Published in final edited form as:

DNA Repair (Amst). 2012 January 2; 11(1): 35–45. doi:10.1016/j.dnarep.2011.10.003.

Reversibility of replicative senescence in *Saccharomyces cerevisiae*: effect of homologous recombination and cell cycle checkpoints

Sandra C. Becerra, Hiranthi T. Thambugala, Alison Russell Erickson, Christopher K. Lee, and L. Kevin Lewis*

Department of Chemistry and Biochemistry, Texas State University, San Marcos, TX 78666

Abstract

Primary human somatic cells grown in culture divide a finite number of times, exhibiting progressive changes in metabolism and morphology before cessation of cycling. This telomere-initiated cellular senescence occurs because cells have halted production of telomerase, a DNA polymerase required for stabilization of chromosome ends. Telomerase-deficient *S. cerevisiae* cells undergo a similar process, with most cells arresting growth after approximately 60 generations. In the current study we demonstrate that senescence is largely reversible. Reactivation of telomerase (*EST2*) expression in the growth-arrested cells led to resumption of cycling and reversal of senescent cell characteristics. Rescue was also observed after mating of senescent haploid cells with telomerase-proficient cells to form stable diploids. Although senescence was reversible in DNA damage checkpoint response mutants (*mec3* and/or *rad24* cells), survival of recombination-defective *rad52* mutants remained low after telomerase reactivation. Telomere lengths in rescued *est2* cells were initially half those of wildtype cells, but could be restored to normal by propagation for ~ 70 generations in the presence of telomerase. These results place limitations on possible models for senescence and indicate that most cells, despite gross morphological changes and short, resected telomeres, do not experience lethal DNA damage and become irreversibly committed to death.

Keywords

telomerase; senescence; homologous recombination; checkpoint; aging

1. Introduction

The ends of chromosomes in most eukaryotic cells are protected by specialized structures called telomeres, which consist of an array of short repeated DNA sequences bound by specific telomere and chromatin-associated proteins. Human telomeres contain repeats of the hexanucleotide sequence TTAGGG and most other eukaryotes have a similar repeat motif

© 2011 Elsevier B.V. All rights reserved.

*Corresponding author: L. Kevin Lewis, Chemistry and Biochemistry, Texas State University, 601 University Drive, San Marcos, TX 78666, LL18@txstate.edu, Phone: 512-245-8594, Fax: 512-245-2374.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

[1,2]. Stable maintenance of chromosome ends requires the presence of telomerase, which is a telomere-specific, RNA-dependent DNA polymerase [1,3–5]. Telomerase is required to overcome the “end-replication problem” in dividing cells, which arises because replication by the major DNA polymerases cannot complete synthesis of the lagging strands at the ends of linear chromosomes.

Cells within most human tissues halt production of telomerase shortly after differentiation and subsequently experience progressive loss of DNA from chromosome ends [1,6]. As a consequence, most cells within older humans have shorter telomeres than those of younger individuals. The precise role of telomere shortening in organismal aging remains unknown, but several studies have associated shorter-than-average telomeric repeats in aged humans with increased risk of mortality and age-associated disease [7–10]. Consistent with the idea that telomere shortening impacts aging, constitutive expression of telomerase in mice was found to increase median lifespans [11].

The time-dependent shortening at chromosome ends has been incorporated into models of human carcinogenesis in two different ways. First, telomere shortening has been interpreted positively based on its ability to limit cell division and act as a tumor suppression mechanism. In contrast to normal somatic cells, approximately 90% of human cancer cells have acquired mutations resulting in expression of telomerase and their chromosome ends are relatively stable. This critical metabolic alteration provides an explanation for why most cancer cells become immortal, though some cancer cells appear to use an alternative mechanism involving homologous recombination between telomeres [1,12]. Other studies have suggested that shortened telomeres may actually promote chromosome instability and carcinogenesis. For example, formation of shortened, dysfunctional telomeres was found to be an early event in development of prostate cancer [13] and telomerase knockout mice with shortened telomeres have an increased incidence of some cancers [14]. A possible explanation for increased chromosome instability caused by shortened telomeres is that the DNA ends lose their protective protein caps, undergo nuclease degradation and become highly reactive. Eventual loss of telomere-associated protein complexes may cause the cell to interpret the exposed ends as a form of DNA damage and activate checkpoint and/or apoptosis-type responses [15–17]. Broken DNA ends are known to be hotspots for induction of recombination and mutation, possibly explaining why shortened chromosome ends promote such events [18–20].

Aging studies have been aided by the demonstration that normal, telomerase-deficient, somatic human cells grown in culture undergo many of the changes seen in cells isolated from aging humans. For example, primary human fibroblasts typically grow in tissue culture for approximately 50 cell divisions, during which time they accumulate morphological and biochemical changes that ultimately lead to replicative senescence, also called telomere-initiated cellular senescence or *in vitro* cell aging [21]. The senescence of fibroblasts (loss of ability to grow in culture) can be averted if human telomerase (hTERT) is ectopically expressed, which demonstrates that telomere shortening is the primary cause of replicative senescence. However, oxidative damage to telomeric DNA can also play an important role in determining senescence kinetics [21–23].

The precise relationship between replicative senescence observed in cell culture and natural human and animal aging is a subject of much debate. In both intact organisms and in cultured cells progressive telomere shortening occurs, cell stress responses are elevated, and intracellular levels of both iron and oxidative byproducts become increased with age (passage) [6,15,21,24,25]. Cells with many of the characteristics of senescent cells have been found in the tissues of aged animals, accounting for between 1% and 15% of the total cell population in different reports. Such cells are detectable at higher levels in older animals

than in younger ones [21,26,27], another indication that the study of senescence of cultured cells has relevance to *in vivo* aging.

A similar process of replicative senescence with associated telomere shortening is observed in cultured cells of other organisms, including telomerase-defective cells of the widely studied model eukaryote *Saccharomyces cerevisiae* (budding yeast) [28–30]. Yeast cells produce a telomerase complex analogous to human telomerase that is composed of both RNA and protein subunits. These components include Est1, Est2 (the polymerase subunit), Est3 and *TLC1*, which is the RNA subunit [31]. *TLC1* RNA contains an internal 17 nucleotide sequence (CACCACACCCACACACA) that is used as a template by the enzyme to synthesize new telomeric DNA repeats. The essential telomere-associated protein Cdc13 is also critical for telomerase function *in vivo* [31]. Inactivation of *EST1*, *EST2*, *EST3* or *TLC1* leads to progressive telomere shortening, degradation of DNA ends by Exo1 and possibly other nucleases, gross cell enlargement and loss of growth capability after approximately 60 cell divisions [29,30,32]. During senescence in liquid culture most yeast cells also undergo cell cycle arrest in G₂ phase that is dependent upon the checkpoint genes *MEC3*, *RAD24* and a subset of other genes known to be involved in normal DNA damage responses [29,30]. Interestingly, although telomerase-deficient cells that also have the checkpoint genes *MEC3* and *RAD24* inactivated do not arrest strongly in G₂, they still undergo senescence (loss of growth capability). Senescence kinetics is also dependent on functional DNA repair genes, especially the RAD52 group of homologous recombination genes [28,33–35]. Rare cells called survivors that bypass senescence have been detected in aging yeast cell cultures. Mechanisms for producing such telomerase-independent survivors include epigenetic effects leading to elevation of recombination between telomeric repeats, circularization of chromosomes to eliminate all ends (observed in fission yeast), and an unusual mechanism detected in *rad52 exo1* mutants that involves formation of expanded palindromes near chromosome ends [34,36–38].

