

Telomere Cap Components Influence the Rate of Senescence in Telomerase-Deficient Yeast Cells

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Cells lacking telomerase undergo senescence, a progressive reduction in cell division that involves a cell cycle delay and culminates in “crisis,” a period when most cells become inviable. In telomerase-deficient *Saccharomyces cerevisiae* cells lacking components of the nonsense-mediated mRNA decay (NMD) pathway (Upf1, Upf2, or Upf3 proteins), senescence is delayed, with crisis occurring ~10 to 25 population doublings later than in Upf⁺ cells. Delayed senescence is seen in *upfΔ* cells lacking the telomerase holoenzyme components Est2p and TLC1 RNA, as well as in cells lacking the telomerase regulators Est1p and Est3p. The delay of senescence in *upfΔ* cells is not due to an increased rate of survivor formation. Rather, it is caused by alterations in the telomere cap, composed of Cdc13p, Stn1p, and Ten1p. In *upfΔ* mutants, STN1 and TEN1 levels are increased. Increasing the levels of Stn1p and Ten1p in Upf⁺ cells is sufficient to delay senescence. In addition, *cdc13-2* mutants exhibit delayed senescence rates similar to those of *upfΔ* cells. Thus, changes in the telomere cap structure are sufficient to affect the rate of senescence in the absence of telomerase. Furthermore, the NMD pathway affects the rate of senescence in telomerase-deficient cells by altering the stoichiometry of telomere cap components.

Telomeres, the chromosome ends, are composed of highly repetitive DNA that is synthesized by telomerase, a specialized reverse transcriptase with an integral RNA template sequence. Both telomeres and telomerase are important determinants of a mitotic clock that regulates the proliferative potential of cells (1). In the absence of telomerase or when telomeres become critically short, cells senesce, exhibiting a progressively slower rate of cell division that involves a cell cycle delay (11, 24). Germ line cells and most somatic stem cells have high levels of telomerase and long telomeres. In most somatic cells, telomerase is inactive and telomere sequences erode at variable rates, depending upon the organism and cell type (18). For example, when primary fibroblasts are cultured, they typically undergo a limited number (50 to 100) of divisions prior to senescence and eventual death of almost all cells in the culture. This point of maximal cell death is termed “crisis.” Rare cells that survive crisis and become immortalized, including the majority of cancer cell types and the rare cultured cells that do not senesce, usually have high levels of telomerase activity (20). Furthermore, expression of the catalytic subunit of telomerase is sufficient to immortalize several different cell types in culture (2, 26, 51, 52), indicating that the lack of telomerase activity in somatic cells limits the proliferative potential of those cells.

In the yeast *Saccharomyces cerevisiae*, telomerase is composed of Est2p (32) and TLC1, the telomerase template RNA (44). Deletion of either *EST2* or *TLC1* causes a progressive

shortening of telomeres and eventual loss of culture viability (35) that is analogous to the replicative senescence of cultured human fibroblasts. The increasing cell cycle delay that characterizes senescence prior to crisis involves a distinct set of checkpoint genes, including *MEC1* and *MEC3* (11). As with fibroblast cultures, continued subculturing of telomerase-deficient yeast gives rise to rare survivor cells that ultimately overtake cultures of primarily senescing and dying cells (29, 35, 44, 49, 50). In *S. cerevisiae*, telomerase-deficient survivor cells have two types of altered telomeric DNA structure. In type I survivors, the subtelomeric repeat sequences (STRs) have been rearranged and amplified (35), while in type II survivors, very long terminal repeat sequences (*C*₁₋₃A) are generated (50). The formation of both types of survivors depends upon the product of the *RAD52* gene (35, 50).

EST1, *EST3*, and *CDC13/EST4* also contribute to telomerase function. While the products of these genes are not required for telomerase activity in vitro (32), mutations in these genes cause telomere erosion and eventual senescence phenotypes like those seen in *est2Δ* or *tlc1Δ* strains. Furthermore, genetic analysis places all four *EST* genes and *TLC1* in the same pathway (29). Est1p and Est3p are tightly associated with telomerase RNA and telomerase activity (12, 22, 31, 47, 55) and are likely components of the telomerase complex in vivo (29). *CDC13* encodes a protein that binds to the telomeric single-stranded DNA (ssDNA) and is crucial in protecting telomere integrity and regulating telomere replication, including telomerase access (4). Cdc13p, along with Stn1p and Ten1p, is proposed to form a “telomere cap” that serves to protect the chromosome end and to regulate access of the telomere to the telomerase enzyme (36). *STN1* encodes an essential protein that limits the recruitment of telomerase to the telomere and protects chromosome ends from homologous recombination (10, 14). *TEN1* encodes an essential gene of

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TABLE 1. Yeast strains used in this study

