Altering telomere structure allows telomerase to act in yeast lacking ATM kinases

Simon W.L. Chan*, Jennifer Chang†, John Prescott‡ and Elizabeth H. Blackburn

Background: Telomerase is a ribonucleoprotein that copies a short RNA template into telomeric DNA, maintaining eukaryotic chromosome ends and preventing replicative senescence. Telomeres differentiate chromosome ends from DNA double-stranded breaks. Nevertheless, the DNA damage-responsive ATM kinases Tel1p and Mec1p are required for normal telomere maintenance in *Saccharomyces cerevisiae*. We tested whether the ATM kinases are required for telomerase enzyme activity or whether it is their action on the telomere that allows telomeric DNA synthesis.

Results: Cells lacking Tel1p and Mec1p had wild-type levels of telomerase activity in vitro. Furthermore, altering telomere structure in three different ways showed that telomerase can function in ATM kinase-deleted cells: *tel1 mec1* cells senesced more slowly than *tel1 mec1* cells that also lacked *TLC1*, which encodes telomerase RNA, suggesting that *tel1 mec1* cells have residual telomerase function; deleting the telomere-associated proteins Rif1p and Rif2p *in tel1 mec1* cells prevented senescence; we isolated a point mutation in the telomerase RNA template domain (*tlc1-476A*) that altered telomeric DNA sequences, causing uncontrolled telomeric DNA elongation and increasing single strandedness. In *tel1 mec1* cells, *tlc1-476A* telomerase was also capable of uncontrolled synthesis, but only after telomeres had shortened for >30 generations.

Conclusion: Our results show that, without Tel1p and Mec1p, telomerase is still active and can act in vivo when the telomere structure is disrupted by various means. Hence, a primary function of the ATM-family kinases in telomere maintenance is to act on the substrate of telomerase, the telomere, rather than to activate the enzymatic activity of telomerase.

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Background

Telomeres are the DNA-protein "caps" that shield eukaryotic chromosome ends from eliciting inappropriate cellular DNA damage responses [1]. In the majority of eukaryotes, telomeric DNA consists of tandem repeats of a short sequence characterized by G clusters in the strand running 5′–3′ to the chromosome end. In diverse organisms, this strand terminates in a 3′ overhang of regulated length. The overhang is maximal in late S phase, when telomere elongation by telomerase occurs [2, 3]. Telomerase is a ribonucleoprotein (RNP) reverse transcriptase that synthesizes the G-rich strand of telomeric DNA by copying a short templating sequence within its intrinsic RNA. This replenishes telomeric DNA to compensate for incomplete DNA replication at chromosome ends. In telomerase-positive cells, telomere length is normally variable but confined within strict limits; *Saccharomyces cerevisiae* telomeres are 350 ± 50 base pairs long, with a degenerate repeat unit sequence abbreviated $TG_{1-3}(TG)_{1-6}$.

The genetic requirements for in vivo telomere maintenance by telomerase in *S. cerevisiae* include the core telomerase enzyme components; the reverse transcriptase Est2p and the telomerase RNA TLC1; and Est1p, Est3p, and the essential telomere binding protein Cdc13p [4]. Cdc13p is thought to bind to the single-stranded telomeric DNA overhang, thereby recruiting telomerase to the telomere [5]. The deletion of one or any combination of these genes (or the use of a Cdc13p point mutation that uncouples its essential function from its role in telomerase recruitment) causes the "est" phenotype, gradual telomere shortening, and replicative senescence. Importantly, to date, only deletions in Est2p and TLC1 RNA have been shown to abrogate telomerase activity in vitro [6–8].

In the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, two other proteins that are essential for telomere maintenance by telomerase are the ATM-family (ataxia telangiectasia mutated) kinases Tel1p and Mec1p (Rad3p in fission yeast) [9, 10]. ATM kinases are also important for DNA damage responses [11]. Although *S. cerevisiae tel1* null mutants are viable, they have very short telomeres

that are stably maintained at a mean length of about 70 base pairs; the kinase activity of Tel1p is required for normal telomere maintenance [12, 13]. Human lymphocytes and fibroblasts lacking ATM, the homolog of *S. cerevisiae* Tel1p, have accelerated telomere shortening, suggesting that ATM is involved in telomere maintenance in mammalian cells [14, 15]. ATM defects cause ataxia telangiectasia patients to be cancer prone. Therefore, understanding the mechanism of ATM kinases in telomere maintenance is important for understanding how telomere maintenance impairment may contribute to cancer.

Mec1p is a homolog of the essential mammalian kinase ATR (ataxia telangiectasia related), and is a central component of the DNA replication and DNA damage checkpoints in budding yeast [16]. *S. cerevisiae* cells with *mec1* lesions have normal telomere maintenance (if cellular nucleotide levels are supplemented by deleting the *SML1* gene), but *tel1 mec1* double mutants show an "est" phenotype, like that of cells unable to maintain telomeres using telomerase [10].

How the ATM-family kinases function in telomere maintenance is not understood. Two different models for ATM-family kinase function can be envisaged: either they are required to activate the telomerase RNP enzyme directly, or they might alter telomere structure, either to allow telomerase access to its substrate or to render the telomeric DNA into a suitable substrate for telomerase, which requires a single-stranded 3′ overhang [17, 18, 19, M.Rivera and E.H.B, unpublished data]. We reasoned that if these kinases modulate telomere structure to allow telomerase action, interfering with normal telomere structure might allow telomerase to act in ATM kinase-deleted cells, since the telomerase RNP itself would not be altered by the absence of kinase signaling. Distinguishing between these two models is important for determining how ATM kinase activation leads to telomeres being acted upon by telomerase, rather than DNA damage repair processes such as nonhomologous end joining or recombination.

