Polymerase Epsilon Is Required To Maintain Replicative Senescence[∇][†]

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Replicative senescence is a permanent cell cycle arrest in response to extensive telomere shortening. To understand the mechanisms behind a permanent arrest, we screened for factors affecting replicative senescence in budding yeast lacking telomere elongation pathways. Intriguingly, we found that DNA polymerase epsilon (Pol ε) acts synergistically with Exo1 nuclease to maintain replicative senescence. In contrast, the Pol ε-associated checkpoint and replication protein Mrc1 facilitates escape from senescence. To understand this paradox, in which DNA-synthesizing factors cooperate with DNA-degrading factors to maintain arrest, whereas a checkpoint protein opposes arrest, we analyzed the dynamics of double- and single-stranded DNA (ssDNA) at chromosome ends during senescence. We found evidence for cycles of DNA resection, followed by resynthesis. We propose that resection of the shortest telomere, activating a Rad24[Rad17]-dependent checkpoint pathway, alternates in time with an Mrc1-regulated Pol ε resynthesis of a short, double-stranded chromosome end, which in turn activates a Rad9[53BP1]-dependent checkpoint pathway. Therefore, instead of one type of DNA damage, different types (ssDNA and a double-strand break-like structure) alternate in a "vicious circle," each activating a different checkpoint sensor. Every time resection and resynthesis switches, a fresh signal initiates, thus preventing checkpoint adaptation and ensuring the permanent character of senescence.

Perhaps the most astonishing feature of replicative senescence is its permanent character. Although adaptation to a persistent stimulus is one of the most common physiological processes in nature, cells like fibroblasts remain senescent indefinitely, spending energy to maintain a state that inhibits their natural potential to divide (5). One hypothesis is that replicative senescence was evolutionary selected to avoid proliferation of cells with unrepairable damage, because such cells would generate chromosomally unstable daughters. This hypothesis seems reasonable for multicellular organisms in which chromosomal instability would lead to cancer. However, unicellular yeast also senesces under experimental conditions in which telomerase is inactivated (24). Since wild-type yeast has telomerase activity (7), it is unlikely that it has developed and selected a robust response to telomerase inactivation, which is presumably a very rare event in the wild. Therefore, replicative senescence may not be very different from a conventional checkpoint response to DNA damage. Understanding how a checkpoint response is maintained for the equivalent of tens, perhaps even hundreds, of generations in yeast will help to uncover mechanisms required to maintain replicative senescence in higher organisms.

Yeast cells adapt to a single unrepaired double-strand break (DSB) within hours, an adaptation meaning that they resume cell cycle progression in the absence of repair (19, 20, 31, 36). However, adaptation does not usually occur in cells arrested in response to telomere attrition (39). One explanation could be

that short telomeres in a senescent cell are sending many more damage signals than a single DSB. However, data suggest that one or perhaps a few critically short telomeres are sufficient to induce senescence in yeast or mammalian cells (1, 41). Another explanation could be that a short telomere mobilizes more checkpoint pathways than a DSB. Intriguingly, the opposite was found in budding yeast. The Rad24[Rad17]-9-1-1 complex, Mec1[ATR], and Ddc2[ATRIP] are essential for replicative senescence, whereas Rad9[53BP1] or Rad53[Chk2] is apparently not required (10, 15). Therefore, only a subset of the checkpoint proteins responding to a DSB is required for cell cycle arrest in response to telomere attrition (10, 15).

A role for Rad9[53BP1] during replicative senescence might have been masked by the emergence of survivors dependent on alternative lengthening of telomeres or subtelomeres (ALTOS). We introduce the term "ALTOS" to describe the outcome of any pathway able to maintain/elongate (sub) telomeres in the absence of telomerase. ALTOS survivors could be either Rad52 dependent or Rad52 independent (13, 17). Budding yeast cells from most-genetic backgrounds do not form ALTOS survivors in the absence of Rad52. Instead, they enter senescence and remain senescent (13). Yet, telomerase- and ALTOS-negative cells can escape from senescence when nucleases like Exo1 or helicases like Sgs1 are inactivated (18, 28). Exo1 nuclease degrades dysfunctional telomeres (26, 27). Escapers are called PAL survivors (palindrome-immortalized survivors).

PAL survivors are different from ALTOS survivors. Unlike ALTOS survivors, PAL survivors do not maintain/elongate (sub)telomeres. Instead, PAL survivors progressively lose telomeres, subtelomeres, and single-gene loci (18, 28). After many postsenescence population doublings, essential genes become endangered by degradation and cells that formed end-chromosomal DNA palindromes (inverted duplications) take over the population (25, 27). Intriguingly, many generations before

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forming palindromes, PAL survivors escape from senescence in the absence of TG repeats or any other specific sequences at their chromosome ends (25, 27). Therefore, PAL survivors must have compromised the mechanism required to maintain replicative senescence in order to escape. Similarly, DNA-damaged mammalian cells in the process of becoming cancerous are thought to evade or to disable the DNA damage checkpoint control before they start reshaping their genome, including by forming DNA palindromes (35).

In this study, we used budding yeast lacking both telomerase and ALTOS pathways to address the mechanisms required to maintain replicative senescence. We show that subunits of DNA polymerase epsilon (Pol ε) are required but that Mrc1, a polymerase epsilon-associated checkpoint protein, is opposing senescence. We integrate these and other results into a model of replicative senescence called the "vicious-circle model."

MATERIALS AND METHODS

Yeast strains and cell culture. All strains are derivates of W303 and *RAD5*^{+}. The *tlcl∆ rad52∆* strains originated from the DLY2151 diploid (*TLC1/tlc1∆::HIS3*, *RAD52/rad52∆::TRP1*, *EXO1/exo1∆::LEU2*). In this diploid, we deleted *POL32*, *DPB3*, *DPB4*, or *MRC1* with G418-MX cassettes, as previously described (22). Diploid cells were then sporulated and haploids selected by random spore analysis. To reconfirm their genotype, we tested all selected haploid strains by PCR and some also by Southern blotting. Germinated spores were initially grown on YPD (yeast extract, peptone, and dextrose) plates for 3 to 4 days to form colonies. After circa 25 population doublings on germination plates, cells were propagated as follows. For experiments requiring large number of cells (single-stranded DNA [ssDNA] and chromatin immunoprecipitation [ChIP] measurements), cells were propagated in liquid YPD and diluted daily before senescence, followed by a daily change of medium during senescence. For experiments analyzing generation of PAL survivors, strains taken directly from germination plates were propagated on YPD plates every second day until most cells stopped proliferating (became senescent) by pooling circa 1×10^7 cells with a toothpick and streaking them onto fresh YPD plates. Strains escaping from senescence (PAL survivors) were propagated every 4 days.

