]) diploid yeast strains. Anti-*SIR4* staining detected by a DTAF-conjugated anti-rabbit antibody (green fluorescence) and anti-*NOP1* is detected by a Cy5-conjugated anti-mouse antibody (blue fluorescence). (i) and (m) show the mergence of the two antibody signals with the nuclear DNA, stained with ethidium bromide. Overlap of the three signals produces white color. The scale bars in (f) and (m) = 2 μm.

UTH4 and Its Relationship to *SIR4-42*

The *SIR4-42* mutation (Kennedy et al., 1995) was isolated in a strain with a frameshift mutation in the *UTH4* gene that truncates this protein after 207 residues (see Experimental Procedures and Figure 2D). The *UTH4-326* allele, also isolated in this screen, contains a 1 bp deletion that restores the frame of the *uth4-14c* allele, creating an allele encoding a *UTH4* protein of 834 residues (Figure 2D). This allele will hereafter be referred to as *UTH4-WT*, and the truncated allele in BKy1-14c will be referred

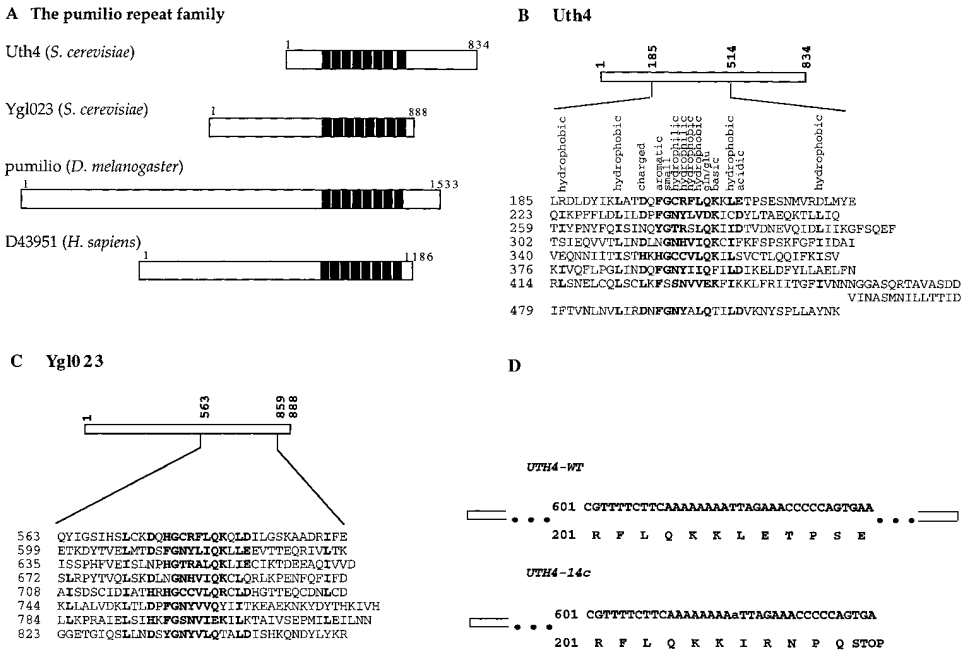

Figure 2. The Pumilio Repeat Family of Proteins

(A) Schematic diagram of the eight Pumilio repeats found in Uth4 and other proteins containing Pumilio repeats.
(B) Protein sequence of the Pumilio repeat region of Uth4.
(C) Protein sequence of the Pumilio repeat region of Ygl023.
(D) Protein sequence of the Uth4 protein encoded by *UTH4-WT* and the new sequence created by the *uth4-14c* allele.

to as *uth4-14c* (Table 1). Thus, *UTH4* is a gene required for longevity of yeast mother cells, and *SIR4-42* may be viewed as a suppressor of the *uth4* frameshift mutation.

To determine if the ability of *UTH4-WT* to extend life span required the Sir complex, *SIR3* or *SIR4* was disrupted in a *UTH4-WT* strain (BKy112, BKy113). The ability of *UTH4-WT* to extend life span was completely abolished in the absence of either *SIR3* or *SIR4* (Figure 3A). This result suggests that *UTH4-WT* increases life span potential by regulating an activity of the Sir protein complex.

We next determined the combined effect of the two life span–extending alleles, *SIR4-42* and *UTH4-WT*. A *UTH4-WT* allele was thus integrated into a *SIR4-42*, *uth4* deletion strain (BKy117). Individually, *SIR4-42* or *UTH4-WT* extended mean life span by approximately 50% relative to BKy1-14c (*SIR4* and *uth4-14c*) (Figure 3B). Life span was not further increased in BKy117. This result further strengthens the theory that *UTH4* and *SIR4* act in a common pathway regulating yeast aging.

If *SIR4* was downstream of *UTH4* in a genetic pathway regulating yeast aging, then the *SIR4-42* mutant strains should retain long life span potential in the complete absence of *UTH4*. Surprisingly, we found that life span extension by *SIR4-42* was completely abolished when the *uth4-14c* frameshift allele was completely deleted (Figure 3C). This finding demonstrates that *SIR4-42* is an allele-specific suppressor of *uth4-14c*, indicative of a close association between Uth4p and Sir4p. Further, this shows that the truncated amino terminus of Uth4p encoded by *uth4-14c* is required for life span extension by *SIR4-42*.

