

Telomerase and ATM/Tel1p Protect Telomeres from Nonhomologous End Joining

Short Article

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Summary

Telomeres protect chromosome ends from fusing to double-stranded breaks (DSBs). Using a quantitative real-time PCR assay, we show that nonhomologous end joining between a telomere and an inducible DSB was undetectable in wild-type cells, but occurred within a few hours of DSB induction in $\sim 1/2000$ genomes in telomerase-deficient cells and in $>1/1000$ genomes in telomerase-deficient cells also lacking the ATM homolog Tel1p. The fused telomeres contained very little telomeric DNA, suggesting that catastrophic telomere shortening preceded fusion. Lengthening of telomeres did not prevent such catastrophic telomere shortening and fusion events. Telomere-DSB fusion also occurred in cells containing a catalytically inactive telomerase and in *tel1 mec1* cells where telomerase cannot elongate telomeres. Thus, telomerase and Tel1p function in telomere protection as well as in telomere elongation.

Introduction

Telomeres, the ends of eukaryotic chromosomes, are protected from nonhomologous end joining so that they do not fuse to a double-stranded break (DSB). This protection of DNA ends was noted in early experiments on broken chromosomes by McClintock, whose cytogenetic analysis indicated that normal chromosome ends lack the “stickiness” of random chromosome breaks, and Muller, who inferred that chromosome ends (the “terminal genes”) have a protective function, based on his inability to isolate terminal deletions (Gall, 1995).

Telomerase, a specialized reverse transcriptase, copies its intrinsic RNA template into telomeric DNA, compensating for telomere shortening that results from incomplete DNA replication. The catalytic core of *Saccharomyces cerevisiae* telomerase comprises telomerase reverse transcriptase Est2p and telomerase RNA *TLC1*, and synthesizes a degenerate telomeric repeat sequence that can be abbreviated TG₁₋₃ (Nugent and Lundblad, 1998). In replicating cells lacking telomerase, gradual telomere shortening eventually leads to loss of chromosome end protection. Loss of chromosome end protection caused by critical telomere shortening provokes a cell cycle arrest that blocks cellular proliferation; telomere-telomere fusion may occur at this stage (Black-

burn, 2000). Thus, the role of telomerase in averting chromosome end-to-end fusion has been attributed entirely to its preventing attrition of telomeres to critically short lengths.

Some evidence, however, points to a role of telomerase in chromosome end protection that is independent of its ability to effect telomere elongation. *S. cerevisiae* cells expressing an active telomerase RNA template mutant grow at wild-type rates but have shorter telomeres than telomerase-negative cells at the point of senescence (Prescott and Blackburn, 1997b). Similarly, human fibroblasts and endothelial cells ectopically expressing telomerase reverse transcriptase can bypass replicative senescence despite a lack of bulk telomere elongation (Yang et al., 1999; Zhu et al., 1999). These observations suggested that the catalytic activity of telomerase at telomeres, rather than bulk telomere elongation per se, may protect very short telomeres from recognition as damaged DNA.

The ATM family of protein kinases contains two members, Tel1p/ATM and Mec1p/ATR, whose functions are critical to both DNA damage checkpoint signaling and telomere maintenance (Ritchie et al., 1999; Zhou and Elledge, 2000). In *S. cerevisiae* cells lacking Tel1p, telomeres are stably maintained at a short length. Tel1p acts through the Mre1p/Rad50p/Xrs2p (MRX) complex to facilitate telomere elongation by telomerase (Ritchie and Petes, 2000). The MRX complex has been localized at human telomeres, suggesting that the role of ATM in telomere maintenance is conserved between yeast and humans (Zhu et al., 2000). Telomeres in *tel1 tlc1* cells shorten at the same rate as those in *tlc1* cells for many generations, consistent with Tel1p exerting its effect on telomere elongation entirely through telomerase (Ritchie et al., 1999). Mec1p plays a more limited role in *S. cerevisiae* telomere maintenance. Telomere length is wild-type in *mec1* cells, but deleting both Tel1p and Mec1p results in the same initial overall rate of telomere shortening and eventual cellular senescence as deleting telomerase (although *tel1 mec1* cells divide for somewhat longer than *tlc1* cells) (Chan et al., 2001; Ritchie et al., 1999). We recently showed that telomerase is enzymatically active in *tel1 mec1* cells but is unable to act on its substrate, the telomere (Chan et al., 2001).

Ataxia telangiectasia patients lacking the Tel1p homolog ATM are cancer-prone, and their cells have a severely impaired DNA damage response. Telomerase-expressing ATM^{-/-} lymphocytes and fibroblasts exhibit cytological telomeric associations (Metcalfe et al., 1996; Wood et al., 2001). The pleiotropy of the human ATM knockout complicates analysis of the telomeric functions of ATM in these cells. However, *S. cerevisiae tel1* mutants grow indistinguishably from wild-type and are not affected in their DNA damage responses (Greenwell et al., 1995; Morrow et al., 1995).

A recent study by Kolodner and colleagues raised the possibility that telomerase and Tel1p have a novel role in chromosome end protection—the rate of “gross chromosomal rearrangements” (the loss of two counterselectable genes on the same chromosome arm) is un-

