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Tel1 and Rad51 are involved in the maintenance of telomeres with capping deficiency

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ABSTRACT

Vertebrate-like T_2AG_3 telomeres in *tlc1-h* yeast consist of short double-stranded regions and long single-stranded overhang (G-tails) and, although based on Tbf1-capping activity, they are capping deficient. Consistent with this idea, we observe Y' amplification because of homologous recombination, even in the presence of an active telomerase. In these cells, Y' amplification occurs by different pathways: in Tel1$^+$ *tlc1h* cells, it is Rad51-dependent, whereas in the absence of Tel1, it depends on Rad50. Generation of telomeric G-tail, which is cell cycle regulated, depends on the MRX (Mre11-Rad50-Xrs2) complex in *tlc1h* cells or is MRX-independent in *tlc1h tel1*Δ mutants. Unexpectedly, we observe telomere elongation in *tlc1h* lacking Rad51 that seems to act as a telomerase competitor for binding to telomeric G-tails. Overall, our results show that Tel1 and Rad51 have multiple roles in the maintenance of vertebrate-like telomeres in yeast, supporting the idea that they may participate to evolutionary conserved telomere protection mechanism/s acting at uncapped telomeres.

INTRODUCTION

Telomeres are evolutionary conserved structures consisting of arrays of G-rich repeats assembled with a complex set of proteins that mark the end of the linear chromosomes of eukaryotic cells. They were discovered by the pioneering works of Muller and McClintock, which distinguished native chromosome ends from broken ends by the ability of the native ends to avoid chromosome rearrangements. Later, it was recognized the telomeres enable cells to overcome the end-replication problem (1). Thus, telomeres are involved in protection of chromosomes from degradation, fusion events and recombination (capping function), and they allow telomerase-mediated telomere elongation. Collectively, telomeric DNA and proteins are responsible of these essential functions. Indeed, telomeric repeats act as primers for telomerase-mediated telomere elongation (2), and telomeric proteins achieve both telomerase recruitment/activation and capping (3). The molecular basis of capping, although extensively studied, still remains poorly understood.

In *Saccharomyces cerevisiae*, telomere capping involves the Cdc13, Stn1 and Ten1 (CST) complex and Rap1/Rif1-2 proteins. CST binds the telomeric G-rich 3′ overhang and protects telomeres from degradation and DNA damage checkpoint activation (4–6). Rap1 depletion from telomeres leads to end resection, primarily occurring by the Exo1 exonuclease in dividing cells (7). Additionally, it has been proposed that Rap1, by the recruitment of Rif proteins, indirectly inhibits MRX-dependent end resection (8). However, Tel1 hyperactivation, as obtained by the *tel1-hy909* mutant, was shown to improve end resection by counteracting the Rif2-mediated inhibition of MRX (9). Other factors seem to participate in telomere capping. The Ku heterodimer (Ku70/80) binds DNA ends and blocks Exo1-mediated resection outside of S phase (10). This activity is carried out at both DSBs and telomeres with only slight differences in the checkpoint activation pathways.

More recently, it has been shown that Tbf1, previously identified as a transcriptional insulator that binds T_2AG_3 repeats at subtelomeric positions, participates in telomere capping and length regulation. Indeed, Tbf1 tethering at telomeres causes telomere shortening (11). Moreover, Tbf1 binding to (T_2AG_3)n telomeric seeds regulates telomere elongation in a length-dependent manner (12). These results are consistent with the finding that telomeres

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consisting solely of T_2AG_3 repeats can be maintained in yeast (13,14) and suggest that Tbf1 capping is an evolved function possibly acting as a back up mechanism that recovers ultra-short telomeres (12). It cannot be excluded that Tbf1 directly modulates the lengths of natural telomeres as suggested by the following data: Tbf1 participates in length regulation of T_2AG_3-$TG_{1\text{-}3}$ mixed telomeres (13); yeast cells with a hypomorphic TBF1 allele have short telomeres (15); Tbf1 cooperates with Rap1 to limit MRX and Tel1 recruitment to telomeres independently of Rif1 or Rif2 (16).

In *S.cerevisiae*, telomeres consist of an irregular array of ~300 bp of TG_{1-3}/AC_{1-3} repeats that terminate with a short 3′ TG_{1-3} single-stranded overhang (G-tail). In vertebrate, telomeres consist of long tracts of T_2AG_3 repeats ending with a long T_2AG_3 single-stranded overhang. Replacement of the endogenous TLC1 telomerase RNA component with the *tlc1h* mutants allele, which directs the synthesis of T_2AG_3 repeats rather than the normal TG_{1-3} repeats, leads to formation of chimeric TG_{1-3}-T_2AG_3 telomeres (17). Additionally, by using (T_2AG_3)n seeds in *tlc1h* cells, *de novo* telomeres consisting entirely of vertebrate repeats can be generated (13,14). Vertebrate-like telomeres, although shorter than wild-type, are functional as suggested by the viability of *tlc1h* cells. However, *tlc1h* cells exhibit meiotic defects (14), a chronic state of checkpoint activation and telomere fusion events, suggesting that telomere capping is partially impaired (18).

Yeast telomeres with a mixture of yeast and vertebrate repeats are different from yeast telomeres in multiple ways (19). Genetic and sequence analyses suggest that vertebrate-like telomeres in yeast are almost free of the shelterin-like proteins Rap1 and Rif as demonstrated by the fact that *rap1-18* or *rif1/2* mutants in *tlc1h* strains do not show telomere lengthening (13,14), and less than one Rap1-binding site was found in the core yeast sequence of these telomeres (18). However, vertebrate-like telomeres bind Tbf1, and this binding mediates capping and length regulation (12,14). Cdc13 also bind to vertebrate-like yeast telomeres, and this binding is even higher than binding to wild-type telomeres (14). This increased Cdc13 binding might be due to longer single-stranded overhangs on vertebrate-like telomeres (18,20). More recently, it has been proposed that telomerase itself is required to protect telomeres ending with T_2AG_3 repeats probably because they need to be elongated more frequently than canonical telomeres (21).

Thus, vertebrate-like telomeric repeats in yeast act as a platform to assemble a non-canonical telomeric chromatin that, although generating functional Tbf1-based telomeres, does not guarantee full protection of telomeres. It has been shown previously that uncapped telomeres undergo homologous recombination (HR) by different mechanisms depending on which capping component is compromised (22). Thus, it is reasonable to propose that recombination contributes to telomere maintenance in *tlc1h* cells.

Here, we analyse the mechanisms involved in the maintenance of vertebrate-like telomeres in yeast. Our data show that vertebrate telomeres are maintained by telomerase or Y' amplification because of HR, and that these two mechanisms co-exist in the cells. As in type I survivors, Y' amplification in *tlc1h* cells is Rad51 dependent, but in cells lacking Tel1, it seems to be Rad50 dependent. Likewise, Tel1 modulates telomere end resection that is MRX dependent in cells with Tel1 and MRX independent in *tlc1h*, *tel1*Δ double mutants. Additionally, we provide strong evidence that Rad51 participates to telomere length regulation possibly by competing with telomerase for binding to G-tails.

