TELOMERE-INITIATED CELLULAR AGING IN *SACCHAROMYCES CEREVISIAE*: IMPACT OF NUTRIENT AVAILABILITY AND FACTORS AFFECTING GENETIC EXCHANGE

by

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CHAPTER I

INTRODUCTION

The ends of linear chromosomes have specialized caps called telomeres. Telomere DNA consists of thousands of repeats of a simple and short sequence (TTAGGG in humans), running to the 3`end of the chromosome (1, 2). These specialized caps are bound by proteins that help chromosome stability by providing protection against nuclease degradation, end-to-end fusions, recombination and bridge-fusion–breakage mechanisms. Therefore, the lack of these essential structures makes the chromosome unstable (3).

In eukaryotes, the DNA polymerases cannot replicate the very ends of linear DNA during replication. This is called the "end-replication problem". The replication of the leading strand extends continuously from a single RNA primer in the 5` to 3`direction to the end of chromosome. The lagging strand is replicated discontinuously, producing a number of Okazaki fragments. The extension of Okazaki fragments requires an RNA primer for each one. The main trouble with the lagging strand is that there is no room for an additional RNA primer at the extreme end of the chromosome and at the end of S phase there is a gap. Also, the size of the gap is increased by removal of the last primer at the end, which can never be filled in by DNA polymerase. As a consequence of this "end-replication problem," cells would lose some of their genetic information and the chromosomes would get shorter and shorter every DNA replication cycle, if not for the presence of a special DNA polymerase called telomerase that can solve this problem.

In 1985, Carol Greider and Elizabeth Blackburn, working at the University of California, provided the first evidence for a terminal transferase-like activity that adds telomeric sequence repeats onto synthetic telomere primers in cell free extracts (4). Telomerase is an enzyme that contains protein and RNA components. This enzyme can bind the ends of chromosomes and synthesize new telomeric repeats (TTAGGG) to extend the 3` end of the chromosome (Figure 1). Adding the telomeric repeats occurs during the synthesis phase (S phase) of the cell cycle by using the RNA component of telomerase as a template and the enzyme's reverse transcriptase activity. Therefore, telomerase has an essential function to maintain the length of chromosomal ends (5).

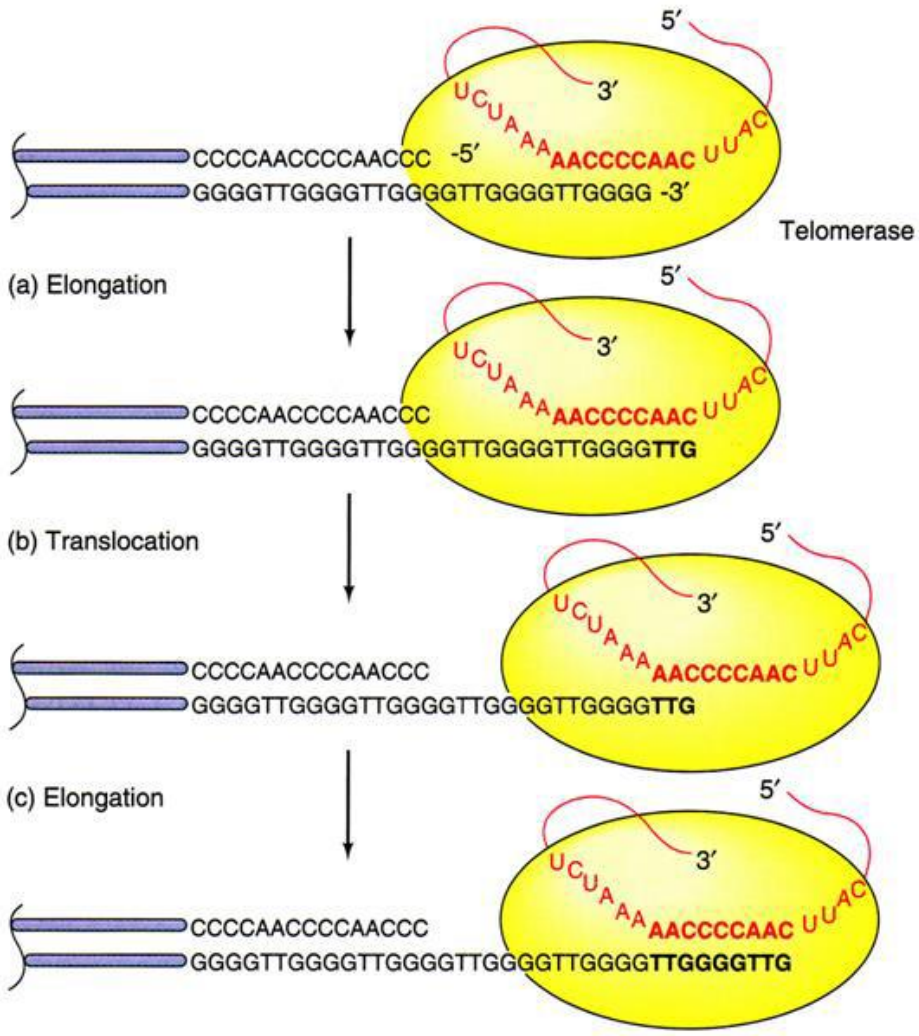


Figure 1. Schematic diagram of telomere DNA replication by telomerase (6).

In most human somatic cells the telomerase activity is undetectable. In cells that experience high turnover rates, telomerase stays active (1, 6, 7). For this reason, the telomeres of cells in younger humans are longer than those of older humans. In the absence of telomerase, human cells lose 30-100 base-pairs (bp) every cell cycle (8). Aging studies have shown, when somatic human cells are grown in culture with all nutrients necessary for growth, the cells grow for approximately 50 cell cycles. Then they stop

growing and eventually die, even though the nutrients are abundant. This is called cellular senescence or aging in cell culture. It occurs due to the telomeres becoming critically short in the absence of telomerase (9). Even with its importance, most human cells do not make telomerase. In contrast, approximately 90% of cancer cells make telomerase and that's why they are considered immortal. Thus, cancer cell formation increases through activity of telomerase. This might be an advantage of non-expression of telomerase by most human cells (10).

The shorter telomeric repeats in older humans and elevated risk of age-associated diseases have been correlated in several studies. The time-dependent shortening of telomeres is associated with human carcinogenesis in two different ways. First, the ability of telomere shortening to limit the number of cell divisions can serve as a tumor elimination mechanism. The other model argues that chromosome instability and carcinogenesis increase with shorter telomeres. Older people with the shortest telomeres are much more likely to die, get heart disease or other age-associated diseases (9).

Oxidative stress is another factor that may affect the rate of telomere shortening and senescence. DNA damage or mutations caused by reactive oxygen species (ROS) can lead to this stress and potentially accelerate cellular senescence (11). During normal cellular metabolism, especially mitochondrial respiration, ROS are produced. Cellular oxidation is caused by many different types of free radicals. The three most common ROS inside cells are superoxide radicals, peroxyl radicals, and hydroxyl radicals. These free radicals react, causing damage to biomolecules such as DNA, proteins, and lipids. Oxidative damage to DNA is elevated with increasing production of ROS. Therefore, DNA damage is increased by two factors: decreasing of DNA repair capacity and

promotion of reactive oxygen species production during aging. These factors and steps are illustrated in Figure 2 below.

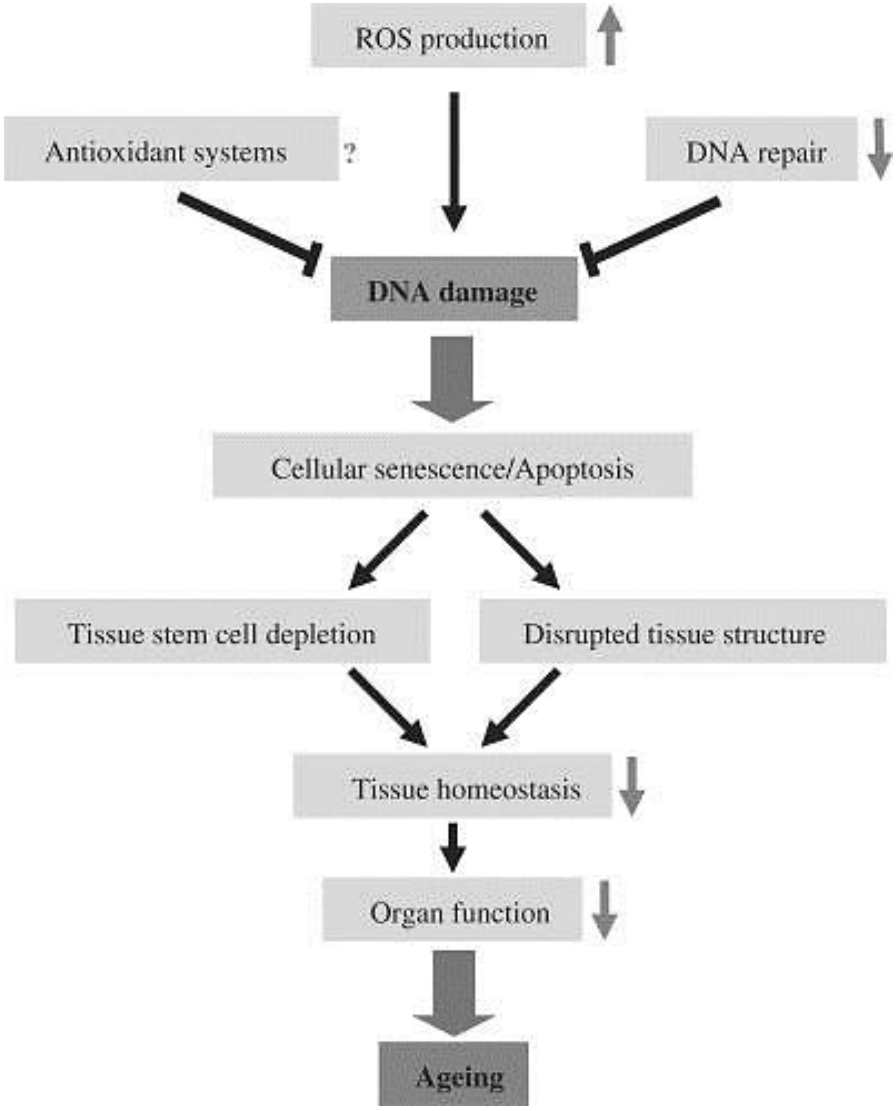


Figure 2. Relationships among DNA damage, cellular senescence and organismal aging (11).

Cells can be preserved from DNA damage that is caused by ROS through antioxidant enzymes. Superoxide dismutases, catalases, and glutathione peroxidases are among the most well-known antioxidants (12).

Several studies have used *Saccharomyces cerevisiae (*budding yeast*)* as a model for genetic studies of aging. Many factors give this model experimental value. First, yeast has a simple life cycle and short generation time (every 90 min at 30 ˚C). Moreover, its ability to grow as either a haploid, which has only one copy of each chromosome, or diploid cell, which has two copies of each chromosome, is an advantage for genetic experiments (13, 14, 15). In addition, telomerase that is produced by *S. cerevisiae* is structurally similar to human telomerase (Figure 3). The telomerase complex of yeast is composed of *TLC1,* the RNA subunit, and Est1, Est2, and Est3 protein subunits. *TLC1* RNA is composed of an internal 17 nucleotide sequence (CACCACACCCACACACA), which serves as a template for synthesis of new telomeric DNA repeats by the enzyme. Inactivation of *EST1, EST2, EST3* or *TLC1* causes telomere shortening and cellular senescence after approximately 60-70 cell cycles. Even though the expression of telomerase has been stopped, a small fraction of cells can acquire a mutation that results in partially stable telomeres, and an increase in homologous recombination between telomeres. These cells are called “survivors” and occur at low frequency in late passage cultures (9).

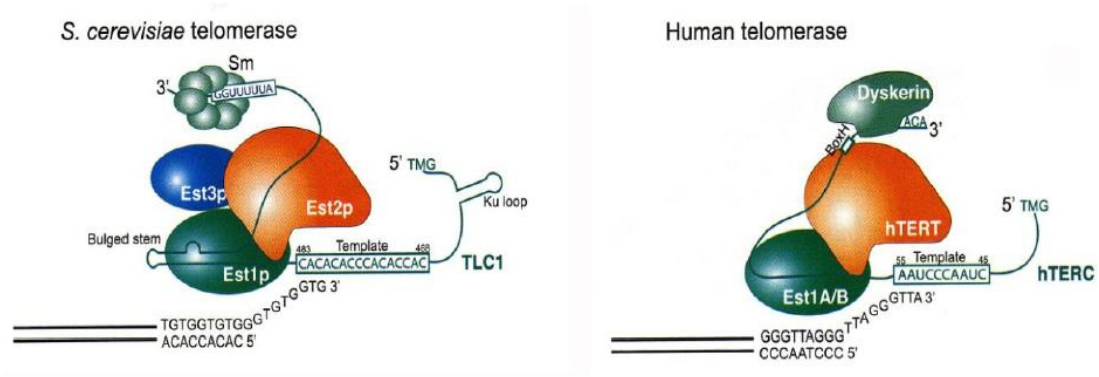


Figure 3. Comparison between the *S. cerevisiae* and human telomerase complexes (13).