Strain	Genotype
YJB195	<i>MATa ura3-1 ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1</i>
YJB334	<i>MATa/α ura3-1 ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1</i>
YJB2213	<i>MATa/α est1Δ::URA3/EST1 upf2Δ::HIS3/UPF2</i>
YJB2659	<i>MATa/α est2Δ::URA3/EST2 upf2Δ::HIS3/UPF2 VR-ADE2-TEL/VR-TEL</i>
YJB2677	<i>MATa ura3-1 ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1 cdc13-1(ts⁻)</i>
YJB2689	<i>MATa/α est3Δ::URA3/EST3 upf2Δ::HIS3/UPF2 VR-ADE2-TEL/VR-TEL</i>
YJB2751	<i>MATa nmd2::his3::LEU2 VR-ADE2-TEL/VR-TEL</i>
YJB2857	<i>MATa/α cdc13-2 (est4)/CDC13::URA3::cdc13-1 upf2Δ::HIS3/UPF2</i>
YJB2937	<i>MATa/α cdc13-2 (est4)/CDC13::URA3::cdc13-1 upf2Δ::HIS3/UPF2 tlc1Δ::LEU2/TLC1 VR-ADE2-TEL/VR-TEL</i>
YJB3194	<i>MATa/α CDC13::URA3::cdc13-1/cdc13-1ts⁻ upf2Δ::HIS3/UPF2</i>
YJB3645	<i>MATa/α est1Δ::URA3/EST1 upf2Δ::HIS3/UPF2 hmrΔA::TRP1/HMR::TRP1</i>
YJB3685	<i>MATa/α tlc1Δ::LEU2/TLC1 upf1Δ::HIS3/UPF1 VR-ADE2-TEL/VR-TEL</i>
YJB3686	<i>MATa/α tlc1Δ::LEU2/TLC1 upf2Δ::HIS3/UPF2 VR-ADE2-TEL/VR-TEL</i>
YJB3993	<i>MATa/α tlc1Δ::LEU2/TLC1 upf3Δ::HIS3/UPF3 VR-ADE2-TEL/VR-TEL</i>
YJB3994	<i>MATa/α est2Δ::URA3/EST2 upf3Δ::HIS3/UPF3 VR-ADE2-TEL/VR-TEL</i>
YJB3996	<i>MATa/α est1Δ::URA3/EST1 upf3Δ::HIS3/UPF3 HMLα/HMLα VR-ADE2-TEL/VR-TEL</i>
YJB5129	<i>MATa/α est1Δ::URA3/EST1 upf2Δ::HIS3/UPF2 rad52Δ::RAD52</i>
YJB5756	<i>MATa/α CDC13::URA3::cdc13-1/cdc13-2 (est4) upf1Δ::HIS3/UPF1</i>
YJB5775	<i>MATa/α est2Δ::URA3/EST2 upf1Δ::HIS3/UPF1 upf2Δ::HIS3/UPF2</i>
YJB5776	<i>MATa/α est2Δ::URA3/EST2 upf1Δ::HIS3/UPF1 upf3Δ::HIS3/UPF3</i>
YJB5935	<i>MATa/α tlc1Δ::LEU2/TLC1 upf2Δ::HIS3/upf2Δ::HIS3 VIII::URA3/VIII VR-ADE2-TEL/VR-TEL</i>
YJB5936	<i>MATa/α tlc1Δ::LEU2/TLC1 upf1Δ::HIS3/UPF1 upf2Δ::HIS3/UPF2 VIII::URA3/VIII VR-ADE2-TEL/VR-TEL</i>
YJB6411	<i>MATa/α mec3Δ::TRP1/MEC3 tlc1Δ::LEU2/TLC1 upf2Δ::HIS3/UPF2</i>
YJB7492	<i>MATa/α est2Δ::URA3/EST2 upf2Δ::HIS3/UPF2 VR-ADE2-TEL/VR-TEL TRP1::P_{GAL1}-STN1/STN1 (YEplac181)</i>
YJB7590	<i>MATa/α est2Δ::URA3/EST2 upf2Δ::HIS3/UPF2 VR-ADE2-TEL/VR-TEL TRP1::P_{GAL1}-STN1/STN1 (YEplac181) p1916 (YEP TEN1 LEU2)</i>
YJB7722	<i>MATa/α tlc1Δ::URA3/TLC1 upf2Δ::HIS3/UPF2 VR-ADE2-TEL/VR-TEL p2022 (2μm P_{GAL1}-TEN1 LEU2)</i>

unknown function that is required for telomere length regulation (16).

In previous work, we identified mutations in the nonsense-mediated mRNA decay (NMD) pathway that affected several telomere functions (30). The NMD pathway targets mRNAs for nuclease destruction (8). Three genes, *UPF1*, *UPF2*, and *UPF3*, contribute to the NMD pathway (8) and affect telomere length regulation, telomeric silencing, and the localization of Rap1p, a telomere binding protein, within yeast nuclei (30). Telomerase-proficient *upf* mutants have telomeres that are ~100 bp shorter than wild-type telomeres, exhibit a loss of telomeric silencing, as well as a loss of TEL+CEM antagonism (30). The NMD pathway affects the steady-state levels of several hundred native yeast transcripts, as determined by analysis of high-density oligonucleotide arrays (HDOAs) (28). Several genes that control telomerase activity, including *EST1*, *EST2*, *EST3*, and *STN1*, are regulated by NMD (9). A human gene encoding a protein with similarity to Est1p (40, 46) recently was identified as a component of the NMD pathway as well (7).

In this paper, we demonstrate that the NMD pathway accelerates the rate of senescence in telomerase-deficient yeast cells. Deletion of either *UPF1*, -2, or -3 causes delayed senescence in cells lacking either *EST1*, *EST2*, *EST3*, or *TLC1*. This delay in senescence does not require *RAD52* and is not due to the increased formation of survivors. Loss of the NMD pathway causes increased expression of *STN1*, a binding partner of Cdc13p. Moreover, when *STN1* expression is uncoupled from NMD regulation, the delay in senescence in *upf* mutants is lost. Thus, *upf* mutants delay senescence by altering levels of the cap component Stn1p. Furthermore, an altered cap structure in *cdc13-2* cells results in delayed senescence that is not dependent upon the NMD pathway. This is consistent with the

fact that the *cdc13-2* mutation affects interactions with Stn1p (4), and levels of CDC13 mRNA are not affected in *upf* mutants. Thus, altering the telomere end protection protein Stn1p or its interaction with Cdc13p affects the rate of senescence. We propose that senescence, triggered by a cellular response to the loss of telomerase or the erosion of telomeres, is an active process that is accelerated by the wild-type NMD pathway through a mechanism that involves the chromosome end protection components *STN1* and *CDC13*.

MATERIALS AND METHODS

Yeast strains, plasmids, and Southern analysis. All strains used in this study were isogenic with W303 and are listed in Table 1. The *TLC1* disruption was made by one-step gene replacement with plasmid pBlue61::*LEU2* (44) in strain YJB334. The *est1::URA3* allele was obtained from B. Futcher in strain KH000 (47). *EST2* and *EST3* disruptions were made by one-step gene replacement with plasmids pVL523 and pVL418, respectively (37), in strain YJB334. The *cdc13-1* allele was introduced into YJB195 by using pVL451 (37), and the *cdc13-2* allele was introduced into YJB2677 by using pVL437 (37). Telomerase-deficient isolates from early passages were used in standard crosses. The *upf1::HIS3*, *upf2::HIS3*, and *upf3::HIS3* alleles were obtained from A. Jacobson in strains HFY871, HFY116, and HFY861, respectively (21). The *rad52::TRP1* allele was obtained from P. Kaufman in strain PKY065 (13). All of these alleles were introduced into the W303 strain background by standard crosses and multiple backcrosses. Sporulation and tetrad dissection were performed according to standard methods (43). Strains were maintained in standard yeast media (42).

P_{GAL1}-STN1 was constructed in YJB2751 by introducing the *GAL1* promoter immediately 5' of the initiation codon of *STN1*. The *GAL1* promoter was amplified by PCR from pFA6a-TRP1MX6-pGAL1 (33) with oligonucleotides 1021 (5' ATAACAACARCGCCTTCTTGATGACCTATATGTCCGTACTTHTC CATTTTGAGATCCGGGTTT3') and 1022 (5' AAAGAAGTAAGCATTGG AATATATGAAATCCTGCTGTGTTGATATTGAATTCGAGCTGTTAA AC). Yep-GAL-TEN1 was obtained from M. Charbonneau (15).

Southern blots of the telomere terminal repeat were performed essentially as described previously (9, 30).

