Subtelomeric proteins negatively regulate telomere elongation in budding yeast

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The Tbf1 and Reb1 proteins are present in yeast subtelomeric regions. We establish in this work that they inhibit telomerase-dependent lengthening of telomere. For example, tethering the N-terminal domain of Tbf1 and Reb1 in a subtelomeric region shortens that telomere proportionally to the number of domains bound. We further identified a 90 amino-acid long sequence within the N-terminal domain of Tbf1 that is necessary but not sufficient for its length regulation properties. The role of the subtelomeric factors in telomere length regulation is antagonized by *TEL1* and does not correlate with a global telomere derepression. We show that the absence of *TEL1* induces an alteration in the structure of telomeric chromatin, as defined biochemically by an increased susceptibility to nucleases and a greater heterogeneity of products. We propose that the absence of *TEL1* modifies the organization of the telomeres, which allows Tbf1 and Reb1 to *cis*-inhibit telomerase. The involvement of subtelomeric factors in telomere length regulation provides a possible mechanism for the chromosome-specific length setting observed at yeast and human telomeres.

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Introduction

Telomeres prevent chromosome termini from being recognized as double-strand breaks and play a regulatory role in cell proliferation and survival (Blackburn, 2000b; Lundblad, 2000). They are also essential for various chromosome functions, including the regulation of gene expression, chromo-

some positioning and dynamics, recombination as well as for proper mitotic and meiotic divisions (Zakian, 1995; Chan and Blackburn, 2004).

Telomeres are generally composed of short tandem repeated DNA sequences (Blackburn, 1991), which are folded into nucleoprotein complexes that are reorganized during the cell cycle (Brunori *et al*, 2005). In budding and fission yeasts, these end-complexes, called telosomes, are characterized by their resistance to nuclease digestion in a pattern that is distinct from the bulk of nucleosomal chromatin (Wright *et al*, 1992; Cooper *et al*, 1998). The budding yeast end-complex requires an array of the essential repressor/activator protein 1 (Rap1) (Gilson and Gasser, 1995), which serves as a nucleation site for the assembly and spreading within the subtelomeric regions of a silent chromatin state (Perrod and Gasser, 2003). The natural yeast subtelomeric chromatin appears to juxtapose active and silent regions, reflecting a chromosome-specific combination of insulator and silencer elements (Fourel *et al*, 1999; Pryde and Louis, 1999). In vertebrates, telomeric nucleosomes present a heterochromatin-like profile of histone modifications (Garcia-Cao *et al*, 2004). In all organisms studied, telomeres are associated with a complex network of non-histone factors including chromatin, DNA repair and telomere-specific components (Brunori *et al*, 2005). At a higher level of organization, telomeres can fold into looped structures, which are likely to be critical for distinguishing natural chromosome ends from accidental double-strand breaks (Griffith *et al*, 1999). Finally, in lower eukaryotes such as yeast, telomeres are found as multitelomeric clusters, often located near the nuclear periphery (Gilson *et al*, 1993; Palladino *et al*, 1993; Gotta *et al*, 1996). The fact that telomeres combine both heterochromatin and telomere-specific features is consistent with their capacity to exert position effects (Gottschling *et al*, 1990) and with the involvement of epigenetic mechanisms in the maintenance of their structure (Sherman and Pillus, 1997; Surrallés *et al*, 1999; Maillet *et al*, 2001; Sadaie *et al*, 2003; Garcia-Cao *et al*, 2004; Perrini *et al*, 2004; Joseph *et al*, 2005).

In many organisms, telomerase, a specialized reverse transcriptase, compensates for the systematic shortening of telomeric DNA that occurs during the conventional replication of chromosomal DNA termini (Blackburn, 2000a). This enzyme uses an internal RNA template to add specific repeats to the terminal 3' overhang. The action of telomerase is cell-cycle regulated, occurring preferentially in late S/G2 but not G1 phase (Diede and Gottschling, 1999; Marcand *et al*, 2000). A switch between telomere extendible states, for example, accessible to telomerase, and non-extendible states determines the probability for the enzyme to act during one cell cycle (Teixeira *et al*, 2004).

The *in vivo* activity of telomerase is inhibited by specific components of the telomeric chromatin (Smogorzewska and de Lange, 2004). In *Saccharomyces cerevisiae*, the accessibility of a productive telomerase to its substrate is restricted proportionally to the number of Rap1–Rif1–Rif2 complexes

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assembled at the telomere, thus creating a negative feedback loop that determines the mean number of telomeric repeat sequences (Marcand *et al*, 1997; Cooper *et al*, 1998; Kanoh and Ishikawa, 2001; Levy and Blackburn, 2004; Teixeira *et al*, 2004). This mode of telomere length regulation was called a 'counting mechanism'. In cells carrying mutations that impair the Rap1-dependent counting, telomeres lengthen in an uncontrolled way (Kyriou *et al*, 1992; Wotton and Shore, 1997). Conversely, increasing the number of Rap1 or Rif1/Rif2 proteins bound in the immediate subtelomeric region induces a *cis*-shortening of the telomere relative to the number of bound proteins (Marcand *et al*, 1997; Ray and Runge, 1999b; Levy and Blackburn, 2004).