CHAPTER II

MATERIALS AND METHODS

I. Materials

General reagents

A 2-log DNA ladder standard was ordered from New England Biolabs (Ipswich, MA). Ethidium bromide (EtBr) was purchased from Shelton Scientific, Inc. (Shelton, CT). Ethylenediaminetetraacetic acid (EDTA) and agarose were obtained from EMD Chemicals, Inc. (Darmstadt, Germany). Sonicated salmon sperm carrier DNA was ordered from Stratagene (La Jolla, CA). Tris base was purchased from VWR International (West Chester, PA). Polyethylene glycol (PEG) 4000, and sodium dodecyl sulfate (SDS) were ordered from Mallinckrodt (Paris, KY). Methylmethane sulfonate (MMS), dimethyl sulfoxide (DMSO), ampicillin, and lithium acetate were ordered from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sodium hydroxide was obtained from Merck (Darmstadt, Germany).

Yeast growth media

D-(+)-glucose, raffinose, and soy peptone were purchased from Amresco (Solon, OH). Amino acids and D-(+)-galactose were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bacto yeast extract was purchased from Becton Dickinson

Microbiological Systems (Sparks, MD). Agar, molecular biology grade was obtained from Teknova (Hollister, CA).

Yeast strains and plasmids

All strains that were used in this study were derived from YLKL803 (BY4742, *est2Δ::HygB*^{r} containing plasmid pLKL82Y [*CEN/ARS URA3 GAL1-V10p::EST2*]) and listed in Table 1. Several mutants were tested to measure their effect on cell senescence including checkpoint mutants (YLKL840, YLKL841, and checkpoint double mutant YLKL844), antioxidant mutants (YLKL820, YLKL821, and antioxidant double mutant YLKL829), and DNA repair mutants including (YLKL807, YLKL1203, YLKL1204, YLKL1234, YLKL1247 and YLKL1248). The sequences of all oligonucleotide primers used in this project are listed in Table 2. Plasmid pSTL11 (*rad55Δ::LEU2)* was a generous gift from Robert Mortimer (16).

Table 1. *Saccharomyces cerevisiae* strains.

Strain	Genotype	Reference
BY4742	*MATa ura3-Δ0 leu2-Δ0 lys2-Δ0 his3-Δ1*	17
YLKL803	BY4742, *est2Δ*::*HygB*^{r} + pLKL82Y (*GAL1-V10p::EST2, URA3*)	17
YLKL807	YLKL803, *rad52Δ::G418*^{r}	17
YLKL1203	YLKL803, *rad51Δ::LEU2*	Lab strain
YLKL1204	YLKL803, *rad54Δ:: LEU2*	Lab strain
YLKL1205	YLKL803, *rad57Δ:: LEU2*	Lab strain
YLKL1534	YLKL803, *rad55Δ:: LEU2*	Thesis work
YLKL1547	YLKL803, *rad59Δ::G418*^{r}	Thesis work
YLKL1548	YLKL803, *rdh54Δ::G418*^{r}	Thesis work
YLKL820	YLKL803, *ctt1Δ*::*G418*^{r}	18
YLKL821	YLKL803, *gpx3Δ*::*G418*^{r}	18
YLKL829	YLKL820, *gpx3Δ*::*Nat*^{r}	18
YLKL840	YLKL803, *rad24Δ::G418*^{r}	18
YLKL841	YLKL803*, mec3Δ:: G418*^{r}	18
YLKL844	YLKL840*, mec3Δ::Nat*^{r}	18

Table 2. Oligonucleotides used for PCR reactions.

primer	*sequence*
5'rad55	CCCTAAGTTCTTCACGTGTTCTCTTCTC
3'rad55	CATACCACTTTAGGTTCGAGCTGCATACC
grad59A	GAGTTCCAAATCATCGAAGATTGGAATGGAAGACCTGCCAGCGCTTGGTCGGTGCAATGTGACTGTCGCCCGTACATT
grad59B	GAGCCATACAAGCCATCGACATAGTAATTATTTGTAATCTTAGTCTCATAATCGAGACAAGTTCTTGAAAACAAGAATC
5-rad59	GGGCAAATTGATTAGAGGAAGCCACAG
3-rad59	CTGTTGATAAAGGTAAGTCGATACCTG
grdh54A	CGAAGCAGGGAAATTTCTTGGGTCAAGTATGCTACCAAGTGATTCAGATTCTCTCTATGTGACTGTCGCCCGTACATT
grdh54B	GACCATCTCTATGAATTCGTGACATCGCTTGCAAATCTACTGAAGGATTCCAATCAACAAGTTCTTGAAAACAAGAATC
5-rdh54	GAGGTATCTCTATGGATGTGGCTTGAA
3-rdh54	TTCTCTGAGACATATCTCGCCGGGCT

Enzymes and PCR reagents

Restriction enzyme HinDIII and Taq DNA polymerase were ordered from New England Biolabs (Ipswich, MA). ExTaq DNA polymerase was purchased from Clontech Laboratories, Inc. (Mountain View, CA). RNase A was ordered from Sigma-Aldrich Chemical Co. (St. Louis, MO).

II. METHODS

Gel electrophoresis

Gel electrophoresis was carried out using 0.7%-0.9% agarose gels in 1X TAE (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) running buffer in a Labrepco Horizon 11-14 gel rig (Horsham, PA) at a voltage of ~130 V. An Alpha Innotech/Protein Simple Imager (San Jose, CA) was used to capture gel images after staining for 15-20 min with 0.5 mg/ml EtBr.

Yeast transformations

Transformations of plasmid DNA and PCR fragments into yeast cells were performed using the high efficiency protocols of Tripp *et al*. (19).

Targeted gene disruption

For the *RAD55* gene knockout, 15 µL of the plasmid pSTL11 was incubated in a digest buffer containing: HinDIII (80 units), 1x Cutsmart buffer and ddH_2O to 65 µl total volume for 2-4 h at 37ºC. The digest was analyzed by gel electrophoresis, then was transformed into yeast cells using the aforementioned protocols of Gietz *et al.* or Tripp *et al*. Proper integration of DNA was tested by first selecting on glucose minus leucine synthetic plates. Since *rad55Δ::LEU2* mutants are MMS sensitive, the next test was by replica-plating onto YPDA plates with 2 mM MMS. The right integration was further verified using PCR in 50 µL reactions containing 1x ExTaq Buffer, 0.5 µM magnesium chloride, 25 µM dNTPs, 5 units ExTaq DNA Polymerase, 1 µM of 5'rad55 and 3'rad55 primers (Life Technologies), and 4 µl of plasmid or chromosomal template DNA in a total

volume of 50 µl. PCR was started with a 2 min 94 °C denaturation, followed by 32 cycles of 95 °C for 40 s, 46 °C for 40 s, 72 °C for 1 m 45 s, and terminated with a 7 min 72 °C extension. Occasionally, the annealing temperature was adjusted to improve the reaction. The PCR product was then resolved using gel electrophoresis. To knockout *RAD59* and *RDH54*, the G418^r gene from the plasmid pFA6MX4 was first PCR-amplified using specific primers for each gene. Primers used were grad59A and grad59B to knock out *RAD59* and grdh54A and grdh54B for *RDH54*. PCR products were then transformed into yeast cells using the stationary phase protocol of Tripp *et al.* (19).

Proper integration of DNA was tested by first selecting on YPDA with G418 plates. For *rad59Δ::G418* mutants, the right insertion was also verified using PCR. Fifty µL reactions involving 1x ThermoPol Reaction Buffer, 25 µM dNTPs, 5 units Taq DNA Polymerase, 1 µM of 5-rad59 and 3-rad59 primers (Life Technologies), and 4 µl chromosomal template DNA, were preformed using the same PCR conditions as previously described. The PCR product was then resolved using gel electrophoresis. For *rdh54Δ::G418*^{r} mutants, DNA integration was tested using DNAs PCR-amplified in 50 µL reactions as previously described, with the exception that 1x ExTaq Buffer, 0.5 mM magnesium chloride, 5 units ExTaq DNA Polymerase and 1 µM 5-RDH54 and 1 µM 3-RDH54 primers were used.

Solid media-based senescence assays

Cell aging can be measured by the creation of a series of streaks of cells on plates. For the first streak, cells were picked from a freshly-grown (galactose minus uracil) stock plate using sterile toothpicks. A new, more quantitative approach was developed during

the course of this project that followed up on preliminary work done by graduate student Naoko Araki (20). Cells were transferred onto two glucose complete (Glu-comp) synthetic plates as double columns to form 8 separate double columns, and then allowed to grow for 3-4 days at 30 ºC. After the first streak, cells were re-streaked from moderate-sized individual colonies onto two fresh Glu-Comp plates and the process was repeated again. In the third streak, colonies were picked with sterile toothpicks onto six Glu-Comp plates in a grid as 48 single columns (8 columns per plate). After the third streak this process was repeated to form another 48 columns. In the third and fourth streak, streaking of each column was always directed from the outer margin of the plate toward its center to reduce the variability caused by some cells growing faster on the outer edge of the plate.

Counting the number of cells in colonies on senescence assay plates

A colony on a senescence assay plate was harvested with a Pasteur pipette and then transferred into a microcentrifuge tube which had 500 µl ddH_2O. The colony was separated from the agar by vortexing for ~10 s and then the cells were sonicated for 8 s using a Sonics Vibra-Cell sonicator (Newtown, CT) which uses sound waves to separate cells from each other. The cells were introduced into a hemocytometer counting chamber, which was used to calculate the number of cells per unit of volume. The hemocytometer was then placed on a United Scope model M837T phase contrast microscope (Hopewell Junction, NY) stage and cells in 25 squares were counted. To calculate the total number of cells in a colony, the following formula was applied. Total number of cells in a colony = number of counted cells in 25 squares × 10.000 × 0.5.

CHAPTER III

RESULTS AND DISCUSSION

The major goal of this project was to use a genetic system developed in Dr. Lewis's lab to study the genetics of cell aging and to improve the way that cell senescence assays are performed in *S. cerevisiae*. Yeast cells normally produce telomerase and are immortal. The catalytic subunit of yeast telomerase is Est2. Inactivation of the *EST2* gene causes cells to exhibit progressive telomere shortening and senescence. For our assay system, *EST2* was deleted from BY4742 yeast cells to create a plasmid-based *EST2* expression system. Then pLKL82Y was transformed into the telomerase-deficient yeast cells. The pLKL82Y plasmid contains *EST2* under the control of a modified galactose-inducible *GAL1* promoter (*GAL-V10*). Therefore, in galactose media the promoter is ON and the expression of Est2 polymerase is activated, which causes the cells to be immortal. However, in glucose, Est2 polymerase is not expressed and the modified yeast cells exhibit no telomerase activity (Figure 4) (21). As a result, telomeres get shorter after each cell cycle and cells will stop dividing after approximately 60-70 generations.

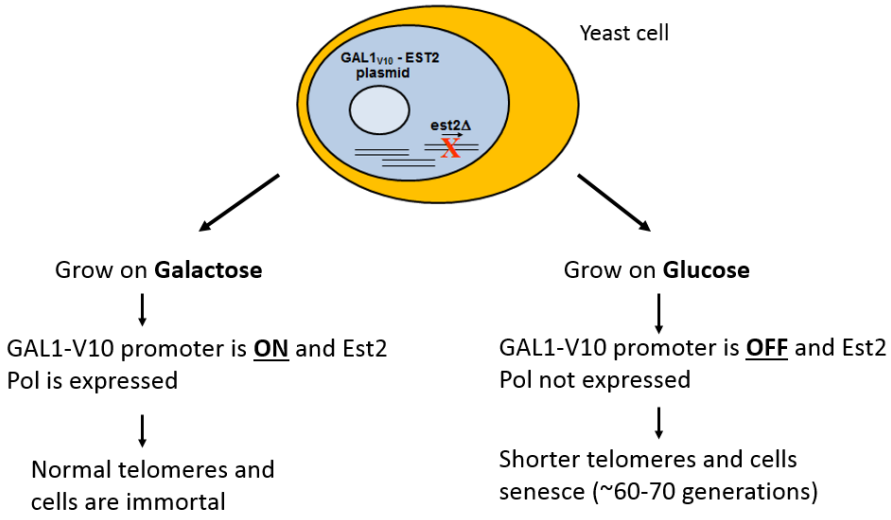


Figure 4. *EST2* polymerase expression system. Cells have normal telomeres in the presence of galactose (left), and shorter telomeres in the presence of glucose (right).