Serial passages of senescing cultures. Telomerase-deficient cells were obtained by sporulation and dissection of the appropriate heterozygous diploids (YJB3685, YJB2213, YJB2659, YJB2689, YJB2857, and YJB7492). After tetrad dissection, spore colonies grew for 2 days on solid medium at 30°C and were suspended in 15% glycerol, and then $\sim 10^5$ cells were inoculated into 1 ml of YPAD medium (42). Liquid cultures were grown for 24 h at 30°C, at which point they had typically completed 10 population doublings (PDs) and had reached stationary phase. The 24-h liquid cultures (passage 1) were diluted 1:1,000 into fresh YPAD medium and grown for 24 h at 30°C (passage 2). Six serial cultures were generated by successive dilutions (1:1,000) of the previous 24-h culture. The optical density at 600 nm (OD_{600}) was measured for each isolate at each passage. The number of population doublings a culture underwent during the 24-h period was calculated by the following formula: $\log_2 [(ending\ OD_{600} \times 1,000)/starting\ OD_{600}]$.

To quantitate the colony size of the senescing colonies, spore colonies isolated immediately after sporulation of *tlc1Δ/TLC1 upf2Δ/UPF2* parents were serially advanced in liquid medium for 10 PDs as described above. Cells from 45, 55, 65, and 75 PDs were then plated on solid medium and allowed to form colonies for 24 h at 30°C, and the colony area of 100 colonies of each genotype was measured. Images of colonies from each passage were captured with a Nikon Cool Pix 900 digital camera mounted on a Zeiss stereoscope Stemi DRC (Sterling Heights, Mich.).

Crisis was defined as the point of minimum PDs per 24-h period. Statistical analysis was performed for comparison of the number of PDs the *UPF* and *upfΔ* telomerase-deficient strains underwent before crisis. The rank sum test was used to compare the medians (45). Median differences with confidence limits were obtained by the method of Hodges-Lehmann (45).

Western analysis. Liquid cultures of exponentially growing wild-type and *upf2Δ* strains containing Stn1-cMyc at its native locus were harvested, washed twice with 1× phosphate-buffered saline, and resuspended in 50 μl of lysis buffer (40 mM Tris-HCl [pH 6.8], 5% sodium dodecyl sulfate [SDS], 8 M urea, 0.1 M EDTA). The tubes were immediately vortexed with 250 μl of glass beads for 90s and heated to 70°C for 3 min, and 250 μl of protein loading buffer was added for 5 min at 70°C. The resulting lysates were centrifuged for 10 min. The protein concentration in the supernatant was determined by A_{280} units, and 50 U was run on an 8% polyacrylamide gel electrophoresis (PAGE) gel. Western analysis was performed with anti-cMyc antibody (1:10,000) followed by horseradish peroxidase-conjugated antimouse secondary antibody (1:45,000). Anti-PGK antibody was used to detect Pgk1p as a control for gel loading. All antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

RESULTS

Loss of nonsense-mediated decay delays senescence in telomerase-deficient cells. In *S. cerevisiae*, deletion of the catalytic subunit of telomerase (*EST2*), the template RNA (*TLC1*), or the accessory factors (*EST1* and *EST3*) causes progressive telomere shortening and eventual senescence of the population (29). Senescence can be observed as a decrease in culture growth upon serial passaging in liquid medium (liquid passages, Table 2). When we isolate telomerase-deficient cells as fresh spore progeny from heterozygous parents (see Materials and Methods for details), they can be passaged for approximately six serial cultures (24 h at 30°C in YPAD medium) before crisis (the state in which very few viable cells are present) occurs. To quantitate senescence in *upfΔ* telomerase-deficient mutants, we used liquid passages to estimate the total number of PDs between sporulation and crisis, defined as the passage of minimum growth. We isolated spore colonies immediately postmeiosis and sporulation and serially passaged the cells in liquid medium for 8 consecutive days (1:1,000 dilutions followed by growth for 24 h at 30°C). During early passages, population growth was limited to approximately 10 PDs per day by crowding of the culture. In the middle passages, cultures did not reach saturation (~ 4 to 9 PDs per day) due to slower growth caused by activation of the telomere checkpoint (11). During the late passages, following the crisis period,

TABLE 2. *upf2Δ* delays senescence in telomerase mutants

Genotype	Median PD	<i>n</i>	Median difference ^a	Lower limit	Upper limit
<i>est1Δ</i>	60	6			
<i>est1Δ upf2Δ</i>	82	6	25*	18	36
<i>est2Δ</i>	61	6			
<i>est2Δ upf2Δ</i>	76	6	23*	11	34
<i>est3Δ</i>	62	6			
<i>est3Δ upf2Δ</i>	91	6	28*	21	29
<i>tlc1Δ</i>	55	6			
<i>tlc1Δ upf2Δ</i>	83	6	26*	19	33
<i>cdc13-2</i>	79	6			
<i>cdc13-2 upf2Δ</i>	81	6	3	-16	11

^a *, significant ($P < 0.05$) median difference (Hodges-Lehmann estimator) (45). *n* = number of independent isolates.

viability increased again due to the formation of telomerase-independent survivors (27, 35, 50), and population growth was again limited by saturation of the culture at 10 PDs per day.

The NMD pathway, which consists of a recognition module encoded by *UPF1*, *UPF2*, and *UPF3* (8), affects the steady-state levels of mRNAs that encode telomerase and telomerase regulators, including *EST1*, *EST2*, and *EST3* (9, 28). To gain further insight into interactions between the NMD pathway and telomere metabolism, we analyzed double mutants defective in both NMD and telomerase function. To generate telomerase-deficient NMD-deficient clones, diploid strains heterozygous for telomerase components or regulators and heterozygous for *UPF* mutations were sporulated, and the growth of cultures that arose from individual spores was monitored through serial passages in liquid medium. We initiated these studies by using a deletion allele of *EST1* (*est1Δ*). When *EST1/est1Δ UPF2/upf2Δ* heterozygous diploids were sporulated, the *est1Δ* UPF spore progeny reached crisis at ~ 60 PDs after meiosis and sporulation (Table 2). Interestingly, *est1Δ upf2Δ* sibling cultures grew and divided significantly longer (82 PDs with a median difference of 25 PDs) than the *est1Δ UPF2*⁺ cultures (Table 2). In both cultures, survivors arose in later passages.

To determine if the delay in senescence observed in *est1Δ upf2Δ* strains was specific for *EST1* or due to loss of telomerase activity in vivo, we constructed the appropriate double mutant strains lacking *upf2* and either *EST2*, *EST3*, or *TLC1* RNA and monitored the growth of these strains in serial liquid passages. As with the *est1Δ upf2Δ* cultures, *est2Δ upf2Δ*, *est3Δ upf2Δ*, and *tlc1Δ upf2Δ* strains senesced 23 to 28 PDs later than the corresponding *UPF*⁺ strains (Table 2), indicating that the delay in senescence due to *upf* mutations is not specific for *est1Δ* strains. *EST1*, *EST2*, and *EST3* mRNA levels are regulated by the NMD pathway (9, 28), while levels of *TLC1* RNA are not (9). Yet the *upf2Δ* mutant had a similar rate of senescence when present with *est1Δ*, *est2Δ*, *est3Δ*, or *tlc1Δ* mutations. Thus, the mechanism by which the *upf2Δ* mutation delays senescence remains intact in all of these telomerase-deficient strains and cannot occur through regulation of the stability of *EST1*, *EST2*, or *EST3* mRNAs.