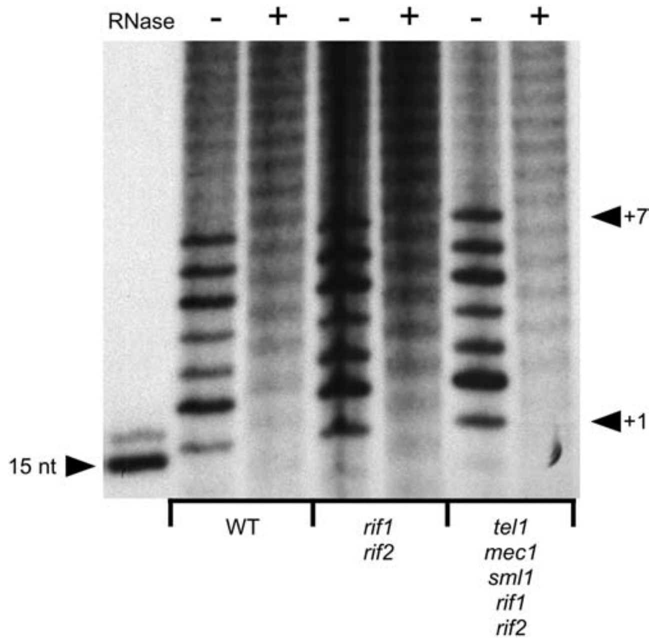
Altering the protein composition of the telomere has shown that the telomeric DNA-protein complex is important for regulating telomerase action at telomeres. Specific mutations in the major double-stranded telomere binding protein Rap1p lead to increased telomere length, as do telomerase template mutations that synthesize repeats with lowered Rap1p affinity [20–22]. Deleting the Rap1p-associated factors Rif1p and Rif2p also causes telomerase-mediated telomere lengthening [23–25].

Certain mutations in the template domain of telomerase RNA, which cause the synthesis of mutated terminal telomeric DNA sequences, can massively disrupt telomere structure and function. In the ciliate *Tetrahymena thermophila* and in the budding yeast *Kluyveromyces lactis*, such mutations caused uncontrolled elongation, degradation, and increased single strandedness of the telomeric DNA [21, 22, 26–28]. Altering terminal telomeric DNA sequences potentially disrupts the binding not only of the double-stranded DNA binding protein Rap1p [21, 29] but also, simultaneously, of the single-stranded telomeric DNA binding proteins Cdc13p, Est1p, and their associated factors. The drastic phenotypes observed with such telomeric DNA sequence alterations suggested that both the protein composition of telomeres and the nature of the single-stranded 3′ DNA overhang at the very terminus were affected.

The single-stranded 3′ overhang is likely to be critical for telomerase action in vivo, since it is required for enzyme activity in vitro. A link between ATM kinase and DNA structure has been shown by genetic experiments, indicating that Tel1p functions in telomere maintenance via the Mre11/Rad50/Xrs2 (MRX) complex [30]. The multifunctional MRX complex is important for recombinational repair of double-stranded breaks, nonhomologous end joining, and meiotic recombination [31]. Its biochemical role may be the processing of double-stranded breaks to generate single-stranded DNA [32]. Phosphorylation of the Xrs2p homolog NBS1 by ATM is important for DNA damage responses in human cells [33, 34]

Here, we address the role of ATM-family kinases in telomere maintenance. We find that in vitro telomerase activity isolated from cells lacking Tel1p and Mec1p is indistinguishable from that in wild-type cell extracts. We also show that altering telomere structure can bypass the requirement for ATM-family kinases in telomere maintenance by telomerase. Three lines of evidence indicate that telomerase is active in vivo in yeast cells lacking both ATM-family kinases. Firstly, although ATM kinase-deleted cells eventually senesce, they do so more slowly than cells lacking both telomerase RNA and the ATM-family kinases. Secondly, deleting the telomere-associated proteins Rif1p and Rif2p prevents the senescence of *tel1 mec1* cells, although telomeres fail to show the elongation that characterizes *rif1 rif2*-deleted strains with intact ATM-family kinases. Thirdly, we report the isolation of a new uncapping point mutation in the template domain of *S. cerevisiae* telomerase RNA, *tlc1-476A*, that synthesizes long, degraded telomeric DNA, causing catastrophic loss of normal telomere structure and increased single strandedness in the G-rich telomeric strand. The *tlc1-476A* telomerase can act in *tel1 mec1* cells, synthesizing overelongated telomeres and bypassing replicative senescence. Thus, the requirement of telomerase action for the ATM-family kinases is bypassed by different kinds of experimental alteration of telomere structure. We conclude that the ATM-family kinases facilitate telomerase

Figure 1



In vitro telomerase activity is normal in cells lacking ATM kinases. Telomerase from isogenic wild-type, *rif1*Δ *rif2*Δ, and *tel1*Δ *mec1*Δ *sml1*Δ *rif1*Δ *rif2*Δ strains was assayed with the 14-nucleotide substrate 5′-GTGTGGTGTGTGGG-3′. Telomerase adds seven nucleotides to this substrate before reaching the end of its template domain.

action by modulating the interaction between the telomere and an active telomerase RNP.

Results and discussion

Telomerase is catalytically active in cells lacking ATM-family kinases

We assayed telomerase activity from wild-type cells, *rif1*Δ *rif2*Δ cells, and *tel1*Δ *mec1*Δ *sml1*Δ *rif1*Δ *rif2*Δ cells (Figure 1; the *sml1* deletion was to suppress the lethality of *mec1*Δ; *tel1*Δ *mec1*Δ *sml1*Δ will be abbreviated *ATM*Δ from here onward). *RIF1* and *RIF2* were deleted to prevent replicative senescence as discussed below; the *rif1*Δ *rif2*Δ mutations did not affect telomerase in vitro activity. RNase-sensitive telomerase activity in extracts from *ATM*Δ *rif1*Δ *rif2*Δ cells was indistinguishable from that in wild-type extracts. Thus, Tel1p and Mec1p are not required for normal levels of telomerase catalytic activity in vitro, and we concluded that they control a different aspect of telomerase function in vivo.

Yeast deleted for ATM kinases senesce more slowly than cells that also lack telomerase RNA

If the ATM-family kinases Tel1p and Mec1p were absolutely required for telomerase to act in vivo, *tel1*Δ *mec1*Δ cells should senesce and lose telomeric DNA in a manner identical to cells lacking telomerase. We compared the rates of senescence and telomere shortening in haploid *tlc1*Δ cells, *ATM*Δ cells, and *tlc1*Δ *ATM*Δ cells; all had been sporulated from the same parent diploid strain. The *tlc1*Δ cells senesced after growth of the first spore colony, followed by two restreaks on YPD plates, a total of about 75 generations of growth (Figure 2a). The absence of both Tel1p and Mec1p did not perceptibly alter the rate of senescence of *tlc1*Δ cells, as *tlc1*Δ *ATM*Δ cells senesced with similar kinetics to *tlc1*Δ cells in this assay. However, *ATM*Δ cells senesced considerably more slowly than either the *tlc1*Δ or *tlc1*Δ *ATM*Δ cells, growing for four restreaks from a spore colony before losing viability, a total of about 125 generations.