In the current study we have expanded the utility of the yeast model for studying cellular senescence by placing expression of the *EST2* polymerase subunit under control of a modified galactose-inducible promoter (*GALI-V10*) that has unusually low basal expression when cells are grown in glucose media [39]. Using this system we demonstrate that nondividing senescent cells can be rescued by reactivation of telomerase expression and also by mating with telomerase-proficient non-senescent cells to form stable diploids. Rescue by telomerase reactivation was also analyzed in mutants defective in DNA damage checkpoint responses and homologous recombination. The results indicate that, although yeast cells undergo many morphologic and DNA metabolic changes during telomere-initiated senescence, they do not become irreversibly committed to cell death.

2. Materials and methods

2.1. Reagents used for DNA manipulation and growth of yeast cells

D-(+)-glucose and ampicillin were purchased from Sigma-Aldrich. Galactose was acquired from Ferro Pfanstiehl Laboratories, Inc. or from Sigma-Aldrich. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Taq Plus Long DNA polymerase was purchased from Stratagene and ExTaq was from Takara Bio Inc. Shrimp alkaline phosphatase (SAP) was purchased from US Biological. 5-fluoroorotic acid (5-FOA) was obtained from ZymoResearch.

For nonselective propagation, yeast cells were grown on YPDA (rich) media (1% bacto yeast extract, 2% bacto peptone, 2% glucose, 0.002% adenine, 2% bacto agar). For plasmid selection, cells were grown on synthetic media with drop-out mix (2% glucose or 2% galactose, 2% bacto agar, plus all essential nutrients minus amino acids or bases used for

selection). Hygromycin B (HygB) (Calbiochem-EMD Biosciences) and G418 sulfate (Cellgro-Mediatech) plates were prepared using 250 ug/ml and 200 ug/ml concentrations, respectively. Nourseothricin (Nat) (WERNER BioAgents) antibiotic plates were prepared using 120 ug/ml concentrations.

Yeast transformations were performed using either the high efficiency method described by Gietz *et al.* [40] or the rapid DMSO-based transformation method of Soni *et al.* [41]. Chromosomal DNA purifications were accomplished using MasterPure Yeast DNA Purification Kits (Epicentre) and plasmid DNA was purified by boiling lysis as described [42].

2.2. Strains and plasmids

The parent strain used for these studies was BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) [43]. The *est2⁻* strain utilized in the assays was YLKL803 (BY4742, *est2Δ::HygB^r* containing plasmid pLKL82Y [*CEN/ARS URA3 GALI-V10p::EST2*]). YLKL807 (*est2Δ::HygB^r rad52Δ::G418^r*) was created from YLKL803 by PCR fragment-mediated gene disruption-deletion. Checkpoint-deficient strains were YLKL840 (*est2Δ::HygB^r rad24Δ::G418^r*), YLKL841 (*est2Δ::HygB^r mec3Δ::G418^r*), and YLKL844 (*est2Δ::HygB^r rad24Δ::G418^r mec3Δ::Nat^r*). Mating assays were performed using laboratory strain YLKL701 (*MATa ade5*).

pLKL82Y was created by PCR amplification of the *EST2* gene from plasmid pVL999 (*ADH1p::EST2*) [44] using primers XbaEst2 (37mer) and BamEst2 (38mer) (sequences available on request) and Taq Plus Long DNA polymerase, followed by digestion with XbaI and BamHI and ligation with pLKL81Y that had been cut with XbaI and BamHI and phosphatased with SAP. pLKL81Y contains the *GALI-V10* promoter cloned into pRS316 (*CEN/ARS URA3*) [39,45].

2.3. Liquid and plate-based senescence assays

Liquid culture senescence assays were performed as previously described with slight modification [33,46]. Briefly, YLKL803 cells were picked from fresh synthetic galactose plates without uracil, counted microscopically by hemacytometer, and inoculated into synthetic or rich glucose liquid media in tall glass tubes at 50,000 cells per ml. After shaking 24 hrs at 30 °C, cell titers were measured and new 5 ml cultures were initiated by inoculation of cells from the first culture at 50,000 cells per ml. Overnight cultures were used to start new cultures repeatedly for up to 8 days. Five replicate cultures were tested for each strain and the results were averaged. To analyze cell cycling, aliquots of the daily liquid cultures were diluted, sonicated, and analyzed by phase contrast microscopy. Unbudded cells were classified as G₁ phase, cells with small buds as S phase, and cells with large buds were in G₂ or M phase (primarily G₂) [47]. Large-budded cells were defined microscopically as cells with buds that were greater than 50% of the size of the mother cell. Cell survival was assessed by making dilutions of each 24 hr culture and spreading appropriate volumes onto rich or selective plates. The spread plates were incubated at 30 °C for 4–6 days. *rad52* mutants were incubated for longer times because they grow more slowly than wt cells. Colonies on each plate were counted and plating efficiencies were calculated. Plating efficiency was defined as the titer (cells per ml) determined from colonies divided by the titer determined microscopically using a hemacytometer.

For plate senescence assays, cells were streaked from synthetic galactose plates without uracil onto synthetic glucose plates lacking uracil essentially as previously described [34]. After incubation at 30 °C for 3 days, cells divided approximately 20 times and formed colonies. Individual colonies were picked and restreaked onto fresh plates and incubated at

30 °C as before. This process was repeated until senescence (loss of ability to grow) was observed. The senescent phenotype was visible by the fourth streak in *est2* cells (~ 60 generations) and by the third streak (~ 40 generations) in certain DNA repair mutants such as *rad52* cells.

EST2 reactivation experiments were performed by streaking *est2* cells containing the *GAL1-VIO::EST2* plasmid pLKL82Y from synthetic galactose plates to glucose plates as described above for the plate senescence assays except that the third streak was allowed to grow four days to ensure that almost all cells had stopped dividing. From the third streak plates, colonies were harvested, diluted, sonicated and counted. Dilutions were spread separately to synthetic glucose and galactose plates without uracil or to YPDA and YPGalactose plates. Reactivation experiments involving recombination-defective strains (*est2 rad52*) and checkpoint single and double mutants (*est2 mec3*, *est2 rad24*, and *est2 mec3 rad24*) were performed similarly.

2.4. Rescue of cellular senescence by mating

To determine if senescent cells could be rescued by mating, senescent *MAT α est2* cells or control *EST2* cells were harvested into 2 ml YPDA broth. After incubation for 60 min at 30 °C to allow the cells to resume growth, cells were diluted, sonicated and counted using a hemacytometer. For mating of YLKL803 (*MAT α est2*) with YLKL701 (*MAT α EST2*) and BY4742 (*MAT α EST2*) with YLKL701, equal amounts of cells were inoculated into YPDA broth in a tissue culture flask. The mating period was optimized empirically (from 0.5 – 6 hrs) and lasted 120 min at 30 °C. Cells were then diluted and spread to either galactose plates without uracil or to diploid selective plates (glucose minus leu, his, trp, and ade) to detect diploids. The rescue efficiency was calculated by measuring plating efficiencies (the number of cells per ml based on colonies formed on selective plates divided by the number of cells per ml in the culture counted microscopically).