MATERIALS AND METHODS

Yeast strains and plasmids

The yeast strains with vertebrate telomere were isolated from the diploid C6.1 (TLC1/*tlc1h*) by tetrads dissection (17); haploid TLC1 and *tlc1h* strains were selected by polymerase chain reaction (PCR) as described in Brevet *et al.* (13). The *tlc1h tel1*Δ, *tlc1-h rad50*Δ, *tlc1-h rad51*Δ, *tlc1h rad52*Δ mutants, the double mutants and the respective controls were obtained as previously described (18). The mutants were obtained from *tlc1h* after five plate passages (P5) and grown further for additional 25 plate passages for a total of 30 consecutive plate passages (P30). Time of incubation at 30°C was extended to 2–4 days depending on the doubling times of the strains. *tlc1h tel1*-HA strain was constructed as reported in Sabourin *et al.* (23). The *de novo* telomere formation assays were performed with the following strains: YSN309, and YSN601 (24), YVR052, YVR090 and YVR116 (12).

Culture conditions and cell synchronization

All cultures were grown in rich YPD medium at 30°C except as noted. Liquid cultures were incubated with rotary shaking at ~150 rpm. For the G_1-arrested cells, overnight cultures were grown to an OD_{660} of 0.3. α-factor was added to a final concentration of 3.2 μg/ml and incubated at 24°C for 3 h. After α-factor removal by filtration, cells were released in culture medium containing protease (Sigma; 150 mg/ml final concentration). Samples were taken at every 15 min, for a total of 90 min, and processed for G-Tail assay and flow cytometry.

To arrest the cells in G_2, 15 μg/ml of nocodazole, from a freshly prepared stock solution (3.3 mg/ml nocodazole in DMSO), was added to the cultures and incubated for 2 h. Samples were taken and processed for telomeric-Oligonucleotide Ligation Assay (t-OLA) and flow cytometry.

Analysis of the telomeric single-strand overhangs

Genomic DNA from DNA samples extracted from α-factor synchronized cultures was digested with the restriction enzyme XhoI (New England BioLabs) and hybridized in denaturing and non-denaturing conditions as described in Dionne and Wellinger (25). The analysis of the single-strand telomeric DNA was performed with $(C_3TA_2)_3$ probe. Negative controls were genomic DNA samples treated with the *Escherichia coli* exonuclease ExoI (New England BioLabs) to degrade G-tails.

Telomeric oligonucleotide ligation assay

T-OLA was performed as previously reported (26) with some modifications. Five picomoles of a telomeric probe $(C_3TA_2)_2$ was labelled with [γ ^{32}P]-adenosine triphosphate (10 mCi/ml) in 1× exchange buffer (50 mM imidazole–HCl, pH 6.4, 12 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 6 μM ADP) containing 10 U of T4 polynucleotide kinase (Invitrogen). The reactions were carried out in a total volume of 10 μl, containing 5 μg of non-denatured, high-molecular weight genomic DNA and 0.5 pmol of labelled probe. After 5 h of incubation at 34°C in 1× T4 DNA ligase buffer, 400 U of T4 DNA ligase (New England Biolabs) was added, and the incubation was carried out for an additional 12 h. A 3-μl aliquot of each sample was mixed to 4 μl of stop solution (USB) containing 95% formamide. Samples were heated at 95°C, immediately cooled down on ice and run onto 6% polyacrylamide/46% urea gels. The gels were dried, and the t-OLA reaction products were visualized as a typical ladder of DNA fragments, which differ from one another for 12 bp, by autoradiography.

To validate t-OLA results, all samples were subjected to a simplified method of the conventional non-denaturing hybridization assay (in-liquid hybridization assay). Indeed, t-OLA is based on hybridization of the $(C_3TA_2)_2$ probe to native DNA, thereby it is expected that the fraction of bound probe is proportional to the amount of the 3′ overhang. In brief, 3 μl of each t-OLA reaction was run on a 1% agarose gel for 1 h at 90 V in 1× TAE buffer. To quantify the amount of DNA and the bound probes in each sample, the gel was stained with ethidium bromide, photographed, dried on nylon membrane (Stratagene) and analysed by Typhoon 9200 (Amersham Biosciences). The total amount of 3′ overhang for each sample was obtained by normalizing the in-solution hybridization signal versus the DNA contained in each reaction mixture and visualized by ethidium bromide staining.

Southern blotting

Terminal restriction fragmets (TRF) were analysed by Southern blotting. XhoI-digested DNA samples were separated on agarose gels; the gels were blotted onto nitrocellulose membranes (Schleicher & Schuell) and hybridized to the $(T_2AG_3)_{10}$ and a 3′-end Y' subtelomeric probe. After washing, hybridization signals were detected using Typhoon 9200 (Amersham Biosciences).

HO cleavage was controlled by Southern blots as previously reported (12,24).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (23) using the following antibodies: polyclonal anti-Rad51 sc-33626 (Santa Cruz); anti-HA or anti-Myc antibodies (Santa Cruz) were used for immunoprecipitation of HA-Tel1 or Myc-Cdc13. Immunoprecipitated DNA samples were quantitated by real-time PCR (Applied Biosystems 7300). Relative enrichment, for proteins binding at telomeric regions, was detected by normalizing signals to input DNA and expressed as the amount of VIIL (telomeric sequence on the left arm of chromosome VII) over endogenous gene (ARO1—internal control). Quantification of proteins bound to *de novo* telomeres after HO cutting was done as previously reported (24). Briefly, enrichment of the amplicons over the internal control (the *PDI1* gene, 50 kb from the telomere of Chr. III-L) was determined after normalization with the efficiency of HO cutting. All the samples were amplified in triplicate, and ChIPs were repeated two to three times for each strain. Data are reported as averages (bars), with standard deviations indicated by lines above.

Y' amplification assay

For the real-time PCR analysis, the Applied Biosystem 7300 PCR system (Life Technologies) with Syber Green technology (Life Technologies) was used. Copy numbers were normalized over the endogenous gene ACT1. The fold increase of the subtelomeric Y' element was calculated by the ΔΔ cycle threshold method. Primers were purchased from Eurofins MWG Operon.

Phleomycin sensitivity assay

For the spot test analysis, overnight cultures were adjusted to an OD_{600} of 0.5, and 5-fold dilutions were spotted on YPD and YPD-phleo plates (2 or 5 μg/ml phleonycin). Cell growth was checked 2 and 4 days after incubation at 30°C.

Statistical analysis

Statistical evaluation of data was done by Student *t*-test.