Cell cycle analysis was performed by fluorescence microscopy after samples were stained with DAPI (4′,6-diamidino-2-phenylindole). The following fractions were counted: cells without buds (in the G_1 phase), cells with small buds (in the S phase), cells with large anucleated buds (at the G_2/M transition), and cells with nucleated buds (in anaphase/telophase). An exponentially growing culture of wild-type budding yeast has cells similarly distributed between these stages of the cell cycle.

ssDNA measurements were performed using quantitative analysis of ssDNA (QAOS), as previously described (3), except that we used asynchronous populations of cells cultivated at 25°C. Genomic DNA was extracted, purified, and quantified at a centromere-proximal locus situated on chromosome 5R (*PAC2*). The ssDNA was quantified in the Y′ subtelomeric repeats, which are found at 2/3 chromosome ends. Quantifications were performed at the "Y′-1000" and "Y′-5000" regions (meaning 1,000 bp and 5,000 bp, respectively, from the chromosome ends) and normalized to the level for Y′ subtelomeric DNA. Additionally, ssDNA was quantified at the *YER188W* single-gene locus (situated circa 8.5 kb from the chromosome 5R end) and normalized to the level for *PAC2* DNA.

ChIP was carried out by standard methods (4, 9). We performed ChIP in parallel to ssDNA measurements on samples collected during midsenescence (passages 9 to 13), rather than during early senescence or presenescence, to obtain the maximum possible number of senescent cells. The number of cells is a limiting factor during replicative senescence experiments. The association of Pol2, Exo1, and Rad9 with chromatin was detected using the following specific antibodies, distributed by Insight Biotechnology: sc-50442 (anti-Rad9), sc-19941 (anti-Exo1), and sc-6753 (anti-Pol2). sc-19941 detects both hExo1 and scExo1. Cells from each cross-linked sample were additionally treated with anti-goat antibodies (sc-2033) to assess the background cross-linking. Input and precipitated DNAs were quantified by real-time PCR with primers and TaqMan probes specific to a centromere-proximal locus (*ERG26*) or to subtelomeres (at about 1-kb from the chromosome ends).

RESULTS

Single-stranded DNA accumulates and declines periodically during senescence. To investigate the mechanisms required to maintain replicative senescence, we induced senescence by generating and propagating budding yeast cells lacking both *TLC1*, encoding the RNA component of telomerase, and *RAD52*, essential for recombination-induced ALTOS. Replicative senescence in this model system is similar to human replicative senescence, because most human somatic cells do not express telomerase and do not undergo alternative lengthening of telomeres (ALT). Cells in the *tlc1∆ rad52∆* background arrest the cell cycle within a few days and remain arrested until death (17). Therefore, the signal(s) for cell cycle arrest in response to telomere attrition would also be expected to last until cellular death. One signal could be the accumulation of ssDNA at telomeres, because ssDNA, a potent checkpoint activator, was previously detected in senescent yeast and mice with short telomeres (25, 32). ssDNA in telomerase-deficient cells is partially dependent upon the Exo1 nuclease (27, 32).

To determine if levels of ssDNA are consistently elevated during senescence, we performed QAOS (43) with cell samples collected from early senescence to midsenescence (passages 4 to 14). The ssDNA was analyzed at distances of 1 kb (Y′-1000) and 5 kb (Y′-5000) from the chromosome ends for the Y′ subtelomeres and also at 8.5 kb from the right end of chromosome V (the *YER188W* single-gene locus). Interestingly, we found that ssDNA did not remain constant during senescence. Initially, ssDNA accumulated up to 6% at Y′-1000 (Fig. 1B and C). However, a gradual decline followed. The lowest ssDNA fraction was about 1.5% (at passage 9). Then, ssDNA reaccumulated, reaching about 3% at passage 11, and declined afterwards (Fig. 1B and C). A similarly oscillatory pattern was observed at Y′-5000 (Fig. 1D and E) and also at *YER188W* (Fig. 1F), except that cells accumulated considerably less ssDNA further from telomeres. In summary, ssDNA accumulates and declines periodically during replicative senescence.

At least two cycles of ssDNA accumulation and decline were detected. Statistical analysis of data from six ssDNA measurements, performed in two independent groups of isogenic strains, indicated an extremely significant difference ($P < 0.0001$) between passages 5 and 9, marking the slope of the first cycle, and also between passages 9 and 11 ($P = 0.006$), marking the slope of a second cycle (Fig. 1G). In conclusion, the oscillations in ssDNA are nonrandom, suggesting the existence of mechanisms reducing the amount of ssDNA during replicative senescence. Such mechanisms are not entirely surprising, considering that ssDNA is a form of damage that cells, including nondividing cells, may have to deal with frequently. For example, UV damage in quiescent cells triggers under certain circumstances an Exo1-dependent ssDNA accumulation that has to be repaired by DNA resynthesis (11). What is surprising is that ssDNA decreases to almost wild-type levels during replicative senescence (Fig. 1G).

