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Cdc13 prevents telomere uncapping and Rad50-dependent homologous recombination

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Cdc13 performs an essential function in telomere end protection in budding yeast. Here, we analyze the consequences on telomere dynamics of *cdc13*-induced telomeric DNA damage in proliferating cells. Checkpoint-deficient *cdc13-1* cells accumulated DNA damage and eventually senesced. However, these telomerase-proficient cells could survive by using homologous recombination but, contrary to telomerase-deficient cells, did so without prior telomere shortening. Strikingly, homologous recombination in *cdc13-1 mec3*, as well as in telomerase-deficient *cdc13-1* cells, which were Rad52- and Rad50-dependent but Rad51-independent, exclusively amplified the TG_{1-3} repeats. This argues that not only short telomeres are substrates for type II recombination. The Cdc13-1 mutant protein harbored a defect in its association with Stn1 and Ten1 but also an additional, unknown, defect that could not be cured by expressing a Cdc13-1–Ten1–Stn1 fusion. We propose that Cdc13 prevents telomere uncapping and inhibits recombination between telomeric sequences through a pathway distinct from and complementary to that used by telomerase.

Keywords: DNA damage checkpoints/Rad50 and homologous recombination/*S.cerevisiae* Cdc13/ senescence/telomere uncapping

Introduction

Telomeres are nucleoprotein structures that protect the ends of linear chromosomes and maintain them at a minimal length required for genome stability (Blackburn, 2000; Evans and Lundblad, 2000). Maintenance of telomere length and structure usually requires telomerase, which is comprised of a reverse transcriptase (TERT in mammals, Est2 in the yeast *Saccharomyces cerevisiae*) and an RNA template (TR in mammals, *TLC1* in *S.cerevisiae*). However, most somatic human tissues possess low or undetectable telomerase activity, thereby leading to progressive shortening of telomeres at each cell division and, eventually, to cellular senescence. Conversely, ectopically expressed hTERT maintains telomere length and allows unlimited growth (Bodnar *et al*., 1998). In mammals, the senescence program appears to be activated via the Rb and p53 checkpoints or related upstream and downstream components (Artandi and DePinho, 2000). In budding yeast, the only known type of senescence is that resulting from a dysfunction of telomerase (Lundblad and Szostak, 1989; Teng and Zakian, 1999).

Human tumor cells or immortalized cell lines can escape senescence and undergo long-term growth by re-activating telomerase, which is found in 80–90% of the cases examined so far. However, in the remaining cases, cells nevertheless were found to have very long telomeres, thus defining an alternative lengthening of telomeres (ALT) pathway (Bryan *et al*., 1997; Dunham *et al*., 2000). Similarly, budding yeast cells can escape senescence by maintaining telomere length through a Rad52-dependent homologous recombination mechanism that generates so-called post-senescence survivors (Lundblad and Blackburn, 1993). Indeed, the repetitive nature of telomeric and subtelomeric sequences makes them a good target for recombination (Horowitz and Haber, 1984), a mechanism for telomere maintenance that has been proposed before telomerase activity was identified (Walmsley *et al*., 1984). Therefore, survivors of death by senescence in yeast can be regarded as functionally similar to some tumor cells in humans.

In yeast, senescence can be observed in telomerase mutants (*est2*Δ or *tlc1*Δ), as well as in strains bearing mutations in telomerase regulators, such as *est1*Δ, *est3*Δ or *est4-1*/*cdc13-2* (Lundblad and Szostak, 1989; Lendvay *et al*., 1996). Senescence has also been observed in the *tel1*Δ *mec1-21* double mutant and presumably is due to a lack of telomerase function (Ritchie *et al*., 1999). Cdc13 is a single-strand telomeric DNA-binding protein that has a dual role, functioning in both telomere end protection and telomere replication (Garvik *et al*., 1995; Lin and Zakian, 1996; Nugent *et al*., 1996). Cdc13 regulates telomerase recruitment at telomere ends both in a positive way through interactions with Est1 (Evans and Lundblad, 1999; Grandin *et al*., 2000) and the *KU* proteins (Grandin *et al*., 2000) and in a negative way through interactions with Stn1 (Grandin *et al*., 2000; Chandra *et al*., 2001). Temperature-sensitive *cdc13-1* mutant cells harbor DNA lesions in telomere-proximal regions of the chromosomes (Garvik *et al*., 1995). In the present study, we have taken advantage of the fact that *cdc13-1* cells can continue to proliferate at restrictive temperature when the DNA damage checkpoint has been inactivated. Under such conditions, *cdc13-1* cells accumulated telomeric DNA damage, which triggered a program of death by senescence. Post-senescence survivors arose via a homologous recombination pathway. Most importantly, senescence in *cdc13-1* cells took place in the presence of functional telomerase. Survivors of *cdc13-1* absolutely required Rad50 but not Rad51. The present data identify a novel pathway of telomere end protection, governed by Cdc13, which functions in parallel with that controlled by telomerase.

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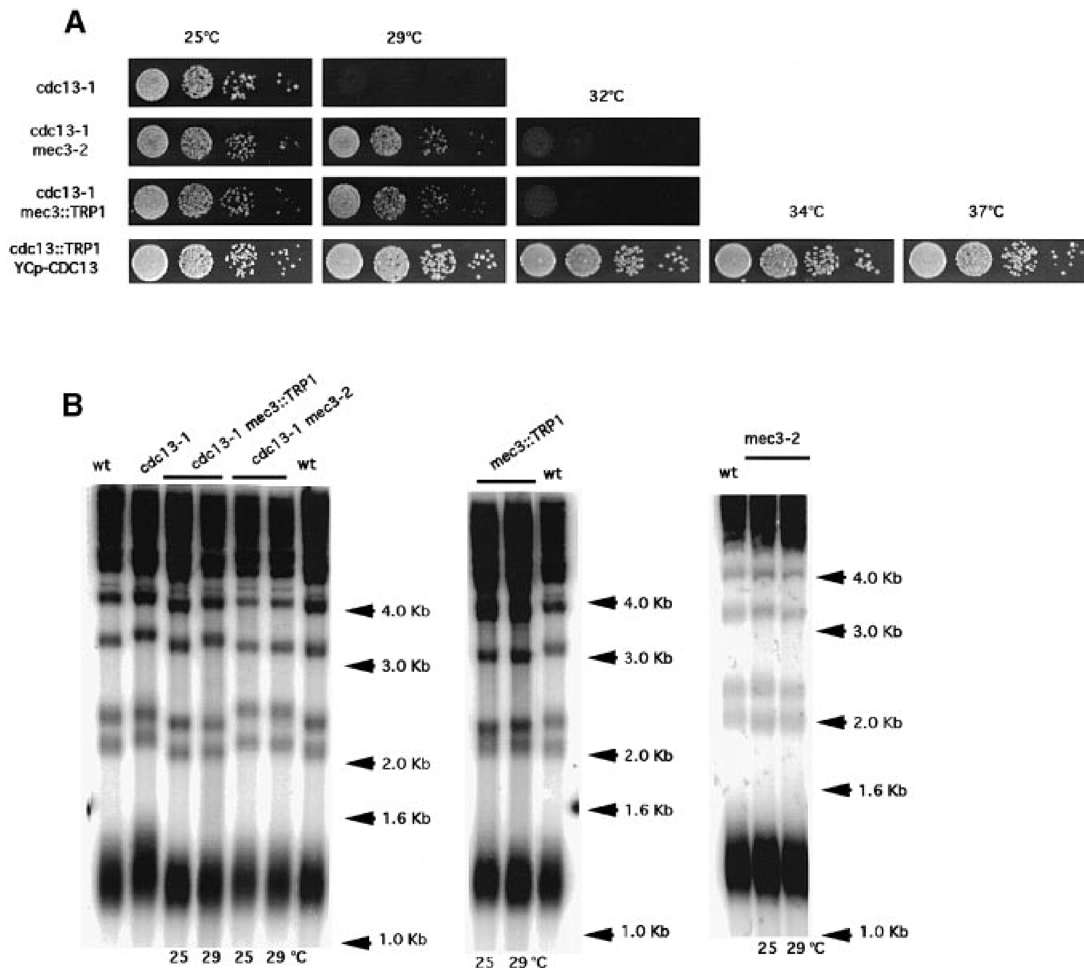
Results

Survivors of checkpoint-deficient cdc13-1 cells undergo Rad52-dependent telomere elongation

At restrictive temperatures, *cdc13-1* mutant cells arrest at G_2/M, due to the activation of the DNA damage checkpoint. Inhibition of this surveillance system, for instance in a *rad9* or *mec3* mutant background, leads to death by mitotic catastrophe (Weinert *et al*., 1994; Garvik *et al*., 1995). In the genetic background used in our laboratory, *cdc13-1* cells stop growing at 27°C. However, when *cdc13-1 mec3* double mutants were grown at 29°C, they initially proliferated at a wild-type rate (Figure 1A), with no apparent morphological defect (not shown). At later passages, however, these cells started to die, but eventually rare survivors appeared (Figure 1C). *cdc13-1* cells bearing a mutation in another checkpoint gene, such as *RAD9*, *RAD24*, *MEC1* or *RAD53*, behaved in a similar way (Figure 1C). The *cdc13-1 mec3* strains were chosen for further analysis, because, in particular, other strains, such as *cdc13-1 rad9*Δ and *cdc13-1 mec1*Δ, displayed synthetic defects between the two mutations at 25°C and during the early divisions at 29°C (Figure 1C).