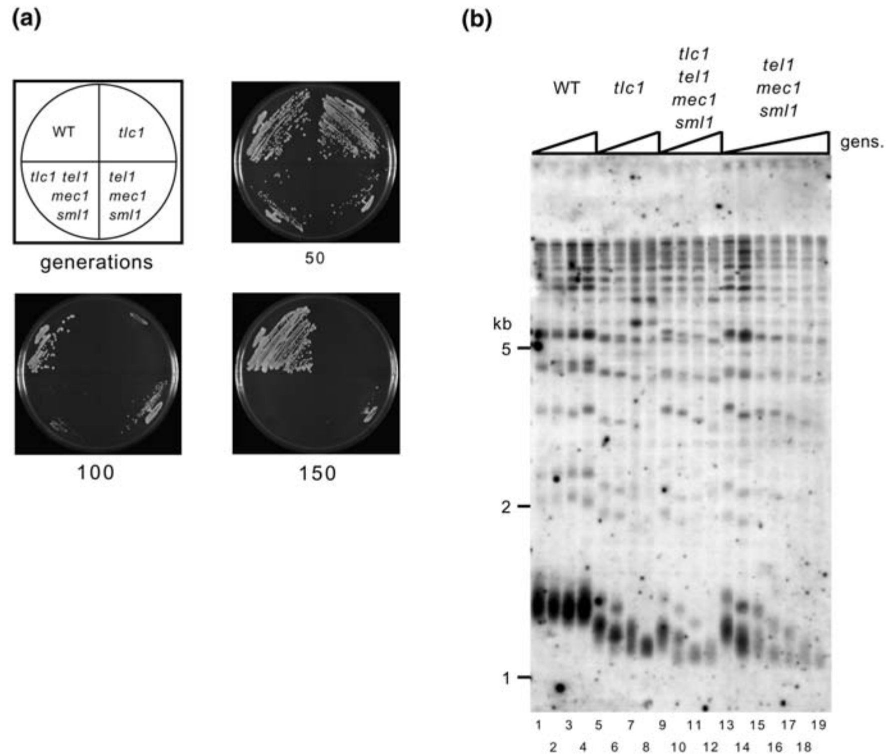
In a previous report, *tel1*Δ *mec1-21* cells senesced at a rate that was more similar to telomerase knockouts than the *tel1*Δ *mec1*Δ *sml1*Δ cells in our experiments [10]. The short telomere phenotype of the hypomorphic *mec1-21* allele and the lethality of *mec1*Δ can both be rescued by deleting *SML1*, a negative regulator of ribonucleotide reductase [10, 35]. This finding indicated that telomerase may require a high level of nucleotide triphosphate substrates for optimal function. Therefore, we propose that the *tel1*Δ *mec1-21* cells senesced more rapidly than *tel1*Δ *mec1*Δ *sml1*Δ cells because of reduced nucleotide pools that lower telomerase activity in vivo.

Initially, the rate of telomere shortening was similar in *ATM*Δ and *tlc1*Δ *ATM*Δ cells (Figure 2b, compare lanes 9–12 to lanes 13–16). This observation differs from findings in ATM$^{-/-}$ human fibroblasts lacking telomerase, which have accelerated telomere shortening relative to wild-type fibroblasts [15]. After about 75 generations (the point at which *tlc1*Δ *ATM*Δ cells senesced), the rate of erosion of the shortest telomeres in *ATM*Δ cells decreased (Figure 2b, lanes 16–19). As described below, telomerase can act on telomeres in *ATM*Δ cells lacking Rad52p-mediated recombination. Taken together with further results presented below, these results are consistent with a model in which telomerase elongates very short telomeres in *ATM*Δ cells, delaying replicative senescence relative to telomerase-minus cells.

Deleting Rif proteins bypasses the requirement for ATM kinases in telomere maintenance

The above results suggest that wild-type telomerase can act on very short telomeres, despite the absence of ATM kinases, and thus the shortness of the telomeres may have been the property that allowed telomerase action. Telomere shortness may diminish the ability of the telomere to assume a structure inaccessible to telomerase [36, 37]. Therefore, we tested whether the need for ATM kinases in telomere maintenance could be bypassed by deleting the telomere-associated proteins Rif1p and Rif2p. Yeast deleted for both Rif1p and Rif2p have markedly elongated telomeres, implying that the Rap1-Rif protein complex negatively regulates telomerase action on

Figure 2



*ATM*Δ cells senesce more slowly than *tlc1*Δ *ATM*Δ cells. **(a)** A diploid strain heterozygous for the *tlc*Δ, *tel1*Δ, *mec1*Δ, and *sml1*Δ mutations was sporulated, and isogenic spores with the genotypes indicated were streaked several times in succession for single colonies on YPD plates. The number of generations postsporulation is estimated at 25 per restreak. **(b)** A Southern blot showing telomere shortening in isogenic spores passaged as described above. Genomic DNA prepared from overnight cultures inoculated from YPD plates was digested with XhoI and probed with a telomeric oligonucleotide. Lanes 1, 5, 9, and 13 were inoculated from spore colonies. Each subsequent lane for a given genotype represents one restreak of growth on YPD plates (∼25 generations).

telomeres. Rif1p and Rif2p interact with the C terminus of Rap1p in a yeast two-hybrid assay and have been shown to be telomere-associated in a one-hybrid assay and by chromatin immunoprecipitation [23, 24, 38, C.D. Smith and E.H.B., unpublished data]. We compared cells grown from *ATM*Δ *rif1*Δ *rif2*Δ spores to isogenic *ATM*Δ cells. Deleting the Rif proteins prevented *ATM*Δ cells from undergoing replicative senescence, even after repeated passaging for over 10 restreaks (data not shown).

Telomere lengths in *ATM*Δ *rif1*Δ *rif2*Δ cells varied between independent isolates (Figure 3a, lanes 22–28 and 3b, lanes 2–7); although, within a given spore culture, it stayed relatively constant over several passages (Figure 3a, lanes 22–28). However, within each *ATM*Δ *rif1*Δ *rif2*Δ isolate, the distribution of telomere lengths was considerably greater than in wild-type cells (Figure 3b, lanes 2–7). Thus, deleting Rif1p and Rif2p in the absence of ATM kinases averted the "ever-shortening telomeres" of *ATM*Δ cells, although the overelongation that normally characterizes *rif1*Δ *rif2*Δ cells did not occur. Bypass of replicative senescence and a similar stable maintenance of telomere length was also seen in *rad52*Δ *ATM*Δ *rif1*Δ *rif2*Δ cells, showing that telomere maintenance in this context does not require RAD52-dependent telomere-telomere recombination (Figure 3b, lanes 9–12 and data not shown). Thus, altering the protein composition of the telomere can allow sufficient telomerase action to maintain telomeres and prevent cellular senescence in the complete absence of ATM signaling.

