

# Tel1 kinase and subtelomere-bound Tbf1 mediate preferential elongation of short telomeres by telomerase in yeast

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**Telomerase enables telomere length homeostasis, exhibiting increasing preference for telomeres as their lengths decline. This regulation involves telomere repeat-bound Rap1, which provides a length-dependent negative feedback mechanism, and the Tel1 and Mec1 kinases, which are positive regulators of telomere length. By analysing telomere elongation of wild-type chromosome ends at single-molecule resolution, we show that in *tel1Δ* cells the overall frequency of elongation decreases considerably, explaining their short telomere phenotype. At an artificial telomere lacking a subtelomeric region, telomere elongation no longer increases with telomere shortening in *tel1Δ* cells. By contrast, a natural telomere, containing subtelomeric sequence, retains a preference for the elongation of short telomeres. Tethering of the subtelomere binding protein Tbf1 to the artificial telomere in *tel1Δ* cells restored preferential telomerase action at short telomeres; thus, Tbf1 might function in parallel to Tel1, which has a crucial role in a TG-repeat-controlled pathway for the activation of telomerase at short telomeres.**

Keywords: Tel1; Mec1; Tbf1; STEX; telomerase; telomere length homeostasis

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## INTRODUCTION

By measuring telomere elongation at single chromosome end molecules at nucleotide resolution, we established that telomerase does not extend every telomere in every cell cycle and that it shows an increasing preference for telomeres as their lengths decrease (Teixeira *et al*, 2004). Rap1–Rif1–Rif2 complexes assemble with the double-stranded region of the telomere and create a negative feedback loop that regulates telomerase in a

length-dependent manner (Kyrion *et al*, 1992; Krauskopf & Blackburn, 1996; Marcand *et al*, 1997; Wotton & Shore, 1997; Teixeira *et al*, 2004), but how this regulatory signal is transmitted to telomerase is not yet known. At the telomeric 3' end, telomerase is recruited during the S phase of the cell cycle through a direct interaction between the telomerase-associated Est1 and Cdc13, which binds to the telomeric 3' overhang (Evans & Lundblad, 1999; Pennock *et al*, 2001; Fisher *et al*, 2004).

The two yeast members of the phosphoinositide-3-kinase-related family—Tel1 and Mec1 (*Saccharomyces cerevisiae* homologues of human ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR), respectively)—are also found at telomeres and are necessary for the correct regulation of telomere length. Yeast cells lacking Tel1 have short but stable telomeres, whereas deletion of *MEC1* yields only a mild telomere shortening phenotype (Greenwell *et al*, 1995; Ritchie *et al*, 1999). The kinase functions of Tel1 and Mec1 seem to be partly redundant in telomere length maintenance, as simultaneous deletion of both *TEL1* and *MEC1* results in a progressive loss of telomeric sequences, leading to cellular senescence (Ritchie *et al*, 1999). In wild-type cells, ectopic tethering of additional Rap1 molecules adjacent to telomere repeats results in telomere shortening, whereas *tel1Δ* cells are not responsive to increased local concentrations of Rap1 (Marcand *et al*, 1997; Ray & Runge, 1999).

Despite the crucial roles of Tel1 and Mec1 in telomere maintenance, their mechanisms of action remain unknown. Tel1 does not directly affect telomerase catalytic activity, but seems to mainly modify the structure of the telomere end complex (Chan *et al*, 2001; Tsukamoto *et al*, 2001). Interestingly, the single-stranded telomere-binding protein Cdc13 was identified as a substrate of these kinases (Tseng *et al*, 2006). Furthermore, very recently, Est2, Est1 and Tel1 have been reported to associate preferentially with short telomeres (Bianchi & Shore, 2007; Sabourin *et al*, 2007), and the association of Est2 and Est1 with telomeres is severely impaired in *tel1Δ* cells (Goudsouzian *et al*, 2006).

To gain deeper insight into the roles of Tel1 and Mec1 in telomere maintenance and length regulation, we analysed telomere elongation of single chromosome end molecules at nucleotide resolution *in vivo*, in yeast strains deleted for *TEL1*, *MEC1* or both.