2.5. Plate holding experiments

Wildtype, *est2* and *est2 mec3 rad24* cells (BY4742, YLKL803 and YLKL844) were streaked onto glucose plates for first and second streaks as before (approximately 40 generations total growth). Seven colonies were harvested into 500 μ l H₂O, counted, and spread to a third glucose plate in order to obtain well separated colonies and to complete ~ 60 generations. After incubation of these plates for 3 days at 30 °C, seven of the resulting colonies were harvested into 500 μ l H₂O, diluted and spread to galactose plates without uracil to measure rescue by telomerase reactivation and to glucose minus uracil plates to confirm senescence. These third glucose growth plates containing unpicked colonies were then returned to 30 °C. Seven more colonies were harvested from the plates and spread to galactose and glucose plates 2, 4, 6 and 8 days later, and the original colony plates were returned to 30 °C immediately after harvesting at each timepoint. Plating efficiencies were reported as averages $\pm$ standard deviations from 7 samples at each timepoint.

2.6. Southern blot hybridization experiments

Assays used a non-radioactive digoxigenin (DIG)-based system from Roche Diagnostics (DIG High Prime DNA Labeling and Detection Kit II). Telomere-specific DNA probes for Southern analysis were synthesized by PCR using plasmid YTCA-1 [48] with M13 forward and M13 reverse primers plus digoxigenin-11-dUTP as recommended by the manufacturer. Chromosomal DNA was digested with XhoI and 1 μ g DNA separated on 1.2% agarose gels run in 1 $\times$ TBE buffer. After gel electrophoresis, DNAs were transferred to an N+ Hybond membrane (Amersham Pharmacia) and crosslinked with a UV-Stratalinker 2400 (Stratagene) at 120,000 microjoules for 20 sec. Hybridization and detection were performed as recommended by Roche using anti-DIG-AP conjugate and disodium 3-(4-

methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) for chemiluminescent detection. Telomere fragment sizes were calculated by measuring the distance traveled by the broad, fastest-migrating telomere band in each blot relative to standard fragments of known size (DIG-labelled DNA Marker III standards – Roche Diagnostics). Three separate gels and blots were performed and the results were averaged.

3. Results

3.1. Use of the GAL1-V10 promoter to regulate telomerase expression and study senescence

A major objective of this work was to develop a system that allowed precise modulation of telomerase expression for analysis of cellular senescence within yeast cells. This was accomplished by creation of the plasmid pLKL82Y (*CEN/ARS URA3 GAL1-V10p::EST2*), which has the *EST2* polymerase gene under the control of a mutant *GAL1* promoter, *GAL1-V10*, that has reduced basal expression in glucose media but is strongly activated in galactose. Expression of a luciferase reporter gene from *GAL1-V10p* was found to be approximately 8-fold lower than expression from the wt *GAL1* promoter when cells were grown in glucose media (the “off” condition) [39].

Growth and senescence of haploid *est2* cells containing pLKL82Y (strain YLKL803) were assessed initially in liquid media containing either glucose or galactose. In these assays, cells were harvested from a galactose plate, inoculated into 3% synthetic glucose liquid media at 5×10^4 cells per ml, and shaken overnight at 30 °C. Every 24 hrs afterward, aliquots of the dense overnight cultures were counted and re-inoculated into fresh media at the same starting cell titer and grown overnight (Fig. 1A). The cells grew at a rate of ~ 11 generations per day for 4 to 5 days after which they began to exhibit senescence, i.e., impaired growth, in which cells could not reach the same density as on previous days (Fig. 1B). In contrast, cells propagated in 3% galactose media consistently achieved high cell titers throughout the time-course (Fig. 1B). Galactose cultures were propagated for as long as 10 days (~ 110 generations) with no indication of senescence (data not shown).

As telomeres get progressively shorter the chromosome ends are thought to become partially uncapped, resembling double-strand break (DSB) ends, and therefore trigger DNA damage response mechanisms and growth arrest. The process is similar to the cell cycle arrest in G₂ phase observed after exposure of cells to DNA damaging agents that cause DSBs and requires some, but not all, of the same DNA damage checkpoint genes [29,30]. Senescing yeast cells undergo G₂ arrest and become greatly enlarged, up to five times the normal size [29], a further indicator of stress. In liquid cell cultures containing the *GAL1-V10p::EST2* expression system, enlarged G₂/M cells became prominent after the fourth day (approximately 40–50 generations) becoming ≥ 70% of all cells (Fig. 1C). This increase was not observed in cells grown in galactose.

Survival, defined as the ability of cells to form colonies when spread onto plates, was also tested by measuring plating efficiency, which is the titer of colony-forming cells in a culture divided by the titer of cells determined by counting in a microscope using a hemacytometer. For the representative experiment shown in Fig. 1D, the percentage of cells that were able to form new colonies on glucose plates decreased sharply after 4 days. Late passage cultures (after day six) showed a slight increase in the amount of cells capable of forming colonies due to the accumulation of survivors. The timing of the appearance of such cells varied among independent cell cultures, becoming apparent on either day 7 or day 8 when cells were grown in synthetic media. These survivors are believed to arise at low frequency via an

epigenetic mechanism that elevates levels of homologous recombination within telomere regions, resulting in partial stabilization of chromosome ends [38,49].

3.2. Telomere-initiated senescence is reversible in yeast

Previous studies have indicated that, in addition to telomere shortening, senescence is accompanied by increases in chromosome rearrangements and end-to-end fusions, as well as extensive exonucleolytic degradation of uncapped telomere ends [18,19,50,51]. Many of these alterations are likely to represent irreversible, lethal events, but the precise association between senescence and cell death has remained unclear. The *GALI-V10p::EST2* system was used to distinguish between two possibilities: whether non-growing, late senescent cells become irreversibly damaged and committed to cell death or, alternatively, the cells are arrested with degraded telomeres but can resume cycling and survive if telomerase expression is turned back on. The experiment was initiated using a plate senescence assay, whereby cells were streaked onto synthetic glucose plates, grown for 3 days at 30 °C, and restreaked from individual colonies onto new plates. After each streak, the cells divide approximately 20 times and form colonies (Fig. 2A). The cells grow into colonies of normal size during each of the first three streaks, but do not form normal colonies on the fourth streak plate (after ≥ 60 generations).

In control experiments for the plate assay, YLKL803 cells (*est2* cells containing pLKL82Y [*GALI-V10p::EST2 URA3*]) were streaked to a glucose plate containing 0.1% 5-FOA to select for colonies that had lost the plasmid [52]. *Ura*⁻ cells from these colonies were then streaked 3 more times to standard synthetic glucose plates as in Fig. 2A and demonstrated senescence kinetics identical to that shown in the figure, i.e., senescence occurred on the 4th streak after 60–65 generations, similar to previous observations of yeast senescence kinetics [29,30]. Furthermore, *est2* cells containing the *GALI-V10p::EST2* plasmid could be streaked and re-streaked onto synthetic galactose plates at least 8 times with no indication of senescence (~ 160 generations; data not shown). These results demonstrate that YLKL803 undergoes typical yeast cell senescence kinetics when grown on glucose and is essentially immortal (*Est*⁺) when grown on galactose plates.

est2 cells containing pLKL82Y were streaked three times on glucose plates except that the cells were allowed to grow an extra (fourth) day after the 3rd streak to ensure that almost all cells had halted growth. At this time cells were harvested, counted, and spread onto glucose plates and galactose plates without uracil, followed by incubation at 30 °C for 4 days. As shown in Fig. 2B, the average plating efficiency of senescent *est2* cells was low on glucose (0.4%). Most colonies that did form were small in diameter. When these colonies were replica-plated to new glucose plates, most did not form colonies again (data not shown). This observation indicated that the colonies were not primarily composed of survivors, but rather were outliers of senescence, a stochastic process, that were able to undergo more cell divisions than average.