Sequence analysis of the 4 kb genomic insert of plasmid pBK4b3 revealed that the clone was identical to the previously isolated *MPT5/HTR1* gene (Kikuchi et al., 1994; Coglievina et al., 1995). *UTH4* has a putative open reading frame of 834 amino acids containing a region of eight 36 aa repeats in the middle of the protein that are highly similar to those of the Drosophila Pumilio protein and two human sequences (Figures 2A and 2B) (Barker et al., 1992; Macdonald, 1992). A search of the sequence database revealed another yeast gene, *YGL023*, that contained a similar repeat structure (Figure 2C) (Chen et al., 1991). The N and C termini of Uth4p display no significant homology to proteins in the data base.

Table 1. Phenotypes of *UTH4* and *YGL023* Alleles

	SIR4	*SIR4-42*	
	Life Span	Life Span	Sir3p Localization
UTH4-WT YGL023	Long	Long	Nucleolus
UTH4-WT Δyg1023	Long	Long	Nucleolus
uth4-14c YGL023	Short	Long	Nucleolus
uth4-14c Δyg1023	Short	Short	Diffuse/Nuclear
Δuth4 YGL023	Short	Short	Nucleolus
Δuth4 Δyg1023	Short	ND	Diffuse/Nuclear

Relative life spans of *SIR4* and *SIR4-42* mutants in combination with various alleles of *UTH4* and *YGL023*. Sir3p localization patterns are also presented for *SIR4-42* alleles. The localization pattern presented reflects the predominant staining pattern observed in that strain (see Table 2 legend for detailed descriptions). All strains presented are in the BKy1-14c background (see Table 3 for detailed genotype).

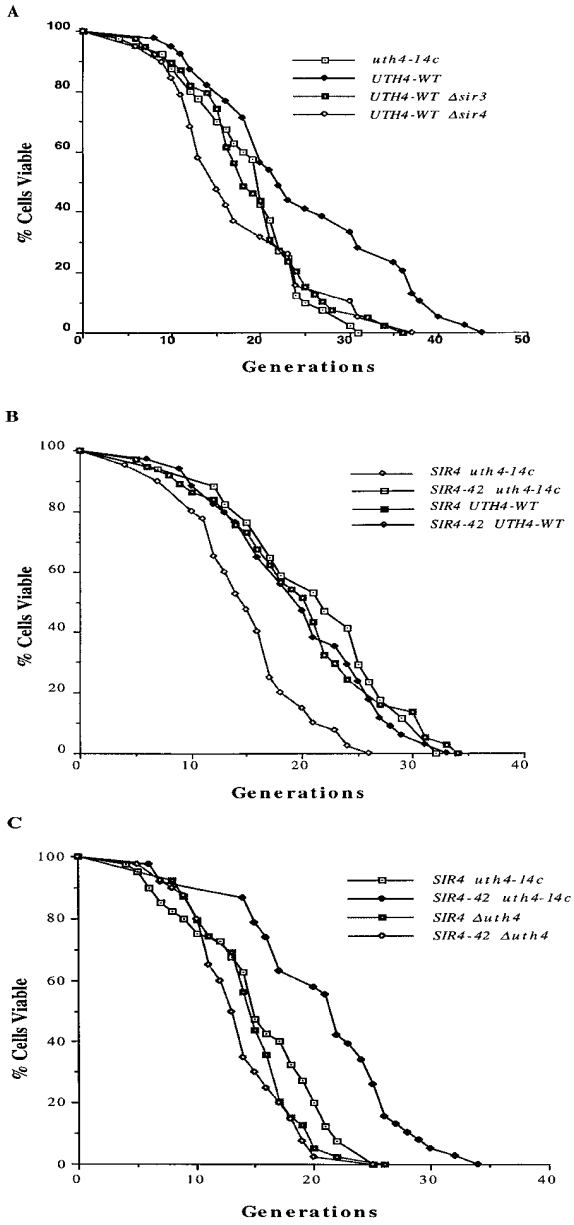

Figure 3. *UTH4* Is Required for Long Life Span in Yeast

Life span analysis was performed by standard methods (see Experimental Procedures). All mortality curves were generated from at least two independent experiments. Life span measurements for a given strain may vary somewhat from one experiment to another. However, within any one experiment, the relative life spans of strains are very constant.

(A) *UTH4* extends life span in a SIR-dependent manner. Sample sizes were as follows: *uth4-14c*, 40 cells; *UTH4-WT*, 39 cells; *UTH4-WT* Δ*sir3*, 39 cells; and *UTH4-WT* Δ*sir4*, 38 cells.

(B) *UTH4* and *SIR4-42* influence the same pathway regulating aging. Sample sizes were as follows: *SIR4 uth4-14c*, 40 cells; *SIR4-42 uth4-14c*, 34 cells; *SIR4 UTH4-WT*, 37 cells; and *SIR4-42 UTH4-WT*, 34 cells.

(C) The N terminus of *UTH4* is required for life span extension by *SIR4-42*. Sample sizes were as follows: *SIR4 uth4-14c*, 40 cells; *SIR4-42 uth4-14c*, 38 cells; *SIR4* Δ*uth4*, 39 cells; and *SIR4-42* Δ*uth4*, 40 cells.

Relationship between *UTH4* and *YGL023* in Determining Life Span and Sir Complex Localization in the *SIR4-42* Mutant

First, we determined the effect of *YGL023* on yeast life span in a *SIR4* wild-type strain background. In the BKy1-14c background, *YGL023* and Δ*ygl023* have identical life spans (Figure 4A and Table 1). Since this strain contains the *uth4-14c* allele and, consequently, is short-lived, we also deleted *YGL023* in the longer-lived *UTH4-WT* but otherwise isogenic strain (BKy3.26). Again, the Δ*ygl023* strain had a life span identical to the corresponding *YGL023* strain (Figure 4A), indicating that *YGL023* also does not affect this strain.