Historically, senescence in yeast using *est2*$^{-}$ yeast cells was measured using a solid media-based assay that utilized a double-column streaking method (22, 23). This method is based on the generation of a series of four streaks of double-columns of yeast cells. Colonies formed 3-4 days after the 1st streak are re-streaked to a new plate. Colonies that form after 3-4 days on the 2nd plate are re-streaked to a 3rd plate and the process is repeated again for a 4th streak. Cells that express telomerase are immortal and continually grow through the first, second, third, and fourth streaks. In contrast, the telomerase-deficient yeast cells stop dividing after ~ 60-70 generations and produce only a few colonies on the fourth streak, which is defined as senescence (Figure 5).

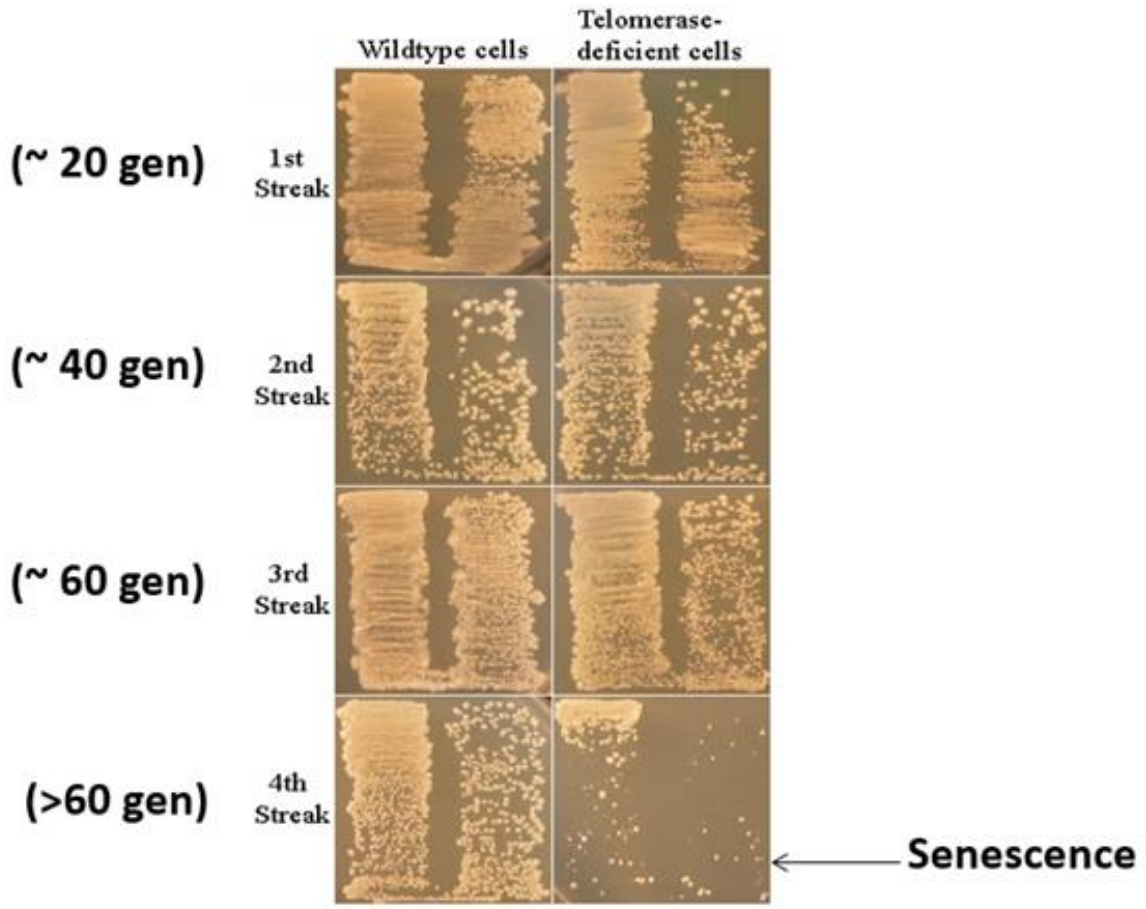


Figure 5. Traditional solid media-based senescence assay using a double-column streaking method.

Senescence occurs when chromosome ends get shorter after each cell cycle and telomere caps become unstable (Figure 6). When telomeres hit a critical length, they lose their protective caps. The resulting uncapped telomeres are subject to 5'-3' nuclease activity, leading to creation of single-stranded DNA. Therefore, certain nucleases, including Exolp and others, play an important role in chromosome stability. In addition, as telomeres reach the critical length and become too short, cells undergo a stress response coupled with a DNA damage response; this causes the arrest of the cells in G_2 phase and the termination of division (22).

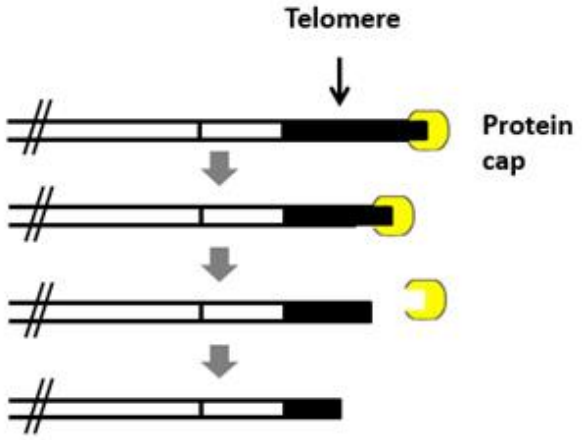


Figure 6. Senescence process. During senescence, chromosome ends shorten and protective cap proteins are lost.

In previous studies in Dr. Lewis' lab and in other labs that tested telomerase-deficient yeast cells, most cells underwent senescence in approximately 60-70 generations. However, some of the cells died in 50 or less cell cycles, while others exhibited no senescence until 90 or 100 generations. This disparity in results is due to the fact that senescence is a stochastic process and the growth on the fourth streak is highly variable in the standard assay (Figure 7). The rate of telomere shortening and uncapping is not identical on every chromosome end or in every cell, some getting shorter very fast and some very slowly. This results in a significant degree of variability in telomere state among the chromosomes in every cell (22).

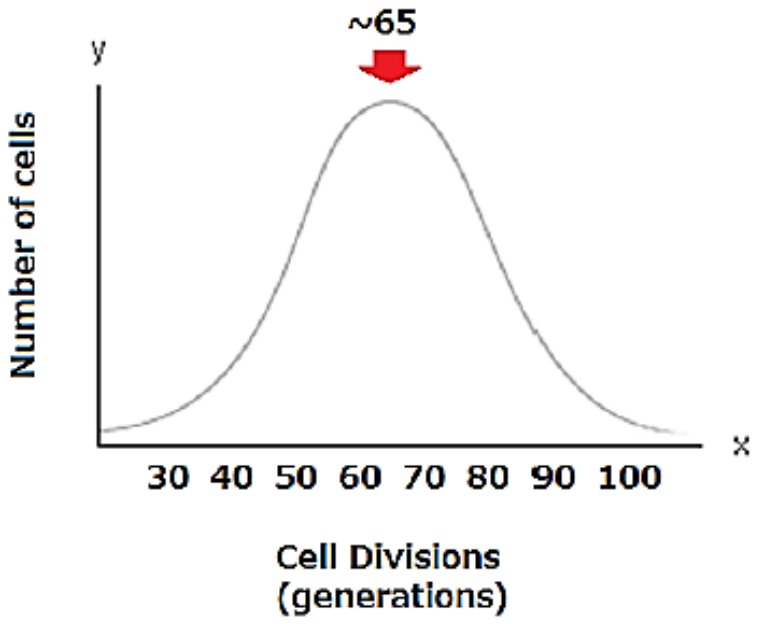


Figure 7. Senescence rate during the aging process. Most cells stop dividing after approximately 65 cell cycles, but some cells senesce much earlier or much later.

A major purpose of this study was to develop a new senescence assay to quantitate senescence in yeast that incorporates strong statistics. The development of this assay was initiated by Naoko Araki, a former graduate student in Dr. Lewis' lab, and we have now further improved it by making certain changes in the previous standard assay which allows the correction for and ultimate reduction in the randomness associated with senescence. Cell aging is normally monitored by repeatedly streaking colonies onto plates following a "double-column" streaking method as mentioned previously, which involves transferring a colony with a toothpick, then using the side of the toothpick to streak the first column. The toothpick is then flipped and used to push cells at the end of the first column twice through to the start of the second column, and then the second column is completed with the same

side of the toothpick. This method is used for the 1st and 2nd streak plates (~ 40 generations of growth). The use of the double-column streaking method in the first and second streak plates is to help reduce the density of cells on the second column and thus generate single colonies that can be used for further streaks. For the third and fourth streak plates, a new single-column streaking method was developed and used to create a set of six plates with each plate containing eight single columns of cells. The third and fourth streaks were performed using a printed, colored grid pattern that was placed under each plate. This allowed the streaking of cells in a uniform pattern, so as to have the same length and width of columns (Figure 8).

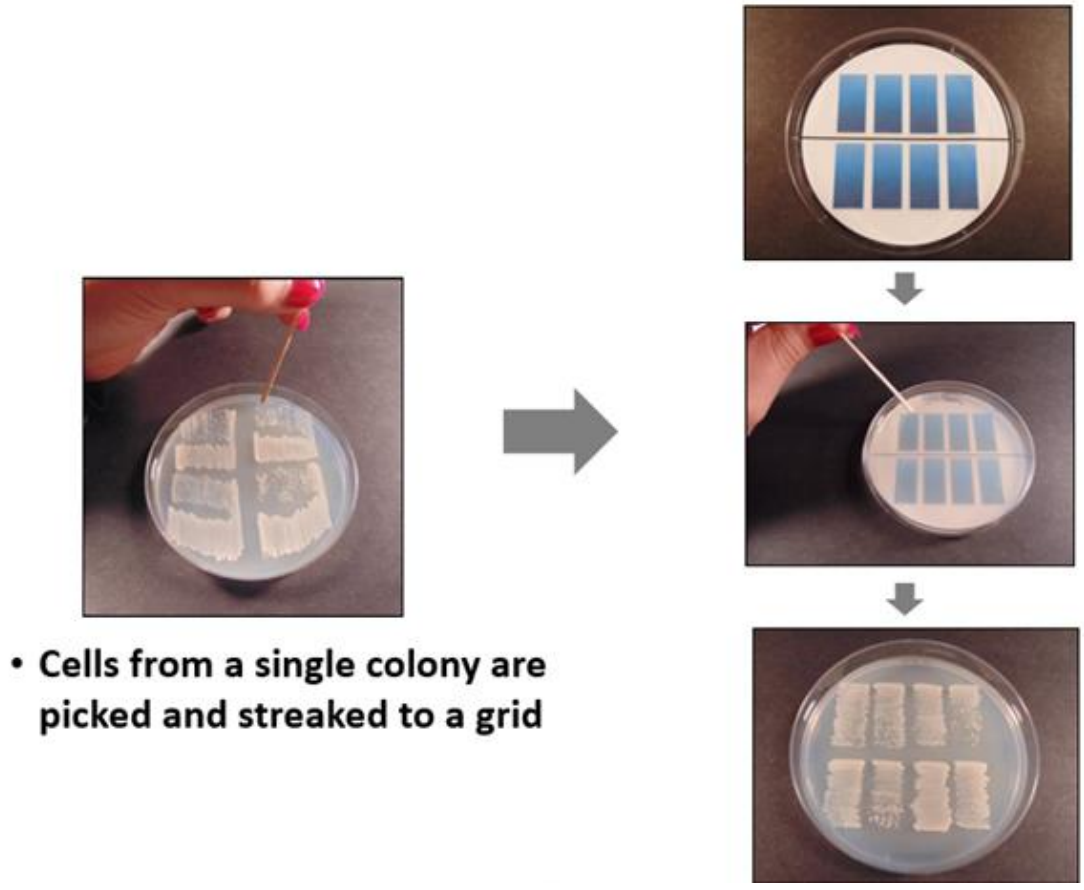
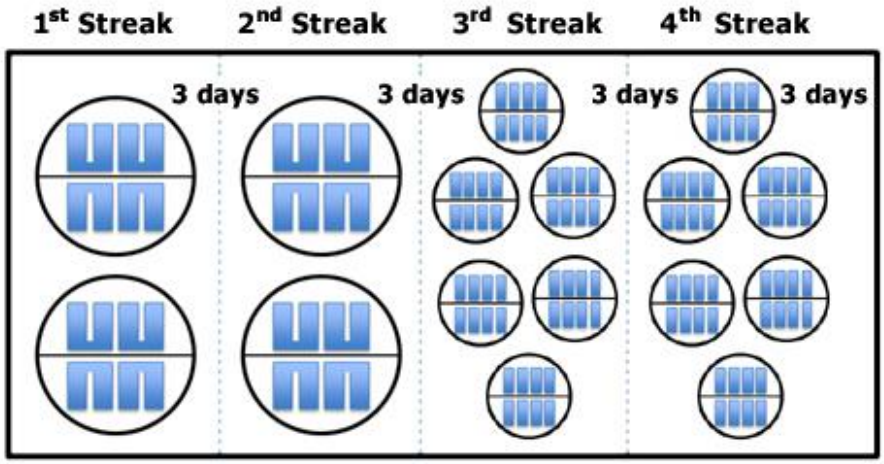


Figure 8. New senescence assay using a grid system for streaking cells.