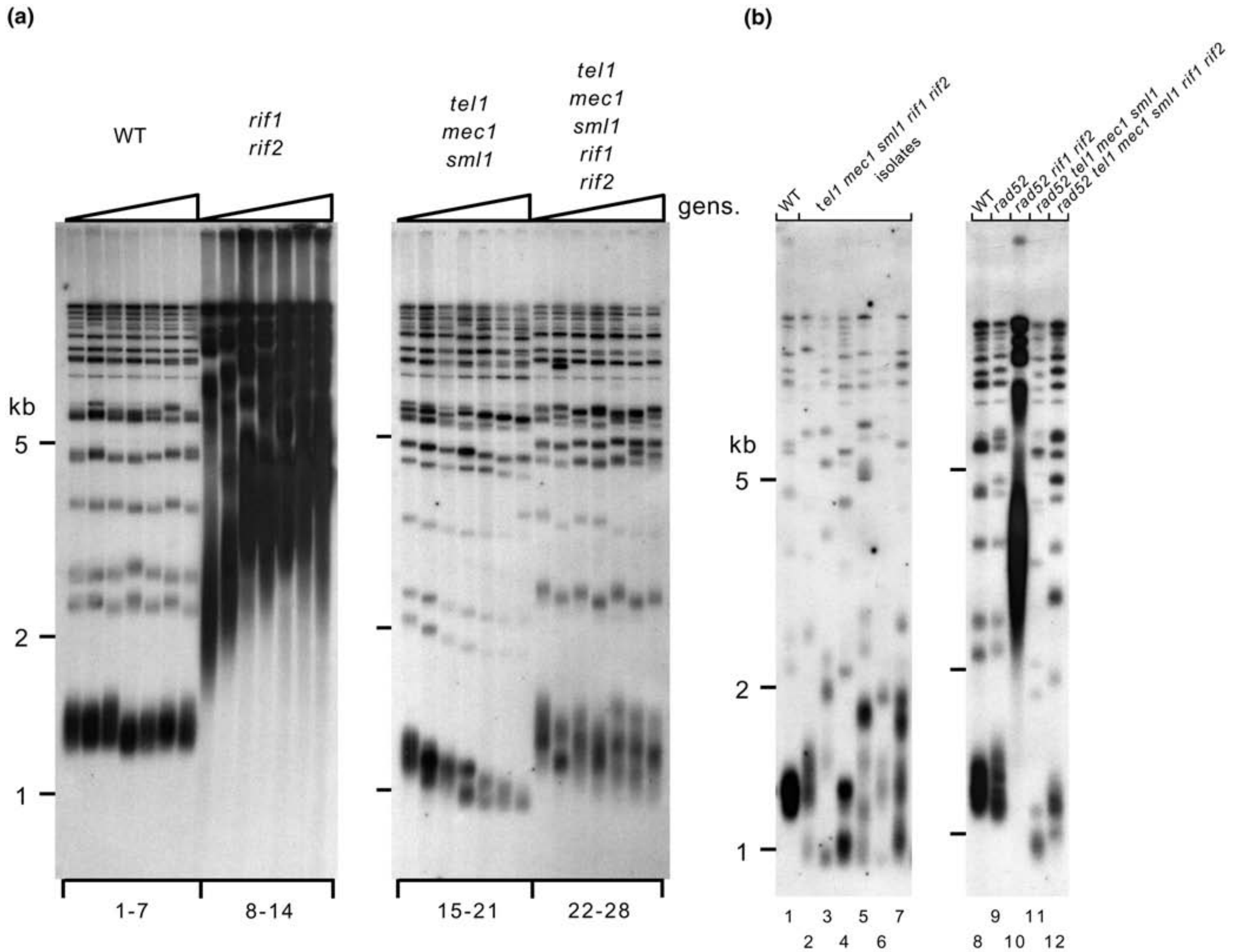
The fact that telomeres in *rif1*Δ *rif2*Δ cells are much longer than in *ATM*Δ *rif1*Δ *rif2*Δ cells (Figure 3a, compare lanes 8–14 and 22–28; Figure 3b, compare lanes 10 and 12) indicates that the ATM kinases are required for telomerase to work at full efficiency in vivo. This finding argues against a model in which the only role of the kinases is to counteract the effects of Rif1p and Rif2p.

These findings, together with those of the previous sections, showed that Tel1p and Mec1p are not absolutely required for catalytic activity of telomerase or for its recruitment to telomeric substrates, as telomerase is able to synthesize telomeric DNA in the absence of these kinases once telomeres shorten or if the normal telomeric DNA-protein complex is disrupted.

An uncapping telomerase in *S. cerevisiae*

Because altering the higher-order telomeric protein complex by deleting Rif proteins only partially stimulated telomerase action in *ATM*Δ cells, we tested whether a more drastic mode of altering telomere structure would allow a more efficient addition of telomeric DNA in the absence of ATM kinases. Altering the sequence of telomeric DNA has the potential to disrupt proteins that bind the double-stranded region of telomeric DNA, as well as

Figure 3



Deleting Rif proteins allows telomere maintenance in *ATM*Δ cells. **(a)** A diploid strain heterozygous for the *tel1*Δ, *mec1*Δ, *sml1*Δ, *rif1*Δ, and *rif2*Δ mutations was sporulated, and isogenic spores of the indicated genotype were passaged on YPD plates. Genomic DNA was digested with XhoI and analyzed by Southern blotting as in Figure 2. Lanes 1, 8, 15, and 22 were inoculated from spore colonies. Subsequent lanes for a given genotype represent one restreak of growth on YPD plates. **(b)** Independent *tel1*Δ *mec1*Δ *sml1*Δ *rif1*Δ *rif2*Δ isolates in lanes 2–7 were generated by sporulating a heterozygous diploid strain as described above. Each spore was passaged seven times on YPD plates (~200 total generations postsporulation) before analysis of genomic DNA by Southern blotting as described above. Haploid strains in lanes 9–12 were generated by sporulating a diploid strain heterozygous for the *rad52*Δ, *tel1*Δ, *mec1*Δ, *sml1*Δ, *rif1*Δ, and *rif2*Δ mutations. Spores of the indicated genotype were passaged twice on YPD plates (~75 total generations postsporulation) before the preparation of genomic DNA. The parent diploid was constructed by mating *rad52*Δ and *tel1*Δ *mec1*Δ *sml1*Δ *rif1*Δ *rif2*Δ cells; long telomeres inherited from the *tel1*Δ *mec1*Δ *sml1*Δ *rif1*Δ *rif2*Δ background cause the slight telomere heterogeneity noticeable in the *rad52*Δ and *rad52*Δ *tel1*Δ *mec1*Δ *sml1*Δ strains.

those specific for the 3′ single-stranded overhang. We therefore screened for mutations in the template domain of telomerase RNA that would radically disrupt telomere structure once altered-sequence telomeric DNA was added to chromosome ends by telomerase.