mec3-2 and *mec3*Δ cells exhibited wild-type length telomeres at 25 or 29°C (Figure 1B). This is in contrast to a previous report showing that *mec3* mutant cells have elongated telomeres (Corda *et al*., 1999), the disagreement possibly being due to different genetic backgrounds. At 25°C, *cdc13-1 mec3* cells also exhibited wild-type length telomeres (Figure 1B). When shifted to 29°C, the length of their telomeres remained constant for ~50 generations before dramatically increasing (Figure 2B). Since we suspected that this might be due to homologous recombination, we constructed a *cdc13-1 mec3-2 rad52*Δ triple mutant (*RAD52*, implicated in all types of homologous recombination, is not essential for growth). In this strain, cell growth was depressed after the second passage at 29°C and totally ceased after the third passage, while in *cdc13-1 mec3-2 RAD52*$^+$ cells, survivors appeared coincident with the acquisition of very long telomeres (Figure 2A and B). The *rad52* mutation had no effect on telomere size and cell growth (data not shown). Therefore, proliferation in *cdc13-1 mec3-2* cells most probably relies on *RAD52*-dependent recombination-induced telomere elongation, as previously described in post-senescence survivors of telomerase-deficient cells (Lundblad and Blackburn, 1993). In different isolates of *cdc13-1 mec3*, senescence did not always occur at the same time (Figure 2A), but in all analyzed cases it was accompanied by telomere elongation. Senescent cells can survive by homologous recombination-dependent amplification of either subtelomeric Y′ sequences or of terminal TG_{1-3} repeats (Lundblad and Blackburn, 1993), now also referred to as type I and type II survivors, respectively (Teng and



Zakian, 1999). Importantly, digestion of genomic DNA from recombinant *cdc13-1 mec3-2* cells with a combination of four-base cutter enzymes demonstrated that the telomeric sequences having recombined were TG_{1-3} and not Y′ (Figure 2C).

Survivors of checkpoint-deficient cdc13-1 cells are not intragenic or extragenic suppressors

We noted that a very small number of suppressors appeared in *cdc13-1* cell populations grown at 29–32°C. However, the finding that a deletion of *RAD52* (Figure 2A)

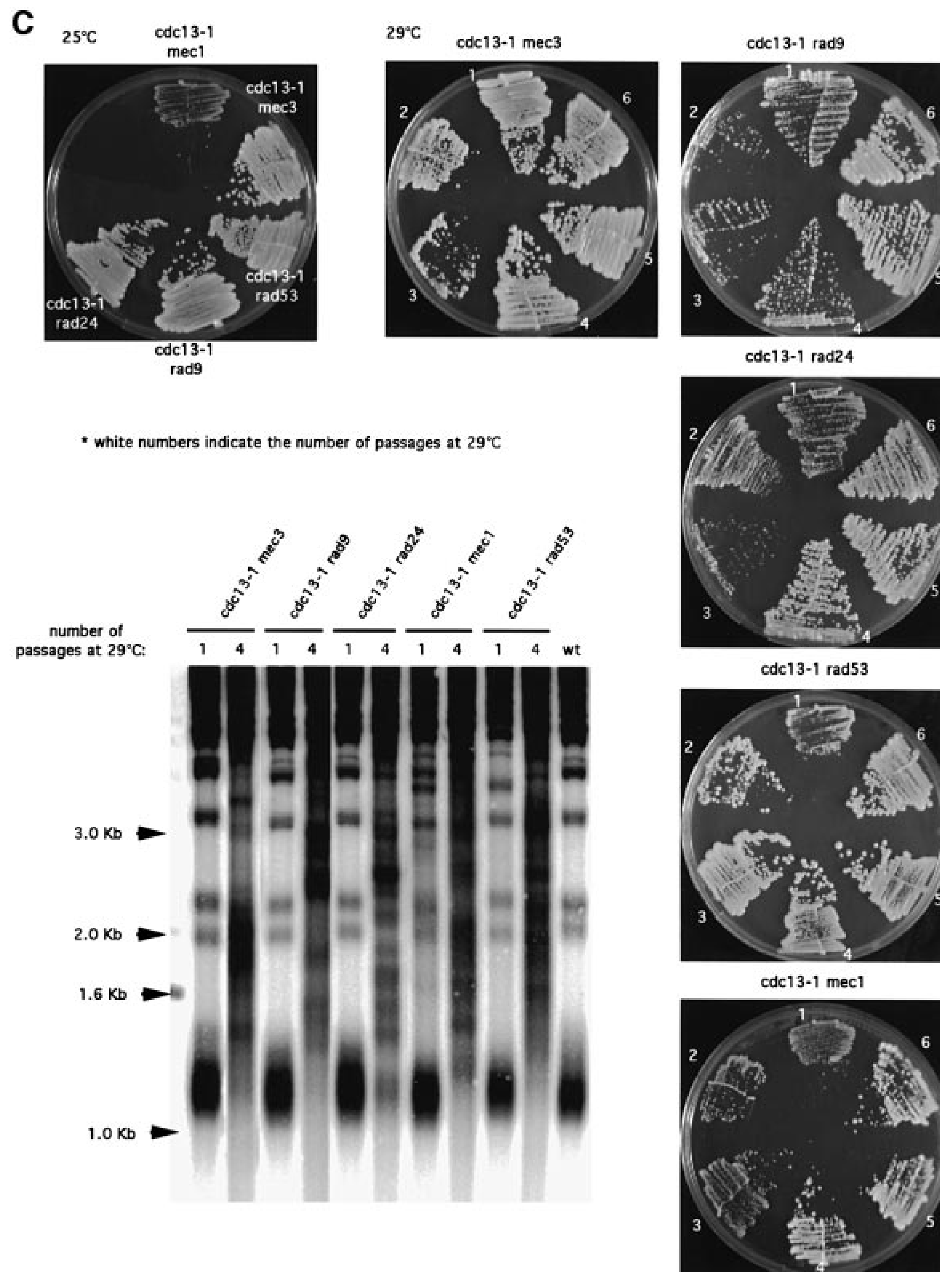


Fig. 1. Checkpoint-deficient *cdc13-1* cells undergo a senescence program followed by survival. (**A**) The *cdc13-1 mec3-2* and *cdc13-1 mec3*Δ double mutant strains could grow at temperatures up to 29°C, whereas the *cdc13-1* single mutant stopped growing above 25°C. A *cdc13*::*TRP1* YCp111-*CDC13* strain was used as a positive control. All cells were first grown at 25°C, and 10-fold serial dilutions (from left to right in each row) were incubated for 2 days at the indicated temperatures and photographed. (**B**) At 25°C, telomeres in the *cdc13-1* mutant cells were slightly longer than those in a wild-type isogenic strain (left panel, compare lanes 1 and 2), while *cdc13-1 mec3*Δ and *cdc13-1 mec3-2* double mutants exhibited wild-type length telomeres at either 25 or 29°C, as indicated. Similarly, *mec3*::*TRP1* (middle panel) and *mec3-2* mutants (right panel) had telomeres of wild-type length at 25 and 29°C. Total DNA was digested with *Xho*I and a TG_{1-3} ^{32}P-labeled probe was used to detect telomeric sequences (see Materials and methods). (**C**) Double mutant strains of the indicated relevant genotype were incubated either at 25°C (upper left panel, photographed here 3 days after re-streaking) or at 29°C (all other panels, with the exception of *cdc13-1 rad9* cells incubated at 28°C) for the indicated number of passages (typically, one passage or re-streak is performed after 20–25 generations). Survivors appeared in all strains, at different times, as rare isolated colonies. *cdc13-1 rad9*Δ cells generated survivors at 28°C but not at 29°C. During passages 1 and 4, cells were prepared for telomere length measurement (*Xho*I digestion, TG_{1-3} probe). All five *cdc13-1* strains bearing a different checkpoint mutation exhibited recombination of telomeric sequences.