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## RESULTS

### Extension analysis of single telomeres

To study the roles of Tel1 and Mec1 in telomere elongation in *S. cerevisiae*, we monitored single telomere extension events at nucleotide resolution at two different chromosome ends *in vivo* as described (Teixeira *et al*, 2004; Fig 1A; Methods). We refer to this method hereafter as STEX, for Single Telomere Extension assay. Telomerase-negative strains—referred to as ‘recipient’—were created from haploid *tel1Δ* or *mec1Δ sml1Δ* yeast cells by deleting one of the essential components necessary for telomerase activity *in vivo*, *EST1* or *TLC1*. *EST1* encodes a telomerase protein subunit that is involved in telomerase recruitment (Lundblad & Szostak, 1989) and *TLC1* encodes the telomerase RNA moiety (Singer & Gottschling, 1994). All *mec1Δ* strains were also *sml1Δ*, to suppress lethality of the *MEC1* deletion (Zhao *et al*, 2001). For simplicity, hereafter we use *mec1Δ* to represent *mec1Δ sml1Δ* double mutants. As the steady-state telomere length in *tel1Δ* cells is short (170 bp at telomere VR (right arm of chromosome V) in our strain background), we had to ensure that recipient telomeres were not lost before mating. Therefore, telomeres were pre-elongated in the recipient by expressing a fusion protein between the DNA-binding domain of Cdc13 and Est1 (Est1-DBD<sub>Cdc13</sub>)—a construct that complements *est1Δ* (and *est1Δ tel1Δ*, this study) and leads to telomere overelongation (Evans & Lundblad, 1999). Before matings, cells that had lost the Est1-DBD<sub>Cdc13</sub> plasmid (pVL1120) were selected to obtain telomerase-negative cells. Functional telomerase was provided by mating the telomerase-negative recipients with telomerase-positive donor strains of the opposite mating type that were also deleted for *TEL1*, *MEC1* or both. Thus, telomeres that had shortened in the telomerase-negative parents became re-extended in the zygote owing to the presence of complementing telomerase (Fig 1A). DNA was isolated from the mating when most zygotes had completed a single S phase, and telomere elongation was detected by cloning and sequencing two different telomeres originating from the recipient strain. Telomere VR was amplified by telomere PCR (Forstemann *et al*, 2000). Native telomere IL (left arm of chromosome I) from the recipients was amplified using a primer complementary to a sequence that was deleted in the donor strains (Teixeira *et al*, 2002, 2004). PCR-amplified telomeres were cloned and sequenced. As yeast telomerase adds imperfect 5'-(TG)<sub>0–6</sub>TGGGTGTG(G)<sub>0–1</sub>-3' repeats (Forstemann & Lingner, 2001), telomere elongation is detectable on sequence alignment by the divergence of telomeric sequences in the telomere-distal region. As expected, telomere sequence determination at VR and IL in the *tel1Δ est1Δ* and *mec1Δ tlc1Δ* recipient strains on clonal expansion before mating revealed differences in size between the telomeres emerging from the different sisters, whereas sequence divergence was rare. At telomere VR, 1 out of 35 sequences (2.8%) diverged in *tel1Δ est1Δ* and 3 out of 45 sequences (6.6%) diverged in *mec1Δ tlc1Δ* cells (supplementary Fig S1A online); at telomere IL, 4 out of 54 sequences (7.4%) diverged in *tel1Δ est1Δ* and 2 out of 34 sequences (5.8%) diverged in *mec1Δ tlc1Δ* cells (supplementary Fig S1B online). These results are similar to the 6.6% of telomerase-independent events at VR seen previously in wild-type cells (Teixeira *et al*, 2004). Thus, *TEL1* or *MEC1* deletion does not significantly affect telomerase-independent modes of telomere elongation.