RESULTS

Telomere recombination occurs in yeast cells with vertebrate-like telomeres in the presence of a functional telomerase

Typically yeast telomeres are maintained by telomerase. However, recombination-driven mechanisms can maintain telomeres in cells lacking telomerase or in telomerase-proficient cells with defective capping activity (5,22).

To detect recombination-based telomere maintenance in *tlc1h* cells, we evaluated the pattern of telomeric XhoI restriction fragments that discriminate between type I and type II survivors. In both wild-type and type I survivors, three major XhoI fragments are detected by a probe from the 3′-end of the subtelomeric Y' element. The smallest of these fragments corresponds to the most distal portion of Y', whereas the two longer fragments identify tandem copies of Y' short and Y' long elements (Figure 1A). Hybridization of Y' short and Y' long is much more intense in type I survivors because of increased numbers of tandemly repeated elements. Type II survivors show many differently sized fragments that hybridize to both a telomeric repeat probe and the 3′ Y' probe.

As previously reported (18), telomere length in *tlc1h* cells was shorter than in *TLC1* cells and did not change

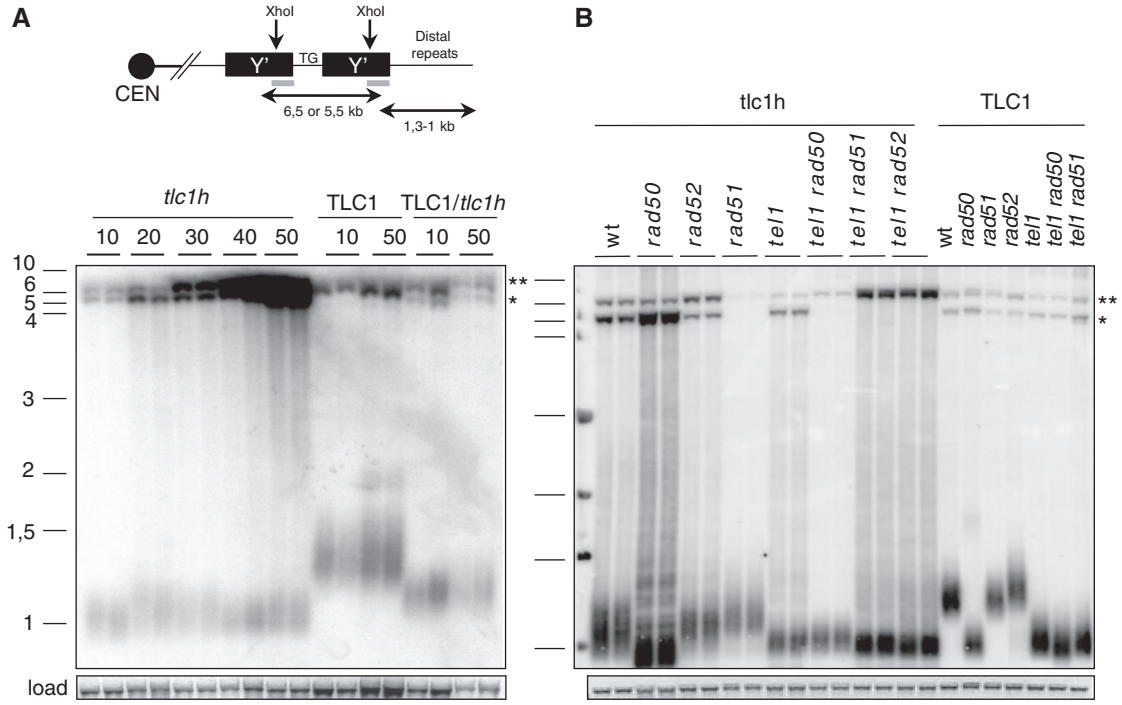

Figure 1. *tlc1h* cells are characterized by short telomeres and amplification of the subtelomeric Y' elements. Southern blots monitoring telomere length and subtelomeric Y' amplification in the indicated strains. Single colonies were inoculated in liquid cultures, grown over night, and DNA was prepared from the resulting cells. XhoI-digested DNA samples were hybridized to a Y' subtelomeric probe. (A) *tlc1h* or TLC1 haploids were picked from sporulation plates and grown by consecutive re-streaks (each re-streak represents ~20 generations). Control TLC/*tlc1h* diploids were also used. Samples at the indicated number of passages were analysed. (B) *tlc1h* or TLC1 cells with the indicated genotypes grown for 30 plate passages. Load control is reported (load); size markers are indicated in kb. **Y' long; *Y' short.

with increasing cell duplications. Heterozygous TLC1/*tlc1h* diploid strains had telomeres of intermediate length, shorter then TLC1 but longer than in *tlc1h* haploid strains (Figure 1A). However, the hybridization profile of *tlc1h* cells showed a progressive increase of the intensity of the subtelomeric Y' fragments, similar to that seen in type I survivors (Figure 1A). Conversely, Y' amplification was not detected in control *TLC1* strains (Figure 1).

To further support this finding, we analysed the variation of Y' copy number by real-time PCR using TLC1 cells as a reference, as the number of Y' elements in these cells is constant. This method was used because it is more quantitative than Southern blotting. Using this method, Y' copy number was 4 (after 600 doublings; P30) or 10 (after 1000 doublings; P50) times higher in *tlc1h* cells (Figure 2A). Overall, these results suggest that yeast cells with vertebrate-like telomeres have amplified subtelomeric Y' elements, even though they are telomerase proficient.

Y' amplification is Rad51 dependent in *TEL1 tlc1h* cells and Rad50 dependent in *tel1Δ tlc1h* cells

The pattern of Y' XhoI restriction fragments suggested that *tlc1h* cells accumulate type I survivors regardless of telomerase activity. To confirm this hypothesis, we determined the Y' copy number in cells lacking Rad51, (required for generation of type I survivors) or Rad50 (required for type II survivors) proteins. *RAD50* or *RAD51* deletion was carried out in fifth passage *tlc1h* cells, before Y' amplification occurs (Figure 2A). Subsequently, mutants were grown for 600 generations (P30). As reported earlier in the text, Y' copy number was assessed by real-time PCR using *TLC1* cells for comparison. The abundance of Y' subtelomeric repeats increased in *tlc1h rad50Δ* cells but not in *tlc1h rad51Δ* cells (Figure 2B). Conversely, in *tlc1h tel1Δ* double mutants, Y' copy number did not increase in the absence of Rad50 (*tlc1h* and *tel1Δ rad50Δ*), but it did so in the absence of Rad51 (*tlc1h tel1Δ rad51Δ*). Moreover, the increase in Y' DNA was lower in *tlc1h tel1Δ rad51Δ* compared with *tlc1h tel1Δ* cells (Figure 2B). These data suggest that Y' amplification was Rad51 dependent in *tlc1h* cells, whereas it was Rad50 dependent in cells lacking Tel1. However, as Y' amplification was detected in *tlc1h tel1Δ rad51Δ* cells, although at lower level (4-versus 8-fold in *tlc1h tel1Δ* cells), it appears that Rad51 contributes to Y' amplification in cells lacking Tel1.