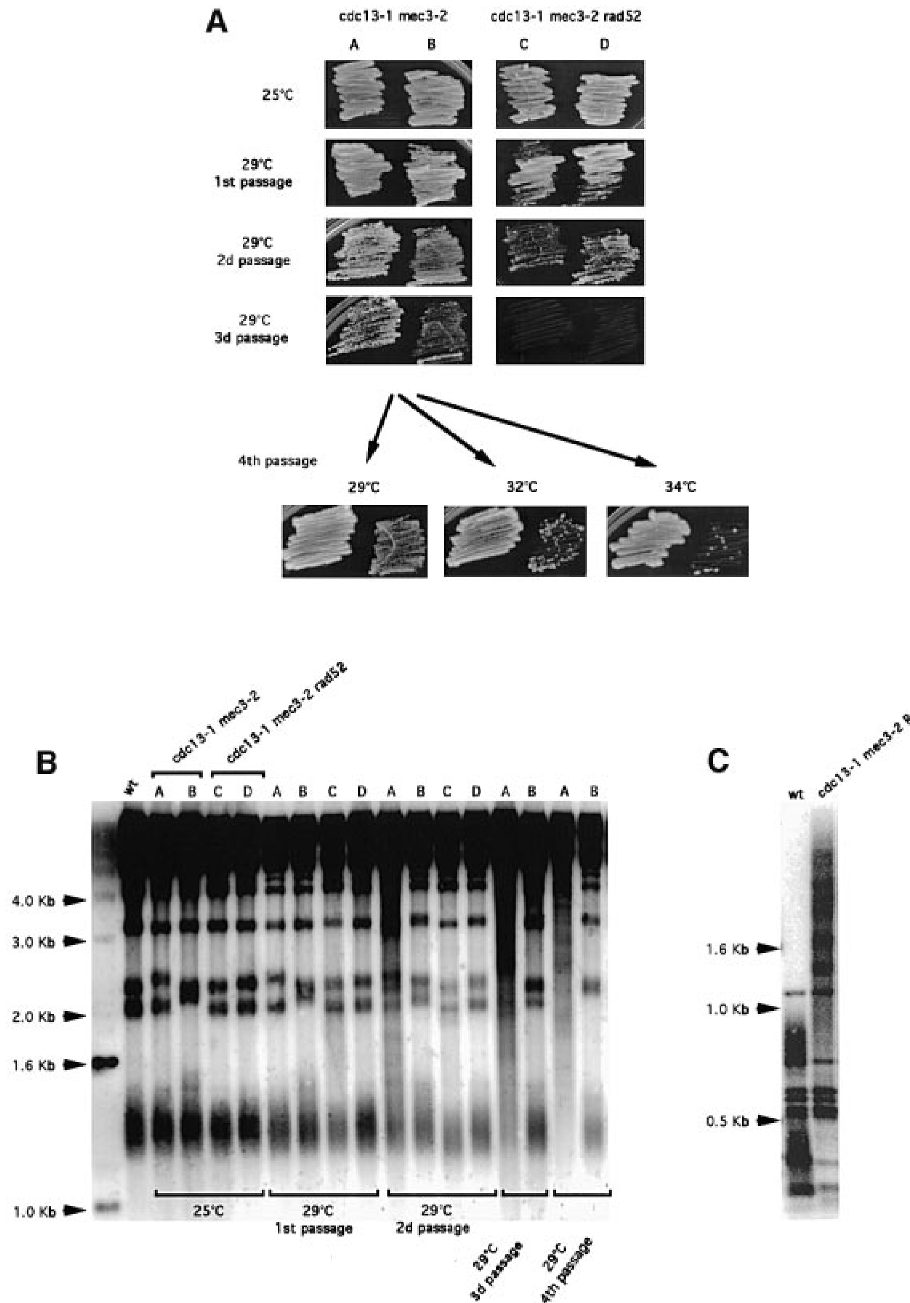


Fig. 2. Continuous proliferation of *cdc13-1 mec3-2* cells at 29°C requires the presence of Rad52 and is accompanied by the acquisition of TG_{1-3} elongated telomeres. In (**B**), telomere lengths (*Xho*I digestion, TG_{1-3} ^{32}P-labeled probe) of the cells shown in (**A**) are displayed at various time points. A and B represent two isogenic *cdc13-1 mec3-2* strains with different kinetics of senescence. In strain A, the crisis began during the second passage, attested by slowing down of the growth rate (A), followed by dramatic telomere elongation during the third passage (B), while strain B has entered crisis during the third passage (A) but has not yet undergone recombination even during the fourth passage (B). In strain A, passage through the crisis and acquisition of elongated telomeres coincided with the acquisition of the capacity of the *cdc13-1 mec3-2* cells to grow at temperatures up to 34°C. Strain B, which has not attained survival yet, is more temperature-sensitive than strain A. Strains C and D, two isogenic *cdc13-1 mec3-2 rad52Δ* triple mutant cells, totally ceased growth during the second passage. (**C**) Genomic DNA from a wild-type strain (lane 1) or from a *cdc13-1 mec3-2* survivor (lane 2), grown on plates ('streak assay' described in Materials and methods), were digested with a mixture of restriction enzymes (*Alu*I, *Hae*III, *Hin*fI and *Msp*I) using a 4 bp recognition sequence, and the Southern revealed with a TG_{1-3} probe. Since these enzymes cut within telomeric Y′ sequences but not within the TG_{1-3} sequences (Teng and Zakian, 1999), the present data show that homologous recombination-induced telomere elongation in *cdc13-1 mec3-2* concerns TG_{1-3} but not Y′ sequences.

totally prevented growth of *cdc13-1 mec3* cells at 29°C was a strong argument against survivors being mere extragenic suppressors. It is very probable that the opportunity for *cdc13-1 mec3* to survive by homologous recombination exerts a strong selective pressure against the appearance of suppressors. As a confirmation, we found that the survivor phenotype was reversible (Figure 3C).

Next, we designed three control genetic experiments to ascertain that the survivors of *cdc13-1 mec3* cells were not revertants of *cdc13-1* (intragenic suppressors). First, reintroduction of *MEC3* into these cells re-established the

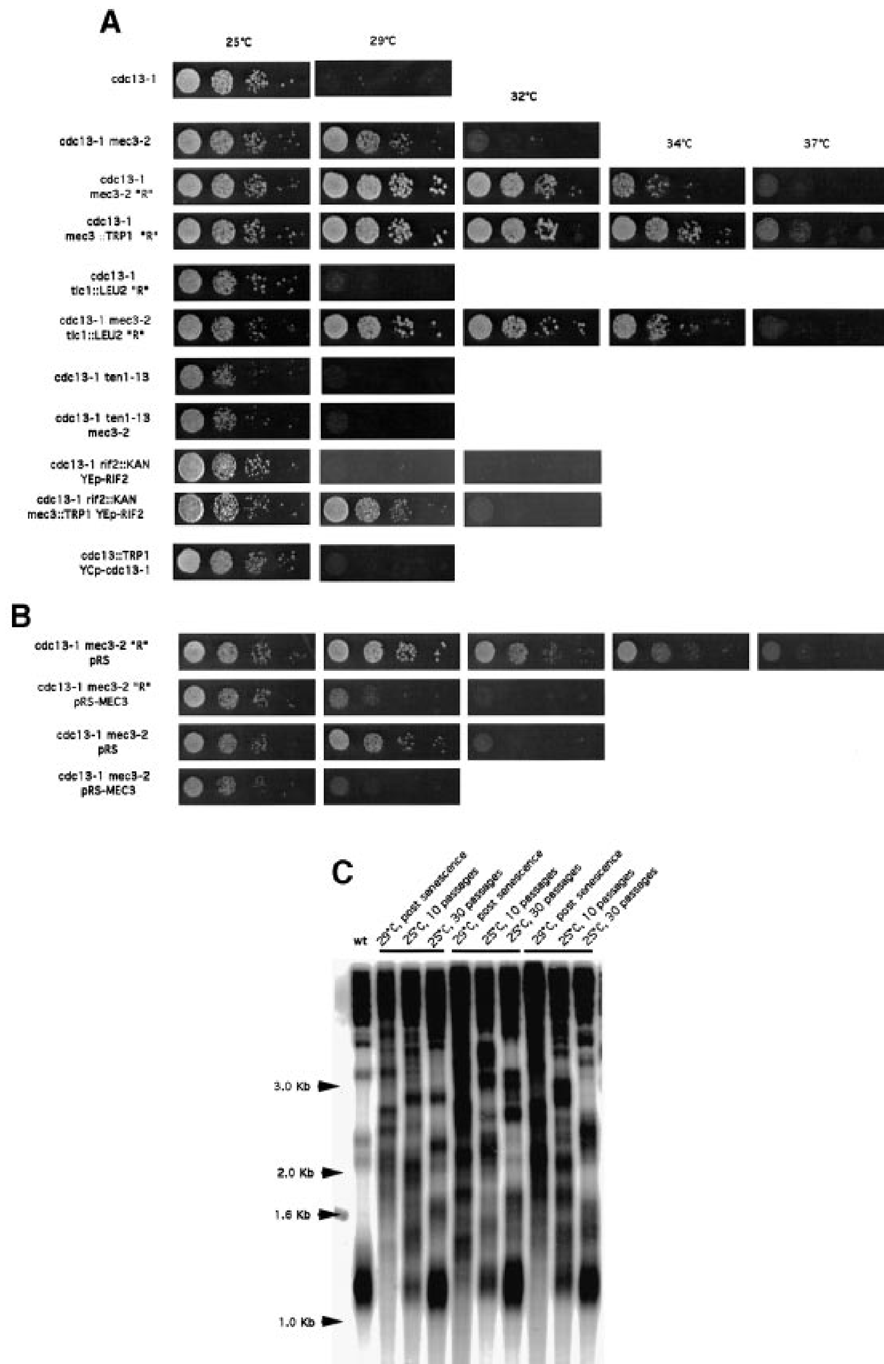


Fig. 3. The *cdc13-1*-induced senescence/survival process is reversible; it also decreases *cdc13-1* temperature sensitivity. (**A**) Growth characteristics of various mutants combining the temperature-sensitive *cdc13-1* mutation and non-temperature-sensitive mutations in *MEC3*, *TLC1*, *TEN1* or *RIF2* (see text for explanations). Ten-fold serial dilutions (from left to right in each row) of transformants were grown for 2 days at the indicated temperatures and photographed. The letter 'R' indicates those of the strains that have overcome crisis and undergone recombination-dependent survival. Only the checkpoint-deficient *cdc13-1* survivors displayed an increase in the permissive temperature of growth from 25°C (in *cdc13-1* cells, row 1) or 29°C (in *cdc13-1 mec3-2* cells prior to survival, row 2) to 34–37°C (rows 3, 4 and 6), while checkpoint-proficient, telomerase-deficient *cdc13-1* cells (*cdc13-1 tlc1::LEU2* 'R', row 5) could not grow above 25°C. In the control experiment shown in row 11, cells disrupted for *CDC13* and harboring a *LEU2*-based single-copy plasmid expressing *cdc13* sequences amplified from genomic DNA from three different *cdc13-1 mec3-2* survivors (only one is shown here) can live only at 25°C after the plasmid containing wild-type *CDC13* had been shuffled out on 5-fluoro-orotic acid-containing medium (*cdc13::TRP1* YCp-*cdc13-1*), thus indicating that the temperature-sensitive *cdc13-1* allele has not reverted to a less temperature-sensitive allele in the original strain. (**B**) Another control experiment shows that reintroduction of *MEC3* on a single-copy plasmid (*cdc13-1 mec3-2* 'R' pRS-MEC3, row 2) restored the arrest at 29°C in *cdc13-1 mec3-2* cells having acquired the ability to grow at temperatures higher than 34°C following survival (*cdc13-1 mec3-2* 'R' pRS, transformed with plasmid alone, row 1), which also suggests that the *cdc13-1* mutation is still present in these cells. (**C**) When survivors of *cdc13-1 mec3*Δ were brought back from 29°C to permissive temperature (25°C), they slowly re-acquired wild-type length telomeres, during the indicated periods of time, as shown here for three independent clones (*Xho*I digestion, TG_{1-3} probe).

cdc13-1 arrest (Figure 3B). Secondly, *cdc13-1 mec3*Δ cells having already undergone homologous recombination and the associated survival process at 29°C were crossed to isogenic wild-type cells. Tetrad analysis showed that

resulting *cdc13-1 MEC3*$^{+}$ cells were unable to grow at temperatures higher than 27–28°C. Thirdly, the *cdc13-1* allele present in survivors could still induce arrest at 27–28°C when transferred into a *cdc13* null strain (Figure 3A, compare rows 1 and 11). These experiments clearly establish that the *cdc13-1* mutation is still present in the *cdc13-1 mec3* survivors.