In contrast to glucose plates, plating efficiency was over 40% after transfer of senescent cells to galactose plates, corresponding to a 92-fold increase when telomerase was turned back on. This plating efficiency is more than half that of wildtype cells, which was approximately 70% on both glucose and galactose plates (Fig. 2B). When individual colonies from the galactose plates were restreaked to fresh galactose plates, colony growth rates (diameters) were normal. Furthermore, rescued cells appeared phenotypically normal when examined by phase contrast microscopy. These results indicate that most of the senescent cells resumed cell division, formed colonies with normal growth rates, and adopted wildtype cell morphologies after reactivation of telomerase.

The use of yeast cells for these studies allowed an alternative test to be conducted to investigate the reversibility of senescence. The experiment was designed to assess whether senescent, growth-arrested haploid cells containing shortened telomeres could be rescued by mating them with healthy telomerase-proficient cells to form stable diploids. Equal numbers of senescent *MAT α est2* cells (YLKL803) harvested from glucose plates and telomerase-proficient *MAT α* cells (YLKL701) were incubated in rich glucose (YPDA) liquid media at 30 °C. After a period of 2 hours of mating, cells were aliquoted onto plates containing diploid selective media. Control matings were also performed by crossing the *EST2* parent strain of YLKL803 (BY4742) with YLKL701. Wildtype *MAT α* cells formed diploids after mating with YLKL701 with 22% efficiency (Table 1). When late senescent cells were spread directly onto glucose plates without mating, plating efficiency was 0.7%. After mating with *EST2* cells, efficiency was raised to 18% (a 26-fold increase), which is similar to the 22% mating efficiency seen with wildtype cells. These findings are in accord with the earlier results involving reactivation of *EST2* expression and provide additional evidence that most cells do not become lethally damaged during senescence.

3.3. Reversibility of senescence in cells defective in homologous recombination and senescence-associated checkpoint responses

Past work has demonstrated that loss of growth potential during replicative senescence is accelerated in cells that are defective in *RAD52*-mediated homologous recombination (e.g., [33,35]). Using the plate assay, we observed faster senescence kinetics in a *rad52* mutant of YLKL803, with most mutant cells halting growth after ~ 40 generations, or only two plate streaks (Fig. 3A). The ability of senescent *est2 rad52* cells to be rescued by resumption of telomerase expression was assessed as before except that (a) cells were harvested from the 2nd streak plate, which had been allowed to grow for an extra (fourth) day, and (b) cells were spread onto synthetic glucose and galactose plates without uracil as before, selecting for the *EST2* plasmid, and were also spread onto rich, nonselective YPDA and YPGalactose plates. The latter step was added to control for possible pLKL82Y plasmid loss that might be enhanced in the *rad52* mutants, potentially confounding the results. Plating efficiencies of the senescent *est2 rad52* cells on both types of glucose plates were more than 100 times lower than for *est2* cells, falling to 0.003% (Fig. 3B). Reactivation of telomerase with galactose increased plating efficiencies by over 2,000-fold, to approximately 8%. The rescued colonies were re-streaked to fresh galactose plates two more times and colony diameters (a measure of growth rate) were observed to be similar to normal *rad52* cells; furthermore, morphology of the rescued cells appeared normal upon examination by phase contrast microscopy (not shown). The plating efficiency of stationary phase, telomerase-proficient *rad52* single mutants was determined to be $44 \pm 17\%$ in this strain background. Thus, reactivation of telomerase increased plating efficiency by three orders of magnitude, but did not restore viability completely to that of normal, non-senescent *rad52* cells (8% vs. 44% plating efficiency). Results were essentially identical using selective and non-selective media, indicating that stability of the *GAL1-V10p::EST2* plasmid in surviving cells did not affect interpretation.

Several genes required for DNA damage checkpoint responses are also involved in the senescence-associated checkpoint response, including *MEC3* and *RAD24* [29,30]. *est2* single mutants and *est2* mutants containing *mec3*, *rad24*, or both genes inactivated exhibited similar kinetics in the plate senescence assay (Fig. 4A). Checkpoint responses were analyzed by harvesting cells from colonies that formed on the third streak (~ 60 generations), sonicating, and counting the number of G₂/M cells microscopically. Colonies from a control *EST2* strain grown similarly for 3 days at 30 °C contained 1% large-budded G₂/M cells, but this proportion increased to 33% in senescent *est2* cells (Fig. 4B). The strong increase in G₂/M cells in senescent versus wt cell cultures during growth on plates was reproducible, but

levels were consistently lower than the 60–70% G₂/M cells observed when senescent *est2* cells were grown in liquid culture (Fig. 1C). The difference in magnitude between cells grown on plates versus liquid media may reflect factors intrinsic to growth of cells on agar surfaces, such as nutrient deprivation and anoxia within the interior of colonies, that do not occur during liquid culture [53]. The percentage of G₂/M cells was reduced approximately 3-fold in *est2 mec3* and *est2 mec3 rad24* mutants and 2-fold in *est2 rad24* single mutants, consistent with previous findings (Fig. 4B) [29,30].

To assess the capability of senescent checkpoint mutant cells to be rescued by telomerase reactivation, *est2 mec3*, *est2 rad24*, and *est2 mec3 rad24* cells were streaked to glucose plates until they had lost the capacity to continue growing as before and spread to glucose and galactose plates. As shown in Fig. 4C, approximately 0.1% of the telomerase-deficient, checkpoint mutant cells were able to form colonies when telomerase expression remained repressed on glucose plates. Thus, plating efficiency on glucose was ~ 4-fold lower in the three checkpoint mutants than in cells deficient in telomerase only (*est2* cells). When the senescent cells were spread to galactose media, colony formation increased 240–340 fold. Similar to *est2* cells, the checkpoint mutant cells were rescued at a high rate (~25% plating efficiency versus 40% for *est2* single mutants). Thus, absence of a strong checkpoint response caused a 4-fold decrease in survival during senescence, but a large fraction of the checkpoint mutant cells could still be rescued by reactivation of telomerase.

3.4. Rescue efficiency decreases slowly in late senescent cells

The *EST2* reactivation experiments indicated that the process of senescence is largely reversible when cells are harvested shortly after most cells have stopped dividing. A plate holding experiment, analogous to liquid holding experiments employed in many DNA repair studies [54] was performed to determine if senescent cells eventually lose the ability to be rescued and if such changes occur suddenly or slowly. *est2* cells were streaked again and again onto glucose plates as before for 3 streaks, or ~ 60 generations, until the cells had stopped dividing and had formed well-separated colonies. Seven isolated colonies from the 3rd streak were harvested, counted, and spread to galactose plates without uracil as before to calculate rescue efficiencies. The original 3rd streak plates were then returned to 30 °C. These plates were kept in the 30 °C incubator for a total of eight days, harvesting 7 colonies every 2 days to test rescue by telomerase. As shown in Fig. 5B, the reversibility of senescence decreased slowly over time, going from 30% plating efficiency immediately after cells stopped growing to 9% after an additional 8 days of incubation at 30 °C. Checkpoint-deficient *mec3 rad24* mutants showed a similar slow decline (Fig. 5C). As a control for these experiments, colonies of wildtype cells that had grown to stationary phase over 3 days at 30 °C were also left in the incubator and plating efficiency tested every 2 days. Viabilities of the non-senescent cells decreased slightly during the time-course, from 60% to 30%. These results suggest that senescent cells, although they are no longer dividing and have experienced stress responses and considerable DNA end degradation, remain metabolically active and lose colony-forming ability slowly, in a manner not dissimilar to that of stationary phase wildtype cells.