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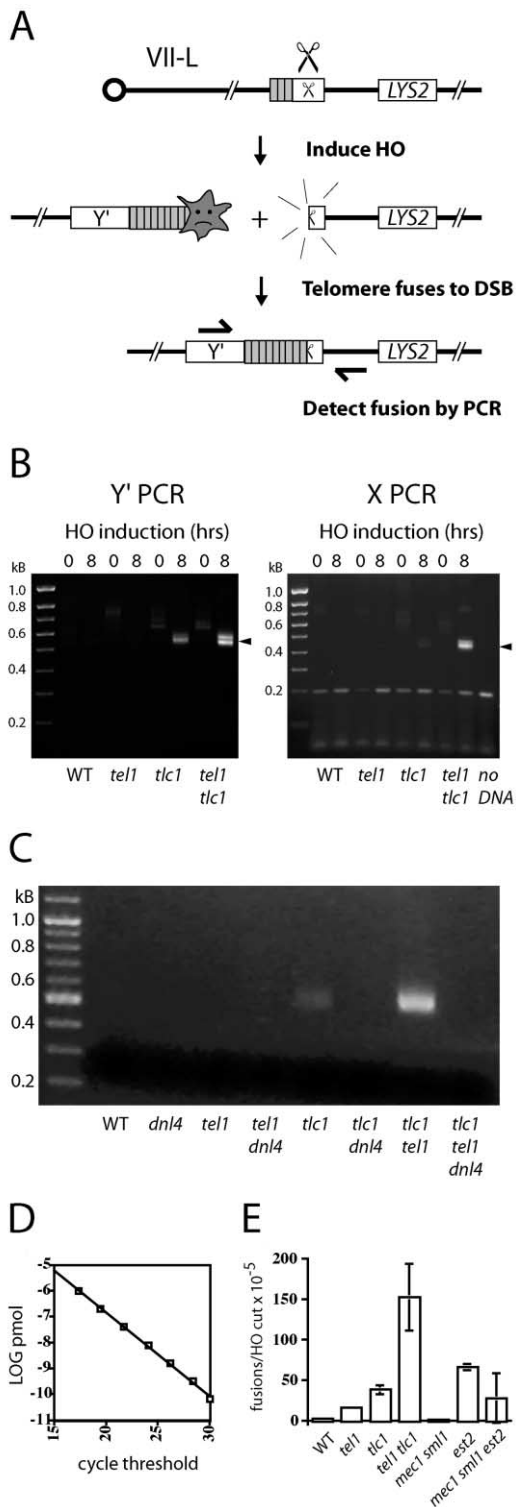


Figure 1. Telomeres Undergo Nonhomologous End Joining with a Double-Stranded Break in Cells Lacking Telomerase and Tel1p

(A) A PCR-based assay for telomere-DSB fusion (see text for details). The centromere-proximal side of the HO cleavage site is adjacent to 81 bp of telomeric DNA. We assayed fusion between a telomere and the centromere-distal side of the HO site. (B) PCR detection of Y'- and X telomere-DSB fusions. Genomic DNAs were prepared 0 or 8 hr after HO induction with galactose. The expected PCR product sizes are arrowed: 487 bp + telomeric

changed in *tlc1* or *tel1* cells but increases nearly 3000-fold in *tel1* *tlc1* double mutants (Myung et al., 2001). Chromosome rearrangements recovered from *tel1* *tlc1* cells showed fusions between a telomere and a variety of internal chromosome sequences. Another genetic assay that is specific for telomere protection defects uses a defined chromosome break generated by the endonuclease HO to initiate telomeric recombination or fusion with a telomere (DuBois et al., 2002). These authors found an increase in telomeric recombination events in *tel1* cells, but saw little effect on the frequency of telomere-DSB fusion. To assay telomere-DSB fusion directly, we modified the procedure of DuBois et al. (2002) to detect fusions between an inducible chromosome break and a telomere using quantitative real-time PCR. Our assay has three advantages for assaying telomere-DSB fusion. Like the genetic assay of DuBois et al., it is specific for telomere-DSB fusions and does not detect other types of chromosomal rearrangement. Second, because the assay is rapid, telomere length will not be changed by repeated cell divisions when cells lack telomerase. Last, this direct assay does not require cellular viability.

We report that telomeres fuse rapidly (within hours) to the HO-generated double-stranded break in cells lacking active telomerase. The frequency of fusion increased when either telomerase or Tel1p was deleted, and was synergistically increased in *tel1* *tlc1* cells. Formation of telomere-DSB fusions occurred by the nonhomologous end joining pathway, as they depended on DNA ligase IV (encoded by *DNL4*). Fusions also occurred in *tel1* *mec1* cells in which telomerase cannot elongate telomeres. Telomere-DSB fusion was assayed well before critical telomere shortening caused detectable cellular senescence, and occurred even in cultures where the vast majority of telomeres were significantly elongated. However, the tracts of telomeric TG₁₋₃ repeat DNA trapped in the telomere/DSB fusions were an average of 200 bp shorter than the bulk telomere length of the cells assayed. In contrast, the nontelomeric, HO-cleaved side of the cloned fusion products was commonly intact and was never resected by more than 17 nucleotides. This suggests that catastrophic loss of most of the telomeric DNA and a consequent defect in chromosome end protection occur at a low frequency in cells lacking telomerase. We propose that telomerase and Tel1p have previously unrecognized roles in chromosome end protection, to prevent catastrophic telomere shortening even when the majority of cellular telomeres are long.

DNA for Y' fusions using the primers VII-HO B and Y' subtelomeric, and 405 bp + telomeric DNA for X fusions using the primers VII-HO A and X subtelomeric.

(C) PCR detection of Y' telomere-DSB fusions from *DNL4* and *dnl4* cells. Genomic DNAs were prepared 8 hr after HO induction. The primers were VII-HO B and Y' subtelomeric.

(D and E) Quantitative real-time PCR of telomere-DSB fusions. (D) Standard curve. (E) Genomic DNA was prepared 8 hr after HO induction. PCR primers were VII-HO B and Y' subtelomeric. Number of fusions per HO cut was calculated from the absolute number of telomere-DSB fusions (derived by comparing the cycle threshold of a given sample with a standard curve for amplification) and the amount of genomic DNA in the amplification reaction. Each genotype represents at least three independent spores from the same parent diploid.