Next, we attempted to find a relationship between recombination-based telomere maintenance and telomere length in *tlc1h* cells and double mutants. As reported previously, *tlc1h* cells had shorter telomeres than *TLC1* cells, whereas telomeres in *tlc1h rad50Δ* cells were even shorter (Figure 1B). However, telomere length in *tlc1h tel1Δ* mutants was not affected by deletion of either *RAD50* or *RAD51* (Figure 1B). These results suggest that Y' amplification in *tlc1h* cells is not simply a consequence of short telomeres. This interpretation is also supported by the

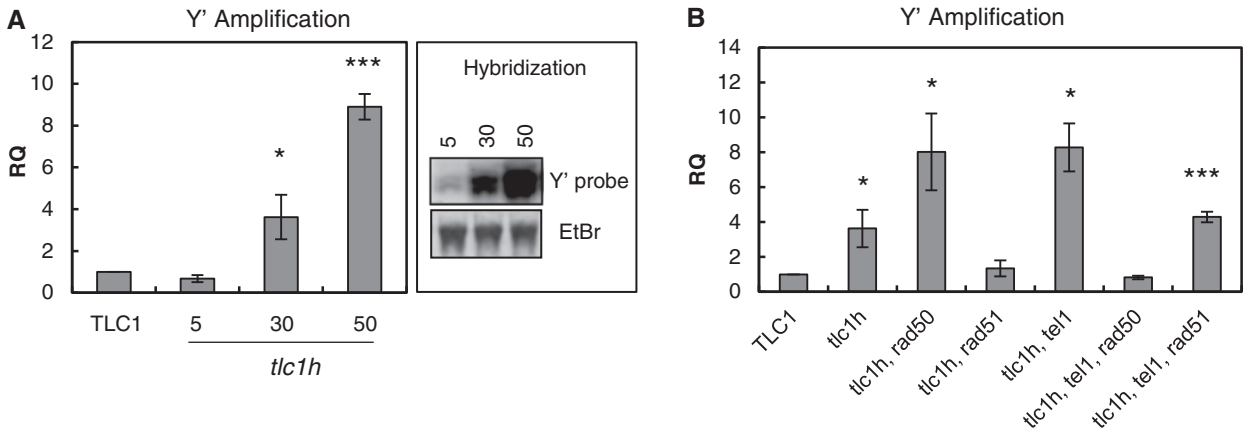

Figure 2. Y' amplification increases in *tlc1h* strains as detected by relative quantification (RQ) of Y' copy number by real-time PCR. (**A**) RQ of Y' subtelomeric elements in the indicated strains. The inset shows the Y' hybridization signals detected in the same *tlc1h* clones. Five, 30 and 50 refer to the number of consecutive re-streaks. Statistical analysis versus *TLC1*: *tlc1h* P30, $P = 0.012$; *tlc1h* P50, $P = 0.001$. (**B**) RQ of Y' elements in the indicated *tlc1h* mutants after 30 re-streaks (P30); TLC1 samples were used as calibrator. Statistical analysis versus TLC1: *tlc1h,* $P = 0.012$; *tlc1h* rad50Δ, $P = 0.023$; *tlc1h* tel1*Δ*, $P = 0.04$; tlc1h tel1Δ *rad51*Δ, $P = 0.0001$.

finding that Y' amplification did not occur in *TLC1 tel1*Δ cells (27), which have very short telomeres (Supplementary Figure S1A).

End resection in *tlc1h tel1*Δ cells is MRX independent

Yeast telomeres ending with vertebrate telomeric repeats have longer single-stranded tails (ssDNA) that may arise from extensive 5′ degradation (12,20). A possible explanation for the high rate of recombination-based telomere maintenance in *tlc1h* cells is that these long overhangs initiate it.

In wild-type cells, G-tail length is cell cycle regulated, ~14-nt long throughout most of the cell cycle and 50–100 nt in late S/G_2 phase (28). To determine whether long telomeric G-tails in *tlc1h* cells were constitutive or cell cycle regulated, *tlc1h* cells were synchronized in G_1 phase by α-factor, subsequently released from the block and G-tails determined during cell cycle progression. Samples were collected at 15-min intervals, and the G-tail lengths were estimated by the ratio between the telomeric hybridization signals in the native gels versus those obtained in the denatured gels (25). As controls, we analysed asynchronous *tlc1h* cells, and samples treated with the exonuclease ExoI to degrade G-tails. Asynchronous cultures revealed hybridization signals under non-denaturing conditions that were eliminated by ExoI treatment (Figure 3A). In synchronized cells, hybridization was low in G_1 with a gradual increase in S and G_2 (Figure 3A). Quantitative analysis of three independent experiments confirmed that G-tails lengthening was restricted to the S phase, and the attained length was maintained in G_2 (Figure 3B). This result was confirmed by the analyses of the G-tail lengths over two consecutive cell cycles that, as expected, showed cyclic G-tails shortening and lengthening (Supplementary Figure S2). Thus, the kinetics of 3′ overhang elongation in *tlc1h* cells is similar to that in *TLC1* cells (28), suggesting that the longer G-tails are not because of impaired cell cycle regulation.

As vertebrate-like telomeres are shorter than wild-type telomeres, it is unlikely that long G-tails are due to a hyperactive telomerase. Rather, we reasoned that end resection might be hyperactive in *tlc1h* cells. This idea is also supported by the lack of telomere-binding proteins that limit nuclease activity, such as Rif1-2 and Rap1, at vertebrate-like telomeres (8,13,14). To test this hypothesis, we analysed the role of MRX complex, which is the major nuclease acting at telomeres, and Tel1 in the resection of *tlc1h* telomeres. Telomeric ssDNA was analysed by a more sensitive assay, the t-OLA that detects single-stranded telomeric DNAs as a ladder of telomeric oligonucleotides (12-nt long) hybridized to the 3′ overhang and self-ligated (26). Additionally, the total amount of 3′ single-stranded DNA was determined by in-liquid hybridization and non-denaturing in-gel electrophoresis assay as the ratio between the hybridization signal and the total amount of DNA loaded (29). The t-OLA assay was performed using a vertebrate-specific $(C_3TA_2)_2$ oligo as a probe, which allows detection of T_2AG_3 single-stranded DNA ≥12 nt. As expected, this probe did not hybridize to TLC1 telomeric DNA but did yield a characteristic telomeric ladder when hybridized to *tlc1h* DNA (Figure 3C). The intensity of the bands in the ladder, as wells as the total hybridization signal, was proportional to the amount of DNA used in the reactions. In addition, pre-treating DNA samples with ExoI eliminated hybridization (Figure 3C), confirming that the ladders were due to T_2AG_3 3′ single-stranded overhangs. This result was confirmed by non-denaturing liquid hybridization showing that T_2AG_3 tails were present in *tlc1h* yeast strains and absent in *TLC1* cells (Figure 3C).