3.5. Chromosomes within rescued cells have short telomeres

Changes in telomere lengths during senescence and after telomerase reactivation were analyzed by Southern blotting using a telomere-specific probe to visualize end fragments in XhoI-digested chromosomal DNA [46,55]. Using the plate senescence protocol to grow cells, DNA was harvested after ~ 20 generations (1st streak), 40 generations (2nd), and 60 generations (3rd), digested and analyzed by gel electrophoresis and hybridization (Fig. 6A). Yeast cells have 16 chromosomes, and therefore 32 separate telomeres, but only 15 bands are seen on the blot because many of the end fragments co-migrate. To assess changes in

telomere lengths, the size of the lowest band in each lane was determined by performing 3 separate blots, measuring migration distances relative to the molecular weight standards in each blot, and using graphical analysis to calculate band sizes, which were averaged. The average fragment length decreased from 1100 bp to 1033, 974 and 927 bp after streaks 1, 2 and 3 (Fig. 6B). Thus, each end lost an average of 173 bp over ~ 60 generations, or approximately half of the average size of the TG₁₋₃ repeat regions, which are typically 350–400 bp [31,56].

Southern blots were next used to analyze telomere lengths in cells rescued by reactivation of telomerase (Fig. 7A). Telomeres from 5 independent colonies of YLKL803 grown only on galactose (lanes 2–6) were compared to isolates of YLKL803 that had undergone senescence until they stopped growing and were then rescued by transfer to galactose (lanes 7–11). For these experiments, the rescued *est2* cells grew into colonies on galactose plates, requiring ~ 20 doublings, and were then shaken overnight in YPGalactose liquid media, conditions that permitted ~ 10 additional generations of growth (30 total). The liquid cultures were performed in order to obtain sufficient DNA for the Southern blots. Interestingly, telomere lengths were not restored to normal in the rescued cells, which had grown for ~ 30 cell cycles with telomerase turned on (Fig. 7A). The average length for the lowest telomere band after this period of growth was only 1008 bp, compared to 1105 bp for *EST2* control cells (Fig. 7B). These results indicate that, although the rescued cells exhibited normal growth rates (based on average colony diameters on plates and growth rates in liquid culture) and appeared normal morphologically, their telomeres remained approximately 100 bp shorter than those of wildtype cells. Colonies on the initial galactose plates were re-streaked to fresh galactose plates and incubated until new colonies formed (corresponding to ~ 40 generations of growth on the two plates) and were then grown overnight in liquid as before (50 generations total). Average telomere fragment lengths increased to 1038 bp (Fig. 7B). Repeating this process once again, resulting in 70 generations of growth in the presence of telomerase, brought telomere lengths up to 1081 ± 37 bp, which is not statistically different than the 1105 ± 7 bp determined for wildtype cell DNA run on the same gels (Fig. 7B). These results demonstrate that senescent cells rescued by telomerase begin to exhibit normal growth and morphological characteristics even though their telomeres are ≥ 100 bp shorter than those of wildtype cells. Restoration of telomere lengths back to wildtype size required ~ 70 cell doublings (or S phases) with telomerase expression reactivated.

4. Discussion

In the current study, the fates of cells undergoing senescence were investigated by placing expression of the *EST2* polymerase subunit under control of a modified galactose-inducible promoter (*GAL1-V10*) that allowed precise modulation of telomerase expression. These experiments revealed that most senescent cells, though they had stopped dividing, were greatly enlarged and contained degraded telomeres, could be rescued by reactivation of telomerase. The efficiency of rescue by telomerase reactivation was high immediately after cells reached late senescence and declined only slowly with time. The results demonstrated that most senescing cells had not accumulated lethal DNA lesions. In further support of this conclusion, haploid senescent cells could also be rescued by mating to telomerase-proficient cells to form diploid strains, with mating efficiencies similar to that of normal haploid cells.

Checkpoint response-deficient *est2* cells exhibited normal senescence kinetics even though G₂-arrest, which increases opportunities for repair of damaged DNA, was impaired. This result is in accord with previous observations [29,30]. A large fraction of the senescent checkpoint-defective cells could be rescued by restoration of telomerase expression (~25% compared to 40% for *est2* single mutants), indicating that they had not accumulated lethal DNA lesions that committed them to cell death. Growth and subsequent replicative

senescence of cultured human cells was originally divided into phases I, II and III by Hayflick [6,57]. Phase III was defined as the stage when cells stop dividing but remain metabolically active and viable for an extended period of time. Shay and Wright referred to this period as M1. Stable ectopic expression of the catalytic subunit of human telomerase (hTERT) overcomes senescence when it is expressed prior to or during M1 phase [22]. Checkpoint-deficient human cells (e.g., cells with p53 inactivated) continue dividing beyond the normal senescence period (proceeding into M2), display increased recombination events and multiple types of chromosomal DNA aberrations, and undergo cell death [22]. Although M1 appears to have similar characteristics in yeast and human cells, the efficient rescue of yeast checkpoint mutants by telomerase reactivation is inconsistent with an M2-equivalent period in these cells.

est2 rad52 mutants exhibited rapid senescence kinetics, halting growth after ~ 40 generations versus ~ 60 for *est2* cells, and displayed much lower plating efficiencies on glucose (0.003% versus 0.5%). Reactivation of telomerase by transfer of cells to galactose media increased the plating efficiency of *est2 rad52* cells by 3 orders of magnitude, but the overall efficiency remained 5-fold lower than that of normal, non-senescent *rad52* cells (8% versus 40%). These results indicate that, in contrast to *est2* cells, a large fraction of the senescent *est2 rad52* cells had become irreversibly growth-arrested during senescence. This finding adds further support to the idea that homologous recombination proteins prolong survival during senescence by promoting repair/stabilization of telomeric DNA ends [28,33–35]. These ends become shortened, partially uncapped, and resected by nucleases such as Exo1 and therefore are likely to generate recombinogenic structures [19]. Interestingly, no survivors were ever detected among *est2 rad52* cells containing the *GAL1-V10p::EST2* plasmid grown in either glucose liquid culture- or plate-based senescence assays. This observation is consistent with previous findings [28,33] and also indicates that mutations within the ~ 650 bp *GAL1-V10* promoter on the plasmid, potentially leading to leaky expression of *EST2* in glucose media, occurred at low frequencies undetectable in the assays.

Interestingly, *est2* cells rescued by resumption of telomerase expression on galactose plates displayed normal growth rates, but chromosomal DNA purified from the colonies retained short telomeres. Thus, after 30 generations of growth with telomerase reactivated, telomeres in the rescued cells were still shorter than normal by approximately 100 bp. Restoration of the shortened telomeres back to normal lengths required propagation of the rescued cells for ~ 70 generations in the presence of telomerase. This finding is consistent with past reports indicating that telomerase extends only a few base-pairs per generation in yeast cells [58]. Observation that the cells were phenotypically normal though their telomeres were short is not unprecedented: several yeast mutants have been identified that have shortened telomeres but do not exhibit obvious growth or cytological abnormalities [59,60].

Past studies in both yeast and human cells have indicated that the presence of telomerase in cells, even if it is catalytically impaired, can stabilize critically short telomeres [61]. Thus, reactivated telomerase may perform two separate functions in the rescued cells: protection of the shortened telomere ends from further degradation, with potential alleviation of the DNA damage checkpoint arrest response, as well as extension of the telomeres by several nucleotides during each cell cycle.