Alternating ssDNA accumulation and decline correspond to alternating checkpoint subpathways. The decline in ssDNA observed during senescence, to values below the threshold required to maintain arrest, may result in a transient cell cycle reentry. Alternatively, a different checkpoint stimulus might be
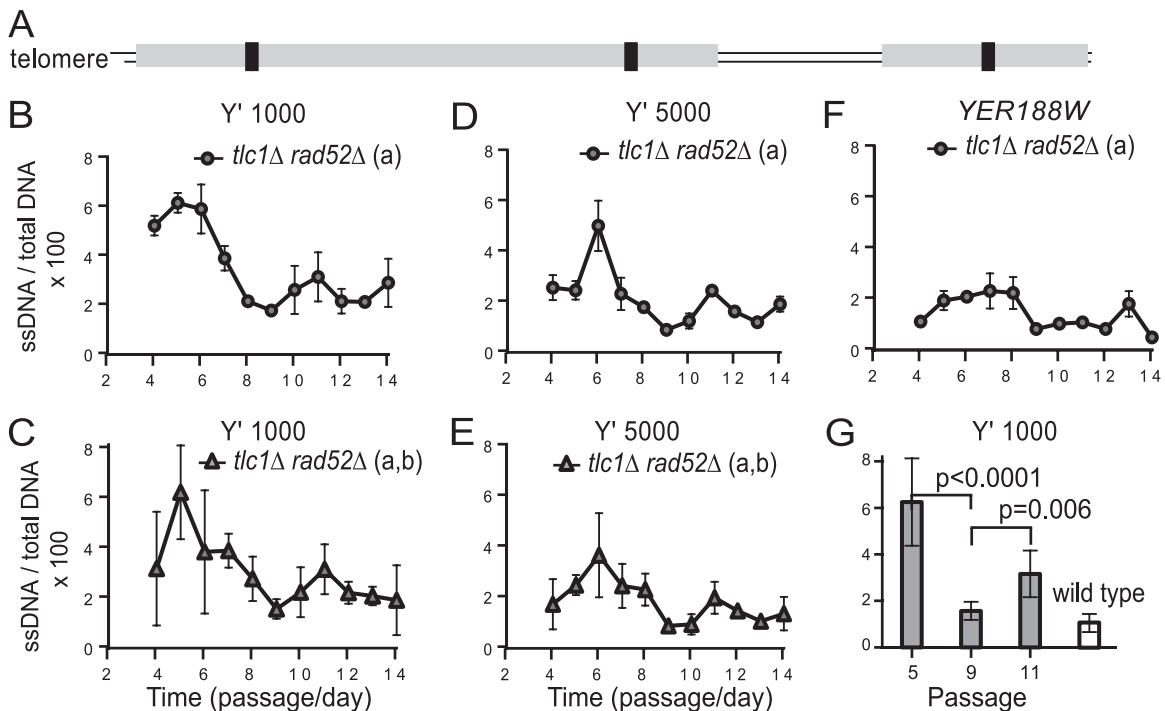
FIG. 1. Dynamics of ssDNA during replicative senescence. (A) Cartoon representing the right arm of chromosome V. (B to G) To ensure a sufficient number of senescent cells, each of three independently generated $tlc1\Delta$ $rad52\Delta$ strains was taken from germination plates, pooled at passage 1, and propagated in groups a and b. (B, D, F) Dynamics of ssDNA during senescence, measured for group a at Y′-1000, at Y′-5000, and at *YER188W*. We called the small sequences in the Y′ subtelomeric repeats situated about 1,000 bp and 5,000 bp from the chromosome ends Y′-1000 and Y′-5000, respectively. Error bars represent the standard deviations (SD) for three measurements. (C, E, G) Average ssDNA calculated for groups a and b at Y′-1000 and at Y′-5000. Error bars represent the SD for six measurements. For group b, the dynamics of ssDNA is presented in Fig. 4 (marked as *DPB*⁺). (G) Statistically significant ssDNA values from senescent strains analyzed for panel E were compared to the average ssDNA values from two wild-type strains treated with nocodazole (to avoid the S-phase-specific ssDNA). Similar *P* values were also obtained for each group.

keeping cells arrested when ssDNA is low. To find out which hypothesis is true, we analyzed ssDNA in correlation with cell cycle distribution in three freshly generated $tlc1\Delta$ $rad52\Delta$ strains, pooled at passage 1, and propagated into senescence. We found that ssDNA (the TG-rich strand) accumulated at passages 1 to 5 and declined at passages 7 to 9 (Fig. 2A), consistent with the pattern observed in Fig. 1. In contrast, ssDNA on the opposite strand (the AC-rich strand) remained constantly below 1%, and therefore, it could be excluded as a potential checkpoint stimulus (Fig. 2A). Accumulation of ss-DNA correlated with cells accumulating in the G_2/M phase of the cell cycle, culminating with over 75% $tlc1\Delta$ $rad52\Delta$ cells arresting in G_2/M at passage 5 (marked as *RAD*⁺ in Fig. 2B). The remaining cells were (arrested?) in G_1. However, the decline in ssDNA at passages 7 to 9 (Fig. 2A) did not correlate with cell cycle reentry (Fig. 2B). Taken together, these data suggest that ssDNA is most likely the trigger for replicative senescence. However, signals other than ssDNA are required to maintain arrest at a later time during replicative senescence, when ssDNA declines.

The hypothesis that ssDNA triggers replicative senescence but that other stimuli replace ssDNA to maintain senescence was further supported by investigations into the checkpoint requirements. We found that a $rad24\Delta$ mutation in the *RAD24* checkpoint gene prevented $tlc1\Delta$ $rad52\Delta$ cells from arresting in G_2/M at passages 1 to 5 (Fig. 2A). However, a high fraction of

cells arrested later on (at passages 7 to 9), despite the $rad24\Delta$ mutation (Fig. 2B). Interestingly, a $rad9\Delta$ mutation in the *RAD9* checkpoint gene had the opposite effect relative to a $rad24\Delta$ mutation: it permitted arrest at passages 1 to 5, whereas it prevented one-third of the strains from maintaining arrest at passages 7 to 9 (Fig. 2B). This indicates that Rad24[Rad17] is essential for the entry into replicative senescence (passages 1 to 5) but that Rad9[53BP1] plays no significant role at that time, consistent with data from the telomerase-negative *RAD52*⁺ background (10, 15). However, a few passages later, arrest becomes Rad9[53BP1] dependent and Rad24[Rad17] independent. In summary, a checkpoint switch takes place during replicative senescence.