TABLE 3. Loss of the NMD pathway delays senescence in telomerase RNA mutants

Genotype	Median PD	<i>n</i>	Median difference ^a	Lower limit	Upper limit
<i>tlc1Δ</i>	55	6			
<i>tlc1Δ upf2Δ</i>	83	6	26*	19	33
<i>tlc1Δ</i>	53	6			
<i>tlc1Δ upf1Δ</i>	71	6	19*	18	20
<i>tlc1Δ</i>	56	7			
<i>tlc1Δ upf3Δ</i>	73	7	16*	11	17
<i>tlc1Δ upf1Δ</i>	71	8			
<i>tlc1Δ upf1Δ upf2Δ</i>	73	8	.4	-0.05	1.3

^a *, significant ($P < 0.05$) median difference (Hodges-Lehmann estimator) (45). *n* = number of independent isolates.

Upf1p, Upf2p, and Upf3p are all required to target mRNAs for NMD-mediated decay (28). To determine if the delay in senescence in *upf2Δ* telomerase-deficient strains was due to a specific function of Upf2p or was the result of loss of the NMD pathway, we analyzed the senescence of telomerase-deficient strains lacking Upf1p or Upf3p. As with *tlc1Δ upf2Δ* strains, *tlc1Δ upf1Δ* and *tlc1Δ upf3Δ* strains exhibited a significant delay in senescence relative to the corresponding *UPF*⁺ telomerase-deficient strains (Table 3). Furthermore, a *tlc1Δ upf1Δ upf2Δ* triple mutant senesced at the same rate as the *tlc1Δ upf1Δ* double mutant (Table 3). Thus, loss of the NMD pathway, rather than loss of *UPF2* specifically, delays senescence in telomerase-deficient strains.

Senescence progresses more slowly in *upf* telomerase-deficient strains. The senescence delay observed in *upfΔ* mutants could be caused by later onset of the senescence program or by slower progression of the senescence process. To distinguish between these two possibilities, we monitored the culture growth rate soon after the loss of telomerase. Quantitation of the number of PDs that a culture underwent allowed us to determine the timing of senescence. Following the loss of telomerase, the cell division rate decreased progressively as telomerase-deficient cells were cultured. This resulted in the formation of progressively smaller colonies, progressively larger individual cells, and an increasing delay in the G₂/M stage of the cell cycle (11). The decrease in colony size can be observed as early as the first passage after sporulation. Measurement of colony area after 24 h of plating on solid medium provides a reproducible measure of the growth rate of the culture (11) and should indicate whether *upfΔ* telomerase-deficient cells senesce more slowly (slope of the curve is more shallow) or initiate senescence later (decrease in colony area begins later) than corresponding *UPF* telomerase-deficient cells.

During the serial passages, both the *tlc1Δ* and *tlc1Δ upf2Δ* colonies formed increasingly smaller colonies. However, the size of the *tlc1Δ* colonies decreased more rapidly (slope = -1.9) than the decrease in the sizes of the *tlc1Δ upf2Δ* colonies (slope = -0.77) (Fig. 1). This indicates that the *tlc1Δ* cells senesce about twice as fast as the *tlc1Δ upf2Δ* cells and implies that the senescence delay in *upf2Δ* cells is due to a slower progression of the senescence program rather than to a delay in the onset of senescence.

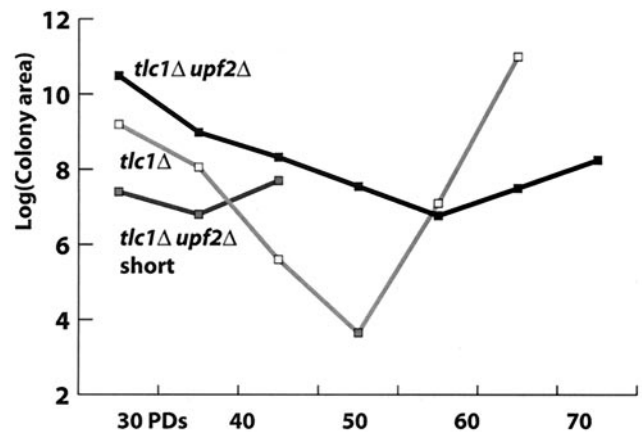


FIG. 1. Kinetics of senescence is altered in *upf* mutants. The log colony area of senescing colonies was measured and plotted against the number of PDs the culture had undergone. *tlc1Δ UPF2* isolates from diploid strain YJB3686 are shown by the light gray boxes and line, *tlc1Δ upf2Δ* isolates from diploid strain YJB3686 are shown by the black boxes and line, and *tlc1Δ upf2Δ* isolates from diploid YJB5935 with short starting telomeres are shown by the medium gray boxes and line. Note that the *tlc1Δ upf2Δ* strain YJB5935 with short starting telomeres formed survivors within one to two passages after meiosis.

Telomere length erosion rates are similar in wild-type and *upfΔ* strains. One possible mechanism for the delay of senescence in *upfΔ* telomerase-deficient strains may be a slower rate of telomere DNA length erosion. We compared the terminal restriction fragment lengths of *est3Δ* and *est3Δ upf2Δ* spore progeny by Southern analysis of serial passages in liquid media. Genomic DNA was prepared from serial passages of *est3Δ* and *est3Δ upf2Δ* spores and digested with *Pst*I, which releases an ~0.8-kb heterodisperse band from the terminus of all chromosomes that have Y' telomere-associated sequences that can be detected with a telomeric (Y' + TG₁₋₃) probe. In telomerase-deficient *UPF* strains (Fig. 2), the terminal *Pst*I fragment became shorter, exhibiting a faster average electrophoretic mobility with increasing numbers of PDs after loss of Est3p. Additional Y'-hybridizing bands, characteristic of type II survivors, were evident in the *est3Δ* survivor culture by ~80 generations (Fig. 2, lanes 7 and 27). In contrast, telomeres in the three *est3Δ upf2Δ* isolates at similar generation times did not undergo obvious rearrangements or amplification of Y' sequences. This indicates that no obvious amplification or rearrangement of TASs or TG₁₋₃ repeats occurred within the PDs analyzed. Like the telomerase-deficient *UPF* strains, the terminal *Pst*I restriction fragment migrated with increasing mobility as cells underwent more PDs. Telomere length erosion in the *est3Δ upf2Δ* strains appeared to occur at rates similar to the rate of telomere erosion in the *est3Δ UPF2* strain (Fig. 2) (data not shown). From our Southern analysis, we cannot rule out the possibility that the rate of telomere erosion in *est upf* strains was slightly slower than the rate in *est* strains. Similar telomere length erosion rates also were observed when *estΔ* mutant strains were compared to the appropriate *estΔ upfΔ* strains (data not shown). Thus, loss of the NMD pathway did not delay senescence by increasing the rate of terminal fragment rearrangements that are characteristic of survivor formation. Furthermore, the obvious shorter telomere length seen in

