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MEC3, *MEC1*, and *DDC2* Are Essential Components of a Telomere Checkpoint Pathway Required for Cell Cycle Arrest during Senescence in *Saccharomyces cerevisiae*

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When telomerase is absent and/or telomeres become critically short, cells undergo a progressive decline in viability termed senescence. The telomere checkpoint model predicts that cells will respond to a damaged or critically short telomere by transiently arresting and activating repair of the telomere. We examined the senescence of telomerase-deficient *Saccharomyces cerevisiae* at the cellular level to ask if the loss of telomerase activity triggers a checkpoint response. As telomerase-deficient mutants were serially subcultured, cells exhibited a progressive decline in average growth rate and an increase in the number of cells delayed in the G2/M stage of the cell cycle. *MEC3*, *MEC1*, and *DDC2*, genes important for the DNA damage checkpoint response, were required for the cell cycle delay in telomerase-deficient cells. In contrast, *TEL1*, *RAD9*, and *RAD53*, genes also required for the DNA damage checkpoint response, were not required for the G2/M delay in telomerase-deficient cells. We propose that the telomere checkpoint is distinct from the DNA damage checkpoint and requires a specific set of gene products to delay the cell cycle and presumably to activate telomerase and/or other telomere repair activities.

INTRODUCTION

Telomeres, the nucleotide-protein structures at the ends of linear chromosomes, serve as a cap to protect the ends of chromosomes (reviewed in Blackburn, 2000). The function of this cap must strike a balance between facilitating and limiting access to the telomere. The cap must allow access to telomeric DNA for DNA replication enzymes, replication forks, and telomerase. In contrast, access to other factors that degrade and/or modify DNA ends must be limited. Thus, the cap must be dynamic, coordinating access to telomere DNA with other cellular events such as DNA replication or mitosis.

Telomere DNA is normally replicated by telomerase, a specialized reverse transcriptase that utilizes an RNA template that is an integral component of the enzyme. Telomerase is activated late in S phase, around the time when telomeres are replicated (Wellinger *et al.*, 1993a, 1993b). Cells that are telomerase-deficient due to mutations in the catalytic component of the enzyme, the template RNA, or other required factors undergo senescence, a progressive loss of viability that is dependent on the number of divisions after loss of telomerase (Lundblad and Szostak, 1989; McEachern and Blackburn, 1996; Nakamura *et al.*, 1997). During senescence, telomeres become progressively shorter as population viability declines (Singer and Gottschling, 1994; Lendvay *et al.*, 1996; McEachern and Blackburn, 1996; Lingner *et al.*, 1997). In yeasts, senescence can be detected as reduced numbers of colony-forming units and decreased colony size on solid media or as a decline in the average culture growth rate in liquid cultures (Singer and Gottschling, 1994; Lendvay *et al.*, 1996; McEachern and Blackburn, 1996; Lingner *et al.*, 1997).

Changes in telomere structure can cause cellular abnormalities. In human cells, overexpression of a dominant-negative form of TRF2, a telomere-regulating protein, causes frequent chromosome rearrangements and apoptosis (Karlseder *et al.*, 1999). Dominant-negative telomerase mutations, which inhibit telomerase activity, also trigger apoptosis (Zhang *et al.*, 1999). In Tetrahymena, a mutation in the telomerase template RNA leads to abnormal mitosis in the micronucleus and abnormally large cells (Kirk *et al.*, 1997). In the yeast *Kluyveromyces lactis*, a mutation in the telomerase template RNA causes slow growth, abnormal karyotypes, and aberrant nuclear divisions (Smith and Blackburn, 1999).

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Table 1. Yeast strains used in this study

Strain	Genotype
YJB195	*MAT***a** *ura3-1 ade2-1 his3-11 leu2-3, 112 can1-100 trp1-1*
YJB209	*MATα ura3-1 ade2-1 his3-11 leu2-3, 112 can1-100 trp1-1*
YJB334	YJB195 × YJB209
YJB2768	YJB334 + *nmd2::HIS3/NMD2 VR-ADE2-TEL/VR-TEL tlc1Δ::LEU2/TLC1*
YJB3867	YJB334 + *VR-ADE2-TEL/VR-TEL tlc1Δ::LEU2/TLC1 rad9Δ::URA3/RAD9*
YJB4565	YJB334 + *tlc1Δ::LEU2/TLC1 mec3Δ::TRP1/MEC3*
YJB6361	YJB334 + *VR-ADE2-TEL/VR-TEL tlc1Δ::LEU2/TLC1 sml1Δ::HIS3/SML1 ddc2Δ::KanMX4/DDC2*
YJB6689	YJB334 + *VR-ADE2-TEL/VR-TEL tlc1Δ::LEU2/TLC1 sml1Δ::HIS3/SML1 mec1Δ::HIS3/MEC1 VIIL::UR3-TEL/VIIL-TEL*
YJB6690	YJB334 + *tlc1Δ::LEU2/TLC1 rad53K227A::KanMX4/RAD53*
YJB6741	YJB334 + *tlc1Δ::LEU2/TLC1 GFP-TUB1::HIS3/his3-11*
YJB6744	YJB334 + *tlc1Δ::LEU2/TLC1 mec3Δ::TRP1/MEC3 GFP-TUB1::HIS3/his3-11 ADE2::URA3/ura3-1*
YJB7448	YJB334 + *tlc1Δ::LEU2/TLC1 tel1Δ::URA3/TEL1 VR-ADE2-TEL/VR-TEL*

These data argue that the loss of telomere cap function leads to abnormal growth and loss of cell cycle coordination (reviewed in Blackburn, 2001).

Cell cycle checkpoints coordinate many processes in which one event must be completed before another is initiated. The DNA damage checkpoint is triggered by single stranded DNA (ssDNA) or broken DNA ends (reviewed in Longhese *et al.*, 1998; Zhou and Elledge, 2000), which trigger a cell cycle delay and the activation of damage repair processes. Failure of this checkpoint results in cells that continue to divide a damaged genome, eventually leading to cell death. The DNA damage checkpoint is mediated by several interdependent pathways that require the products of *MEC3*, *MEC1*, *DDC2*, *TEL1*, *RAD53*, and *RAD9* as well as other genes (Usui *et al.*, 2001, reviewed in Longhese *et al.*, 1998; Zhou and Elledge, 2000).

Normal telomeres terminate with a 3′ ssDNA overhang (Blackburn, 2000), yet they do not appear to activate a DNA damage checkpoint. Furthermore, loss of telomerase does not immediately lead to cell death; rather, senescence occurs (Singer and Gottschling, 1994; Lendvay *et al.*, 1996; McEachern and Blackburn, 1996; Lingner *et al.*, 1997). This suggests that the mechanism that monitors replication or genome integrity functions differently at telomeres than it does at other regions of the genome. Thus, the DNA ends at telomeres are either masked from DNA damage checkpoint detection pathways or they actively signal to the DNA damage checkpoint that telomere structure and/or function is normal.

Despite the fact that intact telomeres do not appear to be recognized as double strand breaks (DSBs), telomeres require several checkpoint genes for their replication and maintenance. In *Saccharomyces cerevisiae*, cells lacking the two ATM-like kinases, *TEL1* and *MEC1*, do not maintain telomere length (Craven and Petes, 1999; Ritchie *et al.*, 1999) and undergo senescence similar to that observed in telomerase-deficient cells, despite having functional telomerase components (Chan *et al.*, 2001). Furthermore, telomerase activation requires the ATM kinase functions of either *TEL1* or *MEC1* in humans, *S. cerevisiae*, and *Schizosaccharomyces pombe* (Vaziri, 1997; Naito *et al.*, 1998; Ritchie *et al.*, 1999; Mallory and Petes, 2000; Chan *et al.*, 2001). This implies that ATM kinases positively regulate telomerase activity either directly or indirectly.