The checkpoint switch from Rad24[Rad9] to Rad9[53BP1] (Fig. 2B and C) corresponded to a switch in ssDNA dynamics, from accumulation to decline (Fig. 2A). It is known that Rad24[Rad17] is among the first checkpoint proteins to detect ssDNA (21) but that Rad9[53BP1] responds to double-strand breaks (DSBs) and the adjacent chromatin modifications (38). Therefore, we propose that resection of the shortest telomere(s) activates a Rad24[Rad17]-dependent pathway that initiates replicative senescence but that the decline in ssDNA generates a short double-stranded chromosome end (resembling a DSB) which in turn activates a Rad9[53BP1]-dependent subpathway to maintain replicative senescence.
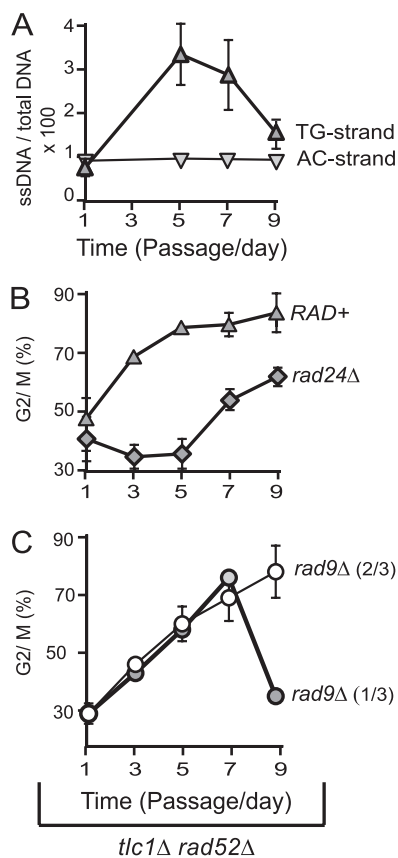
FIG. 2. Alternating ssDNA accumulation and decline correlate with alternating checkpoint responses. (A) ssDNA quantified by QAOS in subtelomeres of the group *RAD*⁺, consistent with three *tlc1Δ rad52Δ* strains taken directly from germination plates, pooled at passage 1, and propagated into senescence. Error bars represent the standard deviations (SD) for three measurements. (B and C) Fraction of cells of *tlc1Δ rad52Δ* strains (with or without *rad24Δ* or *rad9Δ* mutations) accumulating in the G_2/M phase of the cell cycle, once taken from germination plates and propagated into senescence.

Pol ε maintains replicative senescence, acting synergistically with Exo1 nuclease. A decline in ssDNA could be caused either by resynthesis to dsDNA or by ssDNA degradation. If DNA resynthesis was the main cause of ssDNA decline, then a DNA polymerase should be as important as Rad9[53BP1] for maintaining replicative senescence. To test this hypothesis, we propagated into senescence freshly generated *tlc1Δ rad52Δ exo1Δ* yeast strains, with or without mutations in genes encoding the nonessential subunits of DNA polymerases. Examples of this assay are shown in Fig. 3A to C. Each plate was inoculated with eight strains: *tlc1Δ rad52Δ exo1Δ* controls on the left and strains with an additional mutation on the right. At passage 4, all strains arrested proliferation. At passage 7, a fraction of strains escaped from arrest and proliferated afterwards. Such strains are called PAL survivors (28).

We found that strains with a *dpb3Δ* or a *dpb4Δ* mutation in the nonessential polymerase epsilon subunits Dpb3 and Dpb4 escaped from senescence with higher frequency than controls (Fig. 3A). The total fraction of *dpb3Δ* or *dpb4Δ* escapers was 80 to 90%, whereas only about 50% *DPB*⁺ controls escaped (Fig. 3A and D). In contrast, a *pol32Δ* mutation in the Pol32

subunit of Pol δ had no obvious effect in maintaining replicative senescence, since about 50% of either *pol32Δ* or *POL32*⁺ strains escaped (Fig. 3B and F). These data indicate that Pol ε is required to maintain senescence and that its Dpb3 and Dpb4 subunits are important for this function but that Pol δ plays little role. Even in an *EXO1*⁺ background, a *dpb3Δ* or *dpb4Δ* mutation permitted escape from senescence for 10 to 15% of strains (Fig. 3E). In contrast, none of the *DPB*⁺ *EXO1*⁺ cells escaped to generate survivors, consistent with previous experiments (28). These data indicate that (i) Pol ε is essential for maintaining replicative senescence and (ii) Pol ε acts synergistically with Exo1 in the mechanism maintaining replicative senescence.

Mrc1 facilitates escape from replicative senescence. Recent studies demonstrated that Pol2, the catalytic subunit of Pol ε, extensively associates with Mrc1 (23), which is an S-phase checkpoint and replication protein (29) with an additional role in telomere protection against Exo1 (37). Therefore, one possibility is that Pol ε maintains replicative senescence through a checkpoint function conferred by Mrc1. Since Mrc1 is nonessential for viability, we tested its role during replicative senescence, as described for Dpb3 and Dpb4 subunits. Representative plates are shown in Fig. 3C, and the overall outcome from these experiments is presented in Fig. 3F. We found that despite the escape-facilitating *exo1Δ* mutation, only 10% of the *mrc1Δ exo1Δ* strains escaped from senescence but that 50% of the *MRC*⁺ *exo1Δ* controls escaped (Fig. 3C and F). This indicates that unlike a *dpb3Δ* or a *dpb4Δ* mutation, an *mrc1Δ* mutation strongly decreases escape. Conversely, Mrc1 facilitates escape from senescence, which is a paradoxical effect for a checkpoint protein.

Strains with an *mrc1Δ* or *pol32Δ* mutation entered senescence earlier than controls, indicated by the poor growth at passage 2 (Fig. 3B and C). Early senescence could be explained by a requirement for Mrc1 (and potentially for Pol32) in telomere protection (37). Interestingly, the first PAL survivors were identified in strains that entered senescence very early and escaped with high frequency (28). Therefore, early senescence does not imply reduced escape. It is more likely that most strains remained senescent in the absence of Mrc1, because Mrc1 was required to facilitate escape. In conclusion, the hypothesis that Mrc1 confers upon Pol ε a checkpoint activity that contributes to replicative senescence has been disproved. This is because we found that Mrc1 and Pol ε have different, in fact opposing, effects on replicative senescence.