Results

Telomeres Undergo Nonhomologous End Joining with a DSB in Cells Lacking Telomerase and Tel1p

To assay for fusigenic telomeres directly, we exploited a unique site on chromosome VII that was engineered to be cleavable by the HO endonuclease (Diede and Gottschling, 1999) (Figure 1A). In the strain used, the endogenous HO site at *MAT* is deleted, so only one DSB is created (see Experimental Procedures). HO expression from the *GAL* promoter was induced in log phase cells by the addition of galactose. Cells were harvested 2–8 hr after HO induction and frozen for DNA preparation. *S. cerevisiae* telomeres contain either an X or Y' conserved subtelomeric element directly adjacent to the terminal telomeric TG₁₋₃ sequence; in the S288C strain used here, there are 15 X telomeres and 17 Y' telomeres (see complete genome sequence at <http://genome-www.stanford.edu/Saccharomyces/>). Therefore, telomere-DSB fusions were specifically amplified by PCR using one primer adjacent to the HO cleavage site on chromosome VII and a second primer complementary to either the X or Y' subtelomeric element. Telomere shortening in the absence of telomerase can compromise chromosome end protection. For this reason, in all experiments, we assayed telomere-DSB fusion in cells inoculated directly from spore colonies; that is, well before the onset of cellular senescence in cells lacking telomerase.

A PCR product of the expected size for a telomere-DSB fusion was seen with either an X or Y' subtelomeric primer in *tel1 tlc1* cells (Figure 1B). No product was visualized by ethidium bromide staining in assays of wild-type or *tel1* cells, but a faint product was seen with *tlc1* cells. The PCR product depended on induction of HO with galactose. In control reactions, no product was detected when PCR was performed with an X primer alone, a Y' primer alone, or with an X and a Y' primer (data not shown). As will be described below, cloning and sequencing of the PCR products from *tel1 tlc1* DNA confirmed that the assay detects direct end-to-end fusions between telomeres and the HO-cleaved chromosome break.

To ascertain the cellular pathway for telomere-DSB fusion, we assayed the involvement of Dnl4p/DNA ligase IV, the ligase involved in nonhomologous end joining (Wilson et al., 1997). Fusions were reduced to wild-type levels in *dnl4 tlc1* and *dnl4 tel1 tlc1* cells (Figure 1C). Hence, telomeres in *tel1 tlc1* cells fused to the DSB by the canonical nonhomologous end-joining machinery.

In order to quantify the level of telomere-DSB fusion in *tlc1* and *tel1 tlc1* cells, we used real-time PCR with very similar amplification parameters to reactions that yielded ethidium bromide-stained products. The standard for PCR amplification was a linearized plasmid containing a cloned telomere-DSB fusion (wild-type genomic DNA was included in PCR standard reactions, which had the same DNA concentration as experimental samples). Amplification of the PCR standard was linear over five orders of magnitude ($r^2 = 0.998$) corresponding to a range of telomere-DSB fusion frequencies from 0.93 to 5.596×10^{-5} (data not shown). Telomere-DSB fusions were essentially undetectable (i.e., occurred at <1 in 10^5 genomes) in wild-type cells, in agreement with ethidium

bromide staining results (Figure 1D). The frequency of fusion was noticeably increased, to about 1.6 in 10^4 genomes, in *tel1* cells, and was higher still, at about 4 in 10^4 genomes, in cells lacking telomerase RNA (*tlc1*) or telomerase reverse transcriptase (*est2*). The *tel1* and *tlc1* mutations had a synergistic effect on telomere-DSB fusions, which occurred in greater than 1 in 10^3 genomes in *tel1 tlc1* cells. The Tel1p-related kinase Mec1p (homolog of human ATR) was not required for telomere protection; *mec1 sml1* cells did not show telomere-DSB fusions, and *mec1 sml1 est2* cells showed no increase over *est2* cells (*sml1* is a suppressor mutation that rescues *mec1* lethality). We conclude that both telomerase and Tel1p are important in protecting telomeres from fusing to a DSB (Figure 1D).

Telomere-DSB Fusions Contain Very Short Tracts of Telomeric DNA

The size of PCR-amplified telomere-DSB fusion bands indicated that telomeres that fused to a DSB had lost most of their terminal TG₁₋₃ repeat tracts. In every case, the PCR product size was consistent with very little telomeric DNA remaining between the subtelomeric X or Y' elements and HO-cleaved chromosome VII sequence. This was confirmed by cloning and sequencing 77 PCR products (see example in Figure 2A). The average length of telomeric TG₁₋₃ DNA in fusions cloned from *tel1 tlc1* and *tel1 est2-D530A* cells was 33 bp, with a standard deviation of 19 bp and a range from 4 and 130 bp (Figure 2B). There was no significant difference between fusions to X or Y' telomeres. To ensure that the PCR amplification step did not selectively amplify very short telomere-DSB fusions from a pool of fusions containing much longer tracts of telomeric DNA, we amplified cloned telomere-DSB fusions containing 32, 88, and 130 bp of TG₁₋₃ DNA from plasmids. The efficiency of the PCR reaction was similar for all three plasmids (Figure 2C; note that a lower cycle threshold means earlier detection of the PCR product, and thus higher efficiency of amplification). Telomeric DNA in *tel1 tlc1* cells assayed for telomere-DSB fusions had a mean length of $\sim 230 \pm 50$ bp; the shortest telomeres detected by long exposures of Southern blots are about 190 bp long (Figure 3B and data not shown). Hence, the shortest telomeres detectable by Southern blotting in *tel1 tlc1* cells were substantially longer than the TG₁₋₃ tracts retained in the fusions.

This finding suggests that in *tel1 tlc1* cells, a small fraction of telomeres undergoes catastrophic shortening, causing loss of chromosome end protection and fusion to a DSB. As our assay is done a few hours after the HO-cut chromosome break is created, we propose that extremely short telomeres are already present when this inducible break is generated. Two lines of evidence suggest that few *tlc1 tel1* cells contain drastically shortened telomeres: first, *tel1 tlc1* cells at the time of the fusion grew indistinguishably from wild-type, and second, long exposures of Southern blots of *tel1 tlc1* genomic DNA did not detect drastically shortened telomeres. In agreement with these data, telomere-DSB fusion was a rare event.