The telomere checkpoint model (Blackburn, 2000) posits the existence of a telomere-specific checkpoint that arrests cells and activates telomere synthesis in response to the loss of cap function. This loss of cap function is proposed to occur when telomeres become critically short because of lack of telomerase activity or because alteration of telomere components renders the telomere inaccessible to telomerase. In this report, we tested the telomere checkpoint model by analyzing telomerase-deficient cells undergoing senescence.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

All strains used in this study were isogenic with W303 and are listed in Table 1. The *TLC1* disruption was made by a one step gene replacement in diploid YJB334 using plasmid pBlue61::*LEU2* (Singer and Gottschling, 1994). *tlc1* isolates from early passages were used in standard crosses. The *mec3*::*TRP1*, *mec1*::*HIS3*, *ddc2*::*KanMX4*, and *sml1*::*Kanx4* alleles were obtained from M.P. Longhese (Milan, Italy) in strains DMP2145/16C, DMP2952/2B, and DM2995/1B, respectively (Paciotti *et al.*, 1998, 2000). The *rad53-K227A* kinase domain allele was obtained from M. Foiani (Milan, Italy) in yeast strain CY2034 (Pellicioli *et al.*, 1999). The *rad9*::*URA3* allele was obtained from O. Tsuchiya (Higashi-Hiroshima, Japan) in yeast strain W-DR9a (Mizunuma *et al.*, 1998). The *sml1*::*HIS3* and *tel1*::*URA3* alleles were obtained from T. Petes (University of North Carolina) in yeast strain JMY303 and SPY40 (Ritchie *et al.*, 1999). The *GFP-TUB1* allele was obtained from K. Blumer (Washington University) in yeast strain KBY215 (Holly and Blumer, 1999). All of these alleles were introduced into our W303 strain background by standard crosses and multiple backcrosses. Sporulation and tetrad dissection were performed according to standard methods (Sherman and Hicks, 1991). Strains were maintained in standard yeast media (Sherman, 1991).

Serial Plate Passages of Senescing Cultures

Telomerase-deficient cells were obtained by sporulation and dissection of heterozygous diploids (YJB2768, 3867, 4565, 6361, 6689, 6690, 6741, 6744, and 7448). At least six independently derived spores of each genotype were restreaked from the tetrad dissection plate onto fresh solid medium and grown for 24 h at 30°C. The senescing cultures were serially restreaked from the thickest region of the plate for up to 10 passages. Images of each passage were captured on a Nikon Cool Pix 900 digital camera mounted on a Zeiss stereoscope Stemi DRC. To examine the spindle structure of wild-type and *tlc1*Δ senescing cultures, strains containing *GFP-TUB1* were

obtained by sporulation and dissection of heterozygous diploids and were serially passaged by successive restreaking on YPAD plates. Fluorescence microscopy of >400 live cells per passage, (mounted in 15% glycerol), were scored for spindle length and bud size.

Quantitative Measurements of Colony Sizes from Senescing Serial Liquid Cultures

To calibrate the assay, the number of cells in six independent wild-type colonies was determined. We found that estimates of cell number based on colony forming units in early passages was variable and often resulted in significant underestimates (3- to 10-fold) of actual cell number. We established the relationship between measured colony area and cell number by measuring colonies of different sizes for colony area and then manually dissecting and counting the total number of cells in each colony. Measurements of the colony area were reproducible and readily distinguished two-fold differences in cell number in the wild-type strain.

Colony areas were measured for senescing cultures after limiting passages in liquid media and outgrowth on solid media. Telomerase-deficient cells were obtained by sporulation and dissection of the relevant heterozygous diploid strains. Spore colonies were grown for 2 d after dissection on solid medium at 30°C. These colonies were suspended in 15% glycerol, and $\sim 10^5$ cells were inoculated into 1 ml of YPAD. The liquid cultures were grown for 24 h at 30°C, at which point they had typically completed 10 population doublings and had reached stationary phase. The 24-h liquid cultures (passage 1) were diluted 1:1000 into fresh YPAD liquid and grown again for 24 h at 30°C (passage 2). Single cells from each passage were spotted onto solid YPAD media and grown for 24 h at 30°C to quantitate and visualize individual cell growth. Six serial cultures were generated by successive dilution (1:1000) of the previous 24-h culture. Images of colonies from each passage were captured with a Nikon Cool Pix 900 digital camera (Melville, NY) mounted on a Zeiss stereoscope Stemi DRC (Sterling Heights, MI).

Cells that remained from the serial liquid cultures were prepared for DAPI staining by dilution into fresh YPAD medium and growth at 30°C for 6 h. Cells were fixed with 75% ethanol for 30 min, washed one time with water, and stained with 1 ng/ml DAPI for 24 h at 4°C before visualization of nuclear DNA by fluorescence microscopy. For each strain and passage, >400 cells from each independent isolate were scored for nuclear position and bud morphology. Chi square values for each data set were calculated (Snedecor and Cocharan, 1980) to test the null hypothesis that each data set would contain a single mean (i.e., that passaging the cells would not alter their cell cycle distribution).

RESULTS

Progressive Decrease in Colony Size During Senescence Is Due to a Decreased Rate of Cell Division

Yeast strains that incur DNA damage or are deficient in proteins required for DNA replication undergo an abrupt cell cycle arrest (Zhou and Elledge, 2000). Telomerase-deficient yeast, however, undergo replicative senescence and a progressive decline in viability (Lundblad and Szostak, 1989; McEachern and Blackburn, 1996; Nakamura *et al.*, 1997). Senescence may be due to a reduction in the rate of cell division or to an increase in the rate of cell death events. To examine the process of senescence in telomerase-deficient yeast, we isolated mutant cells lacking TLC1, the RNA component of telomerase, by sporulating a diploid *tlc1*Δ/*TLC1* strain (Singer and Gottschling, 1994).

Six independent *tlc1*Δ isolates were serially subcultured by restreaking them on plates every 24 h for 10 consecutive days. We refer to each serial subculture as a "plate passage" to distinguish these experiments from the later assays utilizing liquid cultures. For reference, wild-type cells undergo about six doublings during a single plate passage. As expected, all *tlc1*Δ spores senesced, despite some variation in the timing of senescence. This variation was a property associated with each individual spore because early or late senescence of specific spore progeny was reproducible. Cultures were plated at low density and examined after 24 h to determine if cells in the senescent population were either failing to divide or were dividing at a reduced rate. We followed one representative spore in detail.

*tlc1*Δ cells streaked directly from the tetrad dissection plate (founder cells) formed uniform sized colonies that were nearly wild type in colony size (Figure 1A). However, cells from serial plate passages 1–4 formed colonies that were progressively smaller with each passage. Importantly, the smaller colonies (white arrows) were not accompanied by the presence of significant numbers of dead cells, which could have accounted for the colony size decrease (as seen with *mec3*Δ *tlc1*Δ, Figure 1B). This implies that cell death is not a frequent event during early plate passages in telomerase-deficient cells.

Plate passages 4–5 exhibited the lowest levels of viability, as determined by the growth of the population. On solid medium, a mixture of small colonies and large colonies was evident. One striking feature of the small colonies was that they contained cells that had irregular shapes and very large sizes (Figure 1A, arrow marked M). Some of these individual conspicuous cells were about five times larger than individual wild-type cells, implying that the cells increased in cell size in the absence of cell division. We conclude that the primary reason for senescence during the early passages of telomerase-deficient cells is not due to an increase in cell death but rather appears to be caused by a reduced rate of cell division in the population.

In addition to the small colonies composed of irregular, large cells that began to appear in plate passages 4–5, colonies with wild-type-like size appeared in later plate passages. Eventually, either the culture became inviable or wild-type-like colonies became predominant in the culture. The wild-type-like colonies were "survivors" (Lundblad and Blackburn, 1993) that had undergone recombination events at their telomeres (our unpublished results). We focused our attention on the senescence events that occurred before the appearance of these survivors.