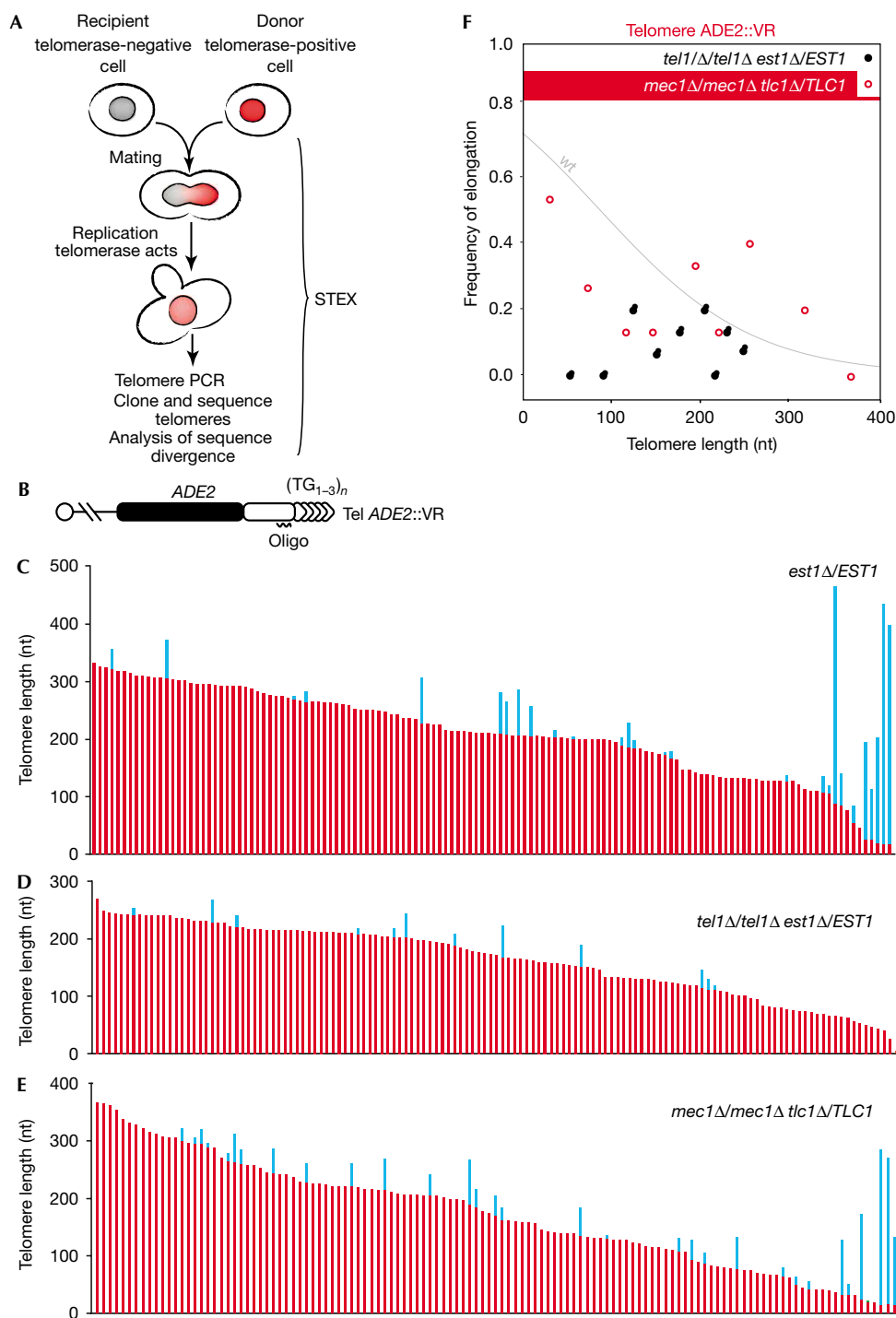
### Tel1 mediates the recognition of short telomeres