Notably, the portion of chromosome VII adjacent to the HO cleavage site and the cleavage site sequence itself was intact in nearly all the cloned fusions (Figure

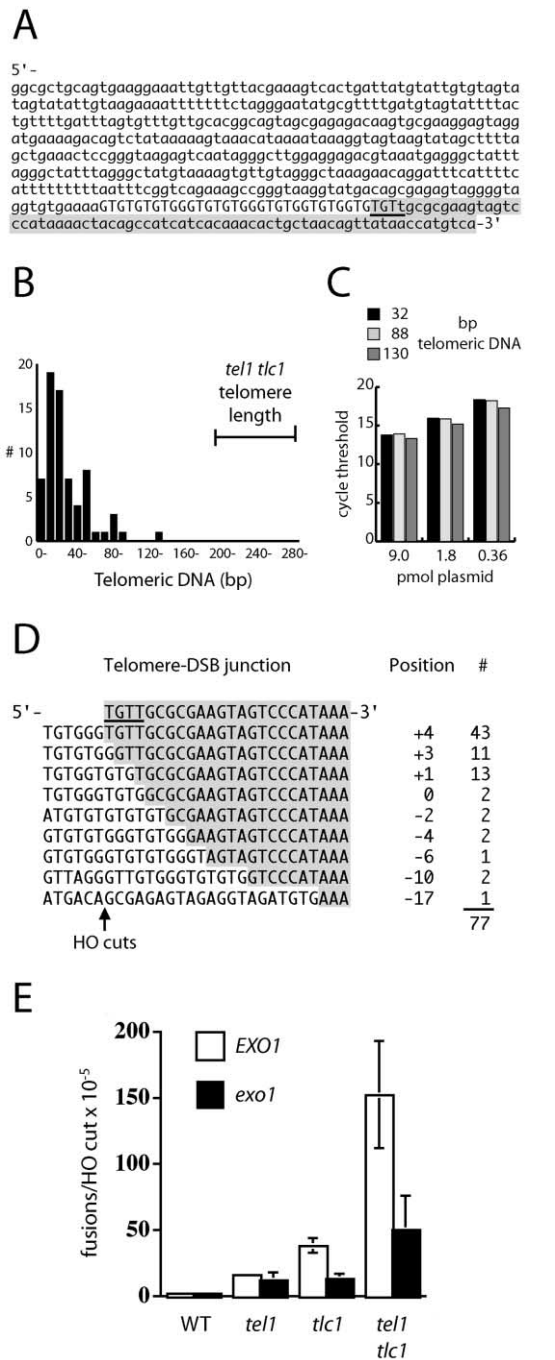


Figure 2. Telomere-DSB Fusions Contain Very Little Telomeric DNA
(A) Complete sequence of one cloned telomere-DSB fusion. Unshaded sequence is derived from a Y' telomere. Shaded sequence is from chromosome VII adjacent to the HO cleavage site. Upper-case sequence conforms to the telomeric DNA consensus TG₁₋₃(TG)₁₋₆. Underlined nucleotides represent the 3' overhang created by HO cleavage, which is preserved in this fusion.
(B) Histogram of cloned telomere-DSB fusions grouped into bins representing 1–10 bp of telomeric DNA, 11–20 bp telomeric DNA, etc. *tel1 tlc1* telomere length is derived from Southern blotting (Figure 3A).
(C) Quantitative real-time PCR of plasmids containing telomere-DSB fusions with different lengths of TG₁₋₃ DNA. Plasmids containing cloned Y' telomere-DSB fusions with 32, 88, or 130 bp of TG₁₋₃ DNA were quantitated with a fluorimeter and amplified by quantitative

real-time PCR using the primers VII-HO C and Y' subtelomeric 2. A lower cycle threshold represents more efficient PCR amplification.
(D) Junction sequences where telomeric DNA is fused to the HO cleavage site. Position "+4" means that the complete 3' overhang (underlined) is preserved; "0" refers to the beginning of duplex DNA at the DSB. A total of 77 telomere DSB fusions were cloned from *tlc1 tel1* (34), *est2-D530A tel1* (34), and *tel1 mec1 sml1* (9) cells.
(E) Quantitative real-time PCR of telomere-DSB fusions. Genomic DNA was prepared 8 hr after HO induction. The PCR primers were VII-HO B and Y' subtelomeric. White bars represent the genotypes shown below the graph. Black bars are the genotypes below the graph with the addition of the *exo1* mutation.

2D). HO cleavage of chromosome VII leaves a 3'-overhang with the sequence 5'-AACA-3', which was fully preserved in 43 of 77 cloned fusions. In 69 of the 77 fusion products, the HO-cut end was not resected beyond this 4-base 3'-overhang.
 In order to investigate the mechanism that generates extremely short telomeres, we tested the possible involvement of Exo1p, a nuclease that degrades telomeric DNA when Cdc13p function is removed (Maringele and Lydall, 2002). Unlike the *dnf4* mutation, *exo1* did not completely suppress telomere-DSB fusions, whose frequency was significantly greater in *exo1 tlc1* and *exo1 tel1 tlc1* than in completely wild-type cells (Figure 2E). Therefore, Exo1p is not solely responsible for catastrophic telomere shortening.

Telomere-DSB Fusion Occurs in *tel1 tlc1* Cells with Long Telomeres

Telomerase preferentially elongates short telomeres, while long telomeres are a poor substrate for the enzyme (Marcand et al., 1999). Therefore, we tested whether telomerase and Tel1p are required to prevent long telomeres from fusing to a DSB. We artificially lengthened telomeres by transiently expressing a fusion between the telomere binding protein Cdc13p and the telomerase protein Est1p. An interaction between these proteins recruits telomerase to the telomere, and fusing them in a single polypeptide causes telomere lengthening (Evans and Lundblad, 1999; Tsukamoto et al., 2001). The Cdc13p-Est1p fusion was expressed from a CEN/ARS plasmid in a *tlc1/TLC1 tel1/TEL1* diploid, causing telomeric TG₁₋₃ DNA to lengthen by approximately 250 bp (Figure 3A, right panel; note that the terminal XhoI fragment also contains ~850 bp of the subtelomeric Y' element). Spores from this diploid that had lost the *CDC13-EST1* plasmid but inherited long telomeres were assayed for telomere-DSB fusion. Gradual loss of telomeric DNA during spore growth produced *tlc1 tel1* cells with an average telomere length of about 480 bp at the time they were assayed (compared to a mean length of 350 bp in wild-type cells; Figure 3A). Lengthening of telomeres to greater than wild-type size did not prevent the appearance of telomere-DSB fusions in *tlc1* and *tel1 tlc1* cells, though their frequency was reduced compared to *tlc1* and *tel1 tlc1* cells with short telomeres (Figure 3B). Importantly, the *tel1 tlc1* cells with short and long telomeres gave the same size PCR products (Figure 3C). These results confirm that it is not bulk telomere shortness per se but rather the absence of telomerase and Tel1p that causes extreme telomere shortening and telo-