The telomere checkpoint model suggests that a cell cycle delay is triggered when one or more telomeres become critically short. It follows that an increased number of short telomeres may trigger a longer cell cycle delay. Late passage cells lacking telomerase should have more critically short telomeres than from earlier passages. To test this model, we measured and compared the rate of cell division in telomerase-deficient cells from different passages. Colony area was used as a quantitative indicator of the number of divisions the *tlc1*Δ cells were able to complete (i.e., growth rate). Analysis of wild-type colony sizes indicated a good relationship between colony area and the number of cells per colony (see MATERIALS AND METHODS). Small colony areas indicate that the senescing *tlc1*Δ cells underwent fewer di-

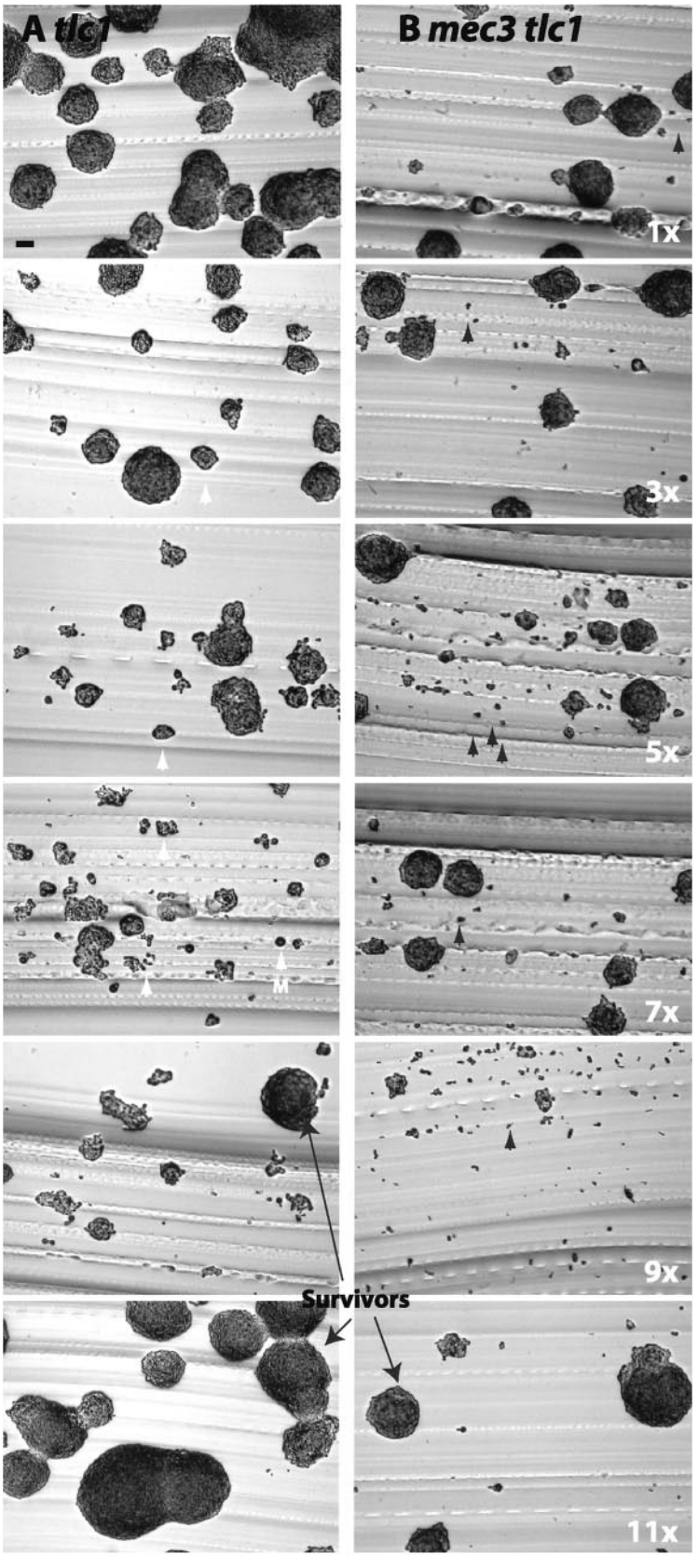

Figure 1. Senescence of *tlc1*Δ and *mec3*Δ *tlc1*Δ strains during serial plate passages. (A) Diploid strain YJB 2768 was sporulated and *tlc1*Δ progeny were isolated. Photomicrographs were taken of successive passages on YPAD plates restreaked onto a fresh plate and grown at 30°C for 24 h. White arrow heads, small senescing colonies; M, monster cells. (B) Diploid strain YJB4565 was sporulated, *mec3*Δ *tlc1*Δ progeny were isolated and grown as in A. Black arrowheads, single cells that did not divide during the course of the plate outgrowth. Note the absence of monster cells in *mec3*Δ *tlc1*Δ cultures. Bar, 0.2 mm. Thin arrows, survivor colonies that form in later plate passages. ×, number of plate passages.

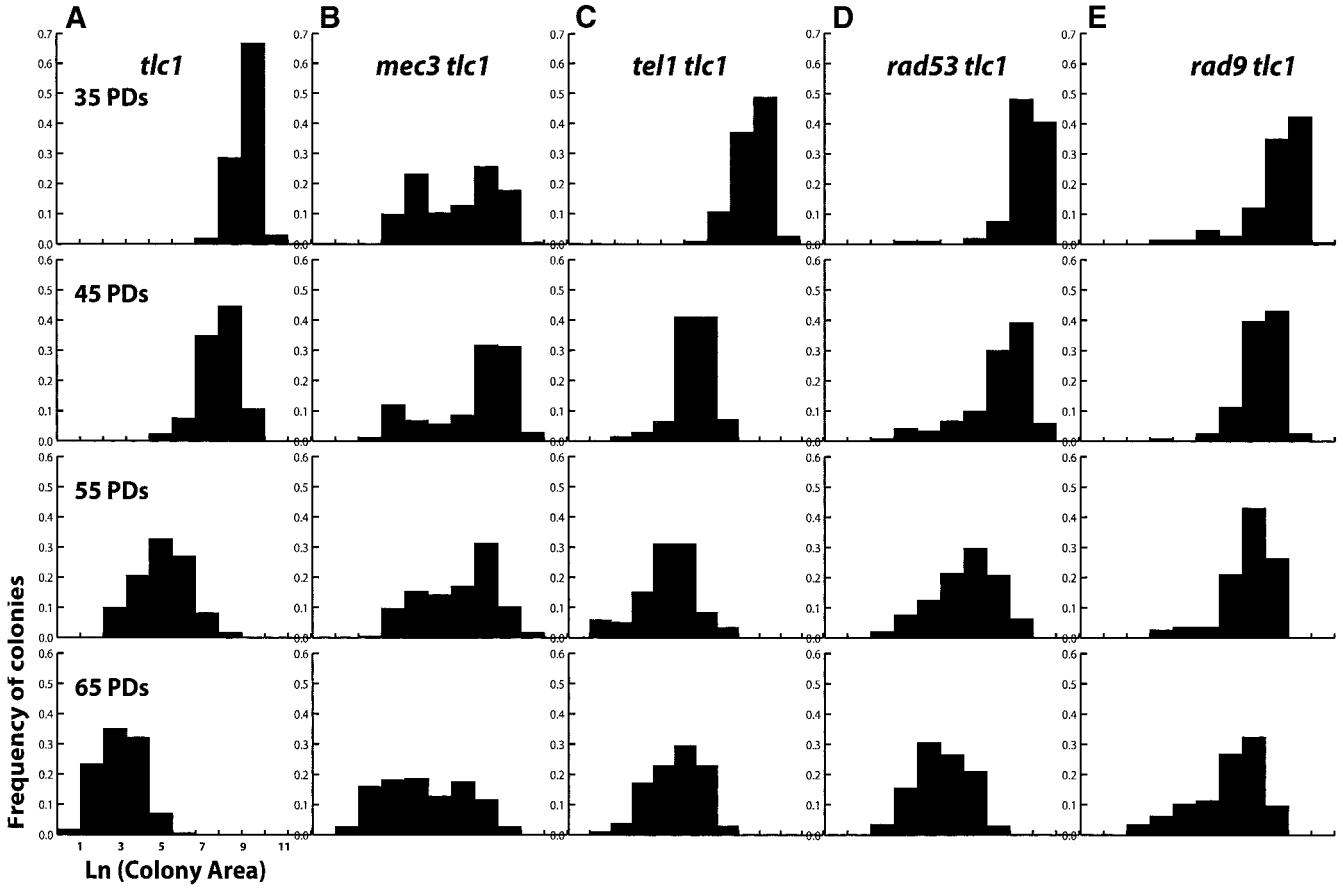

Figure 2. Quantitative measurement of colony area during senescence in liquid passages. Cells were advanced by 10 population doublings in liquid culture and then plated onto YPAD plates. PDs, the average number of population doublings in each culture. Colony area was measured and converted to a natural log scale, expressed as arbitrary units of colony area. Wild-type colonies are typically 4.9 U at 8 h, 5.3 U at 12 h, and 6.8 U at 15.5 h. (A) Colony area after 24 h of growth at 30°C was performed on *tlc1*Δ isolates from diploid strain YJB2768. Note that the *tlc1*Δ median colony area decreases progressively with a Gaussian distribution. (B) Colony area for *mec3*Δ *tlc1*Δ isolates from diploid strain YJB4565. Note the bimodal distribution of colony areas. (C) Colony area for *tel1*Δ*tlc1*Δ isolates from diploid strain YJB7448. (D) Colony area for *rad53-K227A tlc1*Δ isolates from diploid strain YJB6690. (E) Colony area for *rad9*Δ *tlc1*Δ isolates from diploid strain YJB3867.