In the ciliated protozoan *Tetrahymena thermophila* and the budding yeast *Kluyveromyces lactis*, certain telomerase RNA template mutations cause cell division defects in addition to uncontrolled telomere elongation [28, 39]. Such alleles have not been previously reported in *S. cerevisiae*. We screened for mutations in the template of *S. cerevisiae* telomerase RNA (*TLC1*) that caused immediate slow growth, as opposed to the delayed senescence characteristic of telomerase null mutants. The *TLC1* "template cassette" allele (Figure 4a) contains a wild-type template flanked by unique SphI and SalI sites. The mutations creating the template cassette do not alter telomere length

Figure 4

tlc1-476A telomerase synthesizes altered telomere sequences. **(a)** The *TLC1* template cassette allele. Altered nucleotides are shown in cyan. **(b)** The *tlc1-476A* mutation is a single-nucleotide change that disrupts the predicted Rap1p binding site. Template mutations are shown in red. **(c)** Telomeres from *TLC1* and *tlc1-476A* cells were sequenced by the ligation-mediated PCR method. The DNA strand running 5′–3′ to the telomere end is shown. Sequences of TG_n, where n is ≥7, are highlighted in red; TG_n stretches in wild-type telomeres do not exceed n = 6.

or cell growth properties over multiple serial restreaks (J.P. and E.H.B., unpublished data). We mutagenized the TLC1 templating sequence by cloning partially randomized synthetic oligonucleotides into the template cassette. A library of such *tlc1* template mutants genes on a centromere-carrying, self-replicating plasmid was transformed into *tlc1*Δ cells and screened for alleles that caused slow growth as soon as a complementing *TLC1* gene was removed via plasmid shuffle.

Two *tlc1* alleles causing immediate slow growth were isolated from a pool of about 18,000 transformants (Figure 4b). Slow growth was dependent on the presence of the *tlc1* plasmid in both cases. Strikingly, sequencing showed that one allele, *tlc1-476A*, contained only a single C to A transversion (Figure 4b). The second allele caused a similar phenotype but contained additional template base changes and was not investigated further. The slow growth phenotype segregated with the *tlc1-476A* allele when it was integrated at the *TLC1* locus. An increase in the percentage of large-budded cells, loss of viability, and the appearance of misshapen and enlarged "monster" cells (data not shown) suggested that *tlc1-476A* telomerase caused slow growth by inducing telomere defects that lead to mitotic defects. Despite these cellular phenotypic effects of telomere uncapping, *tlc1-476A* cells did not senesce even after ∼400 generations of growth.

The *tlc1-476A* telomerase is predicted to synthesize $(TG)_n$ stretches separated by the sequence TGG but devoid of the TGGG sequences characteristic of wild-type telomeres. In order to sequence the single-stranded, degraded telomeres in *tlc1-476A* cells, we cloned telomeres from haploid mutant cells that had grown for over 30 generations with only the *tlc1-476A* telomerase RNA after sporulation from a *tlc1-476A*/*TLC1* parent diploid. We used a PCR-based method that relies only on the covalent integrity of the "G strand" that runs in a 5′–3′ direction to the chromosome end [40]. All of the 12 telomeres cloned from a *tlc1-476A* strain contained wild-type-like $TG_{1–3}(TG)_{1–6}$ sequences, which may be accounted for by strong selection for functional telomeres. In addition, 4 of the 12 cloned telomeres contained long $(TG)_n$ sequences never seen in wild-type telomeres (e.g., Figure 4c). Individual

$(TG)_n$ sequences were up to 58 base pairs in length and were found toward the distal ends of the cloned telomeres, all of which contained centromere-proximal $TG_{1-3}(TG)_{1-6}$ sequences next to subtelomeric DNA. These sequence features are consistent with the expected action of *tlc1-476A* telomerase at the chromosome ends and indicate that the mutant telomerase is catalytically similar to the wild-type enzyme. Importantly, *tlc1-476A* telomerase is not required to use an unnatural nucleotide precursor, also suggesting that the catalytic properties of the enzyme are probably unaffected. One of the cloned telomeres (#2) contained a wild-type sequence at its terminus that may be accounted for by its *tlc1-476A/TLC1*-derived origin.

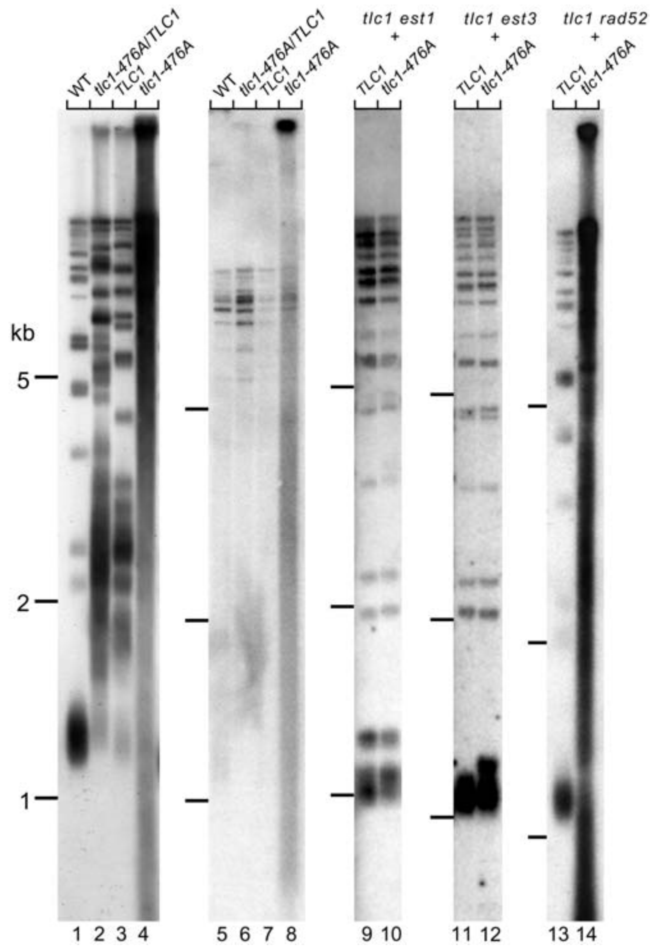
Telomeres are overelongated, degraded, and single stranded in *tlc1-476A* cells