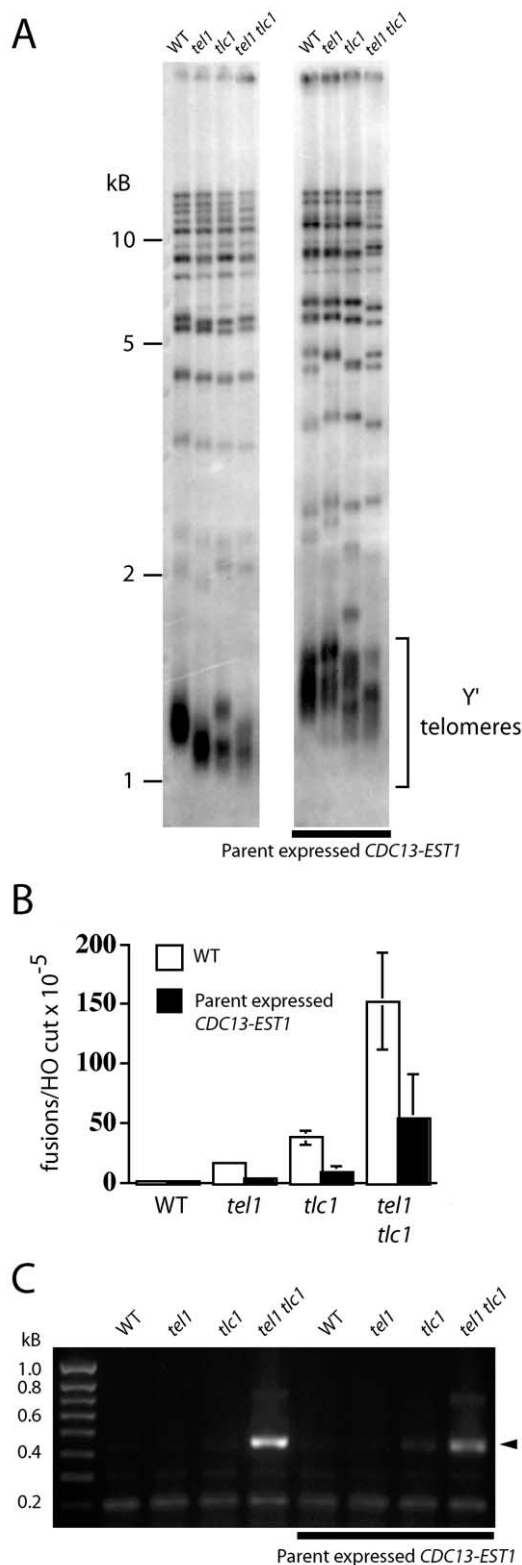


Figure 3. Telomere-DSB Fusions Occur in *tel1 tlc1* Cells with Long Telomeres

(A) Southern blot showing telomere length in strains assayed. Genomic DNA digested with *XhoI* was probed with a telomeric oligonucleotide. Y' telomeres in wild-type cells are the band at about 1.2 kb in wild-type cells.

mere-DSB fusion. Furthermore, they imply that telomerase is still necessary to protect long telomeres from catastrophic shortening. This shows that the telomere-protective function of telomerase is independent of its role in promoting bulk telomere elongation.

The Protective Function of Telomerase Requires Its Catalytic Activity at Telomeres

We tested whether the ability of telomerase to prevent telomere-DSB fusions in the absence of Tel1p requires its catalytic activity. The *est2-D530A* point mutation abolishes the catalytic activity of telomerase (Lingner et al., 1997). As shown in Figure 4A, telomere-DSB fusions were observed by ethidium-bromide staining of PCR products from *est2-D530A* and *tel1 est2-D530A* DNAs. Thus, the presence of an intact but inactive telomerase ribonucleoprotein complex is not sufficient for chromosome end protection. We also examined the effect of a background (*tel1 mec1 sml1*) in which telomerase is catalytically active but telomeres are refractory to its action (telomere shortening in these cells is initially identical to that in telomerase-deficient cells) (Chan et al., 2001; Ritchie et al., 1999). Again, telomere-DSB fusions were detected by ethidium bromide staining of PCR products from *tel1 mec1 sml1* cells, but not from *tel1* or *mec1 sml1* cells, following induction of the HO chromosome break (Figure 4B). Telomere-DSB fusions cloned from *tel1 mec1 sml1* cells contained an average of 29 ± 10 bp TG₁₋₃ DNA, indicating that drastic telomere shortening also occurs in these cells. These results provide evidence that the telomere defect in *tel1 tlc1* cells and in *tel1 mec1 sml1* cells is similar and that chromosome end protection requires telomerase that is both catalytically active and able to access the telomere.

Fusion occurred in *tlc1 tel1-kd* cells, which express a mutant Tel1p predicted to lack kinase activity (Figure 4A). This result implies that a Tel1p phosphorylation substrate is important for telomere end protection. Human ATM phosphorylates NBS1, the homolog of Xrs2p, and Tel1p acts through the MRX complex to promote telomere elongation (Gatei et al., 2000; Lim et al., 2000; Ritchie and Petes, 2000; Zhao et al., 2000). Whether Tel1p also acts through the MRX complex in telomere end protection is unknown. We did not observe fusions in *tel1 tlc1 xrs2* or *tel1 est2 xrs2* cells despite the absence of both telomerase and Tel1p (data not shown). However, telomere-DSB fusions are mediated by nonhomologous end joining (Figure 1C), a process that requires the MRX complex (Boulton and Jackson, 1998). Therefore, addressing the potential telomere-protective role of the MRX complex will require separation-of-function mutations that disrupt its telomeric function but preserve nonhomologous end joining. Mutating the Tel1p phosphorylation sites in the complex may achieve this,

(B) Quantitative real-time PCR of telomere-DSB fusions. Genomic DNA was prepared 8 hr after HO induction. The PCR primers were VII-HO B and Y' subtelomeric.