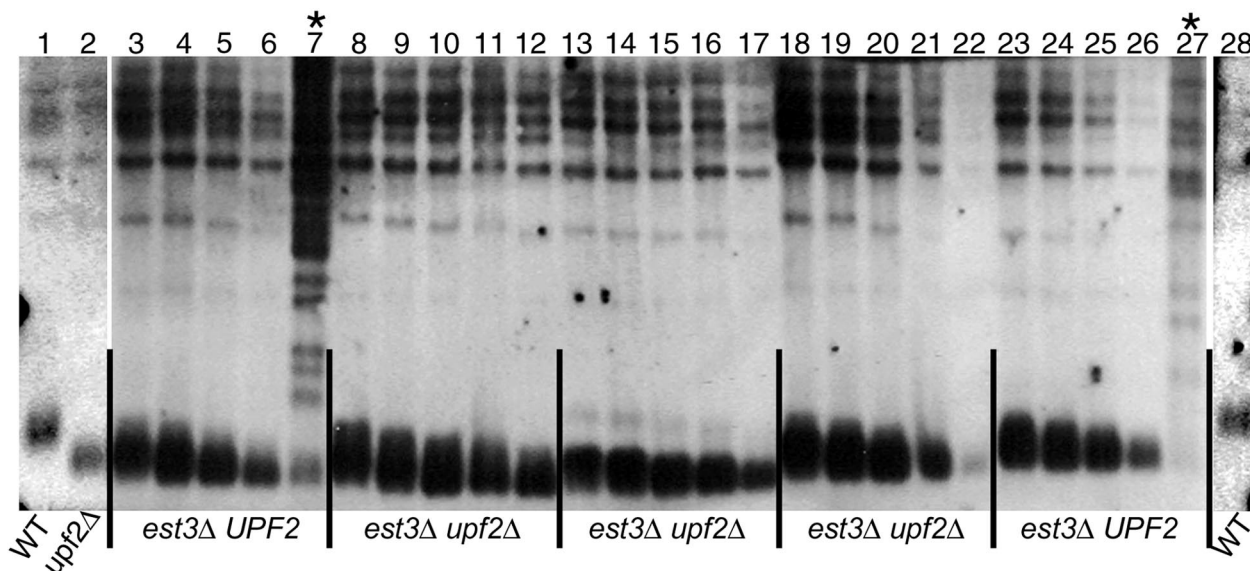


FIG. 2. Rate of telomere length change does not differ between telomerase-deficient and telomerase-deficient NMD-deficient strains. Total genomic DNA was digested with *Pst*I and separated in 1% agarose. Lanes 1 and 28, wild type (WT; YJB209), lane 2, *EST3 upf2Δ* (isolate from diploid strain YJB1274) taken at ~30, 40, 50, 60 and 70 PDs, lanes 3 to 7 and 23 to 27, serial passages of *est3Δ UPF2* taken at ~30, 40, 50, 60, and 70 PDs (isolate from diploid strain YJB2689), lanes 8 to 12, 13 to 17, and 18 to 22, serial passages of *est3Δ upf2Δ* taken at ~30, 40, 50, 60, and 70 PDs (isolate from diploid strain YJB2689). Cultures in lanes 7 and 27 were overtaken by survivors (marked with asterisks).

upfΔ telomerase-proficient cells (28) is not evident in *upfΔ* telomerase-deficient cells. This suggests that the short-telomere phenotype of *upfΔ* mutants requires active telomerase.

Onset of senescence is influenced by telomere length. The telomere length analysis described above demonstrated that *est3Δ upf2Δ* telomeres are similar in size to telomeres in *est3Δ* single mutants. This was surprising because *upfΔ* mutants have shorter telomeres than do wild-type cells (30). The *est3Δ upf2Δ* strains may have wild-type telomere length because the spore progeny are isolated from a heterozygous *UPF2/upf2Δ* parent. To ask if the *upf* status of the parent has an effect on the telomere length of the progeny, we sporulated heterozygous *UPF/upfΔ TLC1/tlc1Δ* and homozygous *upfΔ/upfΔ TLC1/tlc1Δ* parents and analyzed telomere length and senescence in the spore progeny. Telomere length was shorter in the *upfΔ/upfΔ* parents relative to the *UPF/upfΔ* parents (data not shown). This indicates that the initial length of the *upfΔ tlc1Δ* strains is due to the wild-type length of telomeres in the heterozygous parent from which they were derived. *tlc1Δ* derivatives from the *upfΔ/upfΔ* parent grew poorly and reached crisis, forming survivors within one to two passages after sporulation (25 to 35 PDs) (Fig. 1). Progeny derived from the *UPF/upfΔ* parent reached crisis at ~60 PDs. Therefore, initial telomere length appears to be an important determinant of the duration of senescence.

The delayed senescence in *upfΔ* telomerase-deficient strains is independent of *RAD52* and *MEC3*. In yeast strains lacking telomerase, a small subpopulation of cells escape senescence and form “survivors” that are viable, divide with wild-type rates, and rapidly overtake the culture of senescing cells (29, 35). Once this occurs, the viability of the cultures improves dramatically. A possible mechanism for the delay of senescence in *upfΔ* telomerase-deficient strains is an elevated fre-

quency in the generation of survivors relative to that seen in *UPF* telomerase-deficient strains (48). *RAD52* is required for the formation of both type I and II survivors in telomerase-deficient strains (6, 27). To ask if *RAD52* is required for the delay in senescence observed in *upfΔ* telomerase-deficient strains, we compared the senescence of *est1Δ rad52Δ* and *est1Δ rad52Δ upf2Δ* strains immediately following the sporulation of heterozygous diploids. All *rad52Δ* cultures senesced faster than *RAD52* strains. However, the *est1Δ rad52Δ upf2Δ* strains retained viability for significantly more PDs than the *est1Δ rad52Δ UPF2* strains (Table 4), indicating that the delay of senescence in *upfΔ* cells occurs by a mechanism that is independent of *RAD52*. This result is consistent with the idea that *upfΔ* strains do not delay senescence by increasing the rate of survivor formation or by inducing survivor formation at an early time after the loss of telomerase.