Both the t-OLA profiles and hybridization to the telomeric probe did not detect G-tails at telomeres in *tlc1h rad50*Δ cells, which lack MRX activity because of deletion of the Rad50 component of the complex (Figure 3C and D). Additionally, the presence of long single-stranded non-telomeric DNA was ruled out by QAOS (30) at two loci, 600 and 14 500 bp upstream of

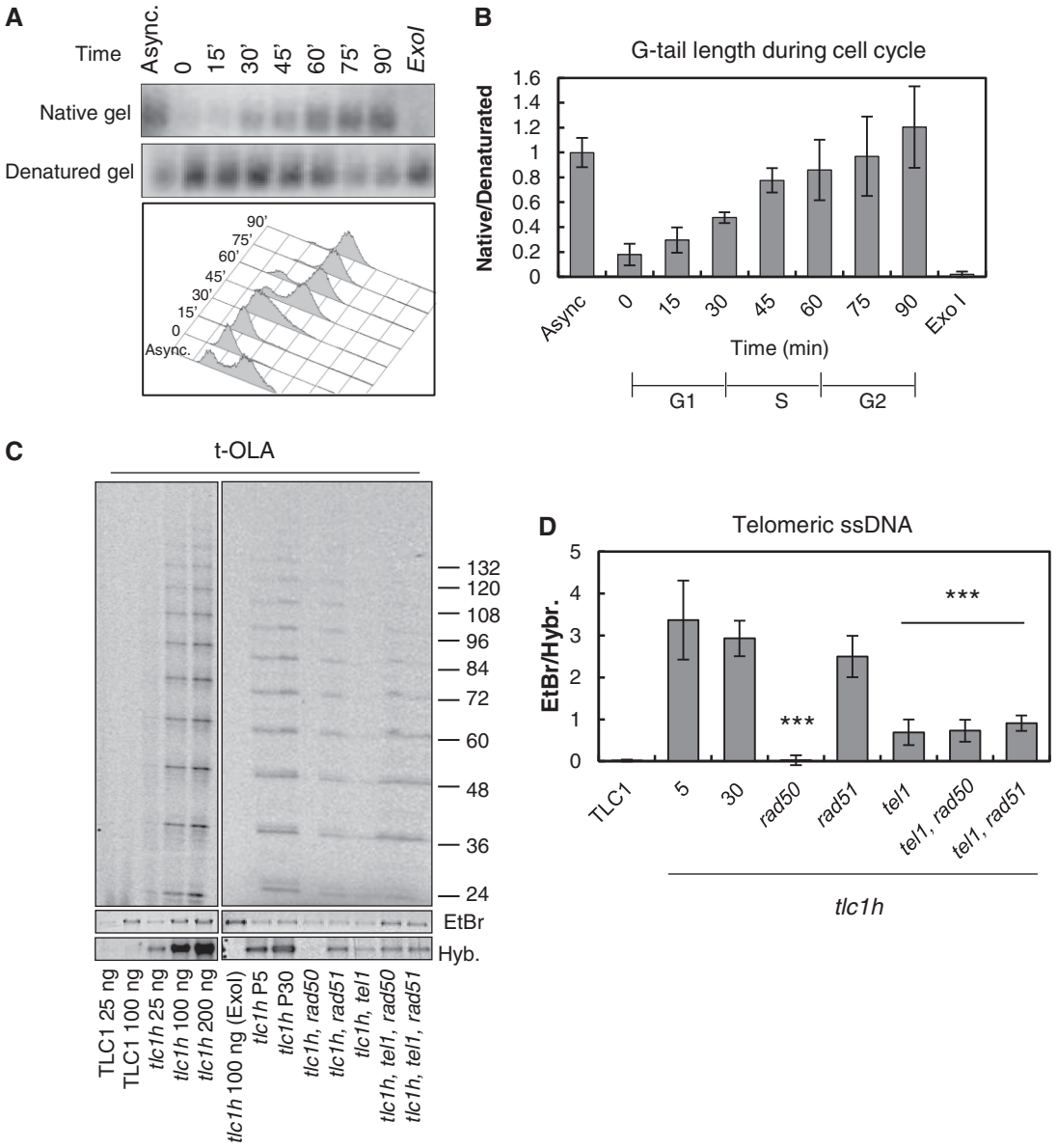

Figure 3. *tlc1h* cells exhibited long and cell cycle controlled, telomeric single-stranded overhang. (**A**) Telomeric ss overhangs were detected during cell cycle by G-tail assay. Cell samples, after α-factor block, were taken at multiple time points throughout the subsequent synchronous cell cycle (fluorescence-activated cell sorting analysis, lower left) and were used to extract genomic DNA. DNA samples were digested with XhoI and analysed by non-denaturing in-gel hybridization (native gel) with the C_3TA_2 probe. After exposure, gels were denatured and re-hybridized to the same probe (denatured gel). Control samples were asynchronous *tlc1h* cultures (Async) and *tlc1h* DNA samples digested with the exonuclease I (ExoI) before analysis. (**B**) Quantitative analysis of the telomeric ssDNA. *y*-axis, ratio between the intensity of telomeric ssDNA as detected by native in-gel hybridization and that obtained by denatured in-gel hybridization. *x*-axis, time (min) following release from α-factor block. Data are mean ± standard deviation of three independent experiments. (**C**) Telomeric ssDNA as detected by t-OLA in the indicated DNA samples. After hybridization and ligation reactions, denaturing gel was performed to separate the t-OLA products. Size length (nucleotides) of ligated products is indicated. The actual amount of DNA used in each reaction was revealed by EtBr staining (EtBr panel); autoradiographs of native agarose gel electrophoresis hybridized by the $(C_3TA_2)_2$ probe showed the overall amount of overhangs for each sample (hyb. panel). (**D**) Quantitative analysis of the telomeric ssDNA as obtained by the ratio between the intensities of the signals because of the amount of DNA (EtBr) and the resulting hybridization. Data are mean ± standard deviation of three independent experiments. Unless otherwise indicated, all *tlc1h* strains were analysed after 30 re-streaks. ExoI refers to DNA pre-treatment with exonuclease I. Statistical analysis versus *tlc1h* P30: *tel1*Δ $P = 0.00014$; *rad50*Δ $P = 1.2.10^{-5}$; *tel1*Δ *rad50*Δ $P = 0.00012$; *tel1*Δ *rad51*Δ $P = 0.00013$.

telomeres (Supplementary Figure S3). Thus, in the absence of MRX activity, tlc1h telomeres terminate with G-tails <12 nt, suggesting that generation of long T_2AG_3 overhangs in *tlc1h* cells is MRX-dependent. Conversely, G-tails were detected at *tlc1h tel1*Δ double and *tlc1h tel1*Δ *rad50*Δ triple mutants, although the signal was less in *tlc1h cells* (Figure 3C and D). Moreover, G-tails of similar length were detected in *tlc1h tel1*Δ and *tlc1h tel1*Δ *rad50*Δ cells, suggesting that they were generated by an MRX-independent mechanism. G-tail occurrence

and length was not affected by *RAD51* deletion in either *tlc1h* or in *tlc1h tel1*Δ cells, confirming that Rad51 does not influence telomere resection. We conclude that vertebrate-like telomeres are resected by MRX in a Tel1-dependent manner; in the absence of Tel1, an MRX-independent mechanism can carry out telomere resection.