(C) Telomere-DSB fusions from cells with long telomeres contain short tracts of telomeric DNA. PCR detection of X-telomere-DSB fusions from *tel1 tlc1* cells with short and long telomeres. The PCR primers were VII-HO A and X subtelomeric.

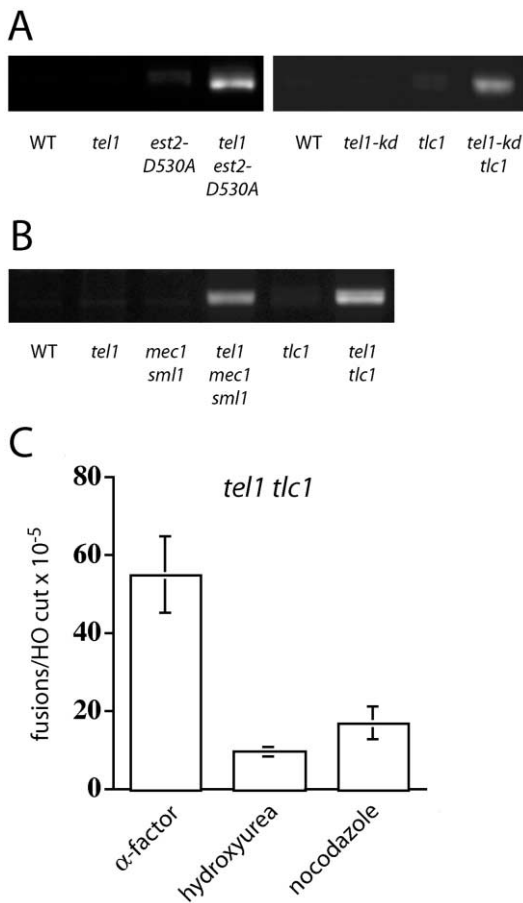


Figure 4. The Protective Function of Telomerase Requires Its Catalytic Activity at Telomeres

(A) PCR for X telomere-DSB fusions performed on genomic DNA prepared 8 hr after HO induction. The primers were VII-HO A and X subtelomeric.

(B) PCR for X telomere-DSB fusions performed on genomic DNA prepared 8 hr after HO induction. The primers were VII-HO A and X subtelomeric.

(C) Telomere-DSB fusions are increased in G1 phase. Quantitative real-time PCR for Y' telomere-DSB fusions. *tel1 tlc1* cells were arrested for 4 hr as indicated, followed by 2 hr of HO induction in the cell cycle arrested state.

as *tel1* cells have no defect in nonhomologous end joining (Boulton and Jackson, 1998).

Telomere-DSB Fusion Occurs in G1 Phase of the Cell Cycle

Telomere elongation by telomerase occurs during S phase and possibly in G2/M and may be coupled to DNA replication as it depends on certain DNA replication factors (Diede and Gottschling, 1999; Marcand et al., 2000). To determine when in the cell cycle telomeres fuse to an induced DSB, we assayed *tel1 tlc1* cells arrested with α factor (G1), hydroxyurea (S), or nocodazole (G2/M). After 4 hr of cell cycle arrest, HO was induced for 2 hr in the arrested state, after which genomic DNA was prepared and analyzed. Interestingly, fusions occurred at a significantly higher frequency in α -factor-arrested cells (Figure 4C). This result implies either that

the frequency of nonhomologous end joining is increased in G1 phase or that telomeres in *tel1 tlc1* are specifically fusogenic in G1.

Previous assays for nonhomologous end joining in *S. cerevisiae* have relied either on healing of transformed linearized plasmids or on repair of the *MAT* locus following HO cleavage (Boulton and Jackson, 1998; Moore and Haber, 1996). Telomere-telomere fusions in *S. pombe* cells lacking the telomere binding protein Taz1p are detectable when cells are arrested in G1 but not in asynchronously growing cells that are primarily in G2/M (Ferreira and Cooper, 2001). Our findings confirm that this preference for nonhomologous end joining in G1 is conserved in budding yeast. Notably, we also observe telomere-DSB fusions in an asynchronous population, where they presumably occur mostly in the G1 cells. Haploid cells may find it advantageous to use nonhomologous end joining in G1, when only one copy of the genome is present. After DNA replication, homologous recombination can use a second copy of a given sequence to achieve a more accurate means of genome repair. The mechanism by which cells select a particular DNA repair pathway depending on their cell cycle state is an interesting question for future research.

Discussion

Here, we have shown that telomerase and the ATM kinase homolog Tel1p are needed to prevent telomeres from fusing to a double-stranded break by nonhomologous end joining. All telomere-DSB fusions analyzed contained very little TG₁₋₃ DNA, so we infer that extreme shortening of the telomeres is the reason that they fused to the DSB. Wild-type telomeres shorten and lengthen within generally well-controlled length boundaries. However, studies of long-term telomere maintenance in the budding yeast *Kluyveromyces lactis* suggest that extreme telomere shortening occurs at a very low frequency in wild-type cells (McEachern et al., 2002). *K. lactis* telomeres are ~400 bp in length, with a range of 250–550 bp, but rare events that shorten the telomeric repeat tract to 25–50 bp were inferred using a silent telomerase RNA template mutation that “marks” the action of telomerase in telomeric DNA.