Homologous recombination decreases the temperature sensitivity of cdc13-1

Following acquisition of Rad52-dependent survival, the *cdc13-1 mec3-2* cells became much less sensitive to temperature than before, as they could now grow at 34°C (Figures 2A and 3A, compare rows 2, 3 and 4). This phenomenon was also observed in the *cdc13-1 mec3 tlc1*Δ mutant after survivors had been generated at 25°C (Figure 3A, compare rows 5 and 6). The difference in the temperature sensitivity of *cdc13-1* in all these strains was not due to the presence of type I or type II survivors, as all of them were of type II (see below). Although they could grow at higher temperatures than before senescence, DNA damage was still present in these post-senescence survivors, as growth arrest at 29°C was restored after *MEC3* was re-introduced on a plasmid (Figure 3B, compare rows 1 and 2).

Because type II recombining telomeres are very long, it was possible that telomere elongation rather than recombination *per se* was responsible for the decrease in the temperature sensitivity of the *cdc13-1* mutation. To test this hypothesis, we constructed the *cdc13-1 mec3 rif2* and *cdc13-1 mec3 ten1* triple mutant strains. Cells of these strains, although they exhibited elongated telomeres at 25°C (data not shown; Wotton and Shore, 1997; Grandin *et al*., 2001), nevertheless were incapable of growing better than the *cdc13-1 mec3* double mutant (Figure 3A, compare rows 2 and 8 for *ten1*). Re-introduction of the wild-type *RIF2* gene into *cdc13-1 mec3 rif2*Δ after the telomeres had elongated (in order to perform analysis in the absence of the additional mutation, *rif2*Δ) gave identical results (Figure 3A, compare rows 2 and 10). These data indicate that telomere elongation alone is not responsible for the growth of post-senescence *cdc13-1 mec3* cells at 34°C. However, it should be noted that telomere elongation resulting from type II recombination is much larger than that conferred by the *ten1-13* or *rif2*Δ mutation.

Senescence/survival in cdc13-1 cells is associated with accumulation of telomeric DNA damage

To correlate the senescence process in *cdc13-1 mec3* cells with telomeric DNA damage due to *cdc13-1* (Garvik *et al*., 1995), we measured the amount of single-stranded DNA in these cells using a technique that measures hybridization of the telomeric probe to non-denatured DNA (Wellinger *et al*., 1993). As expected, DNA damage dramatically increased with both temperature and time (Figure 4A and B). Following digestion of telomeric DNA with *Xho*I, single-stranded DNA appeared not only at the level of the terminal TG_{1-3} repeats (1.2 kb band), but also in subtelomeric regions, as revealed by a large smear at ~1.5–4.5 kb (Figure 4A and B). As the temperature and time of incubation increased, the amount of telomeric DNA actually decreased (inversely proportional to the increase in single-stranded DNA), an effect which was progressive, starting from the distal end of the telomere and continuing in a centripetal direction, as revealed by digesting in parallel with either *Xho*I, *Xba*I or *Eco*RI (Figure 4A and B). Presumably, the concomitant appearance of single-stranded DNA prevented recognition of the restriction enzyme sites, hence the 'disappearance' of the *Xho*I then *Xba*I telomeric bands. This *cdc13-1*-induced degradation of DNA was specific for telomeres, as no degradation was observed in a non-telomeric DNA (Figure 4B). We ruled out the possibility (very unlikely given the short periods of incubation, 1.5–3 h) that degradation of DNA resulted from homologous recombination by making similar observations in a *rad52*Δ background (Figure 4C).

To know whether the appearance of senescence in *cdc13-1 mec3* cells at 29°C could be due to the accumulation of DNA damage at the telomeres, we then performed kinetic experiments using the methodology exposed above. Accumulation of DNA damage during pre-senescence was suggested by the fact that the 1.2 kb *Xho*I digest diminished in intensity when compared with non-telomeric DNA (Figure 4D, middle and bottom left panels, lanes 1–3, 29°C; compare quantitations obtained with the TG_{1-3} probe versus the *CDC15* probe) prior to the occurrence of recombination (Figure 4D, middle left panel, lanes 4 and 5, 29°C). On the other hand, the *Eco*RI digest did not vary in intensity during the pre-senescence period (Figure 4D, upper left panel), thus arguing that degradation of the terminal TG_{1-3} repeats had proceeded during this period of time. This method appeared to be more sensitive than measurement of single-stranded DNA in native gels, which did not allow detection of accumulation of telomeric DNA damage during pre-senescence (Figure 4D, middle right panel, lanes 1–3, 29°C).

cdc13-1 survivors occur independently of telomerase

Since Cdc13 has been implicated previously in regulating recruitment of telomerase at telomere ends, it was possible that the survivors of *cdc13-1 mec3* were similar to those in *cdc13-2* or telomerase-deficient cells. If this was the case, then telomere shortening should be detected prior to the appearance of *cdc13-1 mec3-2* survivors, as in *cdc13-2/est4-1* (Lendvay *et al*., 1996). However, this was not the case, in sharp contrast to the situation in *cdc13-1 mec3-2 tlc1*Δ cells in which disruption of telomerase activity resulted in telomere shortening during the senescence crisis (Figure 5B, compare lanes D and G).

The crisis preceding the appearance of survivors in *cdc13-1 mec3* cells was detectable after 40–60 generations at 29°C, varying somewhat from one strain to the other (Figure 2A, compare strains A and B). In some cases, the crisis was barely visible on the agar plates and was followed by continuous and regular growth, in contrast to that in most *tlc1*Δ senescent cells in which the growth fluctuated in rate, with survivors appearing irregularly with time (not shown), as described previously (Teng and Zakian, 1999). In fact, the growth characteristics of the *cdc13-1 mec3* survivors, which are type II recombinants as shown above, resembled those of type II survivors of *tlc1*Δ cells (Teng and Zakian, 1999). In agreement with recent experiments (Teng *et al*., 2000), the majority of our *tlc1*Δ survivors (28 of 30) were of type I, while all of our *cdc13-1*