visions and therefore had a slower growth rate. For these experiments, we serially cultured *tlc1*Δ cells in liquid media so that they were limited to a maximum of ~10 population doublings per passage ("liquid passage"). Cells from these cultures were then plated on solid medium and grown for 24 h, and the colony area was measured. These measurements were plotted as a histogram on a semilog scale (Figure 2A).

For *tlc1*Δ progeny in the first liquid passage, the colony area after the log transformation resembled a Gaussian distribution with a single peak. The shape of the curve and the narrow range of colony areas suggest that members of the population behave in a similar manner. In subsequent liquid passages, the colony size distribution curve of the *tlc1*Δ spore progeny continued to exhibit a Gaussian distribution with a single peak. However, the mean colony size decreased with each successive liquid passage (Figure 2A). The mean colony size as well as the sizes of the largest and smallest colonies decreased in a gradual, progressive manner with each liquid passage. No abrupt transition from the wild-type growth rate to the reduced division rate was evident. These results indicate that (1) the division rate of the entire population of cells was decreasing and (2) the division rate continued to decrease with increasing numbers of passages.

From these experiments we conclude that senescence is primarily a progressive reduction in cell division rate that correlates with the number of population doublings that have occurred in the absence of telomerase. The progressive nature of the reduction in average cell division rate is consistent with the idea that critically short telomeres signal a need for cell cycle delay and that, as cells continue to divide in the absence of telomerase, more telomeres per cell reach this critically short length. In this case, the signal becomes stronger, resulting in a progressively longer cell cycle delay. It is also possible that the number of cells in the population that undergo a cell cycle delay increases as the number of cell divisions after loss of telomerase increases.

The Decreased Rate of Cell Division in tlc1Δ Cells Is Due to a G2/M Delay

To determine if the *tlc1*Δ cells exhibit a slower average division rate because of a specific cell cycle arrest event, we analyzed the cell cycle distribution of *tlc1*Δ cells during senescence. Unbudded cells, cells with a small bud and a single nucleus, and attached cells with two nuclei (that have completed mitosis) are considered to be in G1 or S phase. G2/M cells are those that contain a large bud with a single nucleus near or spanning the mother-bud neck. Several spores were selected for each strain and followed over serial passages (as indicated below). Greater than 400 *tlc1*Δ cells per passage, from each individual isolate, were stained with DAPI and observed by microscopy.

During plate passages 1–3, the cell cycle distribution of *tlc1*Δ cells was similar to that of wild-type cells (Figure 3A). As cell division slowed in plate passages 4–6, the proportion of *tlc1*Δ cells in G2/M phase increased progressively. By plate passage 6, monster cells made up 10% of the population (Figure 1A and our unpublished results). They often contained multiple large buds and fragmented, condensed DNA. Monster cells were excluded from the cell cycle distribution analysis because they do not have normal cell cycle landmarks. Monster cells are likely the result of cells increasing in volume while dividing only rarely because they are held in a cell cycle-delayed state (Reed, 1980; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989). The population of *tlc1*Δ cells in G2/M increased progressively, and by plate passage 6, 80% of *tlc1*Δ cells (excluding monsters) were in G2/M.

To determine if cells with the nucleus at, or spanning the neck, had initiated anaphase, we used fluorescently tagged tubulin (GFP-Tub1p) to examine spindle morphology in *tlc1*Δ mutant cells. Approximately 60% of cells from passages 3–6 exhibited "partially elongated spindles," spindles with a length intermediate between the typical short (S/G2) and typical long (M phase) spindles of wild-type cells (Figure 3F). These partially elongated spindles suggest that the *tlc1*Δ mutants delay before the metaphase-to-anaphase transition. Monster cells often contained partially elongated spindles as well (our unpublished results).

The decreased colony expansion rate, the accumulation of cells with nuclei near or spanning the mother-bud neck, and the prevalence of partially elongated spindles in *tlc1*Δ cells indicates that telomerase-deficient cells exhibit a significant delay in the G2/M stage of the cell cycle that increases progressively with the number of passages after loss of *TLC1*. This observation is consistent with the idea that a checkpoint triggers a cell cycle arrest in response to telomere defects. Furthermore, this checkpoint functions in the absence of TLC1, the telomerase RNA. We observed similar results with *est1*, *est2*, *est3*, and *cdc13–2* cells (our unpublished results). Thus, neither the components of the telomerase enzyme, nor its regulators, are required to activate this telomere checkpoint.

The DNA Damage Checkpoint Protein, MEC3, Is Required for the G2/M Delay of Senescence

Strains lacking a specific checkpoint component fail to arrest the cell cycle in response to the damage signal that normally activates that checkpoint. Checkpoint-deficient cells die as a consequence of the damage (Weinert and Hartwell, 1988). Accordingly, a strain lacking both telomerase and a component of the telomere checkpoint should fail to arrest the cell cycle and should die as a consequence of the lack of telomerase. Thus, a double mutant lacking both *TLC1* and a telomere checkpoint component should not exhibit the reduced division rate, the G2/M delay, or the prevalence of monster cells seen in *tlc1*Δ cells. Rather, the primary mode of senescence seen in the checkpoint-deficient *tlc1*Δ mutants is expected to be an increase in cell death due to cell division in the presence of critically short telomeres.

Mec3p is a checkpoint protein required for arrest in the G2/M phase of the cell cycle in response to DNA damage. It participates with Ddc1p, Rad17p, and Rad24p to prevent cells from completing cellular division when nuclear DNA has been damaged (Weinert, 1992). To ask if Mec3p is involved in a telomere checkpoint in response to critically short telomeres, we isolated *mec3*Δ *tlc1*Δ strains from a diploid parent heterozygous at both loci. Seven independent *mec3*Δ *tlc1*Δ spores were cultured by serial plate passages (Figure 1B). All *mec3*Δ *tlc1*Δ cultures eventually became inviable or gave rise to survivors after multiple passages in both liquid and solid media, indicating that *mec3*Δ *tlc1*Δ cells, like *tlc1*Δ cells, undergo senescence. Because *mec3*Δ *TLC1* strains are viable, senescence and death are presumably due to the lack of telomerase in *mec3*Δ *tlc1*Δ cells.

We then followed a representative isolate in detail. When observed at the cellular level, senescing *mec3*Δ *tlc1*Δ cultures were clearly different from *tlc1*Δ cells. Immediately after the loss of telomerase, the *mec3*Δ *tlc1*Δ founder cells formed colonies that were indistinguishable in size from the *mec3*Δ, *tlc1*Δ and wild-type sibling progeny (our unpublished results). However, as early as plate passage 1, *mec3*Δ *tlc1*Δ cells formed two distinct types of colonies: large colonies (>1000) cells and microcolonies (<10 cells; Figure 1B). The frequency of microcolonies within the population increased with successive plate passages (Figure 1B, black arrows). This suggests that *mec3*Δ *tlc1*Δ cells were not undergoing the uniform, progressive reduction in division rate that was seen in *tlc1*Δ cells (Figure 1A). Rather, a subpopulation of the *mec3*Δ *tlc1*Δ cells grew at a normal rate, forming colonies that were larger than the corresponding colonies in the *tlc1*Δ cultures, whereas the other subpopulation of cells exhibited a high death rate (as early as plate passage 1). Thus, *MEC3* is required for the high levels of viability in early plate passages of *tlc1*Δ cells.