We provided additional evidence that Tel1 affect vertebrate-like yeast telomeres by testing Tel1 binding to telomeres in *tlc1h* and wild-type cells. This analysis was performed in G_2-arrested cells when Tel1 binding to wild-type telomere is at its highest (23). Tel1 showed low but detectable binding to wild-type telomeres (1.4- to 1.8-fold above binding to non-telomeric control), whereas the binding to vertebrate-like telomeres was higher (2.4- to 2.8-fold above binding to non-telomeric control; Figure 4A).

Rad51 binds vertebrate-like telomeres and inhibits telomerase-mediated elongation

Given that *tlc1h* cells displayed subtelomeric amplification of the Y' element, we speculated that they might bind Rad51, the ssDNA-binding protein that initiates strand exchange during HR. To test this hypothesis, we analysed the binding of Rad51 to vertebrate-like or $TG_{1–3}$ yeast telomeres by ChIP. As predicted, Rad51 binding to telomeres was about two times higher to *tlc1h* compared with *TLC1* telomeres (Figure 4B).

To confirm Rad51 binding to vertebrate-like telomeres, we used the *de novo* telomere formation assay to a T_2AG_3 or $TG_{1–3}$ telomeric seed. The *de novo* telomeres occur *in vivo* after induction of the HO endonuclease that cuts next to an internal tract of telomeric DNA (31). Telomeric repeats of either 60 or 230 bp were used as seeds to mimic short or long telomeres (Figure 5A). This assay was performed in TLC1 background, and telomerase-mediated addition of $TG_{1–3}$ telomeric repeats gives rise to hybrid T_2AG_3-$TG_{1–3}$ telomeres.

Telomere *de novo* addiction was confirmed by Southern blots that showed a rapid elongation of the short telomeric seeds, whereas the long telomeric arrays maintained their length (Supplementary Figure S4). Then, Rad51 binding was determined by ChIP (Figure 5B). Rad51 binding to T_2AG_3 telomeres was higher than that to $TG_{1–3}$. In both cases, preferential binding of Rad51 to short telomeres was detected, suggesting that Rad51 recruitment at telomeres was guided by ssDNA that is more extensive at short rather than long telomeres. Moreover, Rad51 binding to short T_2AG_3 telomere seemed to be similar to non-telomeric DSB, which is consistent with an initial capping defect and nucleolytic attack of short T_2AG_3 telomeres (12). That the tested telomeric regions represented bona fide telomeres was demonstrated by Cdc13 binding

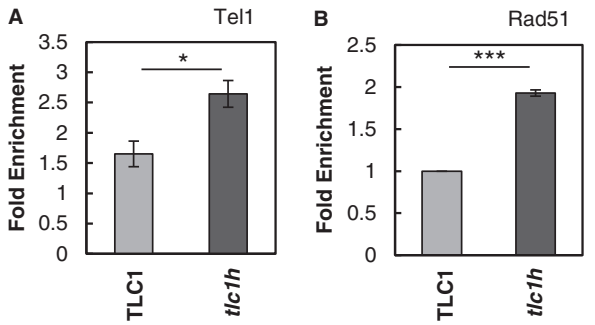

Figure 4. Telomere binding of Tel1 and Rad51 is increased at vertebrate-like telomeres. Tel1 (**A**) and Rad51 (**B**) binding to telomere VIIL was monitored in G_2-arrested cells of TLC1 and *tlc1h* strains by ChIP. *y*-axis, fold enrichment of telomere Tel1 or Rad51 binding over internal control. Data are mean ± standard deviation of three independent experiments. Statistical analysis: Tel1 binding to *tlc1h* versus TLC1 telomeres, $P = 0.044$; Rad51 binding to *tlc1h* versus TLC1 telomeres, $P = 0.0003$.

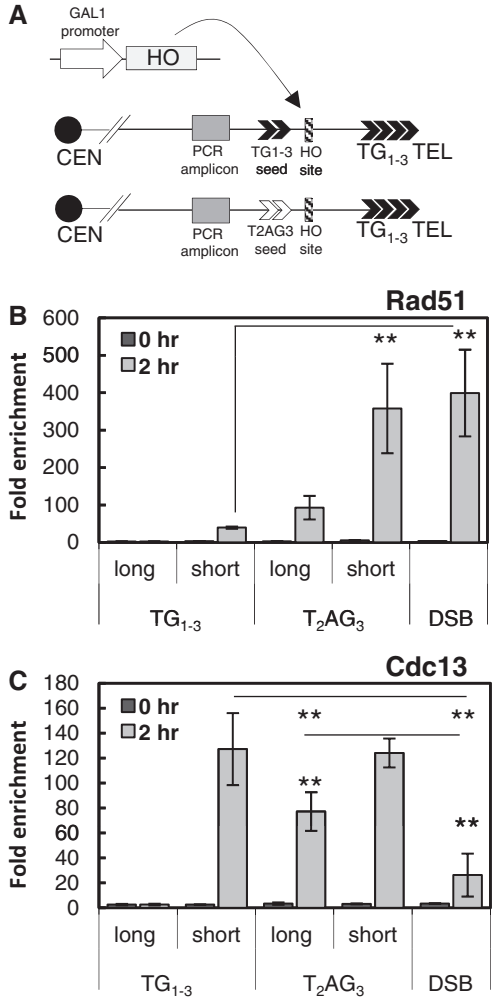

Figure 5. (**A**) Schematic representation of *de novo* telomere assay, telomeric $TG_{1–3}$ or T_2AG_3 seeds are produced after HO cleavage. (**B**) ChIP analysis of the binding of Rad51 after HO induction at $TG_{1–3}$, T_2AG_3 long and short telomere, or at non-telomeric sequence (DSB). Statistical analysis: $TG_{1–3}$ short at 2 h versus T_2AG_3 short at 2 h, $P = 0.005$; $TG_{1–3}$ short at 2 h versus DSB at 2 h, $P = 0.003$. (**C**) Binding of Cdc13 after HO induction at $TG_{1–3}$, T_2AG_3 long and short telomere, or at non-telomeric sequence. Statistical analysis: $TG_{1–3}$ short at 2 h versus T_2AG_3 long at 2 h, $P = 0.003$; $TG_{1–3}$ short at 2 h versus DSB at 2 h, $P = 0.002$. T_2AG_3 short at 2 h versus T_2AG_3 long at 2 h, $P = 0.006$; T_2AG_3 short at 2 h versus DSB at 2 h, $P = 0.002$; average fold enrichment and standard deviation are relative to an internal control and referred to three independent experiments.