In summary, on the first streak, cells from a galactose minus uracil stock plate were picked with a sterile toothpick and streaked using the standard double-column streaking method onto two glucose complete synthetic plates. These plates were then incubated at 30^{o}C for 3 days to allow cells to grow for the subsequent step. For the second streak, colonies from the first plate were streaked following the standard double-column method, then grown at 30^{o}C for 3 days using another two synthetic glucose plates. Single colonies from the second plate were then picked and streaked onto six plates, 8 columns per plate, to form 48 separate columns for the third streak, and these cells were allowed to grow for 3 days at 30^{o}C. The process was repeated for the fourth streak, generating another 6 plates with 8 columns on each plate. The level of senescence was determined by the total number of single columns that did not grow confluently on the 4th streak, i.e., that were unable to form a completely filled column of colonies after 3-4 days.

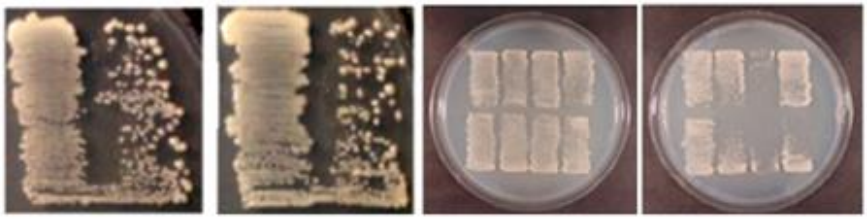
This aging test has a large number of individual assays in order to provide good statistics and reduce variability previously associated with the fourth streak. The new senescence assays, including the number of streaked columns, plates and representative plate pictures for each streak, are shown in Figure 9. Also, a consistent streaking pattern/direction, from the outer margin of the plate toward its center, was used to maintain consistency with nutrient concentrations, since the outer margin has more nutrients than the center. The new method allows wild-type cells to be compared to mutant cells to determine if one undergoes senescence more rapidly than the other. In contrast, the previous method was unable to show with accuracy if wild-type cells differed from mutant cells, due to the high variability of the fourth streak.

(A)



New Method

(B)



(C)

	Number of assays			
New Method	8	8	48	48

Figure 9. (A) Diagram illustrating how the new senescence assays were performed. (B) Plate pictures of the new method. (C) The new approach expands the number of column assays on the 3rd and 4th streaks to 48.

The first experiment using the new method was conducted using telomerase-deficient YLKL803 cells grown in standard synthetic glucose complete vs YPD (rich) media (Figure 10). The purpose of this experiment was to determine if there is a difference in the aging rate between cells grown in these two media. The assay was performed at 30°C, and each streak took approximately 3 days before the plates were ready for the next streak.

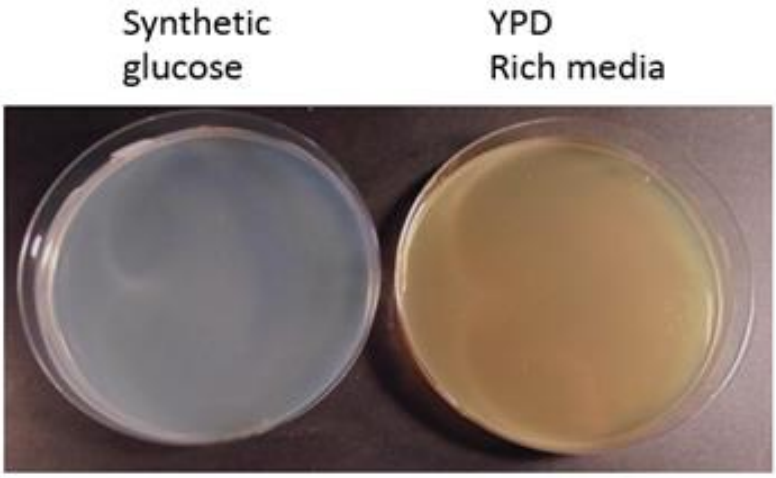


Figure 10. Comparison between synthetic glucose complete plates (defined media) versus YPD plates (rich media).

The results of three trials testing senescence on rich YPD plates versus synthetic glucose plates are shown in Table 3. The results of each trial were analyzed in terms of the number of full columns present, the average number of full columns of growth per plate, the standard deviation, and the median number of full columns of growth per plate. The cells with both synthetic glucose and YPD-rich media grew at relatively the same rate. On both plates there was approximately 100% full columns on the 3rd streak, i.e., ~8 full columns per plate (data not shown). In cells grown on synthetic glucose, the average number of full columns per plate for the fourth streak in each trial was 3.0, 3.25 and 3.5, respectively. In contrast, when cells were grown on rich YPD plates, the average number of full columns per plate for the fourth streak was 1.7, 1.8 and 2. Both sets of plates showed senescence on the 4th streak (less than 8 columns per plate), but growth was reduced more strongly on the YPD plates. The student's T-test indicated that the differences between the growth rates on YPD and synthetic glucose were not significant

(p>0.05). The median values were very similar to the means, suggesting that the distributions were relatively normal.

Table 3. Growth of telomerase-deficient yeast cells on the 4th streak plates using synthetic glucose complete plates and rich YPD plates.

	YLKL803 (4th streak) YPD			YLKL803 (4th streak) Glu-com		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Full Columns	7/32	12/48	11/48	13/32	18/48	21/48
Avg. per Plate (#/8)	1.75	2.0	1.8	3.25	3.0	3.5
Std. Deviation	1.71	1.1	1.8	2.1	2.3	1.9
Median	1.5	2.0	2	3.5	3.5	4.0
Student's T-test	p=0.31	p=0.36	p=0.15			

Although the result of the 3 trials on each type of plate were very similar to each other, indicating high reproducibility, the standard deviations were relatively high (Table 3). This variability is illustrated schematically in Figure 11, which shows the number of full columns per plate for synthetic galactose, synthetic glucose, and YPD plates. On galactose, the cells were making telomerase and did not senesce on the fourth streak; these cells gave approximately 7.5 full columns per plate. However, the glucose and YPD-rich plates both produced a low number of full columns on the fourth streak. The number of full columns per plate on synthetic glucose was higher than on YPD (~3 vs. 2). This may be due to cells growing faster on YPD media, undergoing a slightly higher number of cell

cycles than those on synthetic glucose, therefore passing through more generations. Although an effort was made in these experiments to pick uniformly-sized small colonies, it is possible that, on average, the colonies from YPD plates had slightly more cells than the colonies taken from synthetic glucose plates. Though the overall aging rate on synthetic glucose versus YPD rich media were slightly different, they exhibited overlapping standard deviations and were therefore not statistically distinguishable.

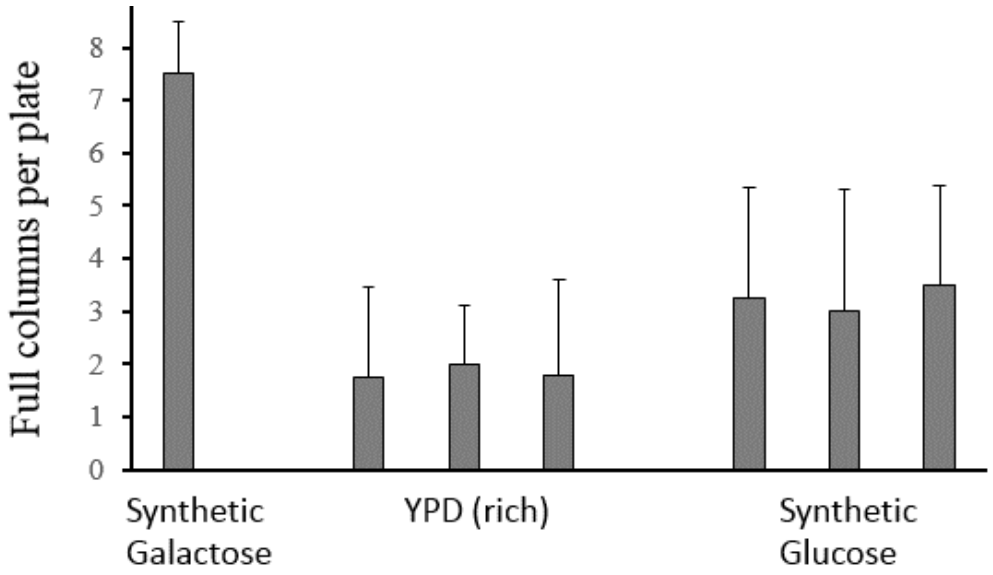


Figure 11. The number of full columns of growth on the 4th streak for telomerase-deficient yeast cells growth in different media. Error bars indicate standard deviations. Results of 3 separate trials shown for YPD and synthetic media.

A second experiment was designed to measure senescence using telomerase-deficient yeast cells at room temperature, 30°C and 38°C. This experiment used only synthetic glucose plates and our new senescence assay. It was found that the yeast cells

had different growth rates at each temperature used. To illustrate this, streaked cells needed 3 days for development of small colonies when they were grown at 30°C. In contrast, they needed about 4 days at 38°C, and 5-6 days at room temperature. The results of the third and fourth streaks are shown in Table 4. The average number of full columns per plate at room temperature and at 30°C for the third streak were 7.5 and 7.8, respectively. For the fourth streak, the average number of full columns per plate was 3.0 at room temperature and 2.3 at 30°C, demonstrating senescence. On the other hand, at 38°C the average number of full columns per plate dropped to 3.5 on the third streak and 0.3 on the fourth streak (shown in boldface in Table 4). This indicated that cells senesced faster at 38°C. The reasons for this acceleration are unclear, but might be because of an increase in oxidative stress and / or may be related to the heat shock response experienced by the cells at this temperature.

Table 4. Effect of temperature on senescence of telomerase-deficient yeast cells.

	YLKL803 (3rd streak)			YLKL803 (4th streak)		
	RT	30°C	38°C	RT	30°C	38°C
Full Columns	45/48	39/40	21/48	18/40	14/48	2/48
Avg. per Plate (#/8)	7.5	7.8	**3.5**	3.0	2.3	**0.3**
Std. Deviation	0.8	0.4	2.4	2.3	1.4	0.5
Median	8.0	8.0	3.5	3.0	2.5	0.0

The results in Table 4 are shown graphically in Figure 10. The number of full columns per plate at 30°C and RT was similar on the 3rd (12A) and the 4th streaks (12B).

However, senescence was accelerated at 38°C when compared to the other two temperatures.

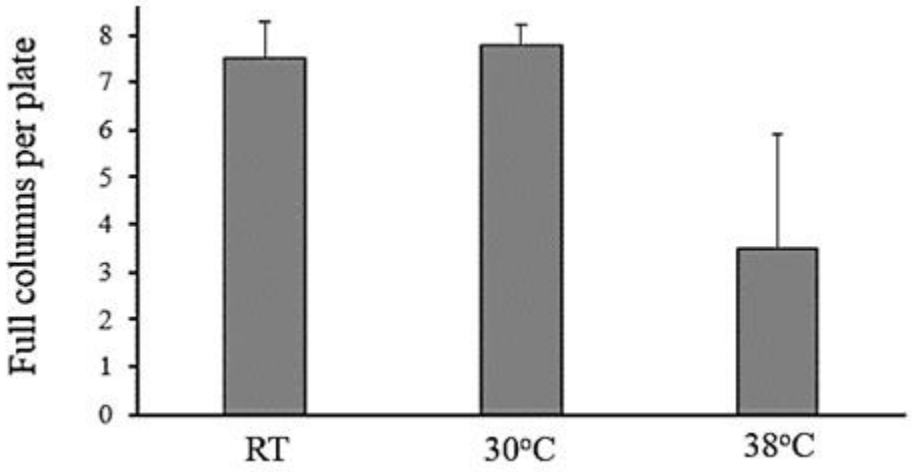


Figure 12A. The number of full columns of growth on the 3rd streak for telomerase-deficient yeast cells grown at different temperatures.