Quantitative analysis of the colony area distribution of serial liquid passages of a single isolate also revealed differences in the *mec3*Δ *tlc1*Δ colonies relative to *tlc1*Δ cultures. In contrast to the Gaussian distribution of *MEC3 tlc1*Δ colony sizes, the *mec3*Δ *tlc1*Δ colonies exhibited a bimodal distribution, even in early liquid passages (Figure 2B). A subset of the population continued to produce colonies with a near-wild-type expansion rate (right side of the distribution curve, Figure 2B). Even in early liquid passages, a large population of the *mec3*Δ *tlc1*Δ population exhibited reduced colony size, eventually producing primarily cells that never divided (left end of the distribution curve). Thus, rather than exhibiting a progressively decreasing division rate, early liquid passage *mec3*Δ *tlc1*Δ cells grew either at a wild-type rate or failed to divide. Furthermore, the population of dead cells in *mec3*Δ *tlc1*Δ cultures (measured by vital staining of

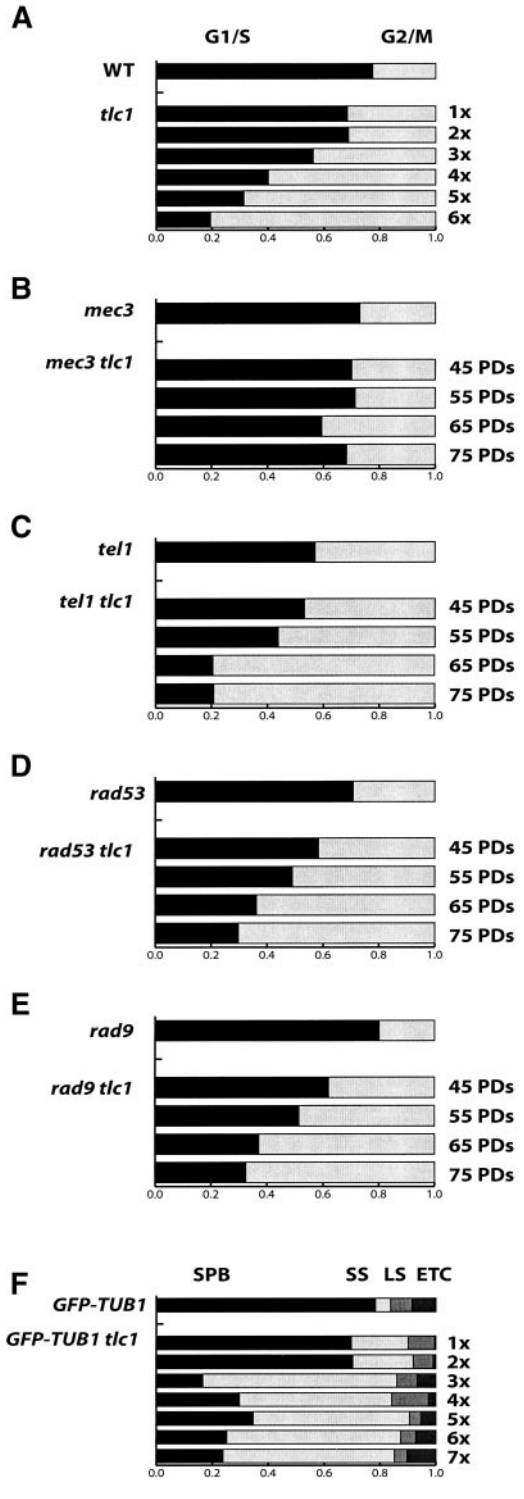

Figure 3. Senescing cultures exhibit an increase in the proportion of cells in the G2/M phase of the cell cycle. ×, the number of plate passages. PDs, the number of culture population doublings in liquid passages. Each bar graph represents the nuclear morphology scored on at least 400 DAPI stained cells from a single passage. Serially passaged cultures were derived from a single spore isolate. (A–E) Proportion of wild-type and *tlc1*Δ cells with a small bud and single nucleus (G1/S, black bar) or a large bud and a single nucleus near or stretched through the mother-bud neck (G2/M, gray bar). (A) Both wild-type and *tlc1*Δ spores were isolated from YJB2768. (B) *mec3*Δ and *mec3*Δ *tlc1*Δ cells isolated from YJB4565. (C) *tel1*Δ and *tel1*Δ*tlc1*Δ cells isolated from YJB7448. (D) *rad9*Δ and *rad9*Δ *tlc1*Δ cells isolated from YJB3867. (E) *rad53K227A* and *rad53K227A tlc1*Δ cells isolated from YJB6690. (F) wild-type and *tlc1*Δ strains expressing GFP-tubulin isolated from YJB 6741. Unduplicated or duplicated spindle pole bodies (SPB, black), short preanaphase spindles (SS, light gray), long anaphase spindles (LS, medium gray), and aberrant spindle forms (ETC, dark gray). Chi square analysis was performed to determine if serially passaged cells altered their cell cycle distribution. *tlc1*Δ, *rad53K227A tlc1*Δ, and *rad9*Δ *tlc1*Δ mutants underwent a significant change in cell cycle distribution during senescence ($p < 0.005$). The *mec3*Δ *tlc1*Δ strain did not exhibit significant change in cell cycle distribution ($p > 0.1$) until after 55 PDs.

YJB4565 spore progeny, our unpublished results) increased with successive passages after the loss of telomerase activity.

The cell cycle distribution of *mec3*Δ *tlc1*Δ cells during senescence was determined from cell shape and nuclear distribution measurements (Figure 3B) as performed on the *tlc1*Δ cells. In contrast to *tlc1*Δ mutants, no dramatic increase in G2/M cells was observed. The *mec3*Δ *tlc1*Δ liquid passages contain ~30% of cells classified as G2/M, and *mec3*Δ *tlc1*Δ cells exhibited similar distributions of cell cycle stages throughout most of the senescence process. We do observe a small but reproducible increase in G2/M cells at ~65 PDs after loss of telomerase, which may indicate that a Mec3p-independent response occurs at a later stage in the senescence process. In plate passage 4 (Figure 1B), the largest cells in *mec3*Δ *tlc1*Δ cultures had a diameter twice that of wild-type cells, whereas *tlc1* monster cells had up to a fivefold increase in diameter, indicating that *mec3*Δ *tlc1*Δ cells do not accumulate monster cells and suggesting that *mec3*Δ *tlc1*Δ do not exhibit the major G2/M delay seen in *tlc1*Δ cells. This is consistent with the idea that *mec3*Δ *tlc1*Δ cells either divided and formed large colonies, died as individual cells, or died after several divisions, forming microcolonies. Taken together with the colony analysis, these data indicate that *MEC3* is required for the progressively reduced colony size, monster cell formation, and the G2/M delay observed during senescence in cells that lack telomerase. Therefore, *MEC3* is a candidate for a component of the telomere checkpoint pathway.

MEC1 and DDC2 Are Also Required for the Cell Cycle Arrest of Telomerase-deficient Cells

MEC1 and *DDC2/LCD1/PIE1* are two essential genes that have a central role in the DNA damage response (Paciotti *et al.*, 2000; Rouse and Jackson, 2000; Wakayama *et al.*, 2001). Mec1p is an ATM-like kinase required for the DNA damage checkpoint response. Ddc2p forms a physical complex with Mec1p and regulates the Mec1p kinase activity (Paciotti *et al.*, 2000; Rouse and Jackson, 2000; Wakayama *et al.*, 2001). When Sml1p, which regulates nucleotide pool levels, is absent, *mec1*Δ *sml1*Δ and *ddc2*Δ *sml1*Δ strains are viable (Zhao *et al.*, 1998). We first asked if Sml1p affects the process of senescence in *tlc1*Δ mutants by measuring colony area after successive passages in liquid medium. Like *tlc1*Δ strains, *tlc1*Δ *sml 1*Δ strains exhibited a normal distribution of colony

sizes that progressively decreased in area with successive liquid passages (Figure 4A). Consistent with previous reports (Ritchie *et al.*, 1999), loss of Sml1p caused a delay in the senescence relative to *tlc1*Δ (our unpublished results). Yet, *tlc1*Δ *sml1*Δ cells, like *tlc1*Δ cells, clearly exhibited a progressive increase in the population of G2/M cells with successive passages (Figure 5A, from 30 to 60% G2/M) when nuclear morphology was examined by DAPI staining. Furthermore, monster cells were evident in passages 5–8 (our unpublished results). Thus, *tlc1*Δ *sml1*Δ mutants, like *tlc1*Δ strains, exhibit a significant G2/M delay during the senescence process, and the *sml1*Δ-mediated delay in the onset of senescence did not affect the process of senescence.