The deleterious effects of *tlc1-476A* telomerase on cell growth suggested that telomere function was greatly perturbed. Minimally, the C476A mutation is predicted to destroy the CACCCA core sequence known to be required for high-affinity Rap1p binding [41]. Southern blotting analysis showed that *tlc1-476A* telomeres were extremely degraded, with a continuous smear of telomeric DNA-hybridizing signal extending from the loading well to the bottom of the gel (Figure 5, lane 4). Simultaneously, *tlc1-476A* telomeres were overelongated, as judged by the accumulation of signal at limit mobility for double-stranded DNA and by the dramatic increase in the overall hybridization signal. An ethidium bromide stain of the same gel revealed no abnormalities in the restriction digest of bulk genomic DNA in the *tlc1-476A* genomic DNA samples (data not shown). Heterozygous *tlc1-476A/TLC1* diploids had elongated telomeres consisting of a mixture of regulated but elongated bands, together with a moderate level of degradation (Figure 5, lane 2). Sporulation of *tlc1-476A/TLC1* cells yielded wild-type spores with considerably lengthened telomeres (Figure 5, lane 3). Wild-type telomerase restored relatively normal length homeostasis to these telomeres; although, in some cases, a smeary signal in the telomere profiles persisted for one or two restreaks. The codominance of *tlc1-476A* further illustrates the loss of control of telomerase activity caused by altered telomeric DNA sequences.

Telomeric DNA migrating above limit mobility indicated that telomeres in *tlc1-476A* cells possess nondouble-stranded or nonlinear character. Probing a nondenaturing Southern blot confirmed that telomeric DNA throughout the lane was single stranded (Figure 5, lane 8). The generation of extensive single-stranded DNA at *tlc1-476A* telomeres resembles the terminal phenotype of *cdc13-1* mutants at the nonpermissive temperature [42], suggesting that the altered telomeres trigger a DNA damage-processing response in *tlc1-476A* cells.

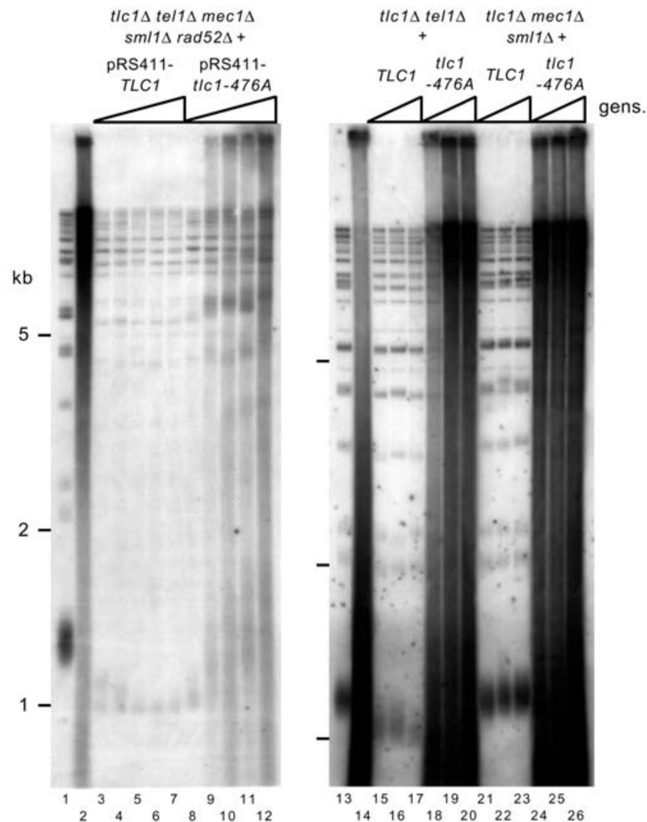
Cells lacking the telomerase reverse transcriptase Est2p, the telomerase recruitment factor Est1p, or another telomerase component, Est3p, showed no telomere lengthening or degradation when transformed with a *tlc1-476A* plasmid (Figure 5, lanes 10 and 12 and data not shown). Thus, the *tlc1-476A* telomeric phenotype was telomerase dependent. In the absence of telomerase, *S. cerevisiae* can generate long, heterogeneous TG_{1-3} tracts at telomeres through a low-frequency, Rad52p-dependent process ("Type II" recombination) [25]. Plasmid-borne *tlc1-476A* also caused the synthesis of degraded, highly elongated telomeres in *rad52* cells (Figure 5, lane 14). These results

Figure 5



The telomere structure is radically disrupted in *tlc1-476A* cells. XhoI-digested genomic DNA was analyzed by Southern blotting as in Figure 1. Lane 1; wild-type haploid. Lane 2; *tlc1-476A/TLC1* diploid, parent to *TLC1* and *tlc1-476A* spores (Lanes 3 and 4, respectively). For lanes 3 and 4, cultures were inoculated from spore colonies. Lanes 5–8 contain the same DNA as that in lanes 1–4, digested with XhoI and analyzed by nondenaturing Southern blotting. Lanes 9 and 10; *tlc1Δ est1Δ* transformed with pRS411-*TLC1* and pRS411-*tlc1-476A*, respectively. Lanes 11 and 12; *tlc1Δ est3Δ* transformed with pRS411-*TLC1* and pRS411-*tlc1-476A*, respectively. Lanes 13 and 14; *tlc1Δ rad52Δ* transformed with pRS411-*TLC1* and pRS411-*tlc1-476A*, respectively. For lanes 9–14, DNA was prepared from cultures inoculated from the transformation plate.

Figure 6



tlc1-476A telomerase is active in *ATM*Δ cells. XhoI-digested genomic DNA was analyzed by Southern blotting as in Figure 1. Lanes 1 and 13; wild-type haploid. Lanes 2 and 14; *tlc1-476A* haploid. Lanes 3–7 and 8–12; *tlc1*Δ *tel1*Δ *mec1*Δ *sml1*Δ *rad52*Δ transformed with pRS411-*TLC1* or pRS411-*tlc1-476A*, respectively. Lanes 15–17 and 18–20; *tlc1*Δ *tel1*Δ transformed with pRS411-*TLC1* or pRS411-*tlc1-476A*, respectively. Lanes 21–23 and 24–26, *tlc1*Δ *mec1*Δ *sml1*Δ transformed with pRS411-*TLC1* or pRS411-*tlc1-476A*, respectively. Lanes 3, 8, 15, 18, 21, and 24 were inoculated from the transformation plate. Each subsequent lane for a given transformation represents one restreak of growth on SC −MET plates (∼25 generations).

show that the aberrant telomere phenotype in *tlc1-476A* cells is mediated by telomerase and neither requires nor can be accounted for by Rad52p-mediated recombination.