Survivor formation is increased in haploid cells expressing both *MATa* and *MATα* information (34). To ask if *upf* mutants were delaying senescence by derepressing a mating information in a *MATα upf* telomerase-deficient strain, we compared

TABLE 4. Delayed senescence is independent of Rad52p and Mec3p

Genotype	Median PD	<i>n</i>	Median difference ^a	Lower limit	Upper limit
<i>rad52Δ tlc1Δ</i>	35	11			
<i>rad52Δ tlc1Δ upf2Δ</i>	47	11	10*	2	19
<i>mec3Δ tlc1Δ</i>	56	6			
<i>mec3Δ tlc1Δ upf2Δ</i>	95	6	39*	22	40

^a *, significant (*p* < 0.05) median difference (Hodges-Lehmann estimator) (45). *n* = number of independent isolates.

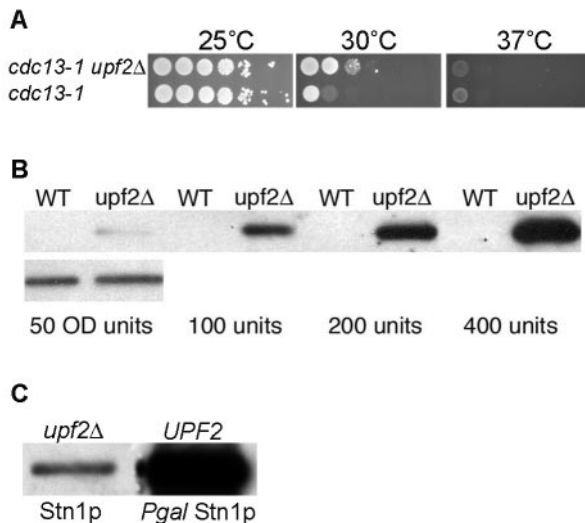


FIG. 3. Higher levels of Stn1p in *upf2Δ* mutants. (A) Liquid cultures were serially diluted, applied as spots to solid medium, and grown at the indicated temperatures for 2 days. *cdc13-1* mutants isolated from diploid strain YJB3194 grow poorly at 30°C and did not form colonies at 37°C. In contrast, *upf2Δ cdc13-1* isolates from YJB3194 formed colonies at 30°C. (B) Western analysis of Stn1p-cMyc in wild-type (WT) and *upf2Δ* strains. (C) Western analysis of Stn1p-cMyc in an *upf2Δ* strain and *GAL1*-Stn1p-cMyc grown in galactose.

the rate of senescence in a *MATα upf2Δ est1Δ* strain and a *MATα upf2Δ est1Δ hmr1Δ* strain. The rates of senescence in these two strains were indistinguishable. Thus, loss of NMD does not delay senescence by altering the expression of silent mating information.

In previous work, we found that senescence in telomerase-deficient cells is characterized by a Mec3-dependent telomere integrity checkpoint (11). To explore whether the *MEC3*-dependent checkpoint was activated in *upf* telomerase-deficient cells, we compared the senescence of *mec3Δ tlc1Δ* cells with that of *mec3Δ tlc1Δ upf2Δ* cells. Both strains exhibited senescence typical of *mec3* strains during the first 75 PDs. As seen previously (11), crisis occurred in the *mec3Δ tlc1Δ* isolates after 56 PDs. In contrast, crisis occurred in the *mec3Δ tlc1Δ upf2Δ* isolates after 95 PDs (Table 4). Thus, the delayed senescence seen in *upf* strains is not due to changes in the Mec3-dependent telomere integrity checkpoint.

Senescence is delayed by constitutive expression of *STN1*.

STN1 is an essential gene that was identified as a dosage suppressor of a temperature-sensitive allele of *CDC13* (*cdc13-1*) (17). Steady-state levels of *STN1* mRNA are 2.55-fold higher in *upf2Δ* strains than in isogenic wild-type strains (7). Stn1p and Cdc13p work cooperatively to regulate the access of telomerase to telomeric DNA (4, 14, 15, 53). *cdc13-1* mutants are temperature sensitive: they grow well at 25°C, poorly at 30°C, and do not grow at all at 37°C (Fig. 3A). Interestingly, *cdc13-1 upf2Δ* strains grow better at 30°C, indicating that loss of NMD can partially suppress the temperature-sensitive phenotype of the *cdc13-1* mutant (Fig. 3A). Since *CDC13* mRNA levels are not altered in *upf2Δ* mutants (9, 28), this result is consistent with the idea that Stn1p levels are increased in an *upf2Δ* mutant and that the extra Stn1p can suppress the *cdc13-1* temperature sensitivity. To ask if Stn1p levels are elevated in *upf2Δ* mutants,

we also measured the levels of an epitope-tagged Stn1p expressed in wild-type and *upf2Δ* strains. Stn1-cMyc is clearly detectable in *upf2Δ* strains, while in W303 *UPF+* strains, it is not detected (Fig. 3B). This may be due to differences between W303 and other strain backgrounds. In other work, Stn1p was detectable only when overexpressed (16). Thus, like *STN1* mRNA levels, the steady-state levels of Stn1 protein are increased in *upf2Δ* mutants.

To determine whether high levels of Stn1p alone could confer a delay in senescence, we first analyzed the senescence of *est1Δ* progeny from a heterozygous *EST1/est1Δ* parent carrying *YEP-STN1*. Surprisingly, these progeny senesced more rapidly than *est1Δ* strains carrying the *YEP* vector alone (data not shown). However, high levels of Stn1p lead to telomere shortening (9). These parents and their progeny had shorter telomeres than the *est1Δ* strain carrying the vector alone. The *est1Δ upf2Δ* strains carrying *YEP-STN1* exhibited delayed senescence relative to the *est1Δ UPF2* strain carrying *YEP-STN1*. This supports the idea that the *STN1* mRNA expressed from this plasmid remains under the control of the NMD pathway (9).

To ask if NMD regulation of *STN1* is necessary for the senescence delay in telomerase-deficient cells, we constructed a strain expressing *STN1* in a *UPF*-independent manner. The 5'-untranslated region (5'-UTR) of *STN1* is sufficient to confer NMD regulation upon *STN1* mRNA (9). To eliminate NMD regulation of *STN1*, we constructed *P_{GAL1}-STN1* by placing the *GAL1* promoter sequences 5' to the *STN1* genomic open reading frame. If NMD regulation of *STN1* is required for the NMD-dependent acceleration of senescence, we expect that a strain expressing *P_{GAL1}-STN1* strain would exhibit a similar senescence rate in *UPF* and *upf2Δ* strains. All diploid parents carrying *P_{GAL1}-STN1* were maintained on glucose medium to ensure there was no effect on telomere length until after sporulation of the progeny. When *P_{GAL1}-STN1-cMyc* strains were grown in galactose, we detected greater than 10-fold more Stn1p than in *upf2Δ* cells (Fig. 3C). In *est2Δ* cells expressing *P_{GAL1}-STN1*, the deletion of *upf2* had little effect on the rate of senescence (Table 5), indicating that *upf* regulation of *STN1* contributes to the delayed senescence of *upf2Δ* telomerase-deficient strains.