To determine if *MEC1* and *DDC2* contribute to the telomere checkpoint pathway that causes a cell cycle delay in telomerase-deficient cells, we compared the senescence of *mec1*Δ *tlc1*Δ *sml1*Δ and *ddc2*Δ *tlc1*Δ *sml1*Δ cells with *tlc1*Δ *sml1*Δ cultures. We examined seven independent spores of each genotype by successive plate passages (our unpublished results) and followed one representative spore of each genotype in detail by serial liquid passages and measurements of colony area. The *mec1*Δ *tlc1*Δ *sml1*Δ and *ddc2*Δ *tlc1*Δ *sml1*Δ mutants senesced more like the *mec3*Δ *tlc1*Δ mutants described above (Figure 1B), with microcolonies and dead cells appearing in early passages and increasing in frequency with serial passage of the cultures (Figure 4, B and C). Some cells continued dividing and formed large colonies throughout the senescence process, indicating that a subpopulation of the *mec1*Δ *tlc1*Δ *sml1*Δ and *ddc2*Δ *tlc1*Δ *sml1*Δ mutants grew at a normal rate (Figure 4, B and C). In addition, neither the *mec1*Δ *tlc1*Δ *sml1*Δ nor the *ddc2*Δ *tlc1*Δ *sml1*Δ cultures exhibited an obvious accumulation of cells in the G2/M phase of the cell cycle (Figure 5, B and C) or the appearance of monster cells. Taken together the reduced colony expansion rate and G2/M accumulation data, these results indicate that *MEC1* and *DDC2* are required during senescence in telomerase-deficient cells for cell cycle arrest and monster cell formation.

TEL1, an ATM Kinase, Is not a Telomere Checkpoint Component

Tel1p is an IP3 kinase that is most similar to *MEC1* and to the human ATM kinase. *TEL1*, along with *MEC1*, is required for maintenance of telomere length: double mutants have extremely short telomeres and undergo senescence despite having functional telomerase (Ritchie *et al.*, 1999; Craven and Petes, 2000). Recent experiments have implicated *TEL1* as a telomere adapter for the MRX complex that helps recruit telomerase to the telomere (Diede and Gottschling, 2001; Tsukamoto *et al.*, 2001). In addition, *TEL1* can act as a DNA damage sensor that activates *RAD9* and *RAD53* independent of either *MEC3* or *MEC1* (D'Amours and Jackson, 2001; Grenon *et al.*, 2001; Usui *et al.*, 2001). Therefore, *TEL1* appeared to be a good candidate for a telomere checkpoint sensor.

We constructed *tel1*Δ *tlc1*Δ heterozygous diploids and examined 10 independent progeny after sporulation. After plate passages, each of the progeny formed colonies that, like the *tlc1*Δ mutant, were progressively smaller with each passage (our unpublished results). One segregant was examined in detail, and colony area was measured after advance in serial liquid cultures. The resulting histogram of colony area (Figure 2C) contained a single peak that, as in *tlc1*Δ cells, moved progressively toward the left side of the distribution (Figure 2A). The cell cycle distribution of this segregant was also measured. The *tel1*Δ *tlc1*Δ cells demonstrated a gradual increase in the proportion of cells in G2/M, with 47% in the earliest passage and 79% in the last passage, compared with 42% in the *tel1*Δ single mutant (Figure 3C). In addition, later passages of *tel1*Δ *tlc1*Δ cells contained monster cells (our unpublished results). Taken together, these data indicate that, in contrast to *MEC1*, *TEL1* is not required for the cell cycle arrest in response to shortening telomeres.

RAD53 and RAD9 Are not Required for the Telomere Checkpoint

Both the telomere checkpoint and DNA damage checkpoint utilize Mec3p, Mec1p, and Ddc2p. To ask if the cell cycle arrest caused by a lack of telomerase is a specific "telomere checkpoint" response or a general DNA damage response that occurs after the telomeres become uncapped (Blackburn, 2001) and the ends become detected as double-strand breaks or single-stranded DNA, we examined the role of the DNA damage checkpoint genes, *RAD53* and *RAD9*, in response to telomere damage caused by a lack of telomerase.

Rad53p is an essential protein kinase that is the central signal transducer in the DNA damage response pathway (Longhese *et al.*, 1998; Zhou and Elledge, 2000). Rad53p activates the transcriptional response to damage and is also required for cell cycle arrest at G2/M, presumably through its kinase activity. The hypomorphic *rad53-K227A* allele contains a point mutation in the protein kinase domain that eliminates the Rad53p-dependent DNA damage response while not eliminating the essential functions of Rad53p (Fay *et al.*, 1997). After sporulation of the appropriate heterozygous diploid strain, *tlc1*Δ *rad53K227A* progeny were passaged on solid (our unpublished results) and in liquid media as described for *tlc1*Δ mutants. Like *tlc1*Δ colonies, the *rad53K227A tlc1*Δ progeny exhibited a progressive decline in colony area that resembled the dynamics of colony area reduction seen in the *tlc1*Δ strains (Figure 2D). In addition, dead cells appeared in passage 2 and increased in numbers with successive passages (Figure 2D). The increased numbers of dead cells may occur because the kinase activity of Rad53p is required to keep the *tlc1*Δ cells alive during the arrest in response to loss of telomerase activity. Nonetheless, the viable *tlc1*Δ *rad53-K227A* cells gave rise to progressively smaller colonies with successive liquid passages, suggesting that the decline in division rate is similar to that seen in *tlc1*Δ cultures (Figure 2A). Like *tlc1*Δ mutants, the *tlc1*Δ *rad53-K227A* strains also accumulated a large proportion of G2/M cells in successive passages (Figure 3D, 70% at 75 PDs), indicating that Rad53p kinase activity is not required for the G2/M cell cycle delay in telomerase-deficient cells.

The *RAD9* signaling response is independent of the *MEC3* pathway, although both pathways activate *RAD53* (Zhou and Elledge, 2000). Previous work demonstrated that *RAD9* was required to arrest cells with an engineered double-strand break located adjacent to a telomere (Sandell and Zakian, 1993). We asked if *RAD9* was also required for the cell cycle arrest that occurs during senescence in telomerase-deficient cells. Like *tlc1*Δ and *rad53-K227A tlc1*Δ strains, *rad9*Δ *tlc1*Δ strains initially formed large colonies that pro-