To create the shortened tracts of telomeric DNA we cloned as telomere-DSB fusions, an average of 200 bp of telomeric DNA would have had to be degraded. Without telomerase, *S. cerevisiae* telomeres shorten at an average of 3–5 bp/generation irrespective of telomere length (Marcand et al., 1999). This rate is consistent with loss through incomplete DNA replication and is similar overall in *tlc1* and *tel1 tlc1* cells. This suggests that the subpopulation of telomeres that fuses to a DSB must shorten considerably faster, although the mechanism for this is not known. In order for a telomere of normal length to give rise to the very short TG₁₋₃ tracts seen in telomere-DSB fusions, both strands of telomeric DNA must have been degraded. This contrasts with strand-specific 5'-3' resection of DNA at a double-stranded break or at a telomere lacking the essential telomere binding protein Cdc13p (Garvik et al., 1995). Notably, strand-specific degradation of the HO-cleaved chromosome did not result in drastic loss of sequence informa-

tion when it fused to a telomere; no more than 17 bp of the HO recognition site were missing in any cloned telomere-DSB fusion, and only 8 of 69 fusions were resected beyond the 3'-overhang of the HO site.

We suggest two models for how telomerase prevents telomere-DSB fusions. In the first, the basal frequency of extreme telomere shortening events is identical in wt and *tlc1* (\pm *tel1*) cells. Telomerase confers end protection to any such extremely shortened telomeres by elongating them. In this case, the failure of *est2-D530A* telomerase to protect telomeres from fusions would result from its inability to synthesize telomeric DNA to re-elongate the extremely shortened telomere. In the second model, the frequency of extreme shortening events increases in *tlc1* (\pm *tel1*) cells because telomerase normally binds telomere ends, sterically protecting them from catastrophic shortening. Supporting a steric protective role for telomerase, Est2p is associated with telomeres in vivo throughout S phase and also in G1, a time in the cell cycle when it cannot elongate telomeres in vivo (Diede and Gottschling, 1999; Taggart et al., 2002). In vivo, telomerase is known to elongate telomeres in late S and G2/M phases of the cell cycle (Diede and Gottschling, 2001; Marcand et al., 2000). Further consistent with this mechanism, in vitro telomerase stably binds its DNA elongation product, the telomeric DNA terminus (Fulton and Blackburn, 1998; Prescott and Blackburn, 1997a). Moreover, conversion of the enzyme to a conformation allowing stable protective association with the telomere depends on a round of polymerization (Prescott and Blackburn, 1997a). Thus, by this model, *est2-D530A* telomerase fails to protect telomeres from fusions because it cannot form a stable association with its telomeric substrate.

Two mechanisms can be envisaged for how Tel1p enhances the protection of telomeres by telomerase. First, telomerase action on telomeres is known to be promoted by Tel1p (Chan et al., 2001; Ritchie et al., 1999), and a protective role for telomerase may be potentiated by the kinase. Tel1p acts through the MRX complex to facilitate telomere elongation. Although it is unknown whether the MRX complex acts downstream of Tel1p to prevent extreme telomere shortening, it has DNA binding, helicase, and nuclease activities in vitro that may modulate telomere structure and act to limit catastrophic shortening (Paull and Gellert, 1998, 1999). Second, a catastrophically short telomere may normally elicit a *TEL1*-dependent cell cycle arrest in either S or G2/M. If cells also lack functional Tel1p, they may proceed into G1, the cell cycle phase when fusion to a DSB is most efficient. However, recent results from Berman and coworkers show that *tlc1* cells near senescence do not require Tel1p to arrest the cell cycle, arguing against this hypothesis (Enomoto et al., 2002).

Our findings may have relevance in human cancer cells, where telomere end protection has two contrasting roles. Early in tumorigenesis, genomic instability caused by critical telomere shortening in the absence of telomerase is thought to facilitate genomic variants that can be selected for enhanced growth properties; subsequently, activation of telomerase becomes essential for unlimited proliferative capacity in most human tumors (Maser and DePinho, 2002). The *ATM* gene has recently been mapped as a breast cancer susceptibility

locus (Chenevix-Trench et al., 2002) and *ATM*^{-/-} ataxia telangiectasia patients have a cancer-prone phenotype. Primary mammary epithelial cells, the progenitors to breast carcinomas, lack active telomerase (Kiyono et al., 1998; Romanov et al., 2001). Furthermore, it was recently shown that mice with mutations in *ATM* and in telomerase RNA show a synergistic increase in telomere dysfunction (Wong et al., 2003). The telomere protection defect we observe in *tel1 tlc1* cells suggests that the absence of telomerase and *ATM* may exacerbate genomic instability early in tumorigenesis. Based on our results in yeast, these effects may manifest themselves well before critical telomere shortening is apparent.

Experimental Procedures

Strain and Plasmid Construction

The diploid strain ySC241 was made by mating UCC5706 (a gift from D. Gottschling) to a *tel1 tlc1 MAT α* derivative of ySC78 (*tel1::HIS3/TEL1 mec1::LEU2/MEC1 sm11::TRP1/SML1 tlc1::TRP1/TLC1 MAT α*) (Diede and Gottschling, 1999). Both parent haploids are in the S288C background. The endogenous HO site at *MAT α -inc* is deleted, so only one DSB is created upon cleavage of the HO site in *MAT α -inc* derivatives of this strain. Subsequent strains were constructed by mating *GAL-HO::LEU2 VII-L::ADE2-TG-HO MAT α -inc* spores from ySC241 to mutant strains isogenic with the S288C strain BY4705 (Brachmann et al., 1998). The *tel1::HIS3, tlc1::TRP1, mec1::LEU2*, and *sm11::TRP1* alleles have been previously described; all are null alleles (Chan et al., 2001). *dn14::URA3, est2::HIS3*, and *exo1::KANMX4* were made by PCR-mediated transformation and delete the entire open reading frame in all cases. *est2-D530A::LEU2* was made by integrating pSC149 into the *est2::HIS3* allele. pSC149 contains nucleotides -524 to 3226 of *EST2* (relative to +1) cloned from *SacI* to *BamHI* into pRS305, and mutagenized by overlapping PCR to introduce the D530A mutation. pSC149 was subcloned from pRS426-*est2-D530A*, a gift from J. Lin. The *tel-*kd*::HIS3* allele was made in a one-step gene replacement with pSC150 and contains the mutations D2612A and N2617K; mutating the corresponding residues in *Mec1p* reduces kinase activity >90% (Mallory and Petes, 2000). To make pSC150, nucleotides 7488 to 9223 of *TEL1* (relative to +1) were cloned as a *XbaI*-*KpnI* fragment into pBluescript and mutagenized by overlapping PCR. The *HIS3* gene from pRS303 was then cloned into the *EcoRI* site at nucleotide 8977 of *TEL1*. For telomere-lengthening experiments with the *Cdc13p*-*Est1p* fusion, the *GAL-HO::LEU2* gene was converted to *GAL-HO::URA3* with plasmid pLU12 (Cross, 1997). The *CDC13-EST1* plasmid pVL1091 (*LEU2* marker) was a gift from V. Lundblad (Evans and Lundblad, 1999). We assayed *Leu*-minus spores from tetrads in which at least one spore contained the *CDC13-EST1* plasmid, to ensure that the plasmid had been lost recently.