TEN1 encodes an essential gene of unknown function that is required for regulation of telomere length and chromosome end protection (16). *TEN1*, like *STN1*, is regulated by NMD through its 5'-UTR sequences (9), but high levels of *TEN1* alone do not affect telomere length. To determine whether Ten1p contributes to the senescence delay in NMD mutants, we constructed a strain containing a high-copy plasmid in which the *TEN1* promoter sequences were replaced with *P_{GAL1}*. In contrast to the *est2Δ P_{GAL1}-STN1* strain, *tlc1Δ YEP P_{GAL1}-TEN1 upf2Δ* strain demonstrated a delay in senescence that was similar to the telomerase-deficient *upf* mutants (Table 5). Thus, NMD regulation of *TEN1* mRNA levels alone does not affect the rate of senescence in telomerase-deficient strains.

TEN1 and *STN1* function together in telomere capping. Furthermore, extra *TEN1* enhances the short-telomere phenotype associated with extra Stn1p (9, 16). Therefore, we asked whether higher levels of Stn1p together with extra copies of *TEN1* would affect the rate of senescence more than higher

TABLE 5. Overexpression of *STN1* but not *TEN1* phenocopies loss of the NMD pathway

Genotype	Median PD	<i>n</i>	Median difference ^a	Lower limit	Upper limit
<i>est2Δ</i> P _{GALI} - <i>STN1</i>	53	6			
<i>est2Δ</i> P _{GALI} - <i>STN1 upf2Δ</i>	65	6	10	-0.6	13.9
<i>tlc1Δ</i> YEP P _{GALI} - <i>TEN1</i>	62	11			
<i>tlc1Δ</i> YEP P _{GALI} - <i>TEN1 upf2Δ</i>	80	11	17*	10	19
<i>est2Δ</i> P _{GALI} - <i>STN1</i> + YEP- <i>TEN1</i>	64	10			
<i>est2Δ</i> P _{GALI} - <i>STN1</i> + YEP- <i>TEN1 upf2Δ</i>	64	8	-0.3	-0.8	0.04

^a *, significant ($P < 0.05$) median difference (Hodges-Lehmann estimator) (45). n = number of independent isolates.

levels of Stn1p alone. For this experiment, *TEN1* was expressed from YEP-*TEN1*, which contains only the portion of the *TEN1* promoter that is not subject to NMD regulation (M. McClellan, unpublished data), while *STN1* was expressed from P_{GALI}-*STN1*. Interestingly, *est2Δ* cells expressing increased levels of both *STN1* and *TEN1* together exhibited no *upf*-dependent change in the rate of senescence. Thus, increasing the levels of both *STN1* and *TEN1* mRNAs is sufficient to phenocopy the *upf* delayed-senescence phenotype and can explain how the NMD pathway affects the rate of senescence in telomerase-deficient strains.

CDC13 effects on senescence are independent of NMD. *CDC13*, along with *STN1* and *TEN1*, is a component of the telomere cap that is important for telomere maintenance. It recruits telomerase and regulates telomere access to telomerase (4, 14, 39). The *cdc13-2* allele was isolated in the same genetic screen that isolated strains carrying mutations in *EST1*, *EST2*, and *EST3* (29). Like the other *est* mutants, *cdc13-2* mutants have short telomeres and undergo senescence (29). However, unlike the *est1*, -2, and -3 alleles isolated, the *cdc13-2* allele retains some residual telomerase activity in vivo and senesces more slowly than other *est* mutants (29). Furthermore, Cdc13-2p does not interact with Stn1p by two-hybrid analysis (4). To determine if the duration of senescence in *cdc13-2* strains is affected by loss of the NMD pathway, we constructed and sporulated the appropriate heterozygous diploids and monitored the senescence of *cdc13-2 upf2Δ* spores in serial liquid culture passages. We found that *cdc13-2* strains, like *upfΔ estΔ* and *upfΔ tlc1Δ* strains, senesced after ~80 PDs. However, unlike the other telomerase-deficient strains, the difference in the timing of senescence between the *cdc13-2* and *cdc13-2 upf2Δ* strains was not significant (Table 2), indicating that senescence of *cdc13-2* mutants is not delayed by loss of the NMD pathway. This is likely due to the fact that Cdc13 mRNA levels are not regulated by NMD (9) and the lack of interaction between Cdc13-2p and Stn1p (4). Thus, alterations in the structure of the telomere cap protein Cdc13p, like alterations in the amount of the telomere cap protein, Stn1p, regulate the rate of senescence in telomerase-deficient cells.

DISCUSSION

Cells that lack telomerase components undergo replicative senescence resulting in a reduction of proliferative potential. The NMD pathway accelerates senescence in otherwise wild-type cells that lack either telomerase holoenzyme components

(TLC1 and Est2p) or accessory factors (Est1p and Est3p). Loss of the NMD pathway causes a delay in the senescence process such that telomerase-deficient *upfΔ* strains live for ~10 to 25 PDs longer than UPF⁺ strains. As in wild-type telomerase-deficient cells, senescence in *upfΔ* strains is accompanied by gradual erosion of telomeric DNA and culminates in crisis.

The extension of proliferative potential in *upfΔ* strains involves neither accelerated survivor formation nor *RAD52*. Yet, as with wild-type strains, survivors arise eventually in later passages of *upfΔ* telomerase-deficient strains. This supports the idea that survivor formation is not due to a stochastic process that occurs continuously. Rather, the *RAD52*-dependent activities that give rise to survivors are induced at a critical point in the senescence process. Interestingly, in *upfΔ* strains, type I survivors are more prevalent, while type II survivors appear with less frequency (data not shown). Furthermore, the delay of senescence in the telomerase-deficient *upfΔ* mutants is not due to the *MEC3*-dependent telomere checkpoint.

STN1 and *TEN1* mRNA levels are regulated by NMD through their 5'-UTR sequences; *upfΔ* strains have elevated levels of *STN1* and *TEN1* mRNAs (28). Increased levels of *STN1* and *TEN1* expression contribute to the short-telomere phenotype of *upfΔ* in telomerase-proficient cells (30). Increased levels of *STN1* and *TEN1* also phenocopy most of the senescence delay seen in *upfΔ* strains (Table 5), indicating that *upfΔ*-mediated senescence delay is primarily due to changes in the telomere cap structure. We propose that the extended proliferative potential of telomerase-deficient *upfΔ* strains is caused by a reinforcement of the telomere cap structure when extra Stn1p is available (Fig. 4). We propose that excess Stn1p strengthens the telomere cap and thus protects the chromosome ends from degradation in telomerase-deficient cells. In cells with active telomerase, extra copies of *STN1* may result in shorter telomeres (9, 30), because the reinforced telomere cap reduces the access of telomerase to the telomeres (Fig. 4).