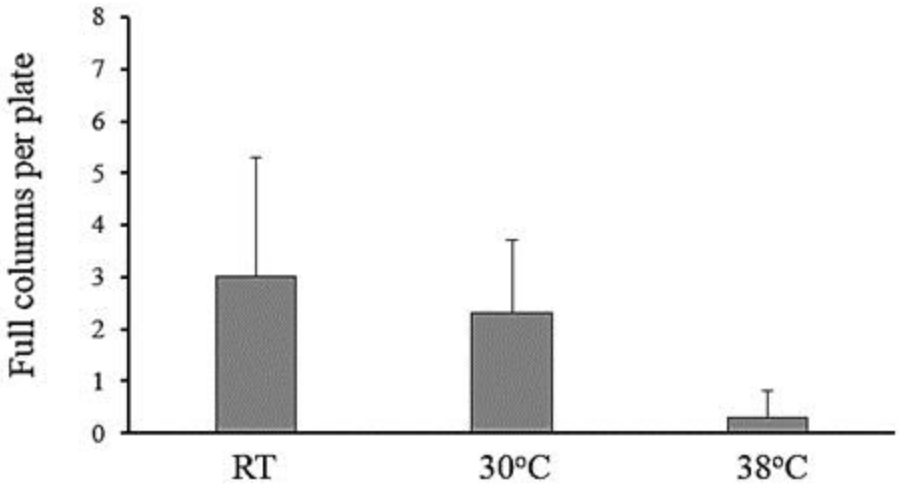


Figure 12B. The number of full columns of growth on the 4th streak for telomerase-deficient yeast cells per plate at different temperatures.

Representative plates for the third streak at each temperature are pictured in Figure 13.

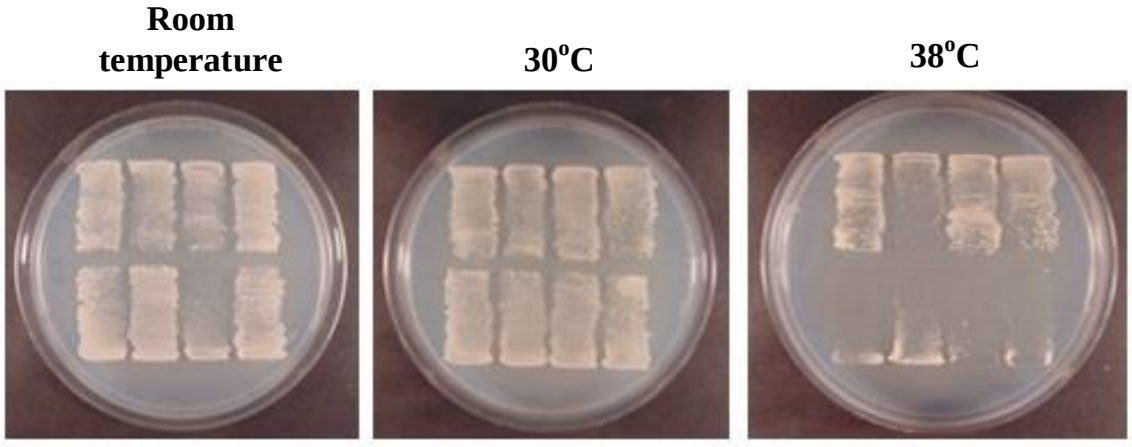


Figure 13. Representative 3rd streak plates of YLKL803 grown at different temperatures.

As previously mentioned, telomeres get shorter after each cell cycle. However, telomeres have similar sequences and undergo genetic exchange with each other. Shorter chromosomes can exchange DNA with longer chromosomes, resulting in an increase in length of the shorter chromosome (Figure 14). This process is called homologous recombination, and it helps to retard aging rates, but is not enough to terminate the shortening process (22, 24). This homologous recombination process is mediated by the RAD52 group of proteins which include *RAD50, RAD51, RAD52, RAD55, RAD54, RAD57, RAD59, MRE11, XRS2* and *RDH54*. It has been shown that inactivation of the *RAD52* gene leads cells to undergo senescence more rapidly when compared to wild-type cells. WT cells underwent senescence after approximately 60-70 generations, while *rad52*

mutants grew for only about 40 generations and then stopped dividing, indicating that *RAD52* plays an important role in telomere shortening and senescence (22, 25).

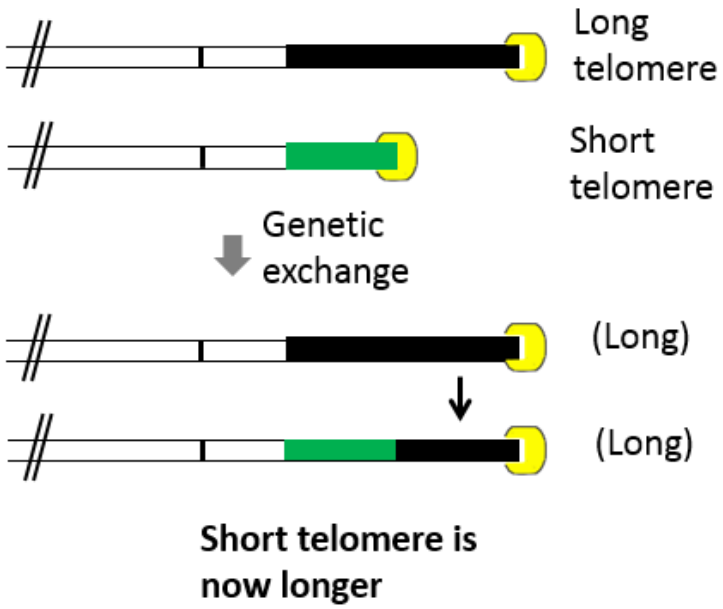


Figure 14. Telomeres undergo genetic exchange (homologous recombination) that slows telomere shortening. Unequal exchange can lead to lengthening of a short telomere.

In the current project, *rad52* mutant cells were tested to determine how the actual aging rate is affected using our new senescence assay. Furthermore, we wanted to determine how different temperatures affect the senescence of *rad52* mutants. This experiment was performed using synthetic glucose plates and three different temperatures: 30^{o}C, 38^{o}C, and room temperature.

The experimental results testing senescence of *rad52* mutants are shown in Table 5. The number of full columns was analyzed, as well as the average number of full columns of growth per plate, standard deviation, and the median number of full columns

of growth per plate. Although wild-type cells exhibit senescence on the 4th streak (after 60-70 generations), *rad52* cells display senescence on the 3rd streak (~40 generations) at 30°C. This can be seen in Table 4 where wild-type cells formed 7.8 full columns per plate on the 3rd streak at 30°C, and Table 5, where *rad52* cells produced only 1.0 full column per plate. The average number of *rad52* mutant columns per plate was reduced (2.7, 1.0, and 0.5) as the temperature increased (RT, 30°C and 38°C).

Table 5. Effect of temperature on senescence of *rad52* mutant yeast cells.

	YLKL807 (3rd streak)		
	RT	30°C	38°C
Full Columns	16/48	6/40	3/48
Avg. per Plate (#/8)	2.7	**1.0**	**0.5**
Std. Deviation	1.8	1.3	0.8
Median	3.5	0.5	0.0

The results with *rad52* mutants at RT, 30°C and 38°C are shown graphically in Figure 15. As seen previously, plates that produced only a small number of full columns per plate exhibited standard deviations that were relatively high compared to the means. Standard deviations at RT and at 38°C overlapped vs the standard deviation error bars at 30°C. Representative plates of *rad52* mutants on the third streak at each temperature are pictured in Figure 16.

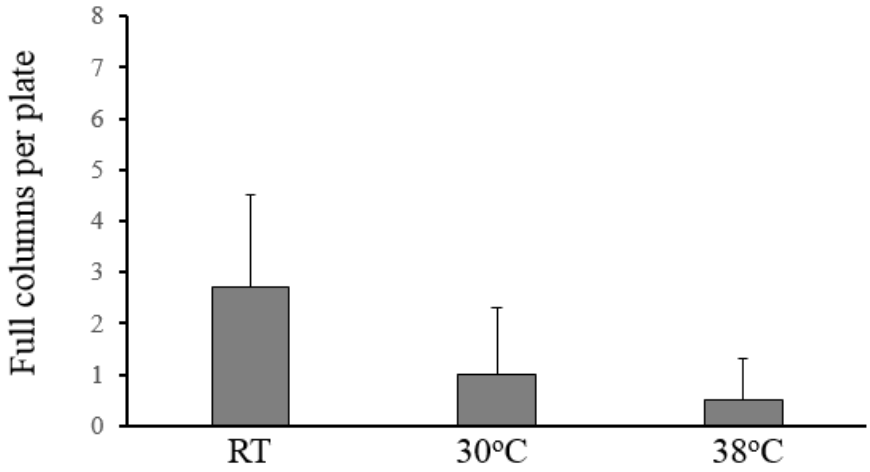


Figure 15**.** The number of full columns per plate produced by *rad52* mutant yeast cells on the 3rd streak.

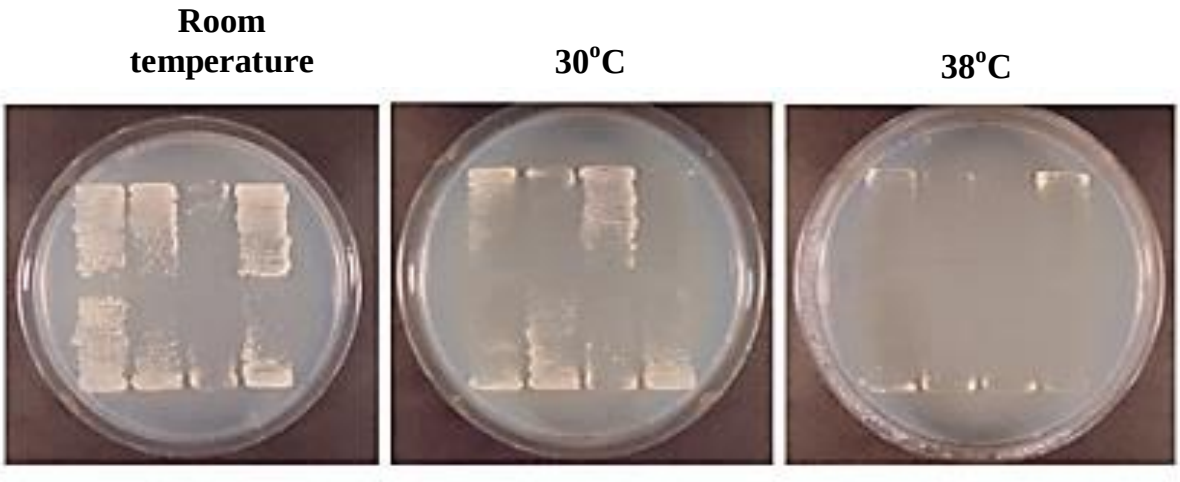


Figure 16. Representative 3rd streak plates of *rad52* mutant cells grown at different temperatures.

Rates of telomere shortening can be affected by many factors, which include nutrient levels and carbon source availability. Past studies have demonstrated that nutrition levels and lifestyle are associated with telomere length in humans and animals

(26). In addition, telomerase activity in peripheral blood mononuclear cells can be modulated by modifying diet and lifestyle (27).

We tested the effect of reducing nutrients on senescence in yeast using different concentrations of YPD-rich media. The results of third and fourth streaks are shown in Tables 6 and 7. All nutrients used to make YPD plates were reduced to 1/5 and 1/20 of normal YPD media nutrient concentrations. Telomerase-deficient yeast cells were grown at 30^{o}C, with those grown on the lowest concentration YPD plates taking 5-6 days to form colonies, versus 3-4 days on standard YPD plates. The average full columns per plate on the third streak as the nutrients were reduced were 6.9, 6.6, and 6.0, respectively, for YPD, 1/5 YPD and 1/20 YPD (Table 6). The average full columns per plate on the fourth streak were 1.9, 0.9, and 3.1, respectively.

Table 6. Effect of nutrient concentration on senescence of telomerase-deficient yeast cells (3rd streak).