HO Induction

Whole spore colonies grown for 3 days postgermination were inoculated into YEP-2.5% raffinose and grown for 12–16 hr at 30°C. Cells were then washed and resuspended in YEP-3% galactose at OD₆₀₀ = 0.3. Prior to HO induction, spore colonies were checked for the presence of the *LYS2* marker indicative of the uncleaved *VII-L::ADE2-TG-HO* allele. Each experiment was repeated with at least three independent spores for a given genotype.

PCR Amplification of Telomere-DSB Fusions

Genomic DNA was prepared as described (Chan et al., 2001) and quantified with a fluorometer and Hoechst 3300 dye with calf thymus DNA as a standard. PCR reactions for ethidium bromide staining (25 μ l) contained 1x PC2 buffer (AB Peptides), 200 μ M dNTPs, 0.5 μ M each oligonucleotide primer, 2.5 units of polymerase (16:1 vol/vol mixture of KlenTaq [AB Peptides] and PfuTurbo [Stratagene]), and 50 ng of genomic DNA. The PCR consisted of thirty cycles of: 94°C for 5 s, 60°C for 30 s, and 72°C for 1 min, and the entire reaction was electrophoresed through a 1.5% agarose gel. Primer sequences: VII-HO A 5'-CGCCATATTGCTAGTTTCG-3'; VII-HO B

5'-CGCGCGCGCCGTCGACATGGTTATAACTGTTAGC-3'; VII-HO C 5'-CGCGCGCGCCGAGGCTTCAATGAGCTTCC-3'; X subtelomeric 5'-AATGGAGGGTAAGTTGAGAGACAGG-3'; Y' subtelomeric 5'-GGCGCTGCAGTGAAGAAATTGTTGTTAGC-3'; Y' subtelomeric 2 5'-GGCGCTGCAGATGTAGAAGTGTGTAGGGC-3'. Consistent results were obtained with the different primer pairs tested.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed with the primers VII-HO B and Y' subtelomeric, using an MJ Research Opticon and the intercalating fluorescent dye SYBR Green to quantify dsDNA accumulation. PCR reactions (50 μ l) contained 1x PC2 buffer (AB Peptides), 200 μ M dNTPs, 0.5 μ M each oligonucleotide primer, 5 units of polymerase (16:1 vol/vol mixture of KlenTaq [AB Peptides] and PfuTurbo [Stratagene]), and 100 ng of genomic DNA. The concentration of SYBR Green dye in the reaction was 0.075 \times relative to the 10,000 \times dye supplied by Sigma.

The PCR reaction cycle was: 5 min at 94°C; 36 cycles of [94°C for 5 s, 60°C for 30 s, 72°C for 1 min, 84°C for 10 s, fluorescence measurement]; 72°C for 5 min; melting curve analysis of PCR reaction products from 70–95°C with fluorescence measurements every 0.2°C. Initial experiments revealed a primer dimer that competed with the product representing telomere-DSB fusion. Measuring fluorescence at 84°C greatly reduced the primer dimer signal ($T_m \sim 77^\circ\text{C}$) without affecting the stability of the telomere-DSB fusion product ($T_m \sim 85^\circ\text{C}$).

Standards for quantitative real-time PCR were reactions containing 100 ng of wild-type S288C genomic DNA plus known amounts of a linearized plasmid containing a telomere-DSB fusion. The plasmid was pSC157, consisting of pCR2.1 (Invitrogen) containing a Y' telomere-DSB fusion with 32 bp of telomeric TG₁₋₃ DNA (total size = 4425 bp). pSC157 was linearized with XhoI, and the enzyme was inactivated by heating at 65°C for 20 min prior to the PCR reaction.

Cell Cycle Synchronization

Cells from a whole spore colony were grown overnight at 30°C in YEP-2.5% raffinose, centrifuged, and resuspended in YEP-2.5% raffinose containing α factor (10 μ g/ml), hydroxyurea (200 mM), or nocodazole (10 μ g/ml). After 4 hr of cell cycle synchronization (after which time >90% of cells had the appropriate morphology), cells were washed, resuspended in YEP-3% galactose containing α factor, hydroxyurea, or nocodazole, and shaken for a further 2 hr. This experiment was repeated three times.

Southern Blotting

Southern blotting of XhoI-digested genomic DNA with a telomeric oligonucleotide has been described previously (Chan et al., 2001).

Cloning of Telomere-DSB Fusions

PCR products cloned by one of two methods: by TA-cloning into pCR2.1 (Invitrogen) or by PstI-EagI digestion into pBluescript using PCR primers containing added restriction sites. Identical PCR results were obtained with and without restriction sites in the primers.

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Note Added in Proof

A report in this issue by Liti and Louis (2003) using telomerase-deficient yeast at a stage much later than ours (after wholesale telomere erosion had caused cellular senescence) showed that *DNL4* is required for fusions in haploid *est2 nej1* cells.