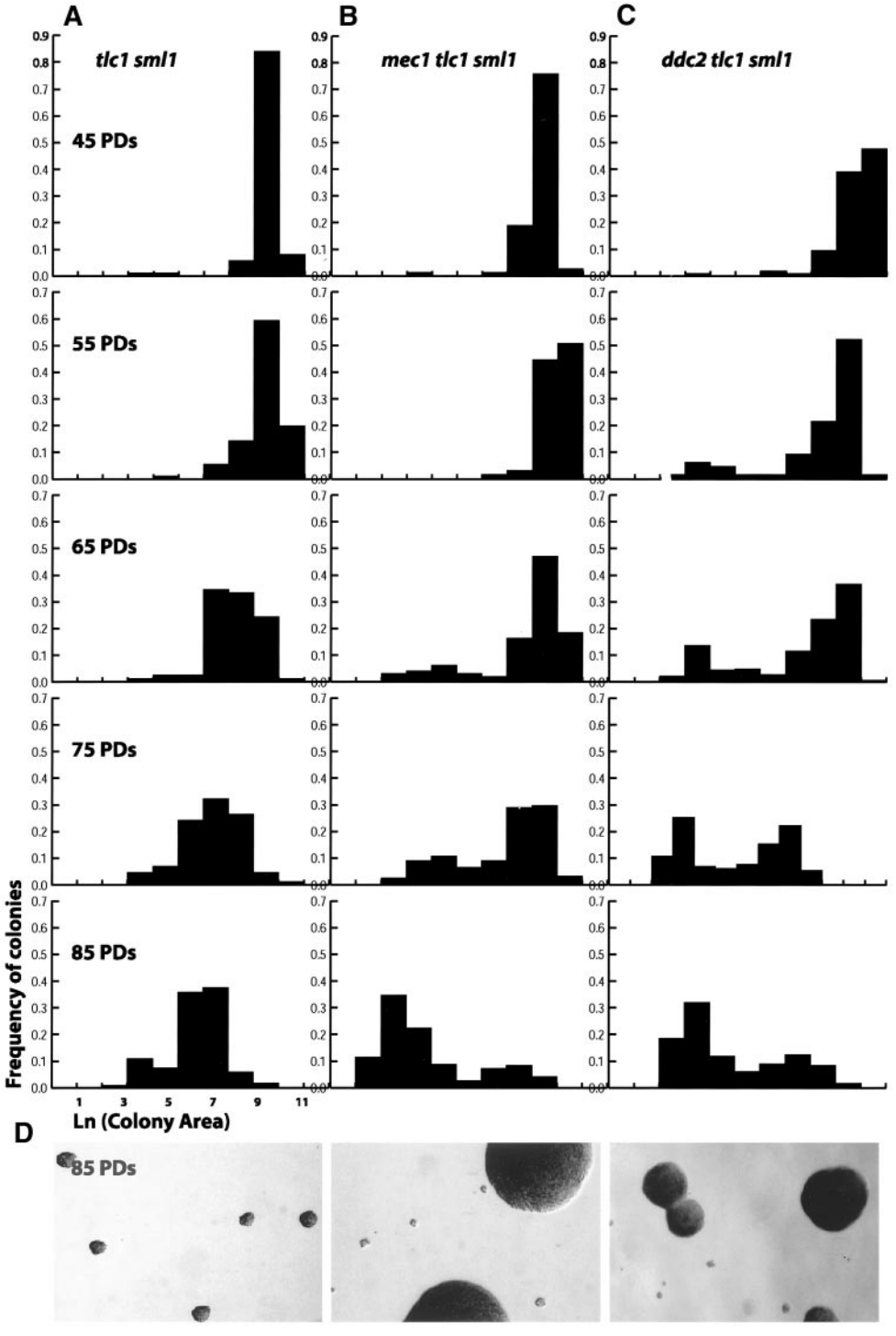

Figure 4. *MEC1* and *DDC2* are required for the cell cycle delay in senescing populations of telomerase-deficient cells. Quantitative measurements of colony areas were determined as described in the legend to Figure 2. (A) *tlc1*Δ *sml1*Δ isolates were spore progeny from strain YJB6689. (B) *mec1*Δ *tlc1*Δ *sml1*Δ isolates were from diploid strain YJB6689. (C) *ddc2*Δ *tlc1*Δ *sml1*Δ isolates were from strain YJB6361. (D) Representative colony populations of *tlc1*Δ *sml1*Δ, *mec1*Δ *tlc1*Δ *sml1*Δ, and *ddc2*Δ *tlc1*Δ *sml1*Δ cultures measured at 85 PDs in the histograms directly above the photomicrographs.

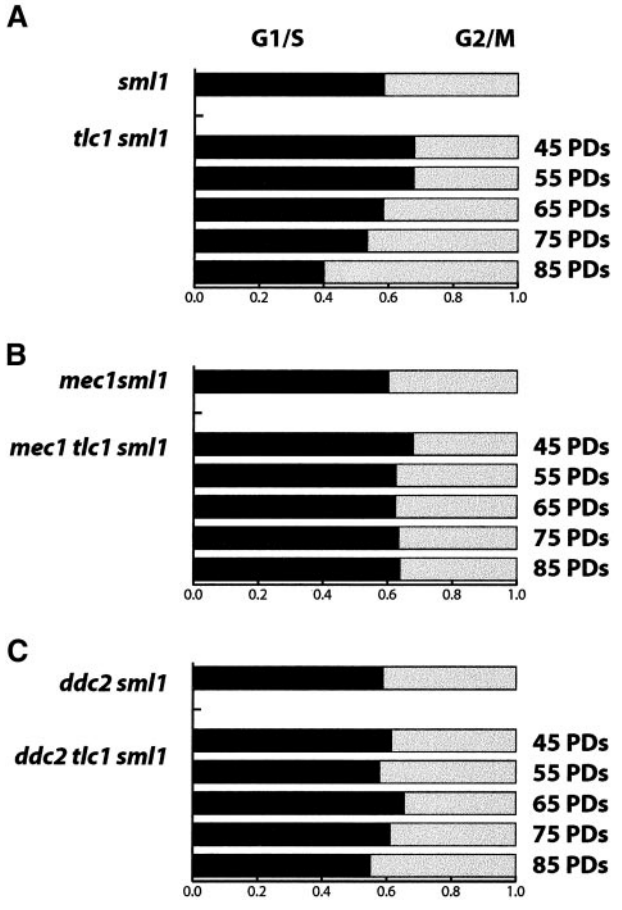

Figure 5. *MEC1* and *DDC2* are required for the G2/M cell cycle delay in telomerase-deficient cells during senescence. The population of cells in G1/S (black bar) or G2/M (gray bar) were determined as described in the legend to Figure 3. (A) *tlc1*Δ and *tlc1*Δ *sml1*Δ isolates were spore progeny from YJB6689. Nuclear morphology was scored on at least 400 DAPI-stained cells for each passage and genotype. (B) *mec1*Δ *sml1*Δ and *mec1*Δ *tlc1*Δ *sml1*Δ isolates were from YJB6689. (C) *ddc2*Δ *sml1*Δ and *ddc2*Δ *tlc1*Δ *sml1*Δ isolates were from YJB6361. Chi square analysis was performed as described in the legend to Figure 3. *tlc1*Δ *sml1*Δ demonstrated a significant ($p < 0.005$) change during serial passages, whereas no significant change ($p > 0.1$) was detected for the *mec1*Δ *tlc1*Δ *sml1*Δ and *ddc2*Δ *tlc1*Δ *sml1*Δ passages.

gressively decreased in average size with successive liquid passages (Figure 2E), indicating that *rad9*Δ *tlc1*Δ strains experience a reduction in cell division rate during senescence. We note that average colony size did not decrease as rapidly in *rad9*Δ *tlc1*Δ strains as it did in the *tlc1*Δ strains. Nonetheless, *rad9*Δ *tlc1*Δ cells accumulated in G2/M with successive passages (Figure 3E, 68% at 75 PDs). Thus Rad9p is not required for the G2/M delay observed during senescence in telomerase-deficient cells. In the *tlc1*Δ *rad9*Δ strains, dead cells were present at levels much higher than in otherwise wild-type *tlc1*Δ cultures. Thus, Rad9p, like the Rad53 kinase activity, appears to be important for maintaining cell viability during senescence. However, neither Rad9p, nor the kinase activity of Rad53p, are required for the cell cycle delay observed in viable telomerase-deficient cells, indicating that the telomere checkpoint is distinct from the general DNA damage checkpoint response.

DISCUSSION

In the absence of telomerase, cells undergo senescence, a process primarily caused by a reduced rate of cell cycle progression during the first several passages of growth. A telomere checkpoint causes a cell cycle delay with the majority of cells exhibiting partially elongated spindles, indicative of a late G2 or early M phase arrest. This delay or arrest is dependent on Mec3p, Mec1p, and Ddc2p, which are also components of the DNA damage checkpoint response. Loss of any one of these checkpoint genes eliminates the progressive increase in cells delayed in G2/M such that cells either continue to divide in the absence of telomerase activity or die, presumably because their telomeres have eroded.

In telomerase-deficient cultures, colonies become progressively smaller and the extent of the G2/M delay in the population increases. Very large monster cells, which continue to increase in size while dividing or budding only rarely, become conspicuous. These observations imply that telomerase-deficient cells spend an increasing amount of time in G2/M as a function of increasing numbers of population doublings after the loss of telomerase. One model to explain this is that a single defective (or critically short) telomere is sufficient to activate a checkpoint-mediated G2/M delay signal and that as more telomeres become defective, the signal becomes proportionally stronger, resulting in a longer G2/M delay. In telomerase-deficient cells, critically short telomeres cannot be repaired. Thus the level of irreparable damage (the numbers of critically short telomeres) increases with increasing passages.