The molecular changes that ultimately lead to the death of cells undergoing replicative senescence are not known, though several mechanisms have been suggested. Proposed models include the possibilities that (i) uncapped, reactive DNA ends may promote lethal chromosome rearrangements such as end-to-end fusions, which have been detected in DNA of senescent cells, (ii) chromosomes may lose essential genetic information due to

shortening and 5'-to-3' exonuclease degradation at uncapped ends, (iii) aneuploidy (chromosome loss or gain) may decrease cellular fitness, or (iv) senescence may initiate an apoptosis-like commitment to cell death [15,18,19,50,51,61,62,63]. Several studies have also pointed to the importance of strand breaks induced in telomeric DNA by oxidation and these findings must also be incorporated into proposed models of telomere-initiated senescence [21,64–67].

Greider and colleagues [18,19] demonstrated that chromosome loss rates and mutations within telomere-proximal genes, caused primarily by terminal chromosomal DNA deletions, are increased during yeast cell senescence. Although such events were increased, they remained rare, and were largely dependent upon the presence of the nucleases Exo1 and Rad1. Based on these results, the authors posited that exonucleolytic end resection is the major mechanism causing chromosome instability during telomere shortening [18,19]. Our experiments demonstrating that most yeast cells can be rescued efficiently by telomerase reactivation, during and long after cells have reached senescence, is in accord with their observation that such potentially lethal mutations and chromosome loss events occur in only a small fraction of the cells.

A model that is consistent with our observations and with those cited above is that replicative senescence involves two classes of events: (i) nonlethal changes that occur at most or all chromosome ends in the cell and are largely reversible upon reactivation of telomerase expression; these phenomena potentially include telomere shortening due to lack of end-replication by telomerase, partial loss of cap proteins, ssDNA resection by exonucleases, and activation of DNA damage checkpoint responses, plus (ii) lethal telomere shortening-induced events that initiate at one or more DNA ends in only a small percentage of cells. The latter events are likely to include end-to-end fusions, recombination-induced genome rearrangements, chromosome loss events and various types of mutations, especially large terminal deletions. The nonlethal changes described above are undoubtedly only a partial list. For example, shortened telomeres exhibit changes in their localization and interactions with nuclear pore complexes [35] and modifications in telomere-associated structures such as T-loops and G-quadruplexes remain unexplored.

The major conclusion of the current work, that cellular senescence is largely reversible by reactivation of telomerase, has implications for higher eukaryotes since cells with characteristics similar to senescent cells have been detected in aged animals [21,26,27]. It is not clear whether such senescent cells have been permanently altered or if they might be induced to regain youthful characteristics by transient or long-term expression of telomerase. Interestingly, constant high-level expression of telomerase has been found to increase median lifespans in transgenic mice [11]. In addition, a recent study demonstrated that short-term telomerase reactivation in telomerase-deficient mice can reverse several measures of tissue atrophy without promoting carcinogenesis [68]. It is possible that the constitutive or temporary production of telomerase in these animals exerts its beneficial effects, in part, by preventing and/or reversing accumulation of senescent cells within critical tissues and organs.

Highlights

- Reversibility of senescence in yeast cells was tested using two approaches.
- Despite telomere damage, most cells were viable after telomerase reactivation.
- Mating of senescent cells with normal cells also restored growth capability.

- Rescue by telomerase differed in checkpoint mutants and recombination mutants.
- Cells rescued by telomerase exhibited shortened telomeres.

Acknowledgments

The authors wish to thank Dan Gottschling for plasmid YTCA-1. LKL was supported in part by National Institutes of Health grant 1R15AG028520-01A1 and a departmental grant from the Welch Foundation.

References

1. de Lange T. How telomeres solve the end-protection problem. *Science*. 2009; 326:948–952. [PubMed: 19965504]
2. Denchi EL. Give me a break: how telomeres suppress the DNA damage response. *DNA Repair (Amst)*. 2009; 8:1118–1126. [PubMed: 19482563]
3. Bianchi A, Shore D. How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex. *Mol. Cell*. 2008; 31:153–165.
4. Linger BR, Price CM. Conservation of telomere protein complexes: shuffling through evolution. *Crit. Rev. Biochem. Mol. Biol.* 2009; 44:434–446. [PubMed: 19839711]
5. Osterhage JL, Friedman KL. Chromosome end maintenance by telomerase. *J. Biol. Chem.* 2009; 284:16061–16065. [PubMed: 19286666]
6. Hayflick L. Living forever and dying in the attempt. *Exp. Gerontol.* 2003; 38:1231–1241. [PubMed: 14698802]
7. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet*. 2003; 361:393–395. [PubMed: 12573379]
8. Honig LS, Schupf N, Lee JH, Tang MX, Mayeux R. Shorter telomeres are associated with mortality in those with APOE epsilon4 and dementia. *Ann Neurol*. 2006; 60:181–187. [PubMed: 16807921]
9. Yaffe K, Lindquist K, Kluse M, Cawthon R, Harris T, Hsueh WC, Simonsick EM, Kuller L, Li R, Ayonayon HN, Rubin SM, Cummings SR. Telomere length and cognitive function in community-dwelling elders: Findings from the Health ABC Study. *Neurobiol. Aging*.
10. Atzmon G, Cho M, Cawthon RM, Budagov T, Katz M, Yang X, Siegel G, Bergman A, Huffman DM, Schechter CB, Wright WE, Shay JW, Barzilai N, Govindaraju DR, Suh Y. Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians. *Proc. Natl. Acad. Sci U S A*. 2010; 107:1710–1717. [PubMed: 19915151]
11. Tomás-Loba A, Flores I, Fernández-Marcos PJ, Cayuela ML, Maraver A, Tejera A, Borrás C, Matheu A, P Klatt, Flores JM, Viña J, Serrano M, Blasco MA. Telomerase reverse transcriptase delays aging in cancer-resistant mice. *Cell*. 2008; 135:609–622. [PubMed: 19013273]
12. Ahmed A, Tollefsbol TO. Telomerase telomerase inhibition. and cancer. *J Anti-Aging Med*. 2003; 6:315–325. [PubMed: 15142433]
13. Meeker AK, Hicks LJ, Platz EA, March GE, Bennett CJ, Delannoy MJ, de Marzo AM. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res*. 2002; 62:6405–6409. [PubMed: 12438224]
14. Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider CW, DePinho RA. Longevity stress response. and cancer in aging telomerase-deficient mice. *Cell*. 1999; 96:701–712. [PubMed: 10089885]
15. Mukherjee AB, Costello C. Aneuploidy analysis in fibroblasts of human premature aging syndromes by FISH during in vitro cellular aging. *Mech Ageing Dev*. 1998; 103:209–222. [PubMed: 9701772]
16. Wong KK, Chang S, Weiler SR, Ganesan S, Chaudhuri J, Zhu C, Artandi SE, Rudolph KL, Gottlieb GJ, Chin L, Alt FW, DePinho RA. Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. *Nat Genet*. 2000; 26:85–88. [PubMed: 10973255]