	YLKL803 (3rd streak)		
	YPD	1/5 YPD	1/20YPD
Full Columns	69/80	66/80	60/80
Avg. per Plate (#/8)	6.9	6.6	6
Std. Deviation	1.5	1.8	1.8
Median	7	7.5	7

Table 7. Effect of nutrient concentration on senescence of telomerase-deficient yeast cells (4th streak).

	YLKL803 (4th streak)		
	YPD	1/5 YPD	1/20YPD
Full Columns	19/80	9/80	31/80
Avg. per Plate (#/8)	1.9	0.9	3.1
Std. Deviation	1.3	0.7	2.3
Median	2.0	1.0	2.0

The average increased slightly on the fourth streak as the nutrient concentration decreased. Overall, all of the plates had similar average full columns per plate and exhibited overlapping standard deviations, indicating that lowering nutrients did not strongly affect senescence.

A similar experiment was also performed to measure the effect of lowering nutrient concentration on senescence in *rad52* mutant cells. Experimental conditions were the same as previously described for wild-type cells. Colonies from *rad52* mutants took longer to form in this experiment, as *rad52* cells take longer to divide when compared to WT cells. Results of *rad52* cells' third streak with normal and reduced YPD nutrient concentration are shown in Table 8. When cells were grown on YPD plates with normal nutrient concentration, the number of full columns was 7/48 and the average per plate was 1.2. In contrast, when cells were grown on 1/5 and 1/20 YPD plates, the number of full columns per plate was 4.2 and 5.3, respectively. The number of full columns per plate

increased about four-fold when nutrients were lowered. Standard deviations overlapped for 1/5 YPD, but not 1/20 YPD plates. Moreover, the result of the Student's T-test indicated that the differences between normal YPD concentration and lowered concentration were significante.

Table 8. Effect of nutrient concentration on senescence of *rad52* mutants.

	rad52 (3rd streak)		
	YPD	1/5 YPD	1/20 YPD
Full Columns	7/48	25/48	32/48
Avg. per Plate (#/8)	1.2	**4.2**	**5.3**
Std. Deviation	1.3	2.1	2.0
Median	1.0	5.0	6.0
Student's T-test		p=0.015	p=0.002

In addition to the *RAD52* gene, *RAD51* is also critical to the homologous recombination process (28). The low-nutrient experiment with *rad52* mutant cells was repeated along with *rad51* mutant cells to confirm that results were reproducible. Since a reduction to 1/20 normal concentration had the greatest effect, it was used alongside the normal concentration in order to measure how aging rates were affected, and to see if it would give similar results to what were observed in the previous experiment. Results of the third streak for both mutants are shown in Table 9. The average per plate of *rad52* and *rad51* cells with 1/20 reduced nutrient concentration was 4.5 and 3.8, respectively, versus

1.8 and 1.7 for cells grown on standard YPD plates. These averages were similar to each other and support what we observed in the previous experiment. These results for *rad52* and *rad51* suggest that lowering the nutrient concentration can slow down aging in these mutants, but once again the results did not reach significance as standard deviations were overlapping.

Table 9. Effect of nutrient concentration on senescence of *rad51* and *rad52* mutants cells.

	rad52 (3rd streak)		*rad51* (3rd streak)	
	YPD	1/20YPD	YPD	1/20YPD
Full Columns	11/48	27/40	10/48	23/48
Avg. per Plate (#/8)	1.8	**4.5**	1.7	**3.8**
Std. Deviation	1.3	2.9	1.9	1.7
Median	2.0	4.5	1.5	3.5

Caloric restriction has been shown to increase lifespan in almost all animals tested. This is the most proven mechanism for making any animal live longer. If they eat approximately 30-40% fewer calories per day they live about 20% longer (29, 30). Our next goal was to determine if glucose percentage could affect the actual aging rate of telomerase-deficient yeast cells. Glucose is the major carbon source in YPD media. This experiment was performed at 30^{o}C using YPD-rich media plates containing four different glucose percentages: 2.0%, 0.5%, 0.05% and 0.01%. All other nutrients were maintained at standard amounts. Results for the third and fourth streaks at different concentrations are

presented in Table 10. The average full columns per plate on the 3rd streak of telomerase-deficient yeast cells as the glucose percentages were reduced were 6.75 (2.0%), 6.5 (0.5%), 6.75 (0.05%), and 5.5 (0.01%), indicating that 3rd streaks were not greatly affected. The average per plate for the fourth streaks were 0.25 (2.0%), 0.25 (0.5%), 3.75 (0.05%), and 2.0 (0.01%). On 0.05% glucose, the average per plate increased by approximately 3.5-fold, and approximately 2 fold with 0.01% glucose (shown in boldface in Table 10). The higher number of full columns on the fourth streak at the two lower glucose concentrations (as compared to the standard 2% glucose concentration) suggested that reducing the glucose might positively affect the rate of telomere shortening. Student's T-tests were not consistent, with p=0.004 and p=0.114 at the lowest concentrations.

Table 10. Effect of glucose concentration on senescence of telomerase-deficient yeast cells.

	YLKL803 (3rd streak)				YLKL803 (4th streak)			
	2% Glu	0.5% Glu	0.05% Glu	0.01% Glu	2% Glu	0.5% Glu	0.05% Glu	0.01% Glu
Full Columns	27/32	26/32	27/32	22/32	1/32	1/32	15/32	8/32
Avg. per Plate	6.75	6.5	6.75	5.5	0.25	0.25	**3.75**	**2.0**
Std. Deviation	2.5	1.3	1.0	1.3	0.5	0.5	1.5	1.8
Median	8.0	6.5	6.5	5.5	0.0	0.0	3.0	2.0
Student's T-test						p=1.0	p=0.004	p=0.114

The experiment was repeated with 2.0% and 0.05% glucose to test whether the previous results were reproducible. The results of the third and fourth streaks are shown in

Table 11. The third streak average with 2% glucose was 7.8 and with 0.05% glucose it was 8.0. The averages on the fourth steak with 2% and 0.05% glucose were 1.4 and 5.5 full columns per plate, respectively, with nonoverlapping standard deviations. The fourth streak averages using 0.05% glucose observed in this experiment (5.5) and the previous experiment (3.75) with p values less than 0.05 strongly suggest that the shortening process of telomeres was slowed down when the percentage of glucose was reduced.

Table 11. Effect of glucose concentration on senescence of telomerase-deficient yeast cells (2nd experiment).

	YLKL803 (3rd streak)		YLKL803 (4th streak)	
	2% Glu	0.05% Glu	2% Glu	0.05% Glu
Full Columns	47/48	48/48	7/48	33/48
Avg. per Plate (#/8)	7.8	8.0	1.4	**5.5**
Std. Deviation	0.4	0.0	0.9	2.1
Median	8.0	8.0	2.0	5.0
Student's T-test				p=0.003

The number of full columns per plate on the 4th streak for both experiments are shown in Table 10 and 11 and graphically shown in Figures 17 and 18.

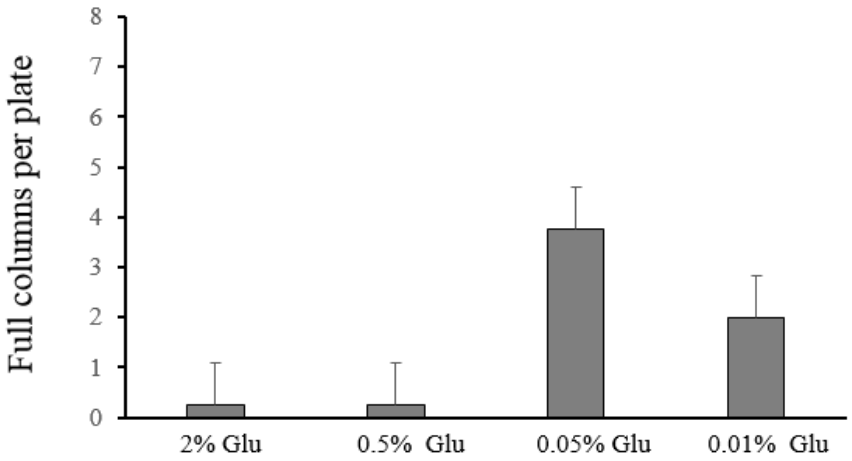


Figure 17. The number of full columns per plate on the 4th streak observed with telomerase-deficient YLKL803 cells grown with different glucose concentrations.

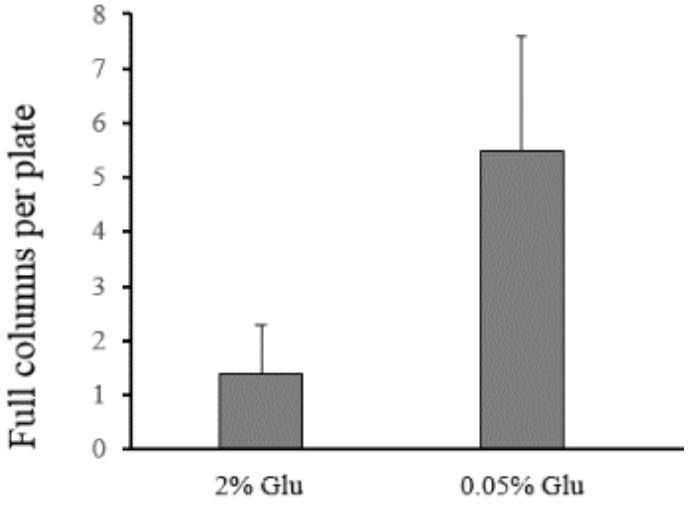


Figure 18. The number of full columns per plate on the 4th streak using plates with different glucose concentrations (second experiment).

Homologous recombination is a method of genetic exchange used by the cell to repair double-stranded breaks in DNA. It requires a homologous chromosome as a template for repair and for this reason it is an error-free pathway. There are several genes in the RAD52 group, all of which are involved in homologous recombination, though some are more critical than others. As mentioned earlier, *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11, XRS2,* and *RDH54* compose this RAD52 group (31). *RAD50, MRE11,* and *XRS2* are involved in both major pathways of DNA double-strand break repair: non-homologous end-joining (NHEJ) and homologous recombination (HR). All other genes are believed to be involved only in HR. As chromosome ends shorten, DNA exchange occurs between the nearly identical DNA sequences found at the telomeres. These DNA exchanges in the telomeric regions occur using only HR, which can delay cell senescence (22).

The roles of all known RAD52 group genes affecting only HR in senescence was tested next. It was necessary to construct new senescence strains with *RAD55, RAD59* and *RDH54* knocked out, but all other RAD52 group mutants were available in the Lewis lab strain collection. For *RAD59* and *RDH54* knockouts, the $G418^r$ gene from the plasmid pFA6MX4 was first PCR-amplified using primers containing homologous sequences at the end of each gene of interest (Figure 19). Each PCR product was resolved using gel electrophoresis in order to ensure that the correctly sized fragment was obtained (bottom left side of Figure 19).

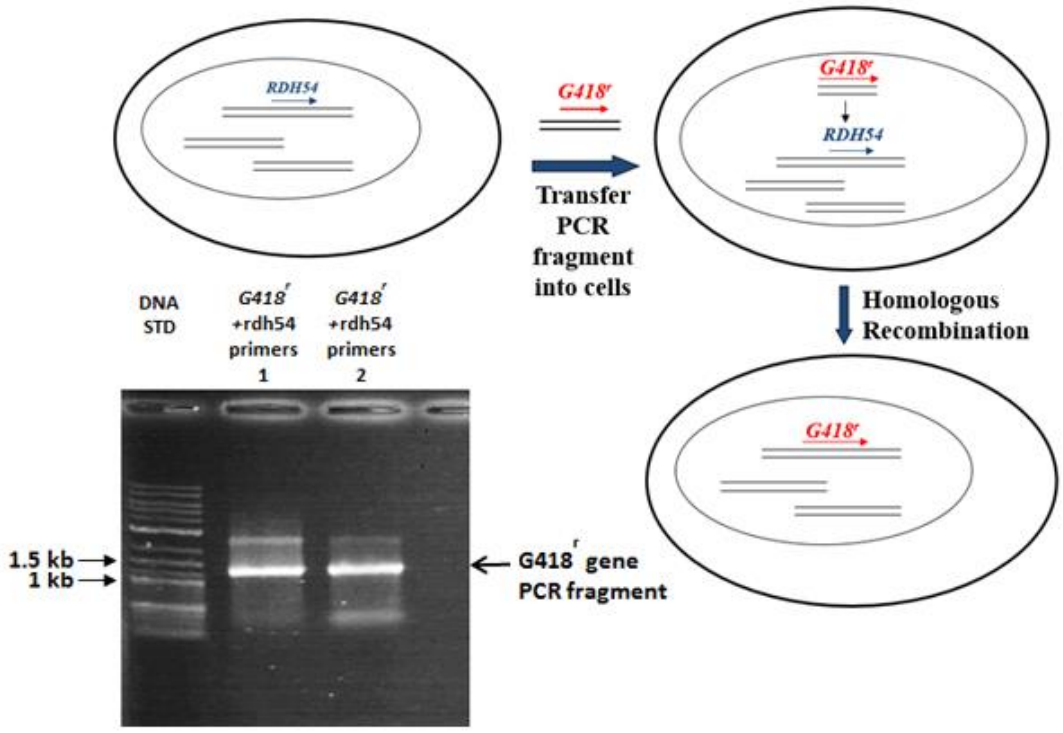


Figure 19. PCR amplification of the G418^r gene using *RDH54* primers and inactivation of a gene (*RDH54*) by insertion of an antibiotic resistant marker (G418^r).