Pol ε is not a checkpoint for uncapped telomeres. We have presented evidence that two checkpoint subpathways, dependent upon either Rad24[Rad17] or Rad9[53BP1], alternate in time to maintain replicative senescence (Fig. 2). Therefore, we analyzed the effects of Rad24[Rad17] and Rad9[53BP1] on the fraction of strains escaping from senescence, as described for Dpb3 and Dpb4. We found that a *rad24Δ* mutation did not affect the fraction of *tlc1Δ rad52Δ exo1Δ* strains escaping from senescence (Fig. 3G). In contrast, a *rad9Δ* mutation increased the fraction of escapers to almost 100%, similarly to the effect of a *dpb3Δ* or a *dpb4Δ* mutation (Fig. 3G). These data suggest that Pol ε may act in the same pathway with Rad9[53BP1] (and a different pathway from Rad24[Rad17]) during replicative senescence.

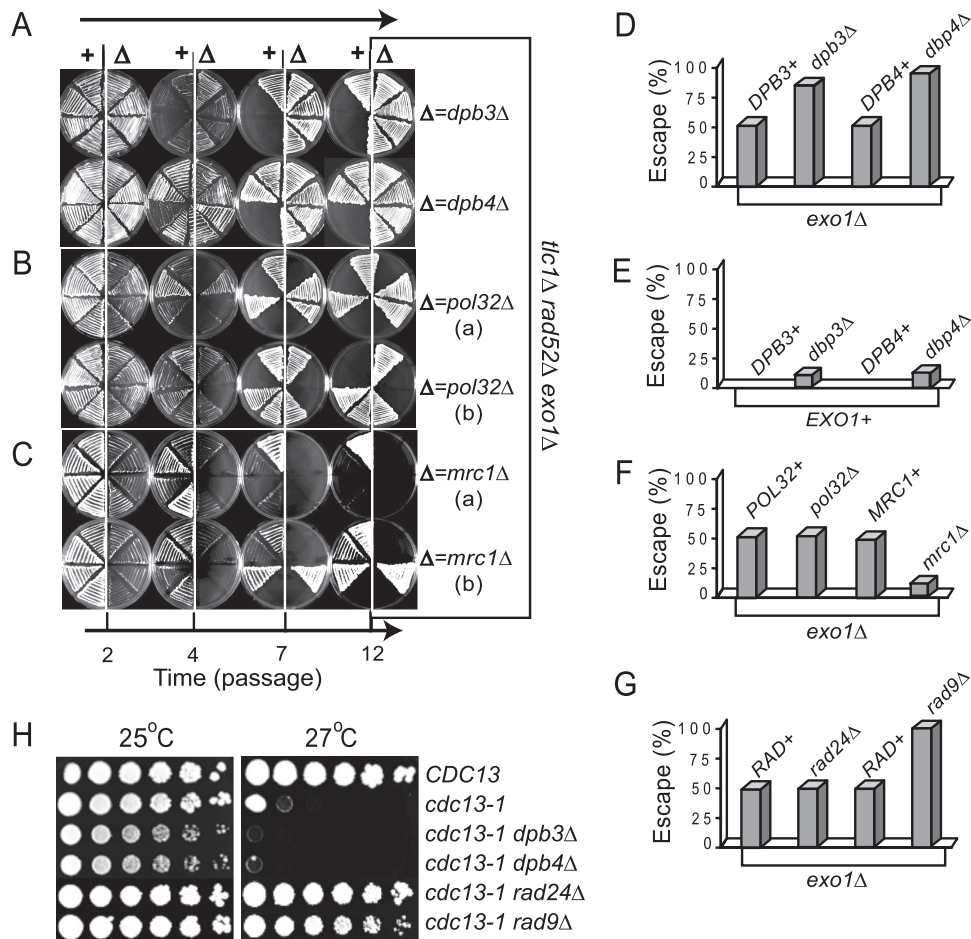One hypothesis is that Pol ε may possess an intrinsic check-

FIG. 3. Effect of nonessential subunits of DNA polymerases and Mrc1 on replicative senescence. (A to C) Representative plates photographed at the indicated time points. Each plate was inoculated with eight independent, freshly generated strains, taken directly from germination plates. Strains on the left side of each plate are *tlc1Δ rad52Δ exo1Δ* controls. Strains on the right side have an additional mutation, indicated at the right side of the plates. (D to G) Columns represent the fraction of isogenic strains that escaped from replicative senescence, from a total of 20 to 40 strains propagated as described for panels A to C. All strains are *tlc1Δ rad52Δ* (with or without other mutations). Mutations in Exo1 are indicated below the columns. All other relevant mutations are indicated above each column. (H) Fivefold serial dilutions of *cdc13-1* strains (with or without other mutations) were spotted onto plates and incubated at 25°C or 27°C. The relevant genotypes are indicated on the right.

point function in response to telomere damage. To test this hypothesis, we assessed the "checkpoint function" of Dpb3 and Dpb4 in a *cdc13-1* background. Similarly to *tlc1Δ rad52Δ* cells, cells with a *cdc13-1* temperature-sensitive mutation in the telomere-capping protein Cdc13[Pot1] accumulate ssDNA at telomeres and require both Rad24[Rad17] and Rad9[53BP1] to maintain the cell cycle arrest (42). We found that a *dpb3Δ* or a *dpb4Δ* mutation did not permit proliferation of *cdc13-1* cells at the restrictive temperature of 27°C. In contrast, a *rad24Δ* or a *rad9Δ* mutation rescued proliferation of *cdc13-1* cells, consistent with previous data (42). This indicates that Pol ε does not behave like a checkpoint protein in response to *cdc13-1* telomere uncapping. Therefore, Pol ε does not possess an intrinsic telomere damage checkpoint function.

Pol ε inhibits ssDNA accumulation during replicative senescence. The archetypal function of Pol ε is to perform DNA synthesis. A Pol ε-dependent DNA resynthesis may explain the periodical decline in ssDNA during senescence. To test this hypothesis, we analyzed the effect of Dpb3 on ssDNA dynam-

ics during senescence of *tlc1Δ rad52Δ* strains in experiments similar to those described in the legend to Fig. 1. We found that strains with a *dpb3Δ* mutation accumulated more ssDNA than *DPB+* controls propagated and analyzed in parallel. The highest difference in ssDNA was about 2-fold at passages 8 and 9 (Fig. 4A) and corresponded to a noticeable difference in the total amount of DNA at that time (Fig. 4B). At Y′-1000, more ssDNA was measured in *dpb3Δ* strains than in *DPB+* strains at passages 4, 7 to 9, and 13 and 14 (Fig. 4A). At Y′-5000, more ssDNA was measured in *dpb3Δ* strains than in *DPB+* strains at passages 4 and 5 and 10 to 12 (Fig. 4C). Therefore, *dpb3Δ* strains had more ssDNA than controls in at least one (sub)telomeric region during replicative senescence.