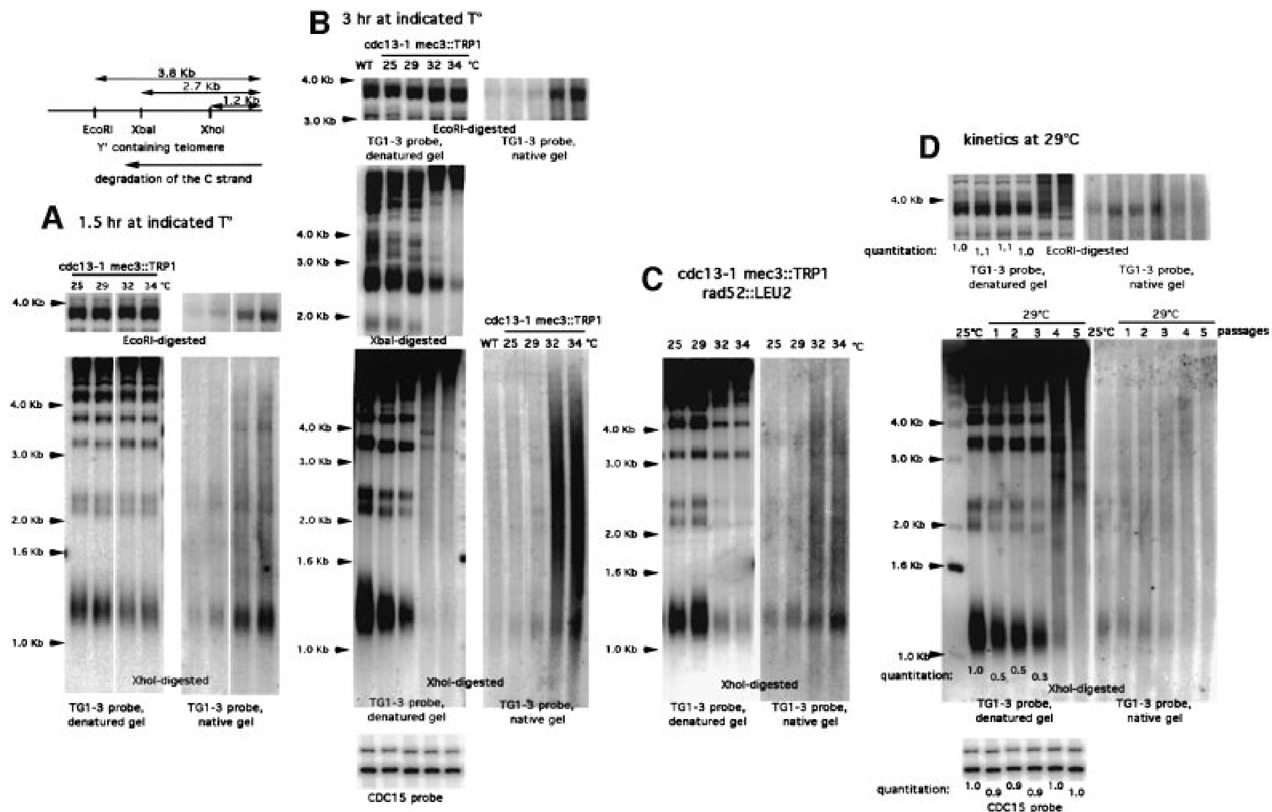


Fig. 4. Accumulation of single-stranded DNA in *cdc13-1 mec3*Δ cells. Non-denaturing (native, right panels in A, B, C and D) or denaturing (denatured, left panels in A, B, C and D) Southern hybridization of genomic DNA to a TG_{1-3} ^{32}P-labeled probe following digestion with either *Xho*I, *Xba*I or *Eco*RI, as indicated. These enzymes cut in subtelomeric regions of Y′ chromosomes, as represented schematically (upper left). (**A** and **B**) Levels of single-stranded DNA in *cdc13-1 mec3*Δ cells increased with both time (1.5 or 3 h of incubation in A and B, respectively) and temperature, as indicated. Meanwhile, the amount of double-stranded telomeric DNA diminished concomitantly (denatured, left panels in A and B), a reaction that progressed centripetally from the telomere end (for instance, in B, degradation at 3 h at 25–34°C in *Xho*I-digested samples, at 32–34°C in *Xba*I-digested samples and no degradation in *Eco*RI-digested samples or in non-telomeric DNA, *CDC15*). (**C**) Accumulation of single-stranded DNA and degradation of double-stranded telomeric DNA were not due to homologous recombination because they still took place in *cdc13-1 mec3*Δ *rad52*Δ cells. (**D**) During the pre-senescence period (lanes 1–3, 29°C), DNA became degraded in telomeric regions, as evidenced by a decrease in the amount of double-stranded DNA exhibiting the *Xho*I site (1.2 kb band in middle left panel), while more internal telomeric DNA was not degraded (3.7 kb band after *Eco*RI digestion in upper left panel). In non-telomeric regions, DNA also remained intact (CDC15 probe, lower left panel). Post-senescence survivors underwent homologous recombination (lanes 4 and 5, 29°C), indicated by dramatic telomere elongation (middle left panel). Quantitations, indicated as numbers under the lanes, were made with a PhosphorImager using ImageQuant. Measurement of single-stranded DNA in native gels (middle right panel) presumably was not sensitive enough to detect the telomeric DNA damage described above.

mec3-2 survivors (60 of 60) were of type II (Figure 5B). Interestingly, *cdc13-1 tlc1*Δ and *cdc13-1 mec3-2 tlc1*Δ survivors were also found to be exclusively of type II (15 of 15 survivors in both strains, and this at 25°C for the former and at 25 or 29°C for the latter; Figure 5C), thus suggesting a dominance of type II over type I survivors in the *cdc13-1* background. This also suggests the existence of a defect due to *cdc13-1* even at permissive temperatures of growth.

Rad52-dependent survival in cdc13-1 mec3 also requires Rad50 but not Rad51

Our next objective was to determine which genes in the yeast *RAD52* epistasis group, besides Rad52 itself (Figure 2A), were required for homologous recombination/survival in *cdc13-1 mec3* cells. Cells of the *cdc13-1 mec3*Δ *rad51*Δ triple mutant could readily generate survivors with kinetics similar to those in the *cdc13-1 mec3*Δ double mutant (Figure 6A). Similarly, *cdc13-1 mec3*Δ *rad55*Δ triple mutant cells (Rad55 is another DNA repair protein essential for DNA damage-induced recombination) gave rise to survivors (data not shown). In contrast, cells of the *cdc13-1 mec3*Δ *rad50*Δ triple mutant were incapable of generating survivors (Figure 6A). Because *rad50*Δ, but not *rad51*Δ or *rad55*Δ, confers telomere shortening (Figure 6B; Kironmai and Muniyappa, 1997) that could have been responsible for the failure to generate survivors, we performed control experiments using three other disruptions in telomeric proteins also known to confer telomere shortening, namely *yku70*Δ, *yku80*Δ (Boulton and Jackson, 1998) and *tel1*Δ (Lustig and Petes, 1986). All three *cdc13-1 mec3*Δ *yku70*Δ, *cdc13-1 mec3*Δ *yku80*Δ and *cdc13-1 mec3*Δ *tel1*Δ triple mutants could readily generate survivors (data not shown). These data demonstrate that Rad50, like Rad52, is necessary for survival in checkpoint-deficient *cdc13-1* cells, while Rad51 and Rad55 are not. Importantly, survivors of *cdc13-1 mec3*Δ *rad51*Δ displayed telomere lengthening of the type exhibited by cells

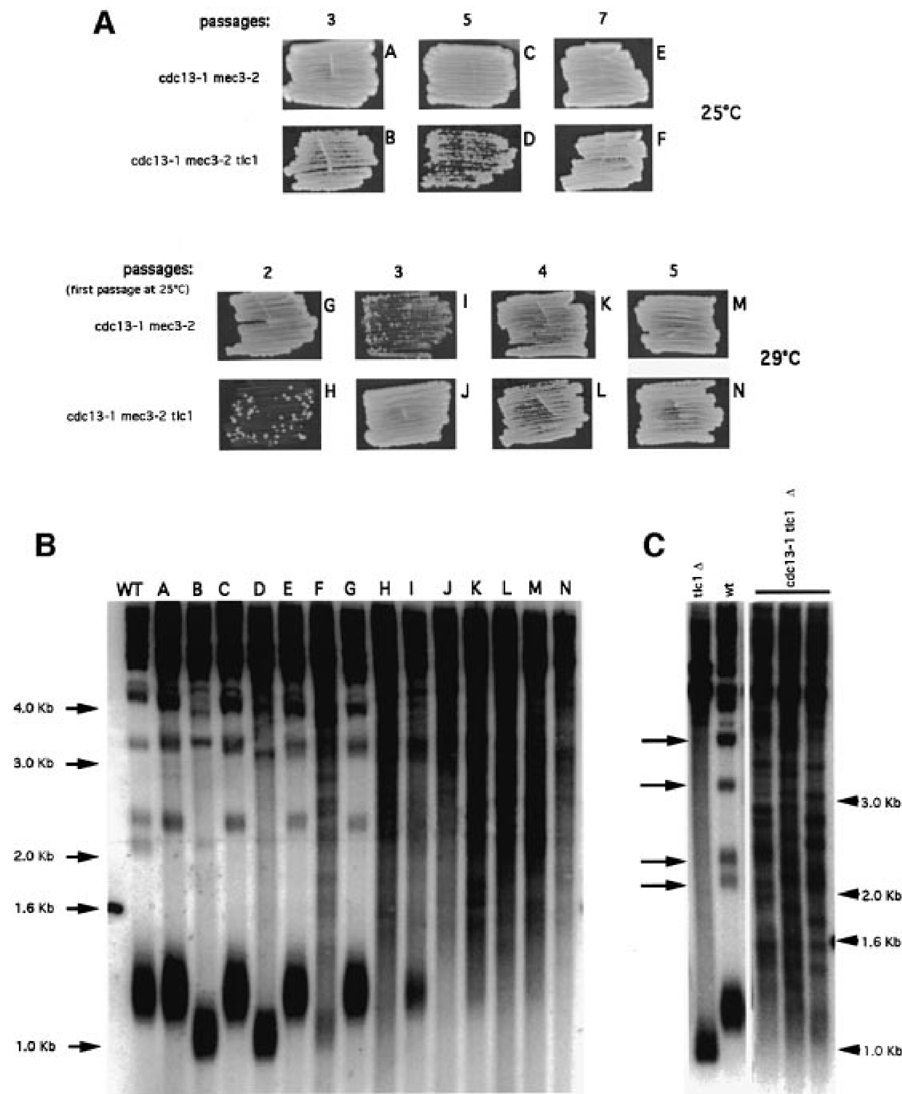


Fig. 5. Post-senescence survival in *cdc13-1 mec3-2* occurs without prior telomere shortening. Growth kinetics (**A**) and corresponding telomere length (**B**) of *cdc13-1 mec3-2* and *cdc13-1 mec3-2 tlc1Δ* cells at 25 or 29°C. Each lane in (B) has been labeled with a letter corresponding to the cell patches shown in (A). At 25°C (A, top panel), *cdc13-1 mec3-2* never experienced senescence, in contrast to *cdc13-1 mec3-2 tlc1Δ* cells which started to senesce by the end of the third passage (patch B), continuing for two additional passages (patch D), accompanied by clear telomere shortening (B, lanes B and D) prior to recombination-induced telomere elongation (lane F). At 29°C (A, bottom panel), *cdc13-1 mec3-2* cells senesced less rapidly than *cdc13-1 mec3-2 tlc1Δ* cells. There was no telomere shortening prior to post-senescence survival in *cdc13-1 mec3-2* cells (B, lane G). Note that type II homologous recombination was observed in both *cdc13-1 mec3-2* (B, lanes I, K and M) and *cdc13-1 mec3-2 tlc1Δ* cells (B, lanes H, J, L and N). Cells were grown at 25 or 29°C for the indicated number of passages (~25 generations per passage) prior to preparation of genomic DNA for telomere structure analysis (*Xho*I digestion; TG_{1-3} probe). (**C**) In telomerase-deficient cells, the type II pattern induced by the presence of the *cdc13-1* mutation (even at permissive temperature for growth) is dominant over type I. Cells were grown at 25°C for ~75 generations prior to preparation of genomic DNA (*Xho*I digestion; TG_{1-3} probe). This also illustrates the very distinctive nature of type II (lanes 3–5, *cdc13-1 tlc1::TRP1*) compared with type I recombination (lane 1, *tlc1::TRP1*) attested here by the disappearance of the non-Y′ bands (arrows), well visible in wild-type cells (lane 2, wt), as described in Materials and methods. The different appearance of type II in (B) and (C) is due to the fact that in (B) the whole patch of cells had to be processed in order to illustrate each time point accurately, hence the smear corresponding to the superimposition of different banding patterns from different colonies, whereas in (C) each type II illustrates a single clone.