After confirming that PCR amplification of pFA6MX4 produced the desired PCR products, they were transformed into telomerase-deficient YLKL803 cells using the stationary phase protocol of Tripp *et al* (19) (Figure 19). Proper integration of DNA was tested by first selecting on YPDA with G418 plates. The correct insertion was verified using PCR with test primers for each gene, then resolved using agarose gel electrophoresis. The size of the PCR fragments of each knocked out gene were compared with WT gene sizes to confirm that the correct size of inactivated gene was obtained. The gel for knockout confirmation of two isolates of *rdh54::G418*^{r} is shown in Figure 20. The WT size is 3056 bp and the size of PCR products made from *rdh54::G418*^{r} DNA is 2500

bp. The lower band appearing at ~ 1500 bp is a nonspecific product appearing in all PCR reactions.

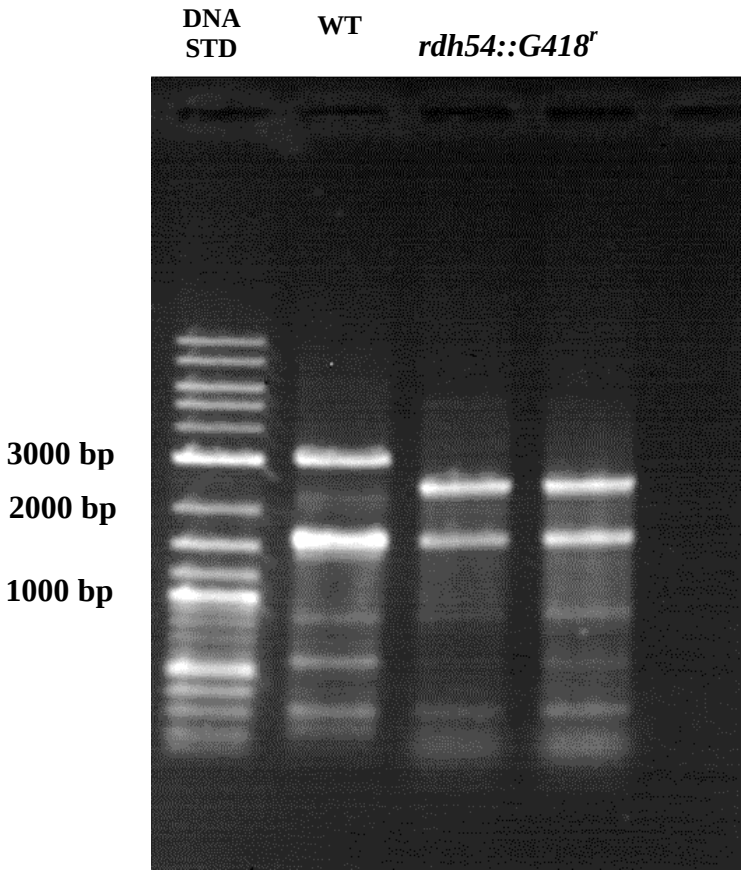


Figure 20. A 0.7% agarose gel run to confirm PCR knockout of *RDH54*.

All genes that are involved in homologous recombination were tested to investigate their effect on senescence. This experiment was performed at 30^{o}C, and all mutants were tested in parallel with WT cells to compare their rates directly to the normal aging rate. The average of full columns per plate on the 3rd streak of repair-proficient WT cells was 7.5 (Table 12). The averages per plate of *rad52*, *rad51*, and *rad54* cells were 0.7, 1.2 and 1.0, respectively (shown in boldface in Table 12). These averages are much lower

than those for the WT cells with nonoverlapping standard deviations. Average full columns per plate for *rad55, rad57, rad59,* and *rdh54* cells were 3.7, 2.7, 3.8 and 5.2, respectively. These averages are low when compared to WT, but not as low as those seen for *rad52*, *rad51*, and *rad54* cells. Based on the third streak results of this experiment, the mutants can be divided into two broad groups: those that are highly important for HR between telomeres and those of lower importance. The most important genes are *RAD52, RAD51* and *RAD54*, while *RAD55, RAD57, RAD59* and *RDH54* appear to be less important. The standard deviations calculated for all mutant averages did not overlap with those of WT cells, except in the case of *rad59* cells. In addition, the p values for all mutants were less than 0.05.

Table 12. Effect of different RAD52 group mutations on senescence.

	3rd streak							
	WT	rad51	rad52	rad54	rad55	rad57	rad59	rdh54
Full Columns	45/48	7/48	4/48	6/48	44/96	27/80	23/48	31/48
Avg. per Plate (#/8)	7.5	**1.2**	**0.7**	**1.0**	3.7	2.7	3.8	5.2
Std. Deviation	0.55	1.8	1.2	1.1	1.9	2.2	3.6	1.6
Median	7.5	0.0	0.0	1.0	3.5	2.5	4.0	4.5
Student's T-test		p=0.00	p=0.00	p=0.00	p=0.00	p=0.00	p=0.03	p=0.01

Oxidative stress is another factor that may affect the rate of telomere shortening and senescence. DNA damage or mutations caused by reactive oxygen species (ROS) can

lead to this stress and accelerate cellular senescence in human fibroblasts (32, 33). During normal cellular metabolism, especially mitochondrial respiration, ROS are produced. Major sources of cellular oxidation are the three common ROS inside cells: superoxide radicals, peroxyl radicals, and hydroxyl radicals. These free radicals react, causing damage to biomolecules such as DNA, proteins, and lipids. DNA oxidative damage is elevated with increasing production of ROS. DNA damage is increased by two factors: decreasing of DNA repair capacity and promotion of reactive oxygen species production during aging. However, antioxidant proteins can protect DNA from oxidative damage that is caused by ROS. Examples are the Ctt1 and Gpx3 proteins. *CTT1* encodes cytosolic catalase T, an enzyme where expression increases in response to oxidative stress. Ctt1 has an important role in detoxification of hydrogen peroxide by breaking it down into dioxygen and water molecules (34). *GPX3* (also called *HYR1*) encodes a thiol peroxidase, which acts a sensor of peroxide inside cells, acting as a peroxidase and a signaling molecule (35).

In the current project, three different senescence strains with different antioxidant genes inactivated were used to measure the effect on senescence. The experiment was performed at two different temperatures, 30^{o}C and 38^{o}C, using synthetic glucose media. The strains used were YLKL820 with *CTT1* inactivated, YLKL821 with *GPX3* (*HYR1*) inactivated, and antioxidant double mutant YLKL829 with both *CTT1* and *GPX3* (*HYR1*) inactivated. The results of the 4th streak at 30^{o}C of each mutant compared to WT cells are shown in Table 13. The expected result was to accelerate senescence in both single and double mutants because of increasing DNA damage due to oxidation by ROS radicals. However, as shown in the table below, the average number of full columns per plate with

each of the mutants was similar to that of WT cells (2.7 for WT cells and 4.3, 2.7 and 4.1 for each of the mutants with overlapping standard deviations).

Table 13. Effect of different antioxidant gene mutations on senescence of cells at 30°C.

4th streak at 30°C	WT	*ctt1*	*gpx3 (hyr1)*	*ctt1 gpx3*
Full Columns	32/96	52/96	27/96	49/96
Avg. per Plate (#/8)	2.7	4.3	2.3	4.1
Std. Deviation	2.3	3.4	1.7	1.7
Median	2.5	5	2.5	4.0

The experiment was done using the same strains with the exception of using 38°C instead of 30°C and the results of the 4th streak are shown in Table 14. There were clear decreases in the average number of full columns per plate at 38^0C comparing to 30°C. However, the averages of all three mutant strains were similar to that of WT cells (0.5 for WT cells versus 0.3, 0.5 and 1.2 for the mutants).

Table 14. Effect of different antioxidant mutations on senescence of cells at 38°C.

4th streak at 38°C	WT	*ctt1*	*gpx3* (*hyr1*)	*ctt1 gpx3*
Full Columns	3/48	2/48	3/48	7/48
Avg. per Plate (#/8)	0.5	0.3	0.5	1.2
Std. Deviation	0.8	0.8	0.5	0.8
Median	0.0	0.0	0.5	1.0

In growing cells a cell cycle checkpoint is activated when there is damaged DNA to give a chance for the DNA to be fixed before starting a new cell cycle. Cycling yeast cells exposed to DNA damaging agents pause cycling predominantly in G_2 phase. This is important to avoid going through mitosis (M phase) with damaged or broken chromosomes. This project focused on two checkpoint genes, *RAD24* and *MEC3*, that are important in the DNA damage-induced cell cycle checkpoint pathway. The results of 4th streak testing of *rad24, mec3,* and *rad24 mec3* double mutants at 30°C using synthetic glucose media are shown in Table 15. We speculated that with checkpoint mutant cells, the damaged cells would not arrest in G_2 phase and keep dividing and therefore go through generations faster than WT cells, which would lead to acceleration of the shortening process. However, as shown in Table 15, the averages for *rad24, mec3* and double mutant cells on the 4th streak were 4.8, 5.2 and 4.3, respectively, versus 1.3 for WT cells. The results indicated an increase in the average with mutant cells, which was not expected.

Table 15. Effect of different checkpoint mutations on senescence of cells at 30°C.

4th streak at 30°C	WT	*rad24*	*mec3*	*rad24 mec3*
Full Columns	16/96	58/96	62/96	52/96
Avg. per Plate (#/8)	1.3	**4.8**	**5.2**	**4.3**
Std. Deviation	1.3	2.6	1.6	2.5
Median	1.0	4.5	5	4.0

The three checkpoint mutants were also tested at 38°C along with WT cells and the results of 3rd and 4th streaks are shown in Tables 16 and 17, respectively. The averages of the 3rd streaks of the mutant cells were 8.0, 7.3 and 8.0, which were similar to the WT cells average of 6.4. There was a slight increase in all mutant cells in the 4th streaks compared to WT cells. As shown in Table 17, the average per plate of the mutants was 3.0, 2.2 and 2.5 which were similar to each other and higher than WT cells, which had an average of 0.0.

Table 16. Effect of different checkpoint mutations on senescence of cells after the 3rd streak at 38°C.

3rd streak at 38°C	WT	*rad24*	*mec3*	*rad24 mec3*
Full Columns	32/40	48/48	44/48	48/48
Avg. per Plate (#/8)	6.4	8.0	7.3	8.0
Std. Deviation	1.3	0.0	1.2	0.0
Median	7.0	8.0	8.0	8.0

Table 17. Effect of different checkpoint mutations on senescence of cells after the 4th streak at 38°C.

4th streak at 38°C	WT	*rad24*	*mec3*	*rad24 mec3*
Full Columns	0/48	18/48	13/48	15/48
Avg. per Plate (#/8)	0.0	**3.0**	**2.2**	**2.5**
Std. Deviation	0.0	1.4	1.8	1.4
Median	0.0	3.0	2.0	3.0

Methyl methanesulfonate (MMS) is a commonly used DNA alkylating agent believed to cause DNA double-strand breaks (DSBs). DSBs are known to stimulate the homologous recombination rate inside cells. We speculated that low dose treatment of cells with MMS might stimulate recombination between telomeres and reduce the rate of telomere shortening and senescence. MMS at different concentrations in synthetic glucose

media was used to test its effect on senescence of telomerase-deficient yeast cells. This experiment was performed at 30^{o}C using two different non-lethal concentrations of MMS (0.1 mM and 1 mM) alongside plates with no MMS added. The results of 3rd and 4th streaks are shown in Table 18. On the 3rd streaks, the average numbers of full columns per plate were similar on plates with and without MMS. On the 4th streaks, the averages were also quite similar (2.4, 2.4 and 2.9). The similar averages indicated that there was no notable effect of MMS on the aging rate of telomerase-deficient yeast cells.

Table 18. Effect of different MMS concentrations on senescence of telomerase-deficient yeast cells.