Next, we determined the effect of *YGL023* in a *SIR4-42* strain background. One potential mechanism by which Sir4-42p could compensate for the absense of the *UTH4* Pumilio region is through use of the Pumilio domain of

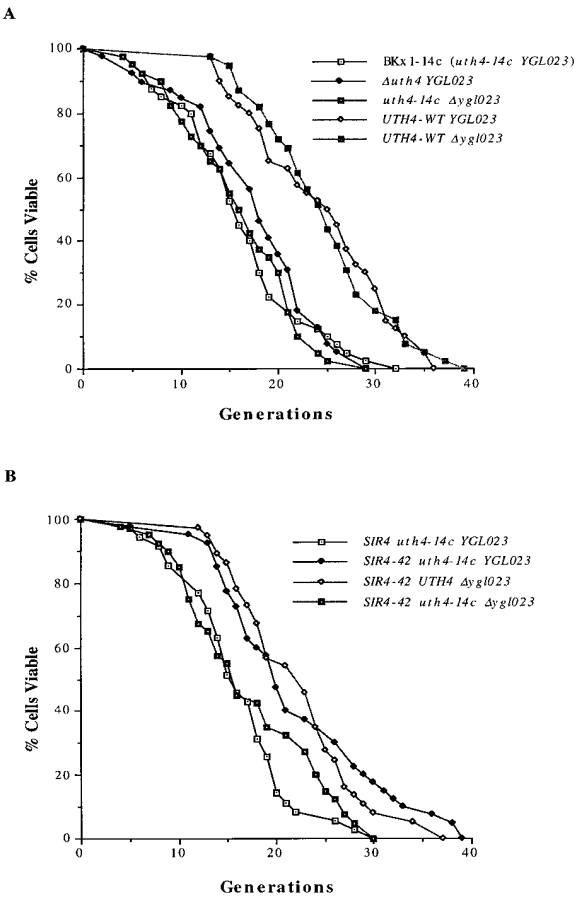

Figure 4. The Role of *YGL023* in Yeast Aging

Life span analysis was performed by standard methods (see Experimental Procedures). All mortality curves were generated from at least two independent experiments.

(A) *YGL023* does not alter life span potential in *SIR4* strains. Sample sizes were as follows: BKx1-14c, 40 cells; Δ*uth4 YGL023*, 39 cells; *uth4-14c* Δ*ygl023*, 40 cells; *UTH4-WT YGL023*, 40 cells; and *UTH4-WT* Δ*ygl023*, 39 cells.

(B) The Pumilio repeat domain of either *UTH4* or *YGL023* is required for life span extension by *SIR4-42*. Sample sizes were as follows: *SIR4 uth4-14c YGL023*, 35 cells; *SIR4-42 uth4-14c YGL023*, 40 cells; *SIR4-42 UTH4* Δ*ygl023*, 37 cells; and *SIR4-42 uth4-14c* Δ*ygl023*, 40 cells.

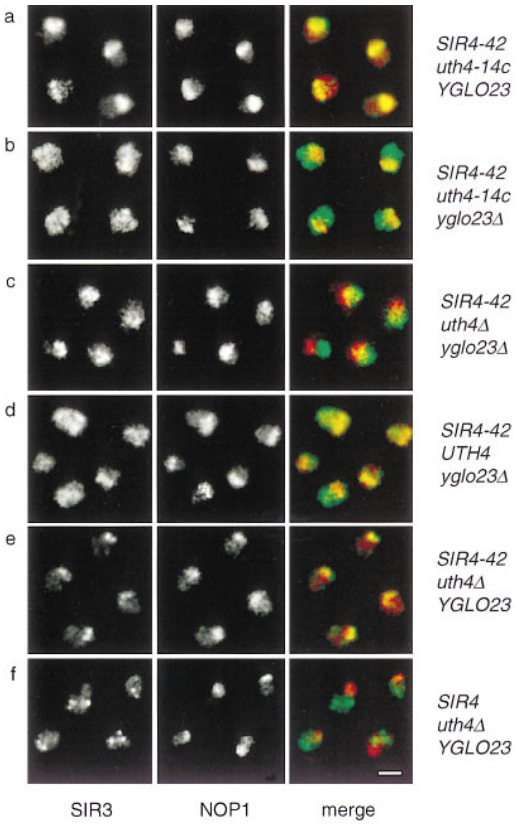

Figure 5. *UTH4* and *YGL023* Both Influence Subnuclear Localization of Sir3p

Haploid strains with the genotypes indicated to the right of the images were subjected to a double immunolabeling with affinity-purified antibodies against *SIR3* and a mouse monclonal against *NOP1*, which were detected by appropriate secondary antibodies and filters, as described in Figure 1. Apart from the relevant mutations in *SIR4*, *UTH4*, and *YGL023* genes, the strains are isogenic and derived from the BKy1-14c background. The left image in each series of three shows the anti-*SIR3* signal, the central image shows the anti-*NOP1* signal, and the right image shows the mergence of the two (anti-*SIR3* in green and anti-*NOP1* in red). Overlap of the two signals indicative of nucleolar staining is yellow. Nonnucleolar Sir3p appears green. In cases in which *NOP1* and *SIR3* colocalize to the nucleolus, the weak nonnucleolar *NOP1* signal allows partial visualization of the rest of the nucleus. To quantitate the distribution of *SIR3* in relation to the nucleolus, the images were uniformly normalized to a signal maximum of 255, and a uniform threshold value for background signal (less than 10% of the total signal) was subtracted. Quantitation of immunofluorescence is presented in Table 2.