Telomerase-mediated elongation of telomeres in a *tel1Δ* background was analysed in four independent STEX assays (Fig 1D). Among the 133 sequences analysed, we detected 12 events in which sequences diverged (9%). This frequency is considerably lower than that observed in wild-type cells for the corresponding length range, in which the frequency increased from approximately 20% at a telomere length of 200 nucleotides to 45% at 100 nucleotides (Fig 1C,F; data pooled from Teixeira *et al*, 2004). Thus, the lower frequency of extension in *tel1Δ* cells provides an explanation for their short telomere phenotype. Furthermore, in marked contrast to wild-type cells, the frequency of elongation at VR did not increase for short telomeres in *tel1Δ* cells. Indeed, not a single telomere extension event was detected for the analysed telomeres that were shorter than 100 nucleotides (Fig 1D). The apparent lack of correlation between frequency of telomere elongation and telomere length was confirmed by statistical analysis. Sequences were pooled into groups of 15 and the frequency of elongation was plotted against the mean telomere length for each subgroup (Fig 1F). In wild-type cells short telomeres were preferentially elongated (Fig 1F, grey curve; Teixeira *et al*, 2004), whereas no correlation was established between divergence frequency and telomere length in the *tel1Δ* background (*tel1Δ/tel1Δ est1Δ/EST1*:  $R=0.34$ ,  $P_{\text{pearson}}=0.3749246$ ;  $P=0.29$ ,  $P_{\text{spearman}}=0.4366237$ ). Thus, Tel1 is necessary for preferential elongation of short telomeres at VR by telomerase. The *ADE2*-tagged telomere VR lacks subtelomeric elements, and telomere length sensing is thought to rely solely on TG repeats and the Rap1-counting mechanism. Thus, our analysis suggests that Rap1-mediated telomerase control relies on the presence of Tel1, at least during the first zygotic cell cycle analysed in this experiment. However, the lack of correlation between telomere elongation frequency and length observed here does not provide an explanation for the residual telomere length regulation at VR, which maintains a short but stable size range in *tel1Δ* cells.

In *mec1Δ* cells, we analysed sequence divergence in *mec1Δ/mec1Δ tlc1Δ/TLC1* diploid cells from five independent STEX assays (Fig 1E). The overall frequency of elongation in the analysed size range was 26%. In contrast to *tel1Δ* cells, *mec1Δ* cells extended very short telomeres at high frequency. However, the inverse correlation between the frequency of telomere elongation and telomere length was also perturbed in *mec1Δ* cells, as the data could not be fitted to the logistic regression model (see the supplementary information online; *mec1Δ/mec1Δ tlc1Δ/TLC1*:  $R=-0.53$ ,  $P_{\text{pearson}}=0.1407485$ ;  $P=-0.42$ ,  $P_{\text{spearman}}=0.2499173$ ; Fig 1F). Thus, Mec1 and Tel1 are both required for optimal regulation of telomerase at chromosome VR, with Tel1 having crucial role.

### Role of a subtelomeric region for length sensing

To monitor elongation events at a natural telomere, we performed STEX for telomere IL in the same *tel1Δ* genomic DNA samples as above. To analyse telomere IL in the *mec1Δ* background, however, a new set of matings were performed using a donor strain that lacked the subtelomeric region at telomere IL. The telomere elongation analyses at IL are shown in Fig 2B–D. Markedly, at this native telomere, and in contrast to the tagged telomere VR, short telomeres were preferentially extended by