	YLKL803 (3rd streak)			YLKL803 (4th streak)		
	No MMS	0.1mM MMS	1mM MMS	No MMS	0.1mM MMS	1mM MMS
Full Columns	70/80	63/72	65/72	24/80	24/80	29/80
Avg. per Plate	7.0	7.0	7.2	2.4	2.4	2.9
Std. Deviation	1.1	1.5	1.0	2.0	2.4	0.5
Median	7.0	8.0	8.0	3.0	2.0	2.1

The next experiments were done to calculate how many generations the cells went through before they underwent senescence. When a single cell on the surface of a plate begins dividing, it goes from 1 cell to 2 cells, and then 2 cells become 4, 4 cells become 8, and so on (Figure 21). The process continues until there are more than a million cells and a small colony becomes visible on the plate. The total number of cells present in the

colony after a given number of generations can be calculated because it follows a simple equation: 2^y = total cells, where y is the number of generations or cell cycles that have occurred. Thus, after 2 generations there are $2^2 = 4$ total cells and after 20 generations there are 2^{20} or 1,048,576 cells. Table 19 shows the total number of cells per colony for each generation. Using the relationships displayed in Table 19, it is possible to count all of the cells in a colony and calculate how many generations of growth have occurred up to that point in time. For example, if a colony is found to have 1,048,576 cells, then it must have undergone exactly 20 cell cycles. Total cell counts that do not match the number in Table 19 exactly indicate that some number of generations plus a fraction of another generation have occurred. Thus, a colony that has a total number of cells between 524,288 and 1,048,576 has undergone 19 generations plus a fraction of one more generation. This fraction can be calculated. For example, a colony with 786,432 cells grew 19 generations to get to 524,288 and then added another 262,144 cells (524,288 + 262,144 = 786,432). To go from the 19^{th} to the 20^{th} generation, cell number must increase from 524,288 to 1,048,576 (Table 19), which is a total increase of 524,288 cells. The example colony did not add 524,288 cells beyond generation 19; instead, it only added 262,144 cells. The fraction of the 524,288 cells needed to get to generation 20 that is contained was 262,144/524,288 = 0.5, or half of another generation. Thus, a colony with 786,432 cells has undergone 19.5 generations.

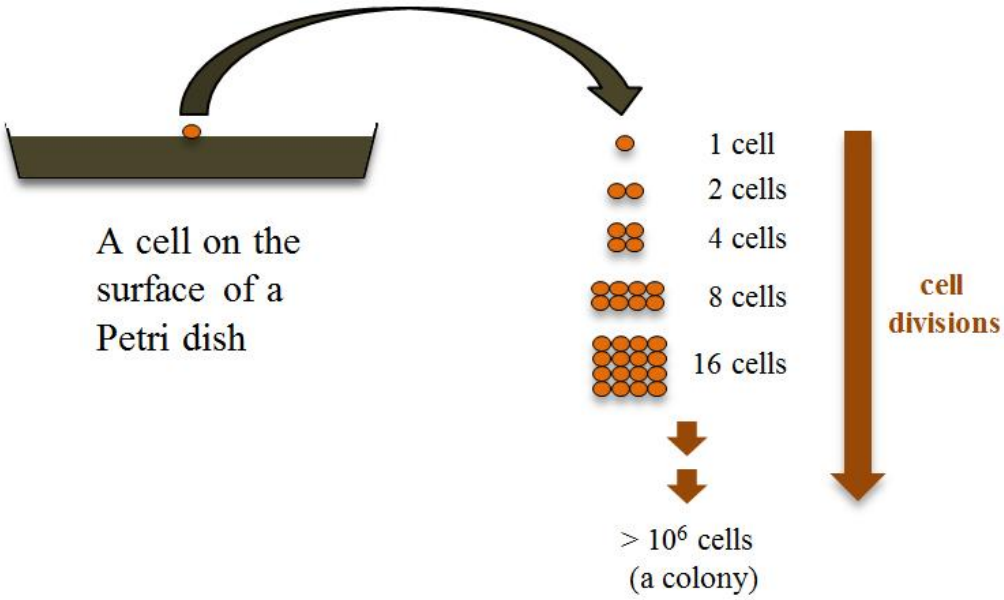


Figure 21. A single cell divides many times to form a colony.

Table 19. The total number of cells per colony for each generation of growth.

Number of generations	Total number of cells per colony
1st generation	2
2nd generation	4
.	.
.	.
.	.
15th generation	32,768
16th generation	65,536
17th generation	131,072
18th generation	262,144
19th generation	524,288
20th generation	1,048,576
21st generation	2,097,152

For this project a method was developed to harvest all of the cells within individual colonies and count them. Long (9 inch) Pasteur pipettes were held vertically over colonies and then pushed into the agar until the bottom of the plastic plate was reached (Figure 22). Pulling the pipette out of the plate resulted in removal of the entire colony plus a small amount of plate agar. The cells plus agar plug were transferred to a 1.5 ml microfuge tube containing 0.5 ml ddH_2O. The cells were vortexed, sonicated and the titer determined using a hemocytometer.

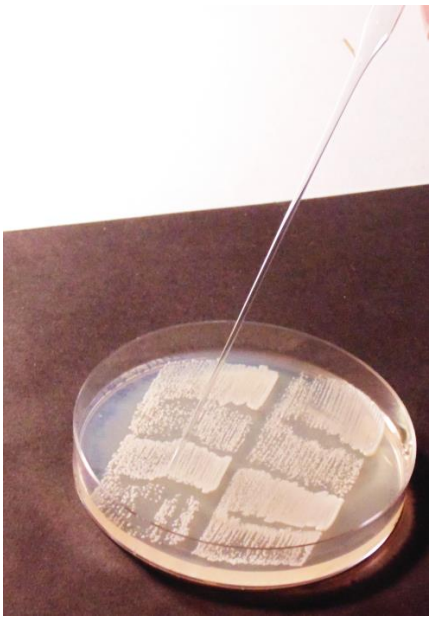


Figure 22. Using a Pasteur pipette to harvest a single colony on a plate surface.

Five colonies from each plate type, YPD and 1/20 YPD, were harvested to count the total number of cells and the number of generations. The results of counting the number of generations of telomerase-deficient cells for each streak using YPD and 1/20 YPD plates (shown previously in Tables 6, 7, 8 and 9) are shown in Table 20. The total

number of generations that cells went through after 3 streaks was 58.1 generations when the cells were grown on normal YPD plates. In contrast, using 1/20 YPD concentration, the number of generations was 57.1. The difference between the number of generations of both types of plates was only one generation and does not appear to represent a big difference. This finding is consistent with the observation that lowering nutrient concentrations did not greatly affect senescence in these cells (Tables 6 and 7). When the same experiment was done with *rad52* cells, the number of generations after 2 streaks was 38.1 with normal YPD and 36.6 with 1/20 YPD, as shown in Table 21, indicating a slightly larger difference. Reducing total nutrients strongly slowed senescence in *rad52* mutants (Table 8 and 9). The modestly lower number of generations of growth on 1/20 YPD plates after 2 streaks is likely to be a contributory factor. Since the cells had undergone fewer generations after 2 streaks, they were able to grow more on the 3rd streak plates, i.e., they formed more full columns on each plate.

Table 20. The number of generations of growth undergone during each streak of telomerase-deficient yeast cells.

	YLKL803					
	YPD			1/20 YPD		
	1st streak	2nd streak	3rd streak	1st streak	2nd streak	3rd streak
Avg. number of cells per colony	8.9×10^5	9.3×10^5	4.25×10^5	7.0×10^5	8.4×10^5	3.2×10^5
Number of generations	19.7	19.8	18.6	19.3	19.6	18.2
Total generations		58.1			57.1	

Table 21. The number of generations of growth achieved during each streak of telomerase-deficient *rad52* mutants.

	rad52 mutants			
	YPD		1/20 YPD	
	1st streak	2nd streak	1st streak	2nd streak
Avg. number of cells per colony	9.1×10^5	3.4×10^5	3.9×10^5	2.4×10^5
Number of generations	19.8	18.3	18.5	18.1
Total generations	38.1		36.6	

CHAPTER IV

SUMMARY AND CONCLUSIONS

Eukaryotic chromosomes consist of several features and the focus of this project was on the telomeric regions. As a result of the end replication problem in linear chromosomes, the chromosomes lose some genetic information each cell cycle and undergo telomere shortening. It has been shown that telomere length can be affected by lifestyle factors and therefore may have an effect on the health and lifespan of a person. In a study of older humans looking at the lengths of telomeres in their blood cells, it has been found that people who are in the lowest 20% of telomere lengths have 3-fold higher mortality from heart disease and a 2-fold increase in overall mortality. Humans of the same age can have telomeres with different lengths and individuals with shorter telomeres are likely to die earlier (36).

Many studies have measured senescence in yeast using a simple streaking method (22, 23). Most cells undergo senescence in approximately 60-70 generations. However, senescence is a stochastic process and the growth on the fourth streak is highly variable. One of the main goals of this project was to develop a new senescence assay to quantitate senescence in yeast that incorporates good statistics by expanding the number of column assays on the 3^{rd} and 4^{th} streaks to 48 separate assays in order to reduce variability previously associated with the fourth streak. This approach differs from one taken in a recent study by Ballew and Lundblad (37). In that work, heterozygous *TLC1/tlc1* diploids were sporulated and the resulting haploids streaked for single colonies again and again

until senescence. The final streaked colony plates were then evaluated and scored on a scale of 1 to 6 to produce relative senescence scores, usually from 20-35 isolates (37).

The second goal of this project was to investigate how different growth media, temperatures, nutrient concentrations and carbon source availability might affect senescence in yeast. The overall aging rates on synthetic glucose versus rich YPD plates were similar with overlapping standard deviations. Growing WT cells at different temperatures (30^{o}C, 38^{o}C and room temperature) to measure their effect on senescence indicated that cells senesced faster at 38^{o}C. On the other hand, the aging rate of *rad52* mutants also increased as the temperature increased. Measuring the effect of nutrient concentration on senescence of wild-type, *rad51* and *rad52* mutants cells indicated that lowering total nutrients did not strongly affect senescence of WT cells. However, the results for *rad51* and *rad52* mutants suggested that lowering the nutrient concentration could slow down the aging rate. Reducing the concentration of glucose, the major carbon source in YPD media, from 2% to 0.05% consistently resulted in more full columns of growth on the 4th streak, suggesting that the senescence rate was reduced. Future studies could measure telomere lengths using Southern blots to determine if rates of telomere shortening are actually affected in these strains.

The third goal was to test the effect of different genes on the aging rate of telomerase-deficient yeast cells. Three groups of genes were tested: RAD52 group genes that participate only in homologous recombination, plus selected antioxidant and checkpoint genes. The first group of genes included *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59* and *RDH54.* Based on the results with our new senescence assay, some of the RAD52 group genes could be classified as highly important for HR between

telomeres, including *RAD51, RAD52* and *RAD54*. In contrast, *RAD55, RAD57, RAD59* and *RDH54* were found to be less critical. These results are consistent with past work that indicated that *RAD51* and *RAD52* play a vital role in homologous recombination (31). The result of measuring the effect of different MMS concentrations on the senescence of yeast cells suggested that, even though it induces recombinogenic DNA lesions that might stimulate HR between telomeres, MMS had no notable effect on the aging rate.

CTT1 and *GPX3 (HYR1)* were the antioxidant genes that were tested at 30^{o}C and 38^{o}C. All antioxidant mutant cells including cells with both genes inactivated showed a similar aging rate to WT cells at both temperatures. However, there was a clear decrease in the average number of full columns per plate at 38^{o}C compared to 30^{o}C.

Three checkpoint mutants were tested: *rad24*, *mec3*, and *rad24 mec3* double mutant cells at 30^{o}C and 38^{o}C. The results showed an increase in growth (more full columns) in all mutants at both temperatures compared to WT cells. The DNA damage checkpoint in G_2 phase normally increases survival of cells because it prevents them from going into mitosis with damaged chromosomes. It is possible that the G_2 arrest response during senescence, which is induced by uncapped short telomeres with increased single-stranded DNA, is less critical than in cells actually experiencing damage throughout the chromosomes, e.g., as caused by exposure to ionizing or ultraviolet radiation. Chromosomes in senescing cells do not have a large number of lesions in interior regions and therefore may survive better when the G_2 arrest response is eliminated or reduced.

The final goal was to develop a way to calculate how many generations the cells went through before they underwent senescence. At the lowest nutrient concentration, WT

cells went through one less generation after 3 streaks than cells grown on normal YPD plates. *rad52* cells went through ~1.5 less generations before they underwent senescence on 1/20 YPD plates. The slightly slower growth might be part of the reason that these cells exhibited more full columns at low nutrient concentrations.

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