(Figure 5C). As previously shown, Cdc13 enrichment to T_2AG_3 telomeres was higher than that to $TG_{1–3}$, but this was true for long telomeres, whereas short telomeres, either $TG_{1–3}$ or T_2AG_3, exhibited similar Cdc13 binding. As previously reported (12), Cdc13 binding to non-telomeric ends was also observed, although at lower level than that to telomeres.

These results confirmed the higher enrichment of Rad51 to T_2AG_3 telomeres than to $TG_{1–3}$ telomeres of similar length and its preferential binding to short over long vertebrate-like telomeres. Rad51 binding to short $TG_{1–3}$ arrays was also detected and exhibited similar enrichment as that obtained with long T_2AG_3 telomeres. This suggests that telomeres are bound by Rad51 not because of their length or sequence but because of the amount of ssDNA.

Our data suggest that Rad51 binds telomeric 3′ overhang and promotes Y' amplification by HR. One consequence of this binding might be to sequester single-stranded G-tails from telomerase, thereby limiting telomerase-mediated lengthening. Indeed, *tlc1h rad51*Δ double mutants had longer telomeres than those in parental *tlc1h* cells, and this telomere lengthening required Tel1, as demonstrated by the fact that the triple mutants *tlc1h tel1*Δ *rad51*Δ had short telomeres (Figure 1B and Supplementary Figure S1B). Rad52 is required for Rad51 loading to ssDNA accordingly, *tlc1h rad52*Δ double mutants showed telomeres longer than *tlc1h* cells and comparable with those in *tlc1h rad51*Δ cells (Figure 1B and Supplementary Figure S1B). These results suggested that Rad51 has an inhibitory effect on telomere length, possibly because it competes with telomerase for binding to G-tails.

Tel1 deletion rescue inviability of *tlc1h rad50* mutants

We previously showed that *tlc1h* cells display a prolonged delay in the G_2 phase because of checkpoint activation in response to partially uncapped telomeres (18). Here, we determine whether MRX, Tel1 and/or Rad51 is needed for viability in *tlc1h* in the absence (complete medium) or presence (phleomycin medium) of a DNA damaging agent. For wild-type cells growing in complete medium, *rad50*Δ, *rad51*Δ and/or *tel1*Δ mutations had little effect on growth rates, except that *tel1*Δ *rad50*Δ cells grew somewhat more slowly than wild-type cells. In contrast, relative to *tlc1h* cells, growth rates of *tlc1h rad50*Δ cells were severely impaired. Unexpectedly, cell viability was rescued by deletion of Tel1, as *tlc1h tel1*Δ *rad50*Δ cells grew faster than *tlc1h rad50*Δ cells.

Deletion of *RAD51* had little effect on growth rates of the different *tlc1h* strains for cells growing in complete medium.

Induction of DSBs by growth in phleomycin (Figure 6, middle and right), which induces a Tel1- and Mec1-dependent checkpoint response (32), had only a minor effect on the growth of TLC1 *tel1Δ cells*, but it severely impaired the growth of TLC1 *tel1*Δ *rad50*Δ and TLC1 *tel1*Δ *rad51*Δ cells. These results suggest that TLC1 cells recover from DNA damage by the action of Tel1- and MRX-dependent pathways, and that Rad51 contributes to cell viability in *tel1*Δ cells.

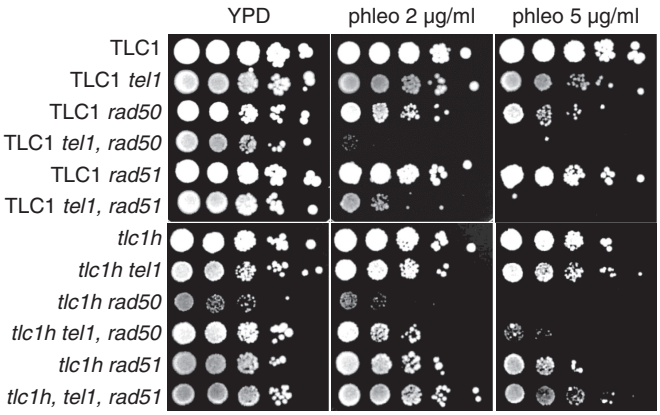

Figure 6. Cells viability of *tlc1h* strains is severely impaired in *rad50*Δ mutants; however, Tel1 deletion rescue growth defects. Growth of TLC1 and *tlc1h* strains on YPD plates without and with DSB-inducing phleomycin. Yeast cultures inoculated with single colonies and grown over night were diluted to the same optical density ad serially diluted; 5 µl samples were spotted on plates, and the growth was detected after 96 h of incubation at 30°C.

tlc1h cells behaved differently from TLC1 cells in phleomycin medium. Although *tlc1h tel1*Δ had fairly normal growth rates, *tlc1h rad50*Δ cells grew poorly in phleomycin. However, the extremely poor growth of the latter cells was recovered by TEL1 mutations as demonstrated by partial rescue of viability of the *tlc1h tel1*Δ *rad50*Δ triple mutants with respect to *tlc1h rad50*Δ.

Similarly, *tlc1h rad51*Δ strains showed ~10 times less viable cells relative to *tlc1h* cells, and this loss of growth was partially rescued by TEL1 as demonstrated by the higher growth of *tlc1h tel1*Δ *rad51*Δ than *tlc1h rad51*Δ cells.

Globally, these results confirmed the contribution of MRX and Tel1 to the viability of TLC1 cells and suggest that they operate in different pathways, both of which are involved in recovery from DNA damage. Similarly, in *tlc1h* cells, MRX and Tel1 belong to different epistasis groups, but the contribution of Tel1 to cell viability seemed different from that observed in TLC1 cells. Indeed, *TEL1* deletion rescued the extremely poor growth of *tlc1h rad50*Δ and *tlc1h rad51*Δ cells both in rich medium and in the presence of DNA damaging agents.

DISCUSSION

Here, we analysed mechanisms involved in the maintenance of vertebrate-like telomeres in *S.cerevisiae*. We show that vertebrate-like telomeres undergo Y' amplification by HR, even though they are telomerase proficient. As in conventional type I survivors, Y' amplification occurs by the Rad51-dependent and Rad50-independent pathway. In contrast, Y' amplification in *tlc1h tel1*Δ requires both Rad50 and, to a lesser extent, Rad51. Rad51 also affects telomere length in *tlc1h* cells, perhaps because it competes with telomerase for DNA ends. Our data also suggest a role for Tel1 in telomeric end resection in *tlc1h* cells.