One explanation for this difference in ssDNA is that a *dpb3Δ* mutation slows down the ability of cells to resynthesize ssDNA. Since the DNA synthesis activity of Pol ε is slowed down in the absence of Dpb3, these data are consistent with a model in which DNA synthesis by Pol ε is responsible for the decline in ssDNA during replicative senescence, converting ssDNA to
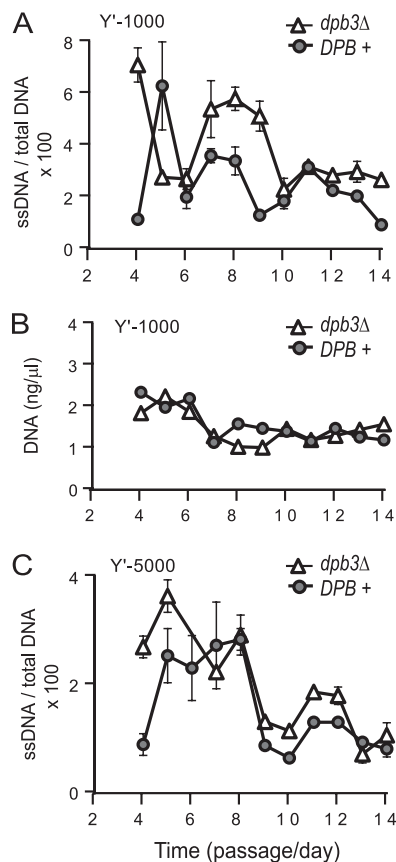
FIG. 4. Effect of Dpb3 on ssDNA dynamics during replicative senescence. Each of three independently generated *tlc1Δ rad52Δ* strains (with or without other mutations) was taken from germination plates, pooled at passage 1, and propagated in two groups: *DPB⁺* (*tlc1Δ rad52Δ* strains) and *dpb3Δ* (*tlc1Δ rad52Δ dpb3Δ* strains). (A and C) ssDNA measured for *DPB⁺* and *dpb3Δ* groups at Y′-1000 (A) and at Y′-5000 (C). Error bars represent the standard deviations (SD) for three measurements. (B) Same as in panel A, except that DNA was quantified at Y′-1000 and normalized to the level for DNA at the centromeric location *PAC2*. The same groups of strains was used for the measurements presented in panels A to C.

double-stranded DNA (dsDNA). A second explanation for more ssDNA in *dpb3Δ* strains could be that Pol ε opposes DNA resection by inhibiting Exo1. This (second) explanation is unlikely, because Pol ε acts synergistically with Exo1 during replicative senescence (Fig. 3D and E).

Dpb4 is required for a Pol2 and Rad9[53PB1] association with (sub)telomeres. If Pol ε is required for DNA resynthesis during replicative senescence, then its catalytic subunit Pol 2 would be expected to associate with regions affected by DNA resection. To test this hypothesis, we measured the association of Pol2 with subtelomeres by chromatin immunoprecipitation in samples collected during midsenescence (passages 9 to 13). Additionally, we measured the association of Rad9[53BP1] and Exo1 with subtelomeres. We found that whereas most Pol2, Exo1, or Rad9[53BP1] proteins did not associate with a centromere-proximal locus during senescence (Fig. 5A), they all associated with subtelomeres.

In *DPB⁺*(a) strains, Pol2 was recruited to subtelomeres at passage 11 (Fig. 5B) during a peak in ssDNA (Fig. 5C) and its

association increased at passage 13. The recruitment of Rad9[53BP1] to subtelomeres took place at passage 13 and coincided with a peak in subtelomeric Pol2 (Fig. 5B). In contrast, Exo1 was recruited at passage 10 (one passage earlier than Pol2) and completely dissociated by the time when Pol2 was recruited, with reassociation at passage 12 (Fig. 5D). In *DPB⁺*(b) strains, Pol2, Exo1, and Rad9[53BP1] associated with subtelomeres during a peak in ssDNA at passage 12 and dissociated thereafter (Fig. 5F to H). These data indicate that in *DPB⁺* strains, Pol2, Exo1, and Rad9[53BP1] associate and disassociate from telomeres during replicative senescence. In contrast, we found no significant association of Pol2 or Rad9[53BP1] with telomeres in *dpb4Δ* strains, despite high levels of Exo1 and ssDNA at telomeres (Fig. 5J to L). In conclusion, these data suggest that (i) Dpb4 facilitates the association of Pol2 and Rad9[53BP1] with DNA damage and (ii) there is a strong correlation between the association of Pol2 and Rad9[53BP1] with (sub)telomeres during senescence.

DISCUSSION

Vicious-circle model of replicative senescence. To explain how replicative senescence is maintained, we start with an analogy from a different signaling pathway: nasal receptors adapt to a persistent odor (meaning they undergo a stimulus-dependent decrease in sensitivity); however, they fail to adapt if the odor comes in pulses separated by odor-free intervals (or by intervals with a different odor) (44). Similarly, we propose that DNA damage checkpoint pathways fail to adapt to replicative senescence, because at least two DNA damage signals are interchanging regularly during senescence. The first signal for replicative senescence is most likely triggered by DNA resection of the shortest telomere(s), performed by Exo1 and other nucleases. Resection generates long 3′ssDNA overhangs and activates a Rad24[Rad17]-dependent checkpoint pathway (Fig. 6A and C). Then, ssDNA is resynthesized to dsDNA by DNA polymerases (Fig. 6B) or is perhaps degraded by nucleases (Fig. 6D). Irrespective of whether ssDNA is resynthesized or degraded, the short chromosome end (too short to acquire efficient protection from telomere-capping proteins) would now resemble a DSB. Therefore, we propose that a DSB-like structure is the second checkpoint signal during replicative senescence, a signal that activates a Rad9[53BP1]-dependent pathway (Fig. 6B and D).