Interestingly, the G2/M arrest in telomerase-deficient cells does not require *RAD9* or *RAD53*. This is surprising because a double-strand break introduced near the telomere triggers a *RAD9*-dependent arrest (Sandell and Zakian, 1993). The rate at which *rad9*Δ *tlc1*Δ colonies become smaller is not as dramatic as the rate of *tlc1*Δ colony size reduction. This could be because cell cycle delays (in addition the G2/M delay that occurs in both strains) occur in *tlc1*Δ but not in *rad9*Δ *tlc1*Δ cells. Furthermore, *rad53-K227A* strains were able to activate the telomere checkpoint (Figure 3C), yet they do not activate the DNA damage checkpoint (Fay *et al.*, 1997). We observed similar results with another *rad53* allele (*sad1-1*, our unpublished results). Thus, the genetic requirements for the senescence response to a loss of telomerase, or to loss of critical telomere components (e.g., Est1p and Est3p) are distinct from the genetic requirements for the response to other forms of DNA damage, including the extensive single-stranded DNA generated in *cdc13-1* mutants (Gardner *et al.*, 1999). This suggests that a critically short telomere is not perceived as a double-strand DNA break (DSB) and that it elicits a response different than the response elicited by a DSB induced only six base pairs away from the telomere (Sandell and Zakian, 1993). The differences between telomere ends and DSBs is likely due to the constellation of telomere-associated proteins, including Ku70/Ku80, the MRX complex, and the Est proteins that ensure that a normal telomere is not a substrate of DNA repair activities (Dubrana *et al.*, 2001).

Because *TEL1* plays a dual role in both telomere maintenance and the DNA damage checkpoint pathway, it might be expected to play a central role in a telomere checkpoint. Surprisingly, *TEL1* is not required for the cell cycle arrest at G2/M in response to eroding telomeres. The MRX complex (*MRE11*, *RAD50*, *XRS2*) acts with *TEL1* to maintain telomere length and an intact DNA damage checkpoint (Ritchie and Petes, 2000; D'Amours and Jackson, 2001; Grenon *et al.*, 2001; Tsukamoto *et al.*, 2001; Usui *et al.*, 2001). We also observed that *rad50*Δ *tlc1*Δ mutants, like *tlc1*Δ cultures, formed progressively smaller colonies during senescence (our unpublished results). This indicates that the MRX complex also is not required for the telomere checkpoint. The MRX complex is thought to activate cell cycle arrest in response to DNA damage by sending signals that feed into the *RAD53-RAD9* pathway (Grenon *et al.*, 2001; Usui *et al.*, 2001). Our results indicate that the telomere checkpoint does not initially utilize *TEL1*, *RAD53*, or *RAD9*. We do not, however, discount the involvement of these proteins in the overall response, because there are differences in colony size distributions between *tlc1*Δ and *tel1*Δ *tlc1*Δ, *rad53*Δ *tlc1*Δ, or *rad9*Δ *tlc1*Δ (Figures 2 and 4). We suggest that initial telomere erosion activates the *MEC3-MEC1-DDC2*--dependent telomere checkpoint, resulting in a G2/M delay. As telomere erosion continues into later passages, a secondary event likely triggers the more conventional DSB damage response.

Our results can be explained by the telomere checkpoint model (Figure 6), which posits that, in wild-type cells, shorter telomeres elicit a checkpoint response that targets them for elongation by telomerase. When telomeres become critically short, the telomere checkpoint delays cell cycle progression, presumably to activate and/or recruit telomerase to the short telomeres. In contrast, the DNA damage checkpoint, mediated by Tel1p, Rad9p, and Rad53p, activates DNA repair activities (Usui *et al.*, 2001, reviewed in Longhese *et al.*, 1998; Zhou and Elledge, 2000). Consistent with the telomere checkpoint model, when a telomere is short, the kinetics of telomere elongation is initially fast, but as telomere length approaches the average wild-type size, the rate of telomere elongation is reduced (Marcand *et al.*, 1999; Ray and Runge, 1999). The telomere checkpoint model may be conserved through evolution because telomere elongation in mouse cells is specifically targeted to the shortest telomeres (Hemann *et al.*, 2001).

Recent work on checkpoints indicates that cell cycle arrest is coordinated with the activation of appropriate cellular responses such as DNA repair pathways (Zhou and Elledge, 2000). For example, Ddc1p and Ddc2p are recruited to sites of double-strand breaks (Kondo *et al.*, 2001; Melo *et al.*, 2001) where they presumably recruit repair activities. For example, in response to DNA damage, the Mec1p-Ddc2p and the Mecp3-Rad17p-Ddc1p complexes are recruited independently of each other, and yet both complexes are found at a DNA lesion (Melo *et al.*, 2001).

Similarly, the telomere checkpoint must have at least two roles at telomeres. First, as demonstrated in this work, it arrests cells at G2/M. Second, we propose that the telomere checkpoint recruits telomerase to chromosome ends, especially those that are critically short, by a mechanism that involves Mec1p, Ddc2p, and Mec3p. Support for the latter role comes from studies of Mec1p, which is required for telomere elongation and the recruitment and/or activation of telomerase in the absence of Tel1p (Ritchie *et al.*, 1999; Tsukamoto *et al.*, 2001), although it does not affect the levels of soluble telomerase activity in the cells (Chan *et al.*, 2001). An intriguing question is how proteins such as Mec1p, Mec3p, and Ddc2p, which are components of both the DNA damage checkpoint and the telomere checkpoint, distinguish between lesions such as DSBs, where DNA repair activities are deployed, and critically short telomeres, where telomerase is recruited and/or activated.

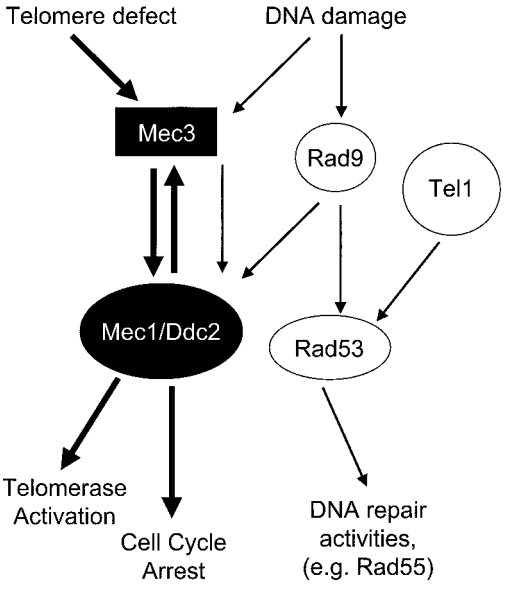

Figure 6. The Telomere Checkpoint model. A defect in the telomere, such as a critically short telomere, triggers the checkpoint pathway (thick black arrows), which requires the activity of Mec3p, Mec1p, and Ddc2p. This leads to cell cycle arrest followed by activation and/or recruitment of telomerase to the short telomeres. The DNA damage pathway (thin black arrows) signals to Mec3p, Mec1p, and Ddc2p and a parallel pathway involving Tel1p is activated. The two DNA damage pathways converge at Rad9p and Rad53p. Activation of the DNA damage checkpoint leads to cell cycle arrest and activation of DNA repair activities but does not activate telomerase.

In telomerase-deficient cells, recombination events that occur in later passages (after 85 PDs) eventually lead to the formation of survivors. Because survivors grow faster than senescing cells, this implies that activities that induce survivor formation are late events that are induced only after failed attempts at telomerase recruitment in the early passages. Furthermore, the induction of survivors, which requires Rad52p as well as Rad51p and/or Mre11p/Rad50p/Xrs2p (Le *et al.*, 1999; Teng *et al.*, 2000; Chen *et al.*, 2001), does not require the telomere checkpoint genes: survivors arose in the later passages of all strains studied in this work (Figure 1). Thus, cells induce these recombination activities, which give rise to survivors, only as a last resort mechanism to maintain viability.

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