17. Mondello C, Scovassi AI. Telomeres telomerase. and apoptosis. *Biochem Cell Biol.* 2004; 82:498–507. [PubMed: 15284903]
18. Hackett JA, Feldser DM, Greider CW. Telomere dysfunction increases mutation rate and genomic instability. *Cell.* 2001; 106:275–286. [PubMed: 11509177]
19. Hackett JA, Greider CW. End resection initiates genomic instability in the absence of telomerase. *Mol Cell Biol.* 2003; 23:8450–8461. [PubMed: 14612391]
20. Pfeiffer P, Goedecke W, Kuhfittig-Kulle S, Obe G. Pathways of DNA double-strand break repair and their impact on the prevention and formation of chromosomal aberrations. *Cytogenet Genome Res.* 2004; 104:7–13. [PubMed: 15162009]
21. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol Cell Biol.* 2007; 8:729–740. [PubMed: 17667954]
22. Shay JW, Wright WE. Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis.* 2005; 26:867–874. [PubMed: 15471900]
23. Richter T, von Zglinicki T. A continuous correlation between oxidative stress and telomere shortening in fibroblasts. *Exp Gerontol.* 2007; 42:1039–1042. [PubMed: 17869047]
24. Killilea DW, L Wong S, Cahaya HA, Atamna H, Ames BN. Iron accumulation during cellular senescence. *Ann. N. Y. Acad Sci.* 2004; 1019:365–367. [PubMed: 15247045]
25. Hwang ES, Yoon G, Kang HT. A comparative analysis of the cell biology of senescence and aging. *Cell Mol. Life Sci.* 2009; 66:2503–2524. [PubMed: 19421842]
26. Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM. Cellular senescence in aging primates. *Science.* 2006; 311:1257. [PubMed: 16456035]
27. Hornsby PJ. Senescence and life span. *Pflugers Arch.* 2010; 459:291–299. [PubMed: 19763609]
28. Lundblad V, Blackburn EH. An alternative pathway for yeast telomere maintenance rescues est1-senescence. *Cell.* 1993; 73:347–360. [PubMed: 8477448]
29. Enomoto S, Glowczewski L, Berman J. MEC3, MEC1, and DDC2 are essential components of a telomere checkpoint pathway required for cell cycle arrest during senescence in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 2002; 13:2626–2638. [PubMed: 12181334]
30. Ijima AS, Greider CW. Short telomeres induce a DNA damage response in *Saccharomyces cerevisiae*. *Mol. Biol Cell.* 2003; 14:987–1001. [PubMed: 12631718]
31. Lundblad V. Telomeres. Cold Spring Harbor Laboratory Press; 2006. Budding yeast telomeres; p. 345-386.
32. Lowell JE, Pillus L. Telomere tales: chromatin, telomerase and telomere function in *Saccharomyces cerevisiae*. *Cell Mol Life Sci.* 1998; 54:32–49. [PubMed: 9487385]
33. Le S, Moore JK, Haber JE, Greider CW. *RAD50* and *RAD51* define two pathways that collaborate to maintain telomeres in the absence of telomerase. *Genetics.* 1999; 152:143–152. [PubMed: 10224249]
34. Lowell JE, Roughton AI, Lundblad V, Pillus L. Telomerase-independent proliferation is influenced by cell type in *Saccharomyces cerevisiae*. *Genetics.* 2003; 164:909–921. [PubMed: 12871903]
35. Khadaroo B, Teixeira MT, Luciano P, Eckert-Boulet N, Germann SM, Simon MN, Gallina I, Abdallah P, Gilson E, Géli V, Lisby M. The DNA damage response at eroded telomeres and tethering to the nuclear pore complex. *Nat Cell Biol.* 2009; 11:980–987. [PubMed: 19597487]
36. Rizki A, Lundblad V. Defects in mismatch repair promote telomerase-independent proliferation. *Nature.* 2001; 411:713–716. [PubMed: 11395777]
37. Maringe L, Lydall D. Telomerase- and recombination-independent immortalization of budding yeast. *Genes Dev.* 2004; 18:2663–2675. [PubMed: 15489288]
38. Makovets S, Williams TL, Blackburn EH. The telotype defines the telomere state in *Saccharomyces cerevisiae* and is inherited as a dominant non-Mendelian characteristic in cells lacking telomerase. *Genetics.* 2008; 178:245–257. [PubMed: 18202371]
39. Lewis LK, Lobachev K, Westmoreland JW, Karthikeyan G, Williamson KM, Jordan JJ, Resnick MA. Use of a restriction endonuclease cytotoxicity assay to identify inducible GAL1 promoter variants with reduced basal activity. *Gene.* 2005; 363:183–192. [PubMed: 16289630]
40. Gietz RD, Woods RA. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* 2002; 350:87–96. [PubMed: 12073338]

41. Soni R, Carmichael JP, Murray JA. Parameters affecting lithium acetate-mediated transformation of *Saccharomyces cerevisiae* and development of a rapid and simplified procedure. *Curr Genet*. 1993; 24:455–459. [PubMed: 8299163]
42. Holmes M, Quigley DS. A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem*. 1981; 114:193–197. [PubMed: 6269464]
43. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*. 1998; 14:115–132. [PubMed: 9483801]
44. Nugent CI, Bosco G, Ross LO, Evans SK, Salinger AP, Moore JK, Haber JE, Lundblad V. Telomere maintenance is dependent on activities required for end repair of double-strand breaks. *Curr Biol*. 1998; 8:657–660. [PubMed: 9635193]
45. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 1989; 122:19–27. [PubMed: 2659436]
46. Singer MS, Gottschling DE. TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science*. 1994; 266:404–409. [PubMed: 7545955]
47. Lewis LK, Kirchner JM, Resnick MA. Requirement for end-joining checkpoint functions, but not RAD52-mediated recombination, after *EcoRI* endonuclease cleavage of *Saccharomyces cerevisiae*. *DNA Mol Cell Biol*. 1998; 18:1891–1902.
48. Singer MS, Kahana A, Wolf AJ, Meisinger LL, Peterson SE, Goggin C, Mahowald M, Gottschling DE. Identification of high-copy disruptors of telomeric silencing in *Saccharomyces cerevisiae*. *Genetics*. 1998; 150:613–632. [PubMed: 9755194]
49. Grandin N, Charbonneau M. Control of the yeast telomeric senescence survival pathways of recombination by the Mec1 and Mec3 DNA damage sensors and RPA. *Nucl Acids Res*. 2007; 35:822–838. [PubMed: 17202155]
50. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell*. 1998; 92:401–413. [PubMed: 9476899]
51. Ducray C, Pommier JP, Martins L, Boussin FD, Sabatier L. Telomere dynamics. end-to-end fusions and telomerase activation during the human fibroblast immortalization process. *Oncogene*. 1999; 18:4211–4223. [PubMed: 10435634]
52. Boeke JD, Trueheart J, Natsoulis G, Fink GR. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol*. 1987; 154:164–175. [PubMed: 3323810]
53. Wrangé M, Roth JR, Hughes D. Accumulation of mutants in “aging” bacterial colonies is due to growth under selection, not stress-induced mutagenesis. *Proc. Natl. Acad. Sci U S A*. 2008; 105:11863–11868. [PubMed: 18701713]
54. Giannattasio M, Lazzaro F, Siede W, Nunes E, Plevani P, Muzi-Falconi M. DNA decay and limited Rad53 activation after liquid holding of UV-treated nucleotide excision repair deficient *S. cerevisiae* cells. *DNA Repair (Amst)*. 2004; 3:1591–1599. [PubMed: 15474420]
55. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*. 1997; 276:561–567. [PubMed: 9110970]
56. Louis EJ, Vershinin AV. Chromosome ends: different sequences may provide conserved functions. *Bioessays*. 2005; 27:685–697. [PubMed: 15954099]
57. Shay JW, Wright WE, Hayflick his limit. and cellular ageing. *Nat. Rev. Mol Cell Biol*. 2000; 1:72–76. [PubMed: 11413492]
58. Marcand S, Brevet V, Gilson E. Progressive cis-inhibition of telomerase upon telomere elongation. *EMBO J*. 1999; 18:3509–3519. [PubMed: 10369690]
59. Askree SH, Yehuda T, Smolikov S, Gurevich R, Hawk J, Coker C, Krauskopf A, Kupiec M, McEachern MJ. A genome-wide screen for *Saccharomyces cerevisiae* deletion mutants that affect telomere length. *Proc. Natl. Acad. Sci. USA*. 2004; 101:8658–8663. [PubMed: 15161972]
60. Gathbonton T, Imbesi M, Nelson M, Akey JM, Ruderfer DM, Kruglyak L, Simon JA, Bedalov A. Telomere length as a quantitative trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast. *PLoS Genet*. 2006; 2:e35. [PubMed: 16552446]
61. Chan SR, Blackburn EH. Telomeres and telomerase. *Philos. Trans. R. Soc. Lond. B. Biol Sci*. 2004; 359:109–121. [PubMed: 15065663]