Resection and resynthesis would continuously alternate at each dysfunctional short chromosome end, providing fresh substrates (ssDNA or a DSB-like structure) for checkpoint activation. Therefore, checkpoint sensors specific to each substrate would also alternate at chromosome ends, thus reducing the possibility that one of them would adapt to a persistent "old" damage. It is easy to imagine that the two substrates would naturally alternate, since ssDNA would attract factors catalyzing its resynthesis to dsDNA, whereas the newly formed dsDNA would attract nucleases to resect it back to ssDNA. Therefore, in the absence of repair, cells would be trapped in a "vicious circle" of resection and resynthesis for as long as they remain alive.

Consistent with the vicious-circle model proposed above, our data indicate that Pol ε is required to maintain replicative senescence and that Mrc1 has the opposite effect, facilitating
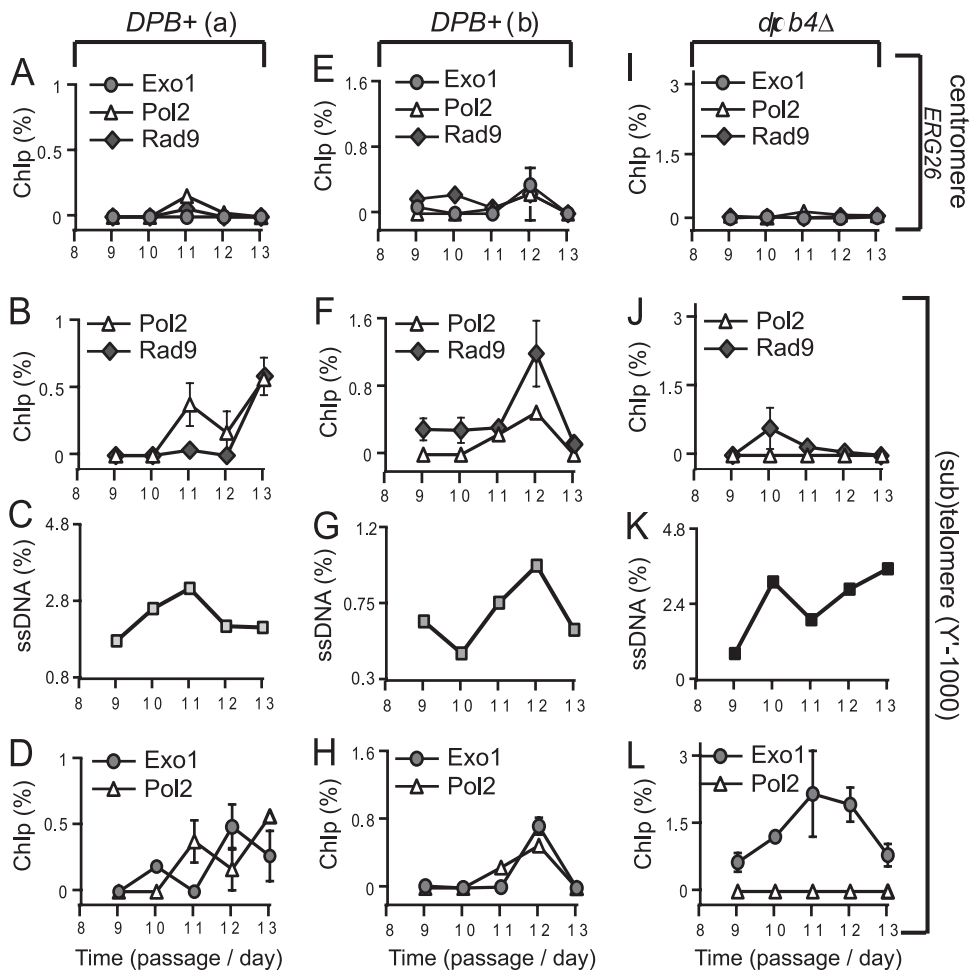
FIG. 5. Association of Pol2, Exo1, and Rad9[53BP1] with sub(telomeres) during senescence. Each of three independently generated *tlc1Δ rad52Δ* strains (with or without other mutations) was taken from germination plates, pooled at passage 1, and propagated in three groups: the *DPB*+(a) and *DPB*+(b) groups are *tlc1Δ rad52Δ*, whereas the *dpb4Δ* group is *tlc1Δ rad52Δ dpb4Δ*. Cell samples collected during midsenescence (passages 9 to 13) were cross-linked and immunoprecipitated. Both the immunoprecipitated and the input DNAs were quantified by quantitative PCR (qPCR) using TaqMan probes. Plotted is the fraction of DNA precipitated with specific antibodies, minus the background precipitation level for each sample; negative values were approximated to 0. The analyzed proteins are indicated above each graph. Regions are indicated on the far right of the figure. (A to D) Group *DPB*+(a) analyzed by ChIP and QAOS. (E to G) Same as in panels A to D, except that group *DPB*+(b) was analyzed. (I to L) The *dpb4Δ* group was analyzed.

escape (Fig. 3). The effect of Mrc1 during senescence is intriguing, since Mrc1 and Pol2 (the catalytic subunit of Pol ε) associate with each other and act in the same pathway(s) during S phase (23). However, the effect of Mrc1 during senescence could be explained as follows. When phosphorylated by the Mec1[ATR] checkpoint protein, Mrc1 is known to partially dissociate from Pol2 (23). Such disassociation would be expected to take place during senescence, since Mec1[ATR] is active (10, 15) and Mrc1 appears phosphorylated (12). Therefore, based upon our observation that Mrc1 has the opposite effect relative to Pol ε during replicative senescence, we propose that a partially dissociated Mrc1 protein renders Pol2 unable to synthesize DNA, therefore acting as an inhibitor of Pol ε. We integrated this hypothesis into the vicious-circle model, as follows. Once replicative senescence is triggered by resection of the shortest telomere(s), activated Mec1[ATR] phosphorylates Mrc1. Phosphorylated Mrc1 partially dissociates from Pol2, leaving it unable to perform DNA synthesis. With

time, the Mec1[ATR] activity decreases, due to a decline in resection rate and/or to checkpoint adaptation. In consequence, dephosphorylated Mrc1 reassociates with Pol2, rendering functional Mrc1-Pol ε complexes able to take over the DNA resynthesis from Pol α (Fig. 6B).