Ygl023p. By this model, *YGL023* would be essential for both the long life span and nucleolar localization of Sir proteins in the *SIR4-42* strain. This would contrast with the *SIR4*, *UTH4* wild-type strain, in which *YGL023* is not required for either (see below). Such a finding would provide evidence that nucleolar localization is required for longevity.

To test this model, we first deleted *YGL023* in the *SIR4-42, uth4-14c* background. Life span was clearly shortened by the deletion (Figure 4B). Thus, Ygl023p has become essential for the life span extension in this strain, in sharp contrast to the *SIR4* strain above (Figure 4A).

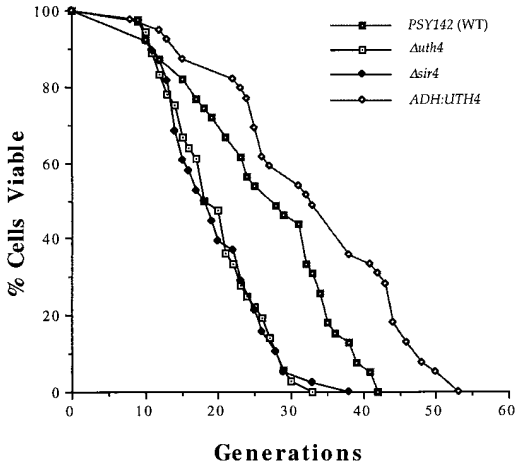

Figure 6. Overexpression of UTH4 Extends Life Span

Life span analysis was performed by standard methods (see Experimental Procedures). All mortality curves were generated from at least two independent experiments. The long-lived PSY142 strain background was used for this experiment. Sample sizes were as follows: PSY142, 39 cells; Δ*uth4*, 36 cells; Δ*sir4*, 37 cells; and *ADH-UTH4*, 39 cells.

Given the findings that *UTH4-WT* (or *YGL023* in the *SIR4-42 uth4-14c* strain) can extend yeast life span in a *SIR4*-dependent manner, we reasoned that these proteins might be involved in redirecting the Sir protein complexes away from telomeres to an *AGE* locus. Since our findings (Figure 1) suggested that the rDNA may be the *AGE* locus, we performed confocal microscopy to determine the effect of mutations in *UTH4* and *YGL023* on Sir protein localization to the nucleolus in the *SIR4-42* mutant. In this experiment, Sir3p is visualized by a fluoroscein-coupled secondary antibody (green in the merged images), and Nop1p is detected by a Cy-5-coupled secondary antibody, indicated as red after merging. Sir4p staining is not shown in this experiment because the Sir4p antibody gave weak staining in strains in which the complex was not concentrated at telomeres or the nucleolus.

Similar to the localization of Sir4p (Figure 1i), Sir3p colocalizes with Nop1p in the nucleoli of the *SIR4-42*, *uth4-14c*, *YGL023* strain, (Figure 5a), as indicated by the yellow color in the merged images. Quantitating the staining patterns in many cells indicated that 87% of cells showed this nucleolar staining (Table 2). Strong nucleolar localization was also observed in *UTH4* Δ*ygl023* or Δ*uth4 YGL023* strains (Figures 5d and 5e, respectively, and Table 2). Strikingly, in the *SIR4-42,* Δ*uth4,* Δ*ygl023* triple mutant, Sir3p was no longer localized to the nucleolus, and, in fact, was generally restricted to the nonnucleolar portion of the nucleus (Figure 5c, and Table 2). This finding clearly demonstrates that Sir proteins are not localized to the nucleolus in the *SIR4-42*, Δ*uth4*, Δ*ygl023* strain and are usually excluded. Thus, *UTH4* and its homolog *YGL023* are required to localize the Sir4-42 protein complex to the nucleolus.

We next stained Sir3p in the *SIR4-42*, *uth4-14*c, Δ*ygl023* strain and found that Sir3p was no longer efficiently localized in the nucleolus (Figure 5b and Table

2). This finding provides evidence that the Pumilio repeat domain is required for robust nucleolar localization and for long life span in the *SIR4-42* mutant. However, nucleolar localization of Sir3p or Sir4-42p is evidently not sufficient for long life span, because the complete deletion of *UTH4* allows at least partial nucleolar localization in a YGL023 strain (Figure 5e) but gives rise to a short life span (Figure 3C). Thus, the N terminus of Uth4p may provide a second essential function in life span extension that is not related to nucleolar localization.

Uth4 Expression Levels Dictate Life Span Potential

All of the prior experiments were performed in a short-lived stress-sensitive strain background (BKy1-14c). To test the generality of *UTH4* as a regulator of yeast life span, we analyzed the PSY142 strain background, which has the *UTH4-WT* allele conferring a long life span and displays no stress sensitivity. When *UTH4* or *SIR4* were deleted, life span was significantly shortened (Figure 6). Moreover, overexpressing *UTH4* from a constitutive *ADH1* promoter extended life span beyond that of the wild-type strain. Thus aging in yeast directly corresponds to the expression level of *UTH4*. A similar extension in life span has been demonstrated by overexpression of *UTH4* in other strain backgrounds (not shown). Overexpression of Uth4p provoked a somewhat higher background of diffuse staining of Sir3p and less well defined telomeric foci (not shown). While these data are consistent with a role of Uth4p in redirecting Sir proteins away from telomeres, we were unable to demonstrate significantly higher nucleolar staining of Sir3p in this strain. This finding may imply that the wild-type Sir complex is driven to the nucleolus only in old cells, a surmise we test below.