62. Bailey SM, Murnane JP. Telomeres chromosome instability and cancer. *Nucl Acids Res.* 2006; 34:2408–2417. [PubMed: 16682448]
63. Williams BR, Prabhu VR, Hunter KE, Glazier CM, Whittaker CA, Housman DE, Amon A. Aneuploidy affects proliferation and spontaneous immortalization in mammalian cells. *Science.* 2008; 322:703–709. [PubMed: 18974345]
64. Furumoto K, Inoue E, Hiyama E, Miwa N. Age-dependent telomere shortening is slowed by enrichment of intracellular vitamin C. *Life Sci.* 1998; 63:935–948. [PubMed: 9747894]
65. Chen JH, Hales CN, EOzanne S. DNA damage, cellular senescence and organismal ageing: causal or correlative? *Nucleic Acids Res.* 2007; 35:7417–7428. [PubMed: 17913751]
66. Passos JF, Saretzki G, von Zglinicki T. DNA damage in telomeres and mitochondria during cellular senescence: is there a connection? *Nucleic Acids Res.* 2007; 35:7505–7513. [PubMed: 17986462]
67. Passos JF, Nelson G, Wang C, Richter T, Simillion C, Proctor CJ, Miwa S, Olijslagers S, Hallinan J, Wipat A, Saretzki G, Rudolph KL, Kirkwood TB, von Zglinicki T. Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol. Syst. Biol.* 2010; 6:347. [PubMed: 20160708]
68. Jaskelioff M, Muller FL, Paik JH, Thomas E, Jiang S, Adams AC, Sahin E, Kost-Alimova M, Protopopov A, Cadiñanos J, Horner JW, Maratos-Flier E, Depinho RA. Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. *Nature.* 2011; 469:102–106. [PubMed: 21113150]

Fig. 1. Senescence of *est2* cells containing the *GAL1-VI0p::EST2* expression system. (A) Illustration of the liquid culture senescence assay. Cells were inoculated at 50,000 cells per ml each day. (B) Cell densities observed in cultures grown in synthetic galactose (*Est2*⁺) or glucose (*Est2*⁻) media. (C) Accumulation of large-budded (*G*₂/*M*) cells during senescence. (D) Fraction of cells grown in liquid synthetic glucose media that were able to form colonies when spread onto glucose plates. The appearance of survivors, indicated by the increase in viable cells on day 7 in this representative culture, typically became apparent on day 7 or 8 but varied among individual cultures. Error bars indicate standard deviations.

Fig. 2.

Use of the plate senescence assay to assess reversibility of *in vitro* cell aging. (A) *est2* cells containing pLKL82Y lose growth capability when repeatedly streaked to glucose plates, but wildtype (*EST2*) cells do not. (B) Plating efficiencies of cells harvested from 3rd streak plates, i.e., after ~ 60 generations of growth, and spread to synthetic glucose or galactose plates. WT, wildtype. Error bars indicate standard deviations.

Fig. 3. Survival of recombination-defective mutants during senescence and after reactivation of telomerase. (A) Acceleration of senescence kinetics in *rad52 est2* mutants using the plate senescence assay. (B) Plating efficiencies of *rad52 est2* cells harvested from 2nd streak plates and transferred to synthetic media (Glu, Gal) or rich media (YPDA, YPGal). Error bars indicate standard deviations.

Fig. 4.

Senescence characteristics of mutants defective in cell cycle checkpoint responses. (A) Use of the plate assay to monitor kinetics of senescence in *mec3*, *rad24*, and *mec3 rad24* strains. The 1st streak is shown at the top and the 4th streak is at the bottom for each strain. (B) Assessment of G₂/M cells present within colonies formed by WT and senescent (3rd streak) cells. (C) Viability of senescent cells with or without reactivation of telomerase expression on synthetic glucose or galactose-containing plates. Error bars indicate standard deviations.

Fig. 5. Assessment of viability within colonies of stationary phase WT cells (A) and senescent *est2* and *est2 rad24 mec3* cells (B and C). Colony plates were maintained in a 30 °C incubator for 8 days and plating efficiencies on galactose plates monitored at the indicated times. Error bars indicate standard deviations.

B

Strain	Generations	Average DNA Fragment Size (bp)
<i>EST2</i>		1100 ± 13
<i>est2</i> (1 st streak)	~ 20	1033 ± 22
<i>est2</i> (2 nd streak)	~ 40	974 ± 26
<i>est2</i> (3 rd streak)	~ 60	927 ± 22

Fig. 6. Quantitative analysis of telomere shortening in *est2* cells containing pLKL83Y grown in glucose media. (A) Southern blot analysis of DNA purified from senescing cells. Bands were visualized using a yeast telomere-specific probe. M, DIG-labelled molecular weight standards. (B) Average size of the lowest telomere band in DNA purified from 1st, 2nd and 3rd streaks of cells. Averages from three blots are shown along with standard deviations.

B

Strain	Generations in galactose	Average DNA Fragment Size (bp)
<i>est2</i> (rescued)	~ 30	1008 $\pm$ 21
<i>est2</i> (rescued)	~ 50	1038 $\pm$ 23
<i>est2</i> (rescued)	~ 70	1081 $\pm$ 37
<i>EST2</i> control		1105 $\pm$ 7

Fig. 7.

Assessment of telomere lengths in colonies formed after transfer of cells to galactose plates and reactivation of telomerase expression. (A) Telomere fragments produced within DNA isolated from telomerase-proficient parent strain BY4742 (lane 1), from YLKL803 cells grown only on galactose (lanes 2–6) or from senescent YLKL803 cells rescued on galactose plates (lanes 7–11). (B) Mean size of the lowest telomere band in DNA purified after rescue and subsequent propagation of senescent cells in galactose for ~ 30, 50 and 70 generations. Averages and standard deviations from three blots are shown.

Table 1Efficiency of rescue of senescent *est2* cells by mating to non-senescent *EST2* cells

Strain	Plating Efficiency ^a (%)	Mating Efficiency ^b (%)
WT	65.8 ± 5.5	21.6 ± 7.0
<i>est2</i>	0.68 ± 0.36	17.6 ± 3.2

^a WT *MATα* cells (BY4742) and senescent *MATα est2* cells were spread onto YPDA (glucose) plates. Numbers represent averages ± standard deviations.

^b Efficiency of diploid colony formation after *MATα* cells were crossed with *MATα* strain YLKL701 and spread onto selective plates.