Tel1 regulates the pathways leading to telomere recombination and end resection

Recombination-dependent telomere lengthening in yeast is mediated by two pathways distinguishable by their dependence on Rad50 (type II recombination) or Rad51 (type I recombination) (33). Typically, type I and II recombination is detected only in telomerase-deficient cells. However, HR at telomeres also occurs in strains with telomere capping defects, because of, for example, mutations in Cdc13, Ku or Tel1 (22).

The *tlc1h* cells, where capping is Tbf1 mediated, had high levels of Rad51-dependent Y' amplification. Nonetheless, telomere length is maintained at a stable but short length, and cells did not senesce, even on prolonged growth, suggesting that Y' amplification is not a surviving mechanism but rather a consequence of imperfect telomere capping. Consistent with this interpretation, *tlc1h rad52* double mutants do not amplify Y' elements, yet their viability is similar to that of *tlc1h* cells [(14) and data not shown]. Unexpectedly, when *tlc1h* cells lack Tel1, Y' amplification switches from Rad51 to Rad50 dependent, suggesting that Tel1 has a role in controlling telomere recombination.

Telomeric recombination pathways seem to be controlled by several factors leading to amplification of telomeric or subtelomeric sequences. Although telomerase negative cells (*tlc1*Δ) mainly generate Rad51-dependent type I survivors (90% of the post-senescence cells), atypical Rad51-dependent type II survivors predominate in *cdc13-1* mutants (34), whereas type I survivors prevail in *yku70*Δ *mre11*Δ double mutants, which are characterized by long overhangs (35). These and our data suggest that the pathways involved in telomere recombination are dictated by the specific capping deficiency of a given strain, which affects end resection and recruitment of recombination proteins.

How Tel1 regulates telomeric recombination is not known, one possibility is that it has an indirect role by regulating end resection. According to the prevailing model, Tel1, the orthologue of the human *ATM* kinase, binds to telomeres, preferentially the short ones, and marks them for telomerase-dependent elongation (23,36). According to the prevailing model, Tel1 binds to telomeres, preferentially the short ones, and marks them for telomerase-dependent elongation. However, it has been recently re-proposed (9,37), the model for Tel1 activity at telomeres put forth by Petes group (38,39) to explain why loss of Tel1 delays the onset of senescence. According to this, MRX recruits Tel1 onto telomeres (31), which in turn exerts a positive feedback loop on MRX itself (9). As MRX generates ssDNA, Tel1 deficiency would reduce telomere resection, thus deferring senescence and would provide a suboptimal substrate for telomerase, leading to telomere shortening.

In summary, we propose that in yeast cells with vertebrate-like telomeres, Tel1 participates to telomere capping by regulating end resection and the recombination pathways leading to Y' amplification.

Rad51 is involved in length regulation of vertebrate-like telomeres

Our data suggest that Rad51 binds telomeres in *tlc1h* cells, possibly because of their long G-tails, which in turn activates strand invasion and Y' amplification. Accordingly, we demonstrated that Rad51-binding to vertebrate-like telomeres is increased relative to wild-type telomeres. Moreover, by using *de novo* telomere formation at T_2AG_3 or control TG_{1-3} seeds, it seems that Rad51 binds T_2AG_3 telomeres in a length-dependent manner, i.e. long telomeres bind little Rad51, whereas short telomeres bind more Rad51. Rad51 binding was also detected at short TG_{1-3} telomeres, although at lower level than that observed at vertebrate-like telomeres, where it approached the levels found at bona fide DSBs. Thus, the binding of Rad51 to both TG_{1-3} and T_2AG_3 telomeric DNA is affected by the length of telomeric DNA. Moreover, Rad51 was more enriched at vertebrate-like telomeres, probably because of their capping deficiency, as demonstrated by similar level of Rad51 binding between short T_2AG_3 telomeres and DSBs.

A key prediction of this model is that Rad51 binding to telomeres may outcompete telomerase for binding to the telomeric overhangs. Accordingly, we show that telomeres in *tlc1h rad51*Δ and *tlc1h rad52*Δ double mutants are longer than that in *tlc1h* cells. Thus, Rad51 recruitment to telomeres may prime a regulatory loop that favours recombination-dependent instead of telomerase-mediated telomere maintenance.

Binding of Rad51 to human telomeres that requires the tumor soppressor protein BRCA2, has also been demostrated (40). BRCA2 acts as a loader of Rad51 to DSBs and is a key component of the DNA repair pathway based on HR. Conditional deletion of BRCA2 and inhibition *Rad51* led to telomere shortening in mouse embryonic fibroblast cells, suggesting that BRCA2-mediated HR has a critical role in the maintenance of telomeric DNA during cell proliferation (40). Therefore, it seems that HR has different roles in the maintenance of mammalian and yeast telomeres. HR may contribute to telomere maintenance in mammalian cells under physiological conditions, whereas in yeast it is normally excluded from telomeres maintenance and engaged only as a salvage pathway when telomerase is absent or telomere capping is perturbed.

It has previously been shown that MRX and Rad51 contribute to viability of yeast cells with uncapped telomeres by opposing effects: MRX contributes to viability of *cdc13-1* mutants (41), whereas Rad51 recruitment to telomeres of telomerase minus cells increases sensitivity to DSBs (42). Consistently, we find that MRX deficiency substantially decreases *tlc1h* cells viability both in the absence and presence of DSBs. Unexpectedly, Tel1 deletion improve the viability of *tlc1h rad50*Δ cells with a more pronounced effect when cells are grown in phleomycin, suggesting that this effect may be related to the capacity of the cells to repair bona fide DSBs. The shift from Rad51-dependent to Rad50-dependent telomere recombination that occur in *tlc1h* cells lacking Tel1 may favour recombination at DSBs, as recombination proteins (Rad51 and Rad52) are no longer sequestered at uncapped telomeres (42).

Alternatively, it may be that the sensitive phenotype to DSBs is related to telomere capping deficiency and end resection. In TLC1 cells, the hypersensitivity of *rad50*Δ mutants to DSBs is suppressed by Exo1 overexpression

(43), suggesting that Exo1 contributes to the DSBs repair in MRX mutants. Similarly, in *tlc1h* cells, Tel1 deletion could activate Exo1, thus improving their capacity to repair damaged DNA.

The more pronounced effect caused by Tel1 deletion in *tlc1h rad50*Δ than that in *tlc1h rad51*Δ cells argue against the possibility that rescue of viability is simply because of the elimination of checkpoint activation. This idea is also corroborated by the fact that residual Rad53 activation is present in *tlc1h tel1*Δ cells.

It has been hypothesized that Tbf1 is an ancestral telomere-binding protein in yeast (44,45), and that it may represent a relic telomeric structure, evolutionary conserved, from a period of transition between T_2AG_3- and $TG_{1–3}$-based telomeres (12). Because of the presence of subtelomeric T_2AG_3 repeats (46), it may be that T_2AG_3-based telomeres act as a backup mechanism that rescues the viability of cells that experienced extensive telomere shortening. In this scenario, Tel1 may be a key regulator of end resection and telomere recombination.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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