The *tlc1-476A* telomerase is active in cells lacking ATM kinases

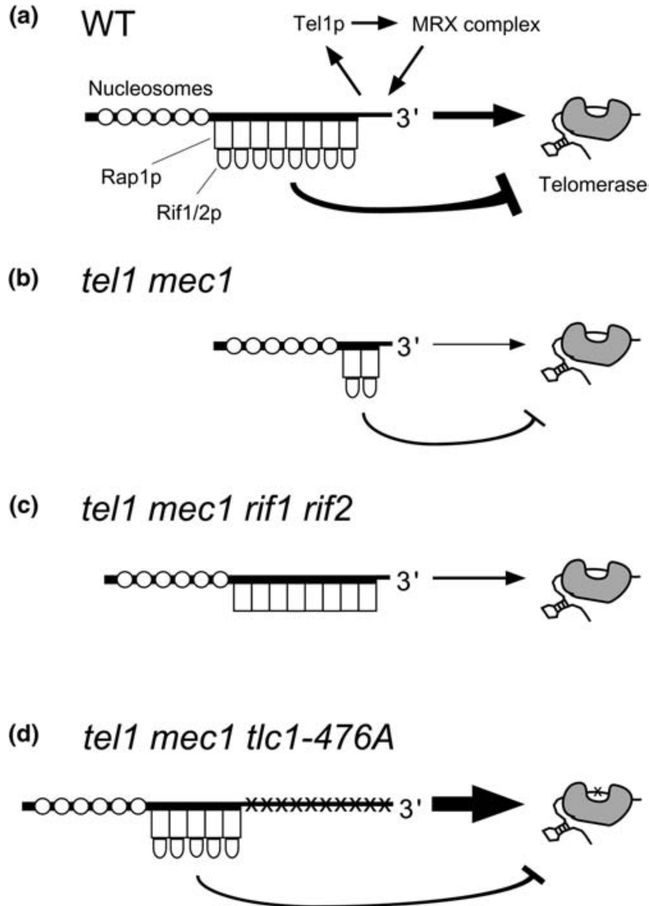
We transformed a *tlc1-476A* plasmid into freshly sporulated *tlc1*Δ *ATM*Δ cells or *tlc1*Δ *ATM*Δ *rad52*Δ cells to assess the effect of the drastically compromised telomere structure caused by *tlc1-476A* on telomerase action in the absence of ATM kinases. After the first ∼30 generations of growth with *tlc1-476A* RNA (cultures inoculated directly from the transformation plate), telomeres had shortened indistinguishably from those in control *tlc1*Δ *ATM*Δ or *tlc1*Δ *ATM*Δ *rad52*Δ cells transformed with wild-type *TLC1* (Figure 6, compare lanes 3 and 8). However, after one or two additional serial restreaks on plates (∼55–80 generations), the telomeres in the cells expressing *tlc1-476A* abruptly became both elongated and simultaneously degraded. The telomere phenotypes appeared to be similar to those of *tlc1-476A* cells with wild-type Tel1p and Mec1p, although the amount of telomeric DNA hybridizing signal was somewhat reduced in the *ATM*Δ cells (Figure 6, compare lanes 2 and 9–12). In contrast, telomeres continued to shorten in the isogenic control cells transformed with *TLC1* (Figure 6, lanes 3–7).

The *tlc1*Δ *ATM*Δ cells transformed with *tlc1-476A* showed no sign of replicative senescence, even after multiple restreaks; telomeres remained long and degraded, with no sign of subtelomeric Y′ element amplification (data not shown). As with *tlc1*Δ cells after senescence, the *tlc1*Δ *ATM*Δ cells that received a *TLC1* plasmid generated "survivor" cells that maintained their telomeres through "Type I" recombination involving amplification of subtelomeric Y′ elements (data not shown). Therefore, by the criterion of bypassing senescence, telomere maintenance by telomerase is independent of ATM kinases and RAD52 function once the telomeres are "uncapped" by *tlc1-476A*.

The length of the delay before *tlc1-476A* telomerase acted in *ATM*Δ cells was variable: abrupt overelongation/degradation occurred between 30 and 55 generations after transformation in different experiments (data not shown). No delay was observed when *tlc1-476A* was introduced into *tlc1*Δ cells lacking either Tel1p or Mec1p alone (*tlc1*Δ *tel1*Δ or *tlc1*Δ *mec1*Δ *sml*Δ cells), showing that either Tel1p or Mec1p is sufficient to allow *tlc1-476A* telomerase to act on telomeres of wild-type length (Figure 6, lanes 18 and 24). The simplest interpretation of these results is that, in cells lacking both ATM kinases, telomerase is only able to act on telomeres that are considerably shortened. This model is consistent with the finding that the initial telomere-shortening rate was similar in *tlc1*Δ and *ATM*Δ cells, despite the fact that senescence is slower in the latter strain. The stochastic nature of the delay hints at a process in which, upon shortening, telomeres randomly underwent a cooperative structural change that increased accessibility to telomerase; the probability of undergoing this change increased as telomeres shortened.

In summary, after a delay of at least 30 generations during which telomeres became very short, *tlc1-476A* telomerase synthesized telomeric DNA despite the absence of the ATM kinases Tel1p and Mec1p. Telomere elongation in these cells was independent of Rad52p-mediated recombination. It is notable that, for over 30 generations, the *tlc1-476A*-transformed *ATM*Δ cells behaved as if they had no telomerase. During that interval, telomeres continued to shorten despite the presence of the mutant telomerase. This further supports the interpretation that telomere

Figure 7



Three situations in which telomerase acts in cells lacking ATM kinases. **(a)** In this model, in wild-type cells, Tel1p and the MRX complex create a 3′ single-stranded telomeric overhang to facilitate telomerase action. Rap1p and the Rif proteins block telomerase action. **(b)** In *tel1 mec1* cells, telomerase can act once telomeres become very short. **(c)** Removing Rif1p and Rif2p lessens the inhibition of telomerase, allowing telomerase to act in *tell mec1* cells. **(d)** In *tlc1-476A* telomeres, the single-stranded character results from adding mutant repeats ("X"s) to telomeric termini, disrupting the binding of sequence-specific protective proteins. Single-stranded telomeric DNA is proposed to bypass the requirement for Tel1p and the MRX complex in telomere maintenance.

elongation by *tlc1-476A* telomerase in the absence of ATM kinases is caused by altered telomere structure rather than by aberrant catalytic properties of the mutant telomerase. The overelongation by *tlc1-476A* telomerase showed that telomere synthesis in vivo can be efficient despite the lack of Tel1p and Mec1p.