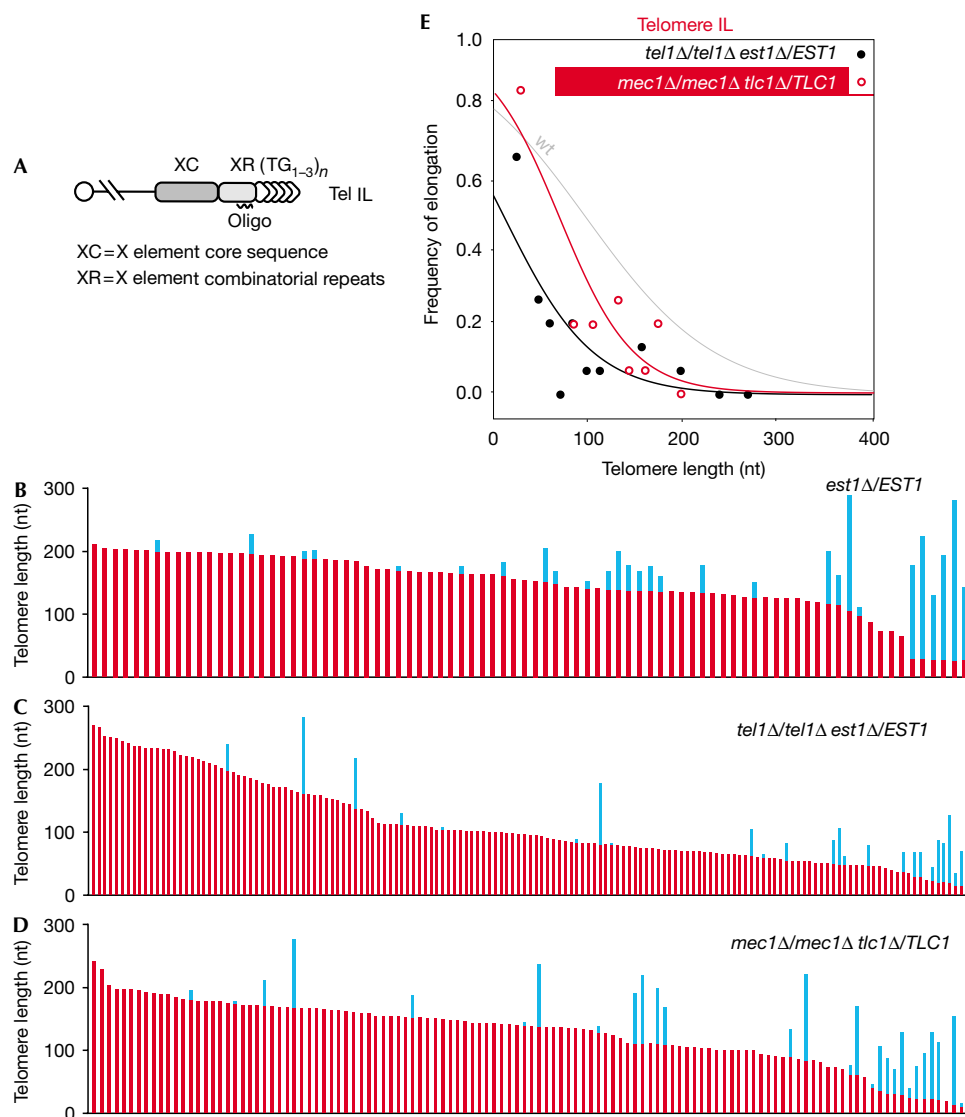


telomerase in *tel1Δ* cells (Fig 2C). The sequences were pooled in groups of 15 and the frequency of elongation was plotted against mean telomere length for each subgroup (Fig 2E). Although the elongation frequencies were lower than in the wild-type cells at telomere IL (grey line in Fig 2E; Teixeira *et al*, 2004), the inverse correlation between the frequency of elongation and telomere length was significant (*tel1Δ/tel1Δ est1Δ/EST1*:  $R = -0.64$ ,  $P_{\text{pearson}} = 0.03462063$ ;  $P = -0.75$ ,  $P_{\text{spearman}} = 0.01115861$ ). Thus, despite

the *TEL1* deletion and a presumed inactivation of the Rap1-mediated counting mechanism, telomere length was still sensed at telomere IL and transmitted to the machinery that regulates telomerase. Thus, our analysis of telomere elongation at telomere IL suggests the existence of a cryptic telomere length-sensing mechanism that seems to rely on chromosomal regions other than the TG repeats.