Redistribution of the Sir Complex from Telomeres to the Nucleolus in Old Wild-Type Cells

To test whether the Sir silencing complex redistributed from telomeres to the nucleolus in old cells, the long-lived strain PSY142 was subjected to two reiterative sorts (Smeal et al., 1996), yielding a population of mother cells that had divided an average of 26 times (approximately 85%–90% of the mean life span of this strain). In this experiment, nuclei were stained with DAPI (blue); Nop1p, with Cy-3-conjugated secondary antibody (red); and Sir3p, with fluorescein-conjugated secondary antibody (green). Cells were examined by CCD microscopy (Experimental Procedures), which compiles nuclear sections yielding images comparable to confocal microscopy.

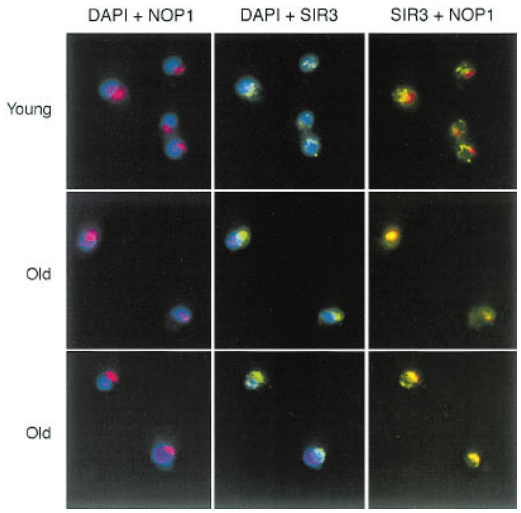

Figure 7. Redistribution of Sir3p from Telomeres to the Nucleus in Old Wild-Type Cells

The haploid strain PSY142 was biotinylated and sorted essentially as described (Smeal et al., 1996; see Experimental Procedures). Cells that were immediately sorted (young) or old mother cells that had divided an average of 26 times (old) were stained as in Figure 5. The blue-purple stain (DAPI) represents nuclei; the red stain, nucleoli; and the green stain, Sir3p. Cells were viewed by CCD microscopy and sectioning (Scanalytics). In all young cells examined, Sir3p exhibited the typical perinuclear staining diagnostic of telomeric localization and was distinct from the nucleolar protein Nop1p. In most old cells examined, significant Sir3p staining was observed in the nucleolus. In a fraction of the old cells, additional staining of Sir3p at telomeres was observed.

Young cells that had been biotinylated and immediately sorted (Figure 7) showed the typical separation of Sir3p telomere staining and Nop1p nucleolar staining. Old cells showed a striking redistribution of Sir3p to the nucleolus, as indicated by the overlap between Sir3p and Nop1p staining (yellow). Significant nucleolar staining was seen in most old cells examined. In some cases, both telomere and nucleolar staining were observed in the same cell (lower panel). These latter cells may be in

Table 2. Quantitation of the Effects of Uth4p and Yg1023p on Sir3p Localization in the *SIR4-42* Mutant

Strain (number of cells)	Dispersed	Nucleolar	Nonnucleolar
SIR4-42 uth4-14c YGL023 (30)	13.0%	87.0%	0.0%
SIR4-42 uth4-14c Δyg1023 (127)	79.5%	19.5%	1.0%
SIR4-42 Δuth4 Δyg1023 (109)	37.6%	0.0%	62.4%
SIR4-42 UTH4 Δyg1023 (146)	35.5%	63.0%	1.5%
SIR4-42 Δuth4 YGL023 (105)	3.9%	96.1%	0.0%

For each strain, between 100 and 150 nuclei were scored, except for *SIR4-42 uth4-14c*, for which 30 were scored. The superposition of the two staining patterns was done manually, and the three categories of distribution were as follows: dispersed, indicating a uniform staining pattern encompassing both nucleolus and nucleoplasm; nucleolar, indicating that Sir3p staining was stronger in the nucleolus than elsewhere, although this does not indicate that there was no background in the nucleoplasm; and nonnucleolar, indicating an enrichment of the Sir3p staining in the nucleoplasm. In the case of the *SIR4-42 Δuth4 Δygl023*, 56% of this class consisted of instances in which Sir3p was excluded from the nucleolus, while the other 44% also had a weak or partial staining in the nucleolus. The percentage is a median of two independent quantitations.

transition from telomere to nucleolar localization of the Sir proteins.

Discussion

We show by immunostaining yeast nuclei that the Sir silencing complex is redistributed from telomeres to the nucleolus in old mother cells of S. cerevisiae. Recent studies also show that localization of the Sir complex to the nucleolus has a profound effect on rates of recombination in the rDNA (D. A. S. and L. G., unpublished data). The redistribution of the Sir complex to the nucleolus is the likely molecular explanation for the loss of silencing at *HM* loci in old cells giving rise to sterility (Smeal et al., 1996). This loss of silencing is first observed about halfway into the life span of mother cells, and the frequency of sterile cells rises progressively thereafter.

The relevance of this redistribution in aging is underscored by molecular analysis of the *SIR4-42* mutation, which prolongs life span in S. cerevisiae (Kennedy et al., 1995). This mutation removes the C terminus of Sir4p, preventing binding of the Sir complex to the telomeric protein, Rap1p (Moretti et al., 1994; Cockell et al., 1995). Prior genetic analysis pointed to a model whereby this mutation redirects Sir protein complexes to a putative *AGE* locus at which they act to delay aging. We show here that Sir proteins concentrate in the nucleolus in a *SIR4-42* strain background, suggesting that the rDNA is the *AGE* locus. We previously viewed *AGE* as a locus that was occupied by the Sir complex in young cells and desilenced in old cells to cause expression of a deleterious protein (Kennedy et al., 1995). Our current findings, rather, suggest that the Sir complex redistributes to the *AGE* locus in old cells to forestall senescence. By this model, the *SIR4-42* mutation phenocopies and strengthens in young cells what normally happens in wild-type old cells. (We cannot rule out that other sites in the nucleus below the resolution of immunofluorescence microscopy are also important for extension of life span.)