with recombining telomeres (Figure 6B), thus demonstrating that Rad51 is dispensable for homologous recombination at telomeres in *cdc13-1* cells.

stn1 mec3 and *ten1 mec3* double mutants do not undergo senescence

The Cdc13–Stn1–Ten1 complex is believed to cap telomere ends (Grandin *et al*., 2001). By two-hybrid analysis, the Cdc13-1 mutant protein was defective in its association with Stn1 and Ten1 (see figure 7 and table I in the Supplementary data available at *The EMBO Journal* Online). However, experiments using a Cdc13-1–Ten1–Stn1 hybrid protein suggested that this binding defect was not sufficient to explain the telomere capping defect conferred by Cdc13-1 (see Supplementary data).

To know more about this, we constructed the *stn1-13 mec3Δ*, *stn1-154 mec3Δ*, *ten1-31 mec3Δ* and *ten1-32 mec3Δ* double mutants (see Materials and methods for strain origin). In the *mec3* null background, at 37°C, the *ten1-32* and *stn1-13* strains showed signs of mitotic

Fig. 6. Rad50, but not Rad51, is required for homologous recombination-based survival in checkpoint-deficient *cdc13-1* cells. (**A**) The indicated strains were propagated at 25°C before being grown at 29°C for the indicated numbers of passages. (**B**) Telomere structure of the strains shown above. Genomic DNA was prepared after growth at 29°C during the indicated periods of time, cut with *Xho*I and revealed with a TG_{1-3} probe. A *cdc13-1 rad51*Δ *mec3*Δ triple mutant behaved exactly like a *cdc13-1 mec3*Δ strain in terms of growth (A) and generation of type II survivors (B), while a *cdc13-1 rad50*Δ *mec3*Δ triple mutant failed to generate survivors after the second passage (A).

catastrophe and died massively, but a few cells continued to divide, as described previously (Grandin *et al*., 1997). *stn1-13 mec3*Δ cells grown at 37°C did not show signs of senescence (see figure 8A in the Supplementary data) and Southern analysis did not allow identification of signs of telomere recombination (supplementary figure 8B). To confirm this finding (*stn1*-associated telomere elongation can easily be mistaken for recombination), we constructed the *stn1-13 mec3*Δ *rad52*Δ triple mutant. This strain behaved exactly like the *stn1-13 mec3*Δ strain at 37°C (supplementary figure 8A and B), thus suggesting that the cells still growing at 37°C are either revertants or cells with sublethal damage, but not recombination-based survivors. On the other hand, the *ten1*Δ YCp-*GAL1-ten1-31 mec3*Δ and *stn1-154 mec3*Δ strains did not grow at all at 30–37°C or 37°C, respectively, and did not exhibit apparent survivors (data not shown). At 34 or 35°C, *stn1-154 mec3*Δ behaved like *stn1-13 mec3*Δ. In conclusion, none of the available *stn1* and *ten1* mutant alleles conferred senescence.

Discussion

In this study, we have shown that senescence, which normally results from telomerase dysfunction (Lundblad and Szostak, 1989; Lundblad and Blackburn, 1993), can take place in the presence of functional telomerase. Abrogation of the DNA damage checkpoint was a prerequisite to senescence/survival in *cdc13-1* cells. Survivors of *cdc13-1* required the presence of Rad52 and Rad50 but not of Rad51. The *cdc13-1* allele is the only known temperature-sensitive allele of *CDC13* that confers a total loss of function (Garvik *et al*., 1995). It is therefore not surprising that no events similar to those described here have been reported for other *cdc13* mutant alleles.

Accumulation of telomeric DNA damage in checkpoint-deficient cdc13-1 cells triggers survival by homologous recombination

Here, we demonstrate that an alternative to cell cycle arrest in *cdc13-1* cells consists of homologous recombination

between telomeric sequences. Several sets of data demonstrated that *cdc13-1* survivors were not revertants or extragenic suppressors. Moreover, the survival process was reversible (Figure 3C). It is probable that in *cdc13-1 mec3* cells, relief from the constraint normally imposed on the cell cycle by the DNA damage checkpoint very efficiently prevents the appearance of revertants and spontaneous suppressors observed in checkpoint-proficient *cdc13-1* cells.

In all analyzed cases, Rad52-dependent telomere elongation was associated with *cdc13-1*-induced senescence and coincided with the appearance of survivors, thus arguing that in these cells, just like in telomerase-deficient cells (Lundblad and Blackburn, 1993), homologous recombination represents the mechanism through which senescent cells can escape death. Therefore, it appears that survival from senescence cannot take place without concomitant telomere–telomere recombination.

Inactivation of *MEC3* or of *RAD24* in *cdc13-1* cells more readily gave rise to survivors than inactivation of *RAD9*, *RAD53* or *MEC1*. *mec1*Δ, *rad53*Δ and *rad9*Δ mutant cells have been found to be more defective in the activation of DNA repair, assessed by measuring phosphorylation of Rad55, than *mec3*Δ and *rad24*Δ mutant cells (Bashkirov *et al*., 2000). It is therefore possible that a mutation in *MEC1*, *RAD53* or *RAD9*, while triggering senescence in *cdc13-1* cells, at the same time indirectly decreases the efficiency of the homologous recombination mechanisms they have contributed to initiate. A more trivial explanation is that synthetic lethality between *cdc13-1* and a mutation in either *MEC1* or *RAD9*, not observed in *cdc13-1 mec3* and *cdc13-1 rad24* cells (Figure 1C), aggravates the status of senescing cells to the point that most of them die before having a chance to undergo survival. The status of *cdc13-1 rad53* cells is unclear, as no synthetic lethality was observed between the two mutations, but yet the rate of survival was unpredictable. Because we find survivors in all five tested *cdc13-1* strains bearing a different mutation in a checkpoint gene, we believe that Mec3 does not play a unique role in these senescence/survival processes.

At 29°C, *cdc13-1* cells generated single-stranded DNA, which, in the absence of the DNA damage checkpoint, failed to arrest cell cycle progression, thus resulting in accumulation of DNA damage. High amounts of single-stranded DNA at 32–34°C were associated with immediate cell death (Figure 4). It is probable that the smaller quantities of DNA damage detectable at 29°C, which slightly increased during passages 1–3 prior to recombination (Figure 4D), provoked only slow death, namely senescence. However, it is possible that senescence has another origin and that the presence of damaged DNA is just an accompanying event. Interestingly, the *cdc13-1*-induced damage, although present and recognized as such upon re-introduction of *MEC3*, was no longer lethal at 34°C after the telomeres had recombined. We propose that the amplification of TG_{1-3} repeats might result in an increase in the number of Cdc13-1 molecules binding to telomeric DNA, thereby decreasing *cdc13-1* temperature sensitivity.

Rad50, but not Rad51, is required for cdc13-1-induced survival

Survivors of *cdc13-1* exclusively resulted from amplification of TG_{1-3} sequences (Figures 2C and 5C) and appear to utilize a single homologous recombination pathway depending on *RAD50* (and *RAD52*), in agreement with mechanisms described recently (Teng *et al*., 2000). On the other hand, survivors of telomerase-deficient cells can arise using two different pathways, one defined by *RAD50* and *RAD59* (and *RAD52*), associated with amplification of TG_{1-3} (type II), and the second one defined by *RAD51*, *RAD54* and *RAD57* (and *RAD52*), associated with amplification of Y′ sequences (type I) (Le *et al*., 1999; Teng *et al*., 2000; Chen *et al*., 2001). The present data, based on agar plate assays, could not allow us to decide whether Rad50 is required for both the formation and maintenance (Teng *et al*., 2000) or only for the maintenance of type II survivors (Chen *et al*., 2001), because experiments in both studies at the origin of this controversy were liquid assays. Single-strand annealing is a process of DNA repair by homologous recombination independent of Rad51 and Rad55, but dependent on Rad52 (Kang and Symington, 2000). Another process of Rad51-independent recombinational repair is break-induced replication, where one end of a broken chromosome invades a homolog and establishes a replication fork all the way to the end of the chromosome (Malkova *et al*., 1996, 2001; Signon *et al*., 2001). Break-induced replication has been proposed to be responsible for telomere elongation in the absence of telomerase (Bosco and Haber, 1998; Le *et al*., 1999).