For STEX on telomere IL in *mec1Δ/mec1Δ tlc1Δ/TLC1* zygotes, three independent matings were analysed (Fig 2D). The overall

**Fig 1** | Single telomere extension analysis at tagged telomere VR in *tel1Δ* and *mec1Δ* cells. (A) Methodology of the STEX assay; see text for details. (B) Schematic of tagged telomere VR. The vector-derived sequence is between the *ADE2* gene and the telomeric tract ((TG<sub>1-3</sub>)<sub>n</sub>). (C) Telomere VR sequence analysis of *est1Δ/EST1* zygotes. Recipient *est1Δ* cells were mated with *EST1* cells for 3 h. Telomere VR was PCR amplified, cloned, sequenced and analysed for sequence divergence as described in the Methods. Individual telomeres are represented by vertical bars. The red bars indicate the telomeric region that is non-diverging; the blue bars indicate the telomeric region in which the sequence diverges from the sisters. Data are pooled from experiments published in Teixeira *et al* (2004). (D) Telomere VR sequence analysis of *tel1Δ/tel1Δ est1Δ/EST1* zygotes. Recipient *tel1Δ est1Δ* cells were mated with *tel1Δ EST1* cells for 3 h and the sequences of the tagged telomere VR were analysed. Results from four individual matings are pooled. (E) Telomere VR sequence analysis of *mec1Δ/mec1Δ tlc1Δ/TLC1* zygotes. Recipient *mec1Δ tlc1Δ* cells were mated with *mec1Δ TLC1* cells for 3 h and the sequences of the tagged telomere VR were analysed. Results from five individual matings are pooled. (F) Frequency of telomere VR extension as a function of telomere length. Sequences obtained from (C) and (D) were ordered according to non-diverging telomere size (as shown in the graphs in (C) and (D)) and pooled into subgroups each containing 15 telomeres. The frequency of elongation in each subgroup was calculated and plotted as a function of telomere length. The grey curve (wild type; wt) describing diverging events at telomere VR in an *est1Δ/EST1* zygote (otherwise wild-type cells) was pre-established (Teixeira *et al*, 2004). STEX, single telomere extension analysis.



**Fig 2** | Single telomere extension analysis at native telomere IL in *tel1Δ* and *mec1Δ* cells. (A) Schematic of native telomere IL (Tel IL). The telomeric X element combinatorial repeat region contains repeats of the D, C, B and A types, as well as Tbf1-binding sites. (B) Telomere IL sequence analysis of *est1Δ/EST1* zygotes. Data are pooled from experiments published in Teixeira *et al* (2004). Methods and labelling are as described in Fig 1. (C) Telomere IL sequence analysis of *tel1Δ/tel1Δ est1Δ/EST1* zygotes. *tel1Δ est1Δ* cells were mated with *tel1Δ EST1* cells lacking the subtelomeric region of telomere IL. Results from four individual matings are pooled. The DNA samples analysed here for telomere IL are the same as in Fig 1C for telomere VR. (D) Telomere IL sequence analysis of *mec1Δ/mec1Δ tlc1Δ/TLC1* zygotes. Results from three individual matings are pooled. (E) Frequency of telomere IL extension as a function of telomere length. Analysis was performed as in Fig 1E. STEX, single telomere extension analysis; wt, wild type.

frequency of elongation (24% for all analysed telomeres) was similar to that observed at telomere VR, although the mating efficiency in the IL experiments was lower (70%). In addition, a significant inverse correlation between divergence events and telomere length was observed at telomere IL (*mec1Δ/mec1Δ tlc1Δ/TLC1*:  $R = -0.84$ ,  $P_{\text{pearson}} = 0.009121891$ ;  $P = -0.75$ ,  $P_{\text{spearman}} = 0.03675595$ ; Fig 2E). Notably, elongation frequency was lower at longer telomeres in *mec1Δ* than in wild type, which cannot be solely explained by lower mating efficiency. This lower overall extension frequency could explain the shorter telomere phenotype of *mec1Δ* cells.