Uth4p and Ygl023p, Proteins Involved in Redistribution of the Sir Complex

It has been proposed that telomeres serve as a reservoir for silencing proteins from which they can be relocalized to other genomic sites when conditions warrant (Maillet et al., 1996; Marcand et al., 1996). Our findings suggest that one physiologically important site is the nucleolus, to which Sir proteins redistribute in old cells.

This model would imply the existence of another set of proteins that would act to direct the localization of Sirs to relevant loci. One existing prototype of this class of proteins may be Sir1p (Ivy et al., 1986; Rine and Herskowitz, 1987). *SIR1* is required for establishment of silencing at *HM* loci, but not for maintenance of the silenced state (Pillus and Rine, 1989). Furthermore, increasing Sir1p activity decreases silencing of reporter genes at telomeres, consistent with the idea that there is a competition between *HM* loci and telomeres for Sir complexes (Marcand et al., 1996). Finally, Sir1p interacts with both Sir4p and a member of the origin recognition complex, which are present at *HM* loci (Triolo and Sternglanz, 1996).

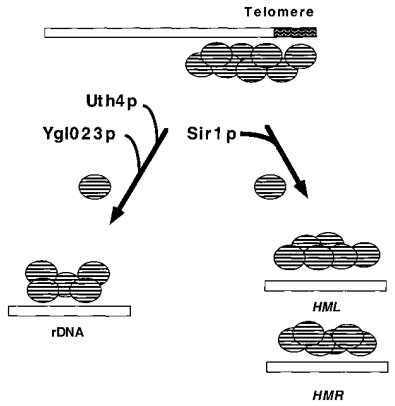

Figure 8. A Model for the Nucleolar Localization of Sir3p and Sir4p by Uth4p and Ygl023p

We propose that Uth4p and Ygl023p act to relocalize Sir proteins to the nucleolus in much the same way in which Sir1p relocalizes the silencing proteins to the silent mating–type loci. The *SIR4-42* mutation facilitates relocalizion of Sir3p and Sir4p to the nucleolus in cycling cells. Either *UTH4* or *YGL023* must be present for this nucleolar localization in the *SIR4-42* mutant. In *SIR4* wild-type strains, we propose that redirection of Sir proteins to the nucleolus by Uth4p and Ygl023p may occur specifically in old cells (see Discussion).

The expression level of the *UTH4* gene determines the life span in yeast. Deletion of *UTH4* shortens life span, and overexpression of *UTH4* extends life span. This longevity function of *UTH4* requires the activity of the SIR complex. We propose that one molecular function of *UTH4* and its homolog, *YGL023*, is to increase the concentration of silencing proteins at the rDNA under certain conditions (Figure 8). Several observations support this theory. First, nucleolar localization of Sir3p and Sir4-42p in a *SIR4-42* strain requires either *UTH4* or *YGL023*. Second, Uth4p and Ygl023p act in concert to reduce silencing at telomeres (B. K. K. et al., unpublished data). Third, mitotic recombination at the rDNA is enhanced by the *SIR4-42* mutation or by overexpression of *UTH4* (D. A. S., and L. G., unpublished data). Fourth, Gotta et al. (1997) have recently found that Sir3p is localized to the nucleolus in Δ*sir4* strains, and that this localization requires Uth4p. The mechanism by which Uth4p and Ygl023p achieve redistribution of Sir3p and Sir4p is not clear. These proteins may bind to the nucleolus themselves and directly recruit Sir proteins to that organelle.

We find that two separate domains of Uth4p are required for its ability to extend yeast life span. The C-terminal 75% of Uth4p, however, contains a region of amino acid repeats also present in Ygl023p (Chen et al., 1991), the Drosophila Pumilio protein (Barker et al., 1992; Macdonald, 1992), and database sequences from several organisms, including humans. This region of Uth4p and Ygl023p participates in the relocalization of Sir proteins to the nucleolus and, thus, is also important for *SIR4*-mediated life span extension. The role of Pumilio in Drosophila is to bind to specific transcripts during development to regulate their translation (Barker et al.,

Table 3. Yeast Strains Used in This Study

Strain	Genotype
BWG1-7A	*MAT**a** ade1-100 his4-519 leu2-3,112 ura3-52 Uth4-14c*
PSY142	*MATα leu2-3,112 ura3-52 Uth4-14c*
BKx1-14c	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c*
BKy3.26	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 UTH4-326 (UTH4-WT)*
BKy4.2	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c SIR4-42*
BKy6	*MAT**a** ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c*
	MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c
BKy28	*MAT**a** ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c SIR4-42*
	MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c SIR4-42
BKy112	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 UTH4-WT Δsir3::URA3*
BKy113	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 UTH4-WT Δsir4::LEU2*
BKy114	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Δuth4::LEU2*
BKy115	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c Δyg1023::hisG*
BKy116	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 UTH4-WT Δyg1023::hisG*
BKy117	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Δuth4::LEU2 SIR4-42 ura3-52:URA3-(UTH4-WT)*
BKy118	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c Δyg1023::hisGSIR4-42*
BKy119	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c Δyg1023::hisG SIR4-42 ARS-CEN-(UTH4-WT)*
BKy120	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Δuth4::LEU2 Δyg1023::hisG SIR4-42*
BKy121	*MATα leu2-3,112 lys2-801 ura3-52 Δuth4::LEU2*
BKy122	*MATα leu2-3,112 lys2-801 ura3-52 UTH4-WT ura3-52::URA3-(ADH-UTH4-WT)*
BKy123	*MATα ade1-100 his4-519 leu2-3,112 lys2-801 ura3-52 Uth4-14c ura3-52::URA3-UTH4-WT SIR4-42*
BKy124	*MATα leu2-3,112 lys2-801 ura3-52 UTH4-WT Δsir4::LEU2*

The following strains were previously described: BWG1-7A (Guarente and Mason, 1983); PSY142 (Kennedy et al., 1994); BKy4.2, BKy6, and BKy28 (Kennedy et al., 1995).