We also propose an alternative model of the replicative senescence void of DNA synthesis, called "the vicious circle of 5′ and 3′ resection" (Fig. 6C and D). In this model, a long 3′ ssDNA overhang forms at the shortest telomere(s) and activates a Rad24[Rad9]-dependent checkpoint pathway. The ssDNA is then degraded, perhaps with help from Pol ε, which has a 3′-to-5′ nucleolytic activity (Fig. 6D). The resulting blunt end activates a Rad9[53BP1]-dependent checkpoint pathway. The blunt end is also a target for 5′-to-3′ nucleases; in consequence, a new 3′ overhang forms, and this overhang is degraded thereafter. It is clear from Fig. 6C that an alternating degradation of 5′ and 3′ strands at the same chromosome end would lead to a rapid chromosome shortening and loss of
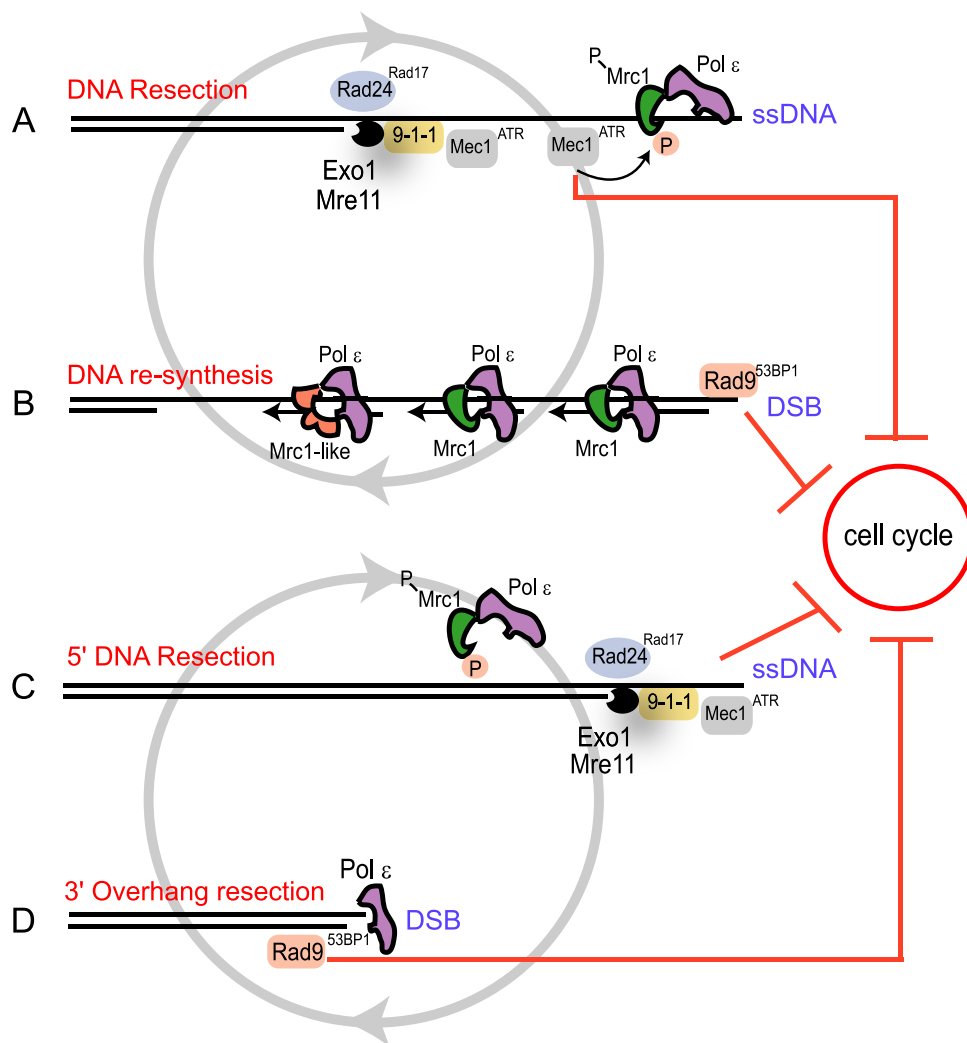
FIG. 6. Cartoon presenting two models of replicative senescence. Vicious-circle model of DNA resection and resynthesis (A) and 5′ and 3′ alternating DNA resection (B). For details, see Results.

essential genes, followed by cell death. Therefore, a DNA resection-based model could not maintain replicative senescence in the long term if completely void of DNA resynthesis.

The loss of the ssDNA overhangs that we detected during the budding yeast replicative senescence could be the equivalent of the ssDNA overhang loss observed at telomeres of human fibroblasts during replicative senescence (6, 16, 30, 34). Could a vicious circle also be responsible for the irreversible character of replicative senescence in mammalian cells? Interestingly, upregulation of the cyclin-dependent kinase (CDK) inhibitors p21^{WAF1} and p16^{INK4a} has been reported to alternate during mammalian replicative senescence (33). However, this was not the case for all senescent cells (14). Moreover, ATM which responds to DSBs (2) is considered more important for replicative senescence than ATR (8, 14), which responds to ssDNA (40). These observations do not exclude a vicious circle during mammalian senescence. For example, if mammalian telomeres recruit DNA polymerases more rapidly than yeast, they will spend more time as ATM-activating DSB-like structures. Adaptation could be prevented through the occasional resection of one chromosome end, leading to a transient activation of a complementary ATR pathway.

It is known that even a very short pause (a few seconds) between exposures to an odor is sufficient to prevent nasal receptors adapting to that odor (44). Similarly, a short switch from ATM to ATR activation during replicative senescence could potentially be sufficient to avoid adaptation and may escape detection during standard experiments. Are ATM foci flickering in senescent cells, similar to light bulbs approaching the end of the "life" span? More focused research is needed to address this question. Our view is that a vicious circle of DNA resection and resynthesis would be sufficient to maintain replicative senescence. Additional factors (oxidative stress, chromosome fusions, etc.) could precipitate, enhance, or delay senescence in response to telomere attrition and eventually kill senescent cells.

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