### Tbf1 tethering provides a backup counting mechanism

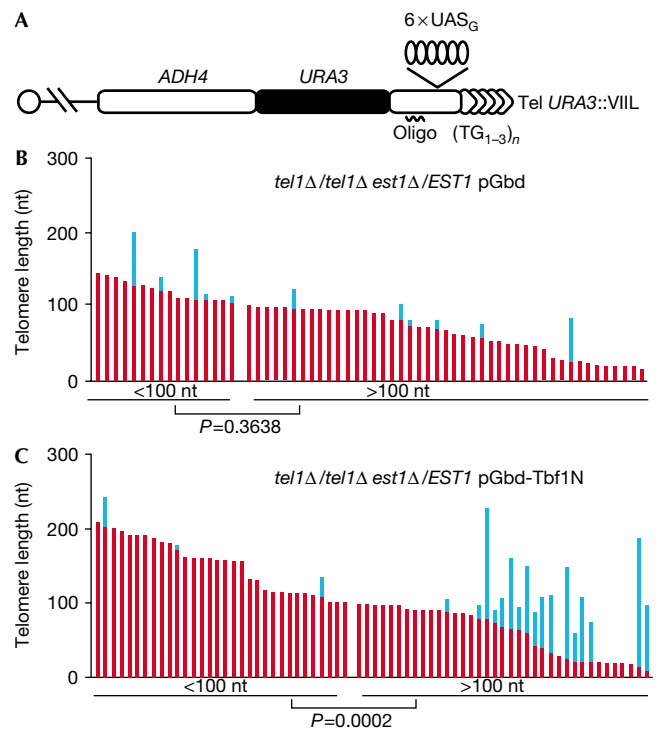
A Rap1-independent telomere length-sensing mechanism, which relies on the presence of subtelomeric DNA bound by Tbf1, was shown in *tel1Δ* cells (Koering *et al*, 2000; Alexander & Zakian, 2003; Brevet *et al*, 2003; Berthiau *et al*, 2006). The ADE2-tagged VR telomere lacks subtelomeric elements and therefore is presumably not bound by Tbf1. To test whether tethering of Tbf1 to a subtelomere-less chromosome end could restore preferential elongation of short telomeres in *tel1Δ* cells, we used plasmids from the Gilson laboratory that express the amino terminus of Tbf1 fused to the Gal4-DNA-binding domain (Gbd-Tbf1N). Effects of expression and telomeric tethering of Gbd-Tbf1N fusion protein or Gbd alone were tested by STEX analysis of a tagged telomere VIII L containing six subtelomeric Gbd-binding sites (Fig 3A). Consistent with the analysis of tagged VR, telomerase did not preferentially elongate short tagged VIII L in *tel1Δ* cells expressing the Gbd domain (Fig 3B). However, on expression of Gbd-Tbf1N in *tel1Δ* cells, short tagged VIII L telomeres were again preferentially elongated over long telomeres (Fig 3C). This analysis therefore shows that Gbd-Tbf1N provides a Tel1-independent mechanism to activate telomerase at short telomeres.

### Telomerase activity requires Tel1 or Mec1

Strains deleted simultaneously for *TEL1* and *MEC1* show an ever shorter-telomere phenotype and senesce (Ritchie *et al*, 1999). It was proposed that the presence of telomerase could delay senescence in *tel1Δ mec1Δ* strains, suggesting residual telomerase activity at telomeres in the absence of both kinases (Chan *et al*, 2001). We analysed three independent *tel1Δ mec1Δ* mutant spores after sporulating heterozygous mutants, and checked for telomere sequence divergence at telomere VR after 29 generations of clonal expansion. The results are pooled in supplementary Fig S2 online. Of 53 telomeres 7 showed divergence (13.2%). This frequency is only slightly higher than the recombinational background seen in *tel1Δ est1Δ* or *mec1Δ tlc1Δ* cells (see above). A slightly elevated recombination frequency in *mec1Δ tel1Δ* cells is consistent with a previous report addressing mitotic recombination and telomeric fusion events (Craven *et al*, 2002). Hence, these results show that telomerase cannot extend telomeres in the absence of Mec1 and Tel1 kinases.