1992; Murata and Wharton, 1995). An RNA-binding activity of the Pumilio domain may be important in localizing *UTH4* and *YGL023* to a nuclear domain, such as the nucleolus, which contains an abundant amount of RNA.

The N-terminal 207 amino acids of Uth4p are also required for long life span. This portion of Uth4p shares no homology with Pumilio, Ygl023p, or any other database sequences. It is possible that this domain functions in concert with the relocalized Sir proteins to carry out some process at the nucleolus.

Aging and the Nucleolus

Several studies have measured changes in both the size of the nucleolus and rDNA content in aged mammalian cells. These studies found a decrease in nucleolar volume with age in murine neuronal cells (Chaconas and Finch, 1973), as well as decreased synthesis of nucleolar RNA in senescent human fibroblasts (Bowman et al., 1976). Experiments have also shown that the number of rDNA copies progressively decreases with age in humans and dogs (Johnson and Strehler, 1972; Strehler, 1986). However, more recent experiments have detected no changes in rDNA content associated with age in mouse myocardial cells (Peterson et al., 1984).

Mutations of the human Werner's gene are associated with premature aging (Epstein et al., 1966). Cloning and sequence analysis of the *WRN* gene indicates that it has high homology to a class of DNA helicases (Yu et al., 1996). The yeast gene most closely related to *WRN* is *SGS1* (Gangloff et al., 1994; Watt et al., 1995). Interestingly, Δ*sgs1* displays large increases in the rate of mitotic recombination at rDNA (Gangloff et al., 1994). We are presently determining the potential role that *SGS1* might play in the regulation of yeast aging.

The findings presented in this report suggest that increased activity of Sir proteins at the nucleolus may be crucial for long life span in yeast. We are not certain of the age-related function of the Sirs at this locus. We suggest that a structural defect accrues at the rDNA in aging cells to limit expression of rRNA and, ultimately, protein synthesis. We speculate that some signal uniquely present in old cells elicits redistribution of Sir proteins to the nucleolus, thereby countering this defect, in some way, and delaying senescence. We do not favor the hypothesis that redistribution of Sir proteins to the nucleolus alters life span by changing the rate of cellular metabolism, because the growth rates of strains with a variety of different alleles of *SIR4* or *UTH4* are all indistinguishable (not shown).

Remodeling of Heterochromatin/Euchromatin Boundaries in Aging

Our findings suggest the possibility that specific redistribution of silencing factors in the genome may be genetically programmed to extend the life span of aging cells and give rise to aging-specific phenotypes, such as sterility in old yeast cells. Is this likely to be a general mechanism in higher eukaryotes? DNA methylation, an indicator of silenced chromatin in mammals, globally decreases with age in mice (Wilson and Jones, 1983; Finch, 1990). Furthermore, a specific gene on the inactive X chromosome was shown to be reactivated in old mice (Wareham et al., 1987). Intriguingly, one locus in which methylation was shown to increase with age in mice is the rDNA (Rath and Kanungo, 1989; Swisshelm et al., 1990). Whether these observations are reflections of specific redistribution of silencing factors, as we observe in yeast, remains to be verified.

One final observation that may relate to our findings is telomere shortening, which occurs with age in human somatic cells (Allsopp et al., 1992). It has been proposed that telomere shortening may be a cause of aging. Our findings suggest that the reverse may be true, i.e., telomere shortening provides a redistribution of silencing

factors that promote longevity. Consistent with this surmise, we have found that shortening yeast telomeres by genetic manipulation actually extends life span (N. Austriaco and L. G., submitted).

Important issues to be addressed are the nature of the defect that accrues in old cells, and how a redistribution of silencing components in the genome ameliorates this problem and forestalls senescence. The molecular analysis of chromatin-remodeling proteins, such as Uth4p and Ygl023p, may aid in this determination.

Experimental Procedures

Strains, Plasmids, and Media

Yeast strains (Table 3) were propagated using standard media and conditions (Sherman et al., 1979). The Δ*sir3* was constructed with *URA3*, using plasmid pDM42 (Mahoney and Broach, 1989). *SIR4* was disrupted and replaced with *LEU2* (Ivy et al., 1986). The *UTH4* disruption plasmid was constructed by replacing the region from 159 base pairs upstream of the putative translational start site through +166 with the *LEU2* gene. The *YGL023* disruption plasmid was constructed by replacing the region from base pair +925 to base pair +1386 of the coding region with hisG-*URA3*. Disruptions were subsequently passed over 5-FOA to eliminate the *URA3* gene (Boeke et al., 1987). All gene disruptions were confirmed by Southern analysis.