It is tempting to suggest that Rad51-independent homologous recombination in *cdc13-1* cells initiates following resection of the telomere end by a putative 5′-to-3′ exonuclease, using mechanisms similar to those proposed recently for healing by break-induced replication of a double-stranded break (Malkova *et al*., 2001; Signon *et al*., 2001). At the telomere, the normal 5′ end resection, which also involves a putative 5′-to-3′ exonuclease (Wellinger *et al*., 1993), might represent the substrate for degradation of the unprotected telomere end in *cdc13-1* cells. Rad50 might be necessary for the creation of a stable intermediate during formation of the replication fork, as proposed by Signon *et al*. (2001), or function on a non-chromosomal substrate such as extrachromosomal circles of TG_{1-3} DNA, as proposed by Teng *et al*. (2000) and Chen *et al*. (2001). Importantly, the present data show that, contrary to what has been proposed recently (Teng *et al*., 2000), telomeres do not need to shorten in order to induce the Rad50-dependent recombinational pathway. Indeed, telomeres of *cdc13-1* cells remained of wild-type size and, although exhibiting a severe degradation of the C-strand at 32–37°C (Figure 4; Garvik *et al*., 1995), they showed only slight degradation at the temperature at which senescence occurred, i.e. 29°C.

Several possible defects are responsible for the generation of post-senescence survivors of cdc13-1

In both telomerase-deficient (Lundblad and Blackburn, 1993) and checkpoint-deficient *cdc13-1* cells (present data), Rad52-dependent homologous recombination events constitute the mechanisms by which survivors arise. Therefore, the pathway described in the present study, but not the mechanism it employs, is novel. It has

been suggested that telomere length should not always be used as a means to predict telomere structure and, hence, the proliferative status of cells (Blackburn, 2000). The present data confirm this view and further establish that telomere stability, contributed here by Cdc13, can be uncoupled from both telomerase activity and telomere length.

In telomerase-negative cells, Cdc13 probably comes off the TG_{1-3} repeats when erosion reaches the Y′ element. In senescing *cdc13-1* cells, the picture is different as the Cdc13-1 mutant protein binds single-stranded DNA as efficiently as wild-type Cdc13 (Hughes *et al*., 2000) and still properly loads telomerase, as evidenced by the slightly elongated telomeres in *cdc13-1* cells (Figure 1B). Since telomeres do not shorten, Cdc13-1 probably remains attached to the telomere throughout senescence. In this view, loss of telomerase and loss of Cdc13 function represent two distinct types of telomere uncapping. However, telomere capping by Cdc13 can also be viewed as a subset of reactions controlled by telomerase. We propose that the 5–10% of type II recombination observed in *tlc1*Δ cells (Teng *et al*., 2000; present data) is due to a direct effect of telomerase loss on Cdc13 function (mimicked by Cdc13-1), while the 90–95% of type I recombination is due to telomere erosion independently of Cdc13. Manipulation of Cdc13 function and of the cellular checkpoints has allowed us to bypass the control by telomerase and separate the role played by Cdc13 from that contributed by telomere length.

One clue to the defect of the Cdc13-1 mutant protein was the finding that it failed to interact with both Stn1, confirming recent data by Wang *et al*. (2000) but contradicting those by Chandra *et al*. (2001), and Ten1 (figure 7, table I in the Supplementary data). However, we note that a Cdc13-1–Ten1–Stn1 triple fusion protein failed to rescue *cdc13*Δ, while the control fusion did rescue *cdc13*Δ. Based on previous experiments of this type on Stn1 and Ten1 (Grandin *et al*., 2001), the present data suggest that the failure of Cdc13-1 to bind Stn1 and Ten1 is not its unique defect. Moreover, available *stn1* and *ten1* mutations did not confer senescence, although both Stn1 and Ten1 are clearly involved in telomere protection (Grandin *et al*., 1997, 2001; Pennock *et al*., 2001). *cdc13-1* cells exhibit a much higher amount of single-stranded DNA than *stn1* or *ten1* mutant cells (Grandin *et al*., 1997, 2001), thus suggesting that Cdc13 has a role in telomere end protection different from that of Stn1 and Ten1. Alternatively, the observed differences might be due to the characteristics of the mutant alleles used. The situation in *cdc13-1* clearly shows that there is only a narrow window of DNA damage triggering senescence/survival (32°C is too drastic a condition, for instance), a window that may not be present in the available *stn1* and *ten1* alleles (for instance, 37°C appears too drastic in the case of *stn1-154* and 34–35°C not drastic enough).

Another clue to the defect of the Cdc13-1 mutant protein is provided by the finding that survivors of *rif1*Δ *rif2*Δ *tlc1*Δ and of *cdc13-1* cells are both 100% type II (Teng *et al*., 2000; present data). Maintenance of normal telomere length in *cdc13-1* cells might explain that type I recombination, which is favored by telomere shortening, cannot take place in these cells. However, this does not explain the dominance of type II over type I survivors in *cdc13-1 tlc1*Δ and *rif1*Δ *rif2*Δ *tlc1*Δ cells in which telomeres do shorten. The recent proposal that Rif proteins might inhibit type II recombination at the telomeres (Teng *et al*., 2000) does not fit with the present finding that *cdc13-1 mec3 RIF*+ cells generate 100% type II survivors. We propose that a functional (physical?) interaction between Cdc13 and Rif1/Rif2 might be required to prevent type II recombination. Cdc13 would play a major role compared with Rif2, because Rif2 is required to protect telomeres only in the absence of telomerase (Teng *et al*., 2000), while Cdc13 is required even in the presence of telomerase (present data). An alternative possibility is that the presence of high amounts of telomeric single-stranded DNA in *cdc13-1* cells might somehow prevent fixation of Rap1 to telomere ends and, hence, of Rif1/Rif2, due to the physical interactions between Rap1 and Rif1/Rif2 (Wotton and Shore, 1997). The very long and heterogeneous length telomeres in type II survivors have been proposed to be reminiscent of those in telomerase-negative immortal cell lines and tumors that utilize the ALT pathway (Bryan *et al*., 1997; Teng and Zakian, 1999). The genetic system described here therefore provides an excellent frame to study these mechanisms not only in biological models such as yeasts but also in human tumor cells.

Materials and methods

Plasmids and strains

General plasmids and media used in this study were as described previously (Grandin *et al*., 1997). Yeast strains used in this study were derivatives of BF264-15Daub (Grandin *et al*., 1997). DNA manipulations were performed according to standard procedures (Ausubel *et al*., 1998). Null mutant strains of *mec3*::*TRP1* (Longhese *et al*., 1996), *rad9*::*LEU2* (Garvik *et al*., 1995), *rad24*::*URA3* (Siede *et al*., 1996), *tlc1*::*LEU2* (Singer and Gottschling, 1994), *rad50*::*hisG-URA3-hisG* (Moore and Haber, 1996), *rad51*::*kanMX4* (Euroscarf, Frankfurt, Germany), *rad52-7*::*LEU2* (Yeast Genetic Stock Center, Berkeley, CA), *rad55*::*kanMX6* (Bashkirov *et al*., 2000), *yku70/hdf1*::*URA3* (Porter *et al*., 1996), *yku80*::*TRP1* (Grandin *et al*., 2000), *tel1*::*kanMX4* (Research Genetics, Inc., Huntsville, AL) or *rif2*::*kanMX4* (Euroscarf, Frankfurt, Germany) were obtained either by transforming the linearized disruption DNA or by backcrossing the original disruption strains five times against the genetic background used in our laboratory. All the strains used in this study were therefore isogenic. The *cdc13-1* strain has been described in Garvik *et al*. (1995). The *mec3-2* mutant, bearing a non-temperature-sensitive allele of *MEC3*, was isolated in a genetic screen as an extragenic suppressor of the *cdc13-1*-induced growth arrest at 30°C (N.Grandin and S.I.Reed, unpublished results). The *ten1-32* (*ten1*::*kanMX4* YCp111-*ten1-32*) strain, bearing a temperature-sensitive mutant allele of *TEN1*, was isolated as described previously (Grandin *et al*., 2001). The temperature-sensitive *stn1-13*, *stn1-154* and *ten1-31* strains have been described previously (Grandin *et al*., 1997, 2001). The *sad1-1* and *sad3-1* strains bearing a mutation in *RAD53* and *MEC1*, respectively, have been described in Allen *et al*. (1994).

Streak assay

For senescence studies, cells were streaked on a YPD plate and incubated at the indicated temperature. Re-streaking of single colonies on a YPD plate was repeated every 48 h, four times or more (typically, cells underwent 20–25 divisions per streakout) to allow loss of viability and appearance of survivors. This procedure allowed the detection of both type I and type II survivors, in contrast to the liquid culture assay in which type II survivors eventually predominate (Teng and Zakian, 1999). At various times during the kinetic studies, single colonies were grown in liquid YPD overnight and cells were then harvested and processed for telomere length measurement.

Measurement of telomere length

Genomic DNA was prepared and separated by electrophoresis as described previously (Grandin *et al*., 1997, 2001). The ^{32}P-labeled

telomeric probe used represented 270 bp of TG_{1-3} sequences. Following digestion with *Xho*I, telomere tracts of wild-type cells appear as a broad band of ~1.1–1.2 kb which represents the average length of most chromosomes, those containing Y′ subtelomeric regions. From non-Y′ chromosomes, *Xho*I cutting typically generates fragments migrating at ~2.1, 2.3, 3.3 and 4.1 kb in Southerns. In senescing cells, the disappearance of the non-Y′ fragments attests to the fact that survivors have arisen by homologous recombination (see, for instance, Teng and Zakian, 1999; Grandin *et al*., 2001). Results were analyzed using a Storm PhosphorImager (Molecular Dynamics).