Increased expression of *TEN1* alone had no effect on senescence rates (Table 5). Similarly, extra *TEN1* had little effect on telomere length or telomeric silencing phenotypes associated with *upfΔ* mutations (9). This is consistent with previous reports that found, in contrast to excess *STN1*, excess *TEN1* had no effect on the suppression of *cdc13-1* temperature sensitivity or on the negative regulation of telomere length (9, 16). However, as in other situations (9, 16, 30), increased *TEN1* expression enhanced the effect of extra *STN1* expression.

Cdc13p is a component of the telomere cap that recruits telomerase to the telomere (12, 14, 23). *cdc13-2* was isolated as

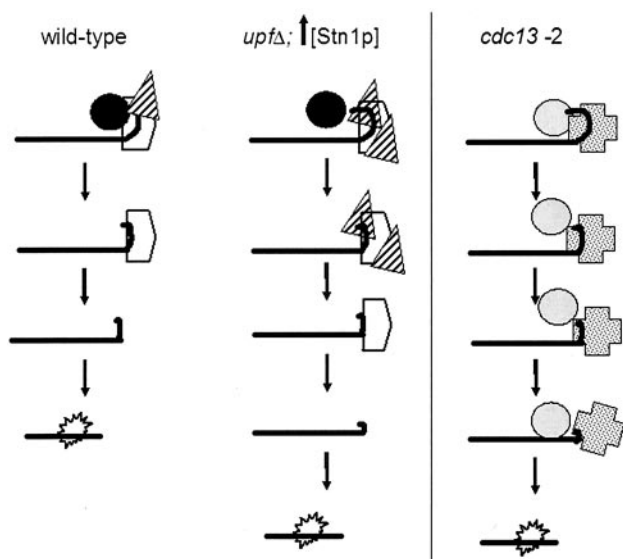


FIG. 4. Model for the role of the telomere cap in senescence in telomerase-deficient cells. The three columns represent three states: wild-type, increased Stn1p levels, and *cdc13-2* strains. The top row in each case indicates the situation in the presence of telomerase. The second row illustrates the situation after the loss of telomerase, and subsequent rows indicate events occurring with increasing PDs. Black and gray circles represent telomerase, striped triangles represent Stn1p, and open pentagons and dotted crosses represent Cdc13p.

an *est* mutant (*est4*) (29, 35). However, *cdc13-2* strains senesce more slowly than *tlc1Δ*, *est1Δ*, *est2Δ*, and *est3Δ* strains, and in *cdc13-2* strains, the rate of senescence is not affected by the NMD pathway. This suggests that the telomere cap structure in *cdc13-2* mutants is different from that in the other telomerase-deficient (*estΔ* and *tlc1Δ*) mutants. The presence of similar rates of senescence in *cdc13-2* and *upf2Δ* strains is consistent with the idea that these mutations delay senescence by altering the telomere cap structure. For *upfΔ* mutants, the cap is altered by increasing levels of Stn1p, which, we propose, also limits access of telomerase and senescence activities to the telomere (Fig. 4). *cdc13-2* alters the cap in a different way that makes it insensitive to *upf* mutations and to levels of Stn1p. Alternatively, because Cdc13p has multiple roles at the telomere, it could affect senescence independent of its role as a telomere cap component.

One function of the telomere cap is to preserve the chromosome ends from degradation. The rate of senescence is influenced by the initial telomere length at the time that cells lose telomerase: strains with shorter telomeres progress through senescence more rapidly than those with initially longer telomeres. Furthermore, telomerase-deficient *upfΔ* strains with shorter telomeres, due to *upfΔ/upfΔ* parents or to high levels of Stn1p in the parent strains, senesce more rapidly than telomerase-deficient *upfΔ* strains with wild-type telomere length. Our results support the assumption that telomere length is a critical factor in determining a cell's proliferative potential. However, it is important to note that the NMD pathway does not affect the rate of senescence by affecting telomere length: telomerase-deficient *upfΔ* cells have average telomere lengths that, at the time of crisis, appear to be shorter

than the average telomere lengths in otherwise wild-type telomerase-deficient cells. Because the telomeric cap components bind telomeric G-overhang structures (3), we cannot rule out the possibility that strains with altered cap proteins may also have altered G-overhang structures. Nonetheless, telomere cap structure, rather than telomere length alone, is an important contributor to the rate of senescence.

Based on our results, it is appealing to consider the idea that increasing telomere end protection may facilitate the modulation of life span in other organisms. This is consistent with the observation that overexpression of mammalian *TRF2* protects shortened telomeres and delays senescence (25). In most human cells, telomerase is not active. Cultured cells exhibit replicative senescence partially due to limited telomerase activity (reviewed in reference 54). Much remains unknown, however, about life span and replicative senescence in whole organisms.

An intriguing connection between telomeres and the NMD pathway was recently revealed in humans. KIAA0732 (hEST1A) was identified by two groups as being a human homologue of Est1p (40, 46). Overexpression of KIAA0732 (hEST1A) caused end-to-end chromosome fusions (40) and altered telomere length (46). At the same time, KIAA0732 was also shown to be similar to SMG5/7a, *Caenorhabditis elegans* genes involved in the NMD pathway. KIAA0732 (hSmg5/7a) copurifies with Upf1p, Upf2p, and Upf3p and may target a protein phosphatase 2A to Upf1p (7). The mechanisms by which NMD affects telomere functions and the role of KIAA0732 in human cell life span remain to be determined.

One approach to extending life span or combating the ill effects of replicative senescence is to reactivate telomerase. A reasonable criticism of this approach is that telomerase activation may be a barrier to cellular immortalization, which would be lost and therefore may lead to an increased risk of carcinogenesis (19, 38), although several experiments have suggested that telomerase activation alone does not increase the occurrence of cancers in mice (5, 41). Our results suggest an alternative approach to increasing life span, without generating immortal cells, by reinforcing the telomere cap structure through increased expression of a cap binding protein. Reinforcing the telomere end structure does not activate telomerase, and at least in yeast, *upfΔ tlc1Δ* cells appear normal for ~20 additional PDs and then undergo crisis in an apparently normal manner that produces rare survivor cells. It has been assumed that in the absence of new telomere synthesis, telomeres would erode during each cell cycle, and thus cell life span would be determined by telomere length. An alternative mechanism to extend cellular replicative potential may be to alter the chromosome end protection complex stoichiometry or structure.

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