Gap repair analysis was conducted to map the mutation in *UTH4-326*. Digestions of pBK4-3 were performed with a variety of restriction enzymes that recognize sites in the *UTH4* coding region. The digested *ARS-CEN* plasmid was then transformed into BKx1-14c. After repair of the plasmid, strains were scored for stress resistance. The mutation was clearly mapped to the region of *UTH4* between base pair +557 and base pair +2155. The *ADH1-UTH4* and construct was made by PCR cloning *UTH4* and inserting it into pDB20 at the NotI site (Becker et al., 1991). To integrate these overexpression constructs, the entire promoter and terminator of *ADH1* including *UTH4* was moved into pRS306 (Sikorski and Hieter, 1989) and integrated at *URA3* by StuI digestion. Integration was verified by Southern analysis.

Strain BKy120 was constructed in the following manner. *SIR4* was disrupted with *LEU2* as described (Kennedy et al., 1995). *LEU2* was then disrupted with *hisG-URA3* and subsequently grown on media containing 5-FOA to recover the *URA3* marker. *SIR4-42* was then integrated at the *URA3* locus by transformation of a StuI-digested plasmid.

Cloning of UTH4

The *UTH4-326* allele was identified as a dominant mutation that conferred stress resistance and increased life span potential (Kennedy et al., 1995). Overexpressing human cdk2 from a *GAL1* promoter causes toxicity in some yeast strains including BKy1-14c. The *GAL1*-cdk2 plasmid was kindly provided by M. Meyerson (Meyerson et al., 1992). In addition to the other previously described phenotypes, the *UTH4-326* mutant allele confers resistance to cdk2 toxicity. Clones that conferred resistance to cdk2 toxicity were isolated from the pJZ1 library and were then tested for their ability to extend life span. The cdk2 toxicity phenotype most likely does not relate to the aging phenotypes. Both Δ*sir4* and *SIR4-42* strains are also resistant to cdk2 toxicity. This finding suggests that the cdk2 phenotype more closely resembles the stress-resistant phenotypes previously described, because Δ*sir4* and *SIR4-42* strains behave differently with regard to life span (Kennedy et al., 1995). One clone, pBK4b3, was isolated that conferred resistance to stress and extended life span when reintegrated into the yeast genome (data not shown). Tight linkage between the cloned DNA and the *uth4* mutation indicated that the genomic DNA present on pBK4b3 contained the *UTH4* gene.

To compare the dominant allele, *UTH4-326*, to the presumed wild-type allele, the *UTH4* allele from strain BKy1-14c was cloned by gap repair (Kennedy et al., 1995). The region of *UTH4* containing the mutation was determined to be in the region of the open reading frame between the BglII restriction site (+557, relative to the putative start of translation) and the BsgI restriction site (+2155) by gap repair of the wild-type plasmid in the *UTH4-326* mutant strain (not shown). This entire region was sequenced. Surprisingly, when the sequences of the two alleles of *UTH4* were compared to the published sequence, the presumed wild-type allele had a 1 bp insertion in a repetitive stretch of adenines. This frameshift led to a stop codon after 207 amino acids, adding an additional 5 amino acids after the frameshift (Figure 2D). To confirm that this sequence alteration in the BKy1-14c allele was not a gap repair artifact, the allele was cloned independently three additional times and sequenced. The same 1 bp insertion was present in all clones. Since BKy1-14c is the product of a cross between BWG1-7A (Guarente and Mason, 1983) and PSY142 (Kennedy et al., 1994, 1995), the *UTH4* alleles from these parent strains were cloned and their sequences analyzed. The BWG1-7A, but not the PSY142, *UTH4* allele contained the 1 bp insertion.

Construction of the Genomic Library pJZ1

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

Immunofluorescence

Immunofluorescence experiments were performed as described (Gotta et al., 1997). Antibodies directed to Rap1 were generated as described (Klein et al., 1992). Antibodies were made that recognize Sir3p and Sir4p as described (Gotta et al., 1997). Anti-Sir4p was raised against a Sir4–β-galactosidase fusion in the Gasser lab (A. Formentin, unpublished data). All rabbit sera were affinity-purified prior to use. Anti-Nop1p is a mouse monoclonal antibody (mAb) that was a generous gift of Ed Hurt (Heidelberg, Germany).

Images were scanned and processed as described in Gotta et al. (1997), unless otherwise noted. For Figure 7, optical sections were obtained using the CELLscan System (Scanalytics, Billerica, MA) equipped with a CCD camera, piezoelectric focus device, and computer-controlled exitation shutter.

Old cells were obtained by the method of Smeal et al. (1996), with the following modifications. Between 10^6 and 10^7 biotinylated cells were grown in 4 liters of YPD medium supplemented with 1% glucose and 40 mg/liter auxotrophic requirements for 16 hr. Cultures were harvested at an OD of 0.8, sorted, and grown for another 16 hr in the same medium before sorting a second time. To avoid old cell lysis, sonication was not performed. Unbound magnetic beads were washed from the preparation with YPD after fixation of cells to the slide.

Life Span Procedure

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

Statistical Analysis

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

Acknowledgments

We thank N. Silverman and Y.-H. Tu for technical advice, J. Zhang for constructing the library used to clone *UTH4*, and members of the Gasser and Guarente laboratories for helpful insights. We also thank Ed Hurt for anti-Nop1p antiserum, Oscar Aparicio for pURADE2, and Gerry Fink for pNL47. D. S. was supported by a Helen Hay Whitney postdoctoral fellowship; K. M., by a National Institutes of Health predoctoral training grant. The Guarente lab is supported by a National Institutes of Health grant (AG11119). S. Gasser thanks the Swiss National Science Foundation, Human Frontiers, and the Swiss Institute for Experimental Cancer Research for support.

Received November 27, 1996; revised March 20, 1997.

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