Detection of single-stranded DNA

To detect single-stranded TG_{1-3} DNA, genomic DNA was prepared as described in Grandin *et al*. (1997), run in a 0.7% agarose gel and subjected to non-denaturing Southern hybridization, using the TG_{1-3} probe, as described in Wellinger *et al*. (1993).

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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References

Allen,J.B., Zhou,Z., Siede,W., Friedberg,E.C. and Elledge,S.J. (1994) The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes Dev.*, **8**, 2416–2428.

Artandi,S.E. and DePinho,R.A. (2000) A critical role for telomeres in suppressing and facilitating carcinogenesis. *Curr. Opin. Genet. Dev.*, **10**, 39–46.

Ausubel,F.A., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1998) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.

Bashkirov,V.I., King,J.S., Bashkirova,E.V., Schmuckli-Maurer,J. and Heyer,W.-D. (2000) DNA repair protein Rad55 is a terminal substrate of the DNA damage checkpoints. *Mol. Cell. Biol.*, **20**, 4393–4404.

Blackburn,E.H. (2000) Telomere states and cell fates. *Nature*, **408**, 53–56.

Bodnar,A.G. *et al*. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science*, **279**, 349–352.

Bosco,G. and Haber,J.E. (1998) Chromosome break-induced DNA replication leads to non-reciprocal translocations and telomere capture. *Genetics*, **150**, 1037–1047.

Boulton,S.J. and Jackson,S.P. (1998) Components of the Ku-dependent nonhomologous end-joining pathways are involved in telomeric length maintenance and telomeric silencing. *EMBO J.*, **17**, 1819–1828.

Bryan,T.M., Englezou,A., Dalla-Pozza,L., Dunham,M.A. and Reddel,R.R. (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat. Med.*, **3**, 1271–1274.

Chandra,A., Hughes,T.R., Nugent,C.I. and Lundblad,V. (2001) Cdc13 both positively and negatively regulates telomere replication. *Genes Dev.*, **15**, 404–414.

Chen,Q., Ijpma,A. and Greider,C.W. (2001) Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events. *Mol. Cell. Biol.*, **21**, 1819–1827.

Corda,Y., Schramke,V., Longhese,M.P., Smokvina,T., Paciotti,V., Brevet,V., Gilson,E. and Géli,V. (1999) Interaction between Set1p and checkpoint protein Mec3p in DNA repair and telomere functions. *Nature Genet.*, **21**, 204–208.

Dunham,M.A., Neumann,A.A., Fasching,C.L. and Reddel,R.R. (2000) Telomere maintenance by recombination in human cells. *Nature Genet.*, **26**, 447–450.

Evans,S.K. and Lundblad,V. (1999) Est1 and Cdc13 as comediators of telomerase access. *Science*, **286**, 117–120.

Evans,S.K. and Lundblad,V. (2000) Positive and negative regulation of telomerase access to the telomere. *J. Cell Sci.*, **113**, 3357–3364.

Garvik,B., Carson,M. and Hartwell,L. (1995) Single-stranded DNA arising at telomeres in *cdc13* mutants may constitute a specific signal for the *RAD9* checkpoint. *Mol. Cell. Biol.*, **15**, 6128–6138.

Grandin,N., Reed,S.I. and Charbonneau,M. (1997) Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13. *Genes Dev.*, **11**, 512–527.

Grandin,N., Damon,C. and Charbonneau,M. (2000) Cdc13 cooperates with the yeast Ku proteins and Stn1 to regulate telomerase recruitment. *Mol. Cell. Biol.*, **20**, 8397–8408.

Grandin,N., Damon,C. and Charbonneau,M. (2001) Ten1 functions in telomere end protection and length regulation in association with Stn1 and Cdc13. *EMBO J.*, **20**, 1173–1183.

Horowitz,H. and Haber,J.E. (1984) Subtelomeric regions of yeast chromosomes contain a 36 base pair tandemly repeated sequence. *Nucleic Acids Res.*, **12**, 7105–7121.

Hughes,T.R., Weilbaecher,R.G., Walterscheid,M. and Lundblad,V. (2000) Identification of the single-strand telomeric DNA binding domain of the *Saccharomyces cerevisiae* Cdc13 protein. *Proc. Natl Acad. Sci. USA*, **97**, 6457–6464.

Kang,L.E. and Symington,L.S. (2000) Aberrant double-strand break repair in *rad51* mutants of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **20**, 9162–9172.

Kironmai,K.M. and Muniyappa,K. (1997) Alteration of telomeric sequences and senescence caused by mutations in *RAD50* of *Saccharomyces cerevisiae*. *Genes Cells*, **2**, 443–455.

Le,S., Moore,J.K., Haber,J.E. and Greider,C.W. (1999) *RAD50* and *RAD51* define two pathways that collaborate to maintain telomeres in the absence of telomerase. *Genetics*, **152**, 143–152.

Lendvay,T.S., Morris,D.K., Sah,J., Balasubramanian,B. and Lundblad,V. (1996) Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes. *Genetics*, **144**, 1399–1412.

Lin,J.-J. and Zakian,V.A. (1996) The *Saccharomyces CDC13* protein is a single-strand TG_{1-3} telomeric DNA-binding protein *in vitro* that affects telomere behavior *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 13760–13765.

Longhese,M.P., Fraschini,R., Plevani,P. and Lucchini,G. (1996) Yeast *pip3/mec3* mutants fail to delay entry into S phase and to slow DNA replication in response to DNA damage, and they define a functional link between Mec3 and DNA primase. *Mol. Cell. Biol.*, **16**, 3235–3244.

Lundblad,V. and Blackburn,E.H. (1993) An alternative pathway for yeast telomere maintenance rescues *est1*$^{-}$ senescence. *Cell*, **73**, 347–360.

Lundblad,V. and Szostak,J.W. (1989) A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell*, **57**, 633–643.

Lustig,A.J. and Petes,T.D. (1986) Identification of yeast mutants with altered telomere structure. *Proc. Natl Acad. Sci. USA*, **83**, 1398–1402.

Malkova,A., Ivanov,E.L. and Haber,J.E. (1996) Double-strand break repair in the absence of *RAD51* in yeast: a possible role for break-induced DNA replication. *Proc. Natl Acad. Sci. USA*, **93**, 7131–7136.

Malkova,A., Signon,L., Schaefer,C.B., Naylor,M.L., Theis,J.F., Newlon,C.S. and Haber,J.E. (2001) *RAD51*-independent break-induced replication to repair a broken chromosome depends on a distant enhancer site. *Genes Dev.*, **15**, 1055–1060.

Moore,J.K. and Haber,J.E. (1996) Cell cycle and genetic requirement of two pathways of nonhomologous end-joining repair of double-strand breaks in *S.cerevisiae*. *Mol. Cell. Biol.*, **16**, 2164–2173.

Nugent,C.I., Hughes,T.R., Lue,N.F. and Lundblad,V. (1996) Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science*, **274**, 249–252.

Pennock,E., Buckley,K. and Lundblad,V. (2001) Cdc13 delivers separate complexes to the telomere for end protection and replication. *Cell*, **104**, 387–396.

Porter,S.E., Greenwell,P.W., Ritchie,K.B. and Petes,T.D. (1996) The DNA-binding protein Hdf1p (a putative Ku homologue) is required for maintaining normal telomere length in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **24**, 582–585.

Ritchie,K.B., Mallory,J.C. and Petes,T.D. (1999) Interactions of *TLC1* (which encodes the RNA subunit of telomerase), *TEL1*, and *MEC1* in regulating telomere length in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **19**, 6065–6075.

Siede,W., Nusspaumer,G., Portillo,V., Rodriguez,R. and Friedberg,E.C. (1996) Cloning and characterization of *RAD17*, a gene controlling cell

cycle responses to DNA damage in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **24**, 1669–1675.

Signon,L., Malkova,A., Naylor,M.L., Klein,H. and Haber,J.E. (2001) Genetic requirements for *RAD51*- and *RAD54*-independent break-induced replication repair of a chromosomal double-strand break. *Mol. Cell. Biol.*, **21**, 2048–2056.

Singer,M.S. and Gottschling,D.E. (1994) *TLC1*: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science*, **266**, 404–409.

Teng,S.-C. and Zakian,V.A. (1999) Telomere–telomere recombination is an efficient bypass pathway for telomere maintenance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **19**, 8083–8093.

Teng,S.-C., Chang,J., McCowan,B. and Zakian,V.A. (2000) Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif1-inhibited recombinational process. *Mol. Cell*, **6**, 947–952.

Walmsley,R.W., Chan,C.S.M., Tye,B.-K. and Petes,T.D. (1984) Unusual DNA sequences associated with the ends of yeast chromosomes. *Nature*, **310**, 157–160.

Wang,M.-J., Lin,Y.-C., Pang,T.-L., Lee,J.-M., Chou,C.-C. and Lin,J.-J. (2000) Telomere-binding and Stn1p-interacting activities are required for the essential function of *Saccharomyces cerevisiae* Cdc13p. *Nucleic Acids Res.*, **28**, 4733–4741.

Weinert,T.A., Kiser,G.L. and Hartwell,L.H. (1994) Mitotic chekpoints genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes Dev.*, **8**, 652–665.

Wellinger,R.J., Wolf,A.J. and Zakian,V.A. (1993) *Saccharomyces* telomeres acquire single-strand TG_{1-3} tails late in S phase. *Cell*, **72**, 51–60.

Wotton,D. and Shore,D. (1997) A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in *Saccharomyces cerevisiae*. *Genes Dev.*, **11**, 748–760.

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