### DISCUSSION

The mechanisms by which telomerase is preferentially activated at short telomeres have been poorly defined. In this study, we have shown that Tel1 is a crucial mediator of this control. Indeed, at an artificial telomere lacking subtelomeric repeats, telomerase was unable to distinguish short from long telomeres in the absence of the Tel1 kinase. Thus, it seems that Tel1 recognizes short telomeres and mediates their preferential elongation by telomerase. Mec1 has a less pronounced but still important role in the



**Fig 3** | Tethering of the Tbf1 amino-terminal domain to the subtelomere restores preferential elongation of short telomeres on tagged telomere VIII L in *tel1Δ* cells. (A) Schematic of tagged telomere VIII L. Six UAS<sub>G6</sub> sites are present between the *URA3* reporter gene and the telomeric tract ((TG<sub>1-3</sub>)<sub>n</sub>). (B) Telomere VIII L sequence analysis of *tel1Δ/tel1Δ est1Δ/EST1* diploids expressing Gbd alone. *tel1Δ est1Δ* pGbd cells were grown and mated with *tel1Δ EST1* cells. Diploids were propagated for five generations after initial mating, and then telomeres were analysed for sequence divergence. Results from three individual matings are pooled. (C) Telomere VIII L sequence analysis of *tel1Δ/tel1Δ est1Δ/EST1* diploids expressing Gbd-Tbf1N fusion. *tel1Δ est1Δ* pGbd-Tbf1N cells were grown and mated with *tel1Δ EST1* cells. Diploids were propagated for five generations after initial mating, and then telomeres were analysed for sequence divergence. Results from three individual matings are pooled. Gbd, Gal4-DNA-binding domain.

regulation of telomerase activation; the underlying mechanisms are not yet known. Interestingly, Tel1 can phosphorylate Cdc13 *in vitro* (Tseng *et al*, 2006); thus, it is conceivable that Tel1 regulates the telomerase recruitment step. Consistent with this idea is the recent finding that Tel1 is required for normal levels of Est1 and Est2 association with telomeric DNA (Goudsouzian *et al*, 2006). In addition, it has been recently shown that short telomeres are more avidly bound by Est1, Est2 and Tel1 (Bianchi & Shore, 2007; Sabourin *et al*, 2007).

Curiously, the extension frequency at a natural telomere was still regulated in a length-dependent manner even in the absence of *TEL1*. This shows a crucial difference between the ADE2-tagged telomere VR and the natural telomere IL. We have shown using the *URA3*-tagged telomere VIII L that tethering of Gbd-Tbf1N, but not Gbd alone, can rescue the *TEL1*-deletion phenotype, with regard to the activation of telomerase at short telomeres. Interestingly, the Tbf1-mediated mechanism seems active only

upon *TEL1* deletion, as changing the number of 5'-TTAGGG-3' repeats internally to yeast telomeric repeats influenced telomere length in *tel1Δ* but not in wild-type cells (Brevet *et al*, 2003). We estimate that the Tbf1-mediated backup mechanism might be important for the efficient healing of telomeres that have accidentally lost most or all of the telomeric repeats and Rap1-binding sites, either owing to a double-strand break near the base of the telomere or replication fork collapse. Indeed, it is well established that telomeric DNA provides a stumbling block for the semi-conservative DNA replication machinery (for example, Cooper *et al*, 1997; Ivessa *et al*, 2002; Crabbe *et al*, 2004), and entire telomere tract loss might occur at a low but significant frequency.

## METHODS

**Single telomere extension analysis (STEX).** Yeast cells were mated as described previously (Teixeira *et al*, 2004). Mating efficiency was measured using a spot dilution assay on synthetic media to select for growth of recipient, donor or mated cells, or by plating a mixture of approximately 200 recipient or mated cells on plates selective for recipients, and then replica plating them on double selective media, specific for mated cells. To determine the effects of Gbd-Tbf1N tethering to the subtelomere in *tel1Δ* cells, mated cells were grown in medium selective for diploids for five cell-cycles after initial mating, before sequence divergence was analysed at the tagged telomere VIII (Berthiau *et al*, 2006). These yeast strains mated with a lower efficiency (5–15%), but the selection of diploids for five generations allowed the analysis of cultures in which diploids prevailed, although preference for elongation of short versus long telomeres could still be distinguished. See the supplementary information online for further details and a description of yeast strains.

**Supplementary information** is available at *EMBO reports* online (<http://www.emboreports.org>).

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