How does altered telomere structure bypass the requirement of telomerase action in vivo for Tel1p and Mec1p?

Here, we have demonstrated that in ATM kinase-deleted cells, the catalytic activity of telomerase was unchanged, yet the cells senesce. We showed three experimental situations in which telomerase adds telomeric DNA in vivo in the absence of ATM kinases (Figure 7): first, when telomeres are very short in *tel1*Δ *mec1*Δ *sml*Δ (*ATM*Δ) cells; although, in these cells, the wild-type telomerase activity at telomeres is insufficient to prevent eventual senescence (Figure 7b); second, when Rif1p and Rif2p are absent (Figure 7c); and third, when the addition of mutant DNA by *tlc1-476A* telomerase uncaps telomeres (Figure 7d). Although deleting Rif1p and Rif2p allows enough telomere elongation to prevent senescence, the extent of telomerase action in vivo is much less than when both ATM kinases are present. Only by making an altered telomeric DNA sequence, with the *tlc1-476A* mutation, that disrupted both the predicted telomeric protein composition and the telomeric DNA structure was telomerase able to act efficiently in cells lacking Tel1p and Mec1p.

Our ability to bypass the requirement for Tel1p and Mec1p through these experimental alterations of telomere structure raises the question of how the kinases function in wild-type cells. Tel1p acts through the Mre11/Rad50/Xrs2 (MRX) complex [30], which we propose creates a single-stranded overhang that renders the telomeric terminus into a suitable substrate for telomerase and/or its recruiting factor Cdc13p (Figure 7a). We suggest that the extensive single strandedness in *tlc1-476A* telomeres freed them from requiring Tel1p/MRX action (Figure 7d). Since *tel1* cells or MRX-defective cells maintain short and stable telomeres, Mec1p and/or other factors apparently can supply the function required for such maintenance.

This work shows that experimentally altering the telomere in three different ways allows telomerase to act in the absence of ATM kinases. Our findings imply that the ATM kinases allow telomerase action in vivo by modulating the interaction between telomerase and its telomere substrate, rather than by altering the catalytic properties of the telomerase RNP enzyme. Understanding how ATM kinases act at telomeres will be an important step toward addressing how the response to a short telomere differs from the response elicited by a double-stranded break.

Materials and methods

Yeast strains and methods

All strains were isogenic with BY4705 [43]. The *tlc1::TRP1* allele and *rad52::LEU2* alleles were made by one-step gene replacement with the plasmids pSC21 and pBRΔHS*LEU2*, respectively (nucleotides 450–917 of *TLC1* [including the template domain] and nucleotides −10–1494 of *RAD52* were removed). The *tel1::HIS3*, *mec1::LEU2*, *sml1::TRP1*, *rif1::TRP1*, *rif2::kanMX4*, *est1::HIS3*, *est2::HIS3*, and *est3::HIS3* alleles were made by PCR-mediated transformation [44]; in each case, the complete open reading frame was deleted. *TLC1* was replaced with *tlc1-476A* by two-step gene replacement [45] using the plasmid pSC82. Yeast were transformed with the lithium acetate procedure [46]. Sporulation and tetrad dissection were performed according to standard methods [47].

In vitro telomerase reactions

Extract preparation and in vitro telomerase assays were performed as described previously [48], except that fractions from the DEAE-agarose column were desalted and concentrated with a vacuum dialysis apparatus and Colloidion nitrocellulose membranes (Schleicher and Schuell).

Cellular senescence assays

Freshly dissected spores were streaked for single colonies on YPD plates and incubated at 30°C. Single colonies were restreaked every 48 hr and compared to isogenic wild-type strains for growth properties. The analysis was repeated with several spores. For cells transformed with pRS411-*TLC1* or pRS411-*tlc1-476A* plasmids, colonies from the SC −MET transformation plate were restreaked on SC −MET plates, incubated at 30°C, and restreaked every 72 hr.

Southern blotting

Genomic DNA was isolated, digested with XhoI, separated on a 0.8% agarose gel, transferred to Hybond-N+, and hybridized to a ^{32}P-labeled oligonucleotide (5′-TGTGGTGTGTGGGTGTGGTGT-3′) as described previously [29]. Nondenaturing Southern blots were prepared according to the method of Wellinger and Zakian [2]. DNA separated in 0.8% agarose gels was depurinated in 0.25 N HCl for 30 min, transferred to Hybond-N+ in 20 × SSC as described, then hybridized with a ^{32}P-labeled oligonucleotide (5′-CACACACACACACACACACA-3′) under the same conditions as denaturing Southern blots.

Plasmid and library construction

pJP152 (a CEN/ARS plasmid containing the *TLC1* template cassette allele) was constructed from pRS313-*TLC1* by overlap PCR [29]. Randomized mutant oligonucleotides (5′-TTACCATGGCATGCCTACCAT CACCACACCCACACACAAATGTCGACA-3′; the second strand was synthesized with the complementary oligonucleotide 5′-AATTAGCTGT CGACATTT-3′) were cloned SphI-SalI into pJP152 to create a library of *tlc1* variants. The underlined region (the template domain of *TLC1*) was synthesized with 5% of each incorrect nucleotide at each position. The *tlc1-476A* allele was cloned BamHI-XhoI into pRS306 to create pSC82. A BamHI-XhoI fragment from pJP152 or pRS313-*tlc1-476A* was cloned into pRS411 to create pRS411-*TLC1* and pRS411-*tlc1-476A*.

Mutant screen

We screened for *tlc1* alleles that caused slow growth by transforming *tlc1*Δ cells with the library of *tlc1* variants in pJP152 (the *tlc1*Δ mutation was complemented by the plasmid pRS316-*TLC1*). Colonies were replica-plated from SC –URA −HIS plates to SC −HIS 5′-FOA plates to select against pRS316-*TLC1*. Plasmids that caused poor growth when wild-type *TLC1* was removed were rescued from colonies on the SC −URA −HIS plates.

Telomere cloning

Telomere fragments were cloned using the ligation-anchored PCR strategy [40]. Genomic DNA (150 ng) ligated to a 3′-amino-modified anchor oligonucleotide was amplified by PCR using an upper primer containing a subtelomeric sequence from the *S. cerevisiae* Y′ subtelomeric element and a PstI restriction site (5′-GGGGCTGCAGAATGGAGGGTAAGTT GAGAGACAGG-3′), and a lower primer complementary to the anchor primer and containing an EagI restriction site (5′-CGACGCGGCCGCT TATTAACCCT-3′). PCR products were extracted from an agarose gel, cloned into a Bluescript vector, and sequenced.

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