In cells lacking *TEL1* (a gene encoding a PI3-kinase involved in DNA damage response and telomere integrity), the mean telomere length is shorter than in wild-type cells and appears relatively insensitive to mutations that impair the Rap1-counting pathway (Craven and Petes, 1999; Ray and Runge, 1999a). In *tel1Δ* cells, the rate of elongation of a telomere that has been specifically shortened progressively declines upon the return to the equilibrium length (Brevet *et al*, 2003). Hence, a factor different from the Rap1–Rif1–Rif2 complex should limit telomere lengthening in *cis*. This process might be more potent in *tel1Δ* cells than in wild-type cells, thus explaining their shorter mean length. Such a notion is in agreement with the fact that the lack of *TEL1* does not directly affect telomerase activity, but appears to modify the structure of the end-complex in such a way that telomerase accessibility is impaired (Chan *et al*, 2001).

A distinctive feature of the Rap1-independent counting mechanism of telomere length regulation is its capacity to accommodate both yeast and vertebrate telomeric sequences. For example, in the absence of *TEL1*, vertebrate repeats inserted at the internal side of a single telomere are taken into account for telomere length regulation (Brevet *et al*, 2003). In *tlc1-h* cells, also known as humanized yeast (Henning *et al*, 1998), a telomere formed only by vertebrate repeats can be maintained at a regulated mean length (Alexander and Zakian, 2003; Brevet *et al*, 2003). It is noteworthy that, in *tlc1-h* cells containing a functional *TEL1* gene, the number of vertebrate repeats at a chromosome end is determined in a Rap1-independent manner, indicating that *TEL1* does not completely abolish the ability of vertebrate repeats to regulate telomere length. Overall, one can predict in budding yeast the existence of factors distinct from the Rap1–Rif1–Rif2 complex that are capable of binding yeast and vertebrate telomeric repeats and that inhibit in *cis* telomerase function.

In this study, we searched in budding yeast for factor(s) that upon their association to vertebrate telomeric repeats limit telomere elongation. We showed that the binding of Tbf1 to vertebrate repeats, a subtelomeric protein previously reported to act as a transcriptional insulator, shortens telomere length. Strikingly, we also discovered that Reb1, another insulator protein present in yeast subtelomeric regions, behaves similar to Tbf1 in terms of telomere length regulation.

Results

Subtelomeric DNA binding sites for Tbf1 or Reb1 are taken into account for length regulation in *tel1Δ* cells

We have previously described the ability of an array of 10 vertebrate telomeric repeats, inserted at a newly formed

URA3-tagged truncated 7L telomere beyond the *ADH4* gene (named hereafter Tel 7L^{tr}), to be taken into account for telomere length regulation in *tel1Δ* but not *TEL1* cells. Their presence leads to a *cis*-shortening of Tel 7L^{tr} (Brevet *et al*, 2003). Here, we investigate the nature of the factor(s) responsible for this effect. As Tbf1 binds to vertebrate repeats and DNA binding sites for Tbf1 are often combined with those for Reb1 in natural yeast subtelomeric regions (Fourel *et al*, 1999; Koering *et al*, 2000), we asked whether a DNA sequence containing three binding sites for either Tbf1 or Reb1 (named (Tbf1)₃ and (Reb1)₃) could recapitulate the *cis*-shortening activity of the vertebrate repeats. The sequence of these Tbf1 or Reb1 sites are designed in accordance with the results of a selection procedure using random oligonucleotide pools (Koering *et al*, 2000) and their specificity has been previously confirmed (Fourel *et al*, 1999). We note that the (Tbf1)₃ and (Reb1)₃ sequences do not exactly match the Tbf1 and Reb1 sites found in natural subtelomeric regions. These sites were inserted next to the distal TG₁₋₃ repeats at Tel 7L^{tr}. As a negative control, in the same location we also inserted a portion of the *RAD52* gene, which was previously shown to be neutral with regard to telomere length regulation (named NR) (Brevet *et al*, 2003) (Figure 1A).

In *TEL1* cells, there is shortening (roughly 40 nt) of the distal repeats for the (Tbf1)₃-containing telomere but not for those with either (Reb1)₃ or the control NR sequence (Figure 1B and C). The *tel1Δ* cells revealed a *cis*-shortening effect of the (Reb1)₃ insert, which is now similar to that of (Tbf1)₃ (Figure 1B and C). As expected, the NR control sequence does not lead to a *cis*-shortening in this context. The shortening effect of the non-telomeric Tbf1 and Reb1 sites used in these experiments appears to reflect the property of the sites found in natural subtelomeric regions. Indeed, natural sequences from the X and Y' subtelomeric elements containing multiple Tbf1 and Reb1 sites also lead to telomere shortening when inserted at Tel 7L^{tr} (data not shown). Overall, these results show that an array of DNA binding sites for Reb1 or Tbf1 behaves similar to vertebrate telomere repeats, being taken into account for length regulation in *tel1Δ* cells. Only Tbf1 binding sites appear to lead to *cis*-shortening in the presence of *TEL1*.

Identification of an allele of *TBF1* that alleviates the Tbf1-dependent telomere shortening

It is conceivable that additional proteins binding to vertebrate repeats or (Tbf1)₃, or some special features of these sequences themselves, and not the binding of Tbf1, were responsible for the length regulation effect. Therefore, we repeated the above experiments in cells expressing a viable allele of *TBF1* named *tbf1Δi*. This allele encodes a mutated form of Tbf1 lacking a sequence of 90 amino acids (Tbf1I) located within the transcriptional insulation domain of the protein (Tbf1N) (Fourel *et al*, 2001) (Figure 2A). Upon expression of Tbf1Δi instead of Tbf1, the (Tbf1)₃ element no longer downregulates telomere length in either *TEL1* or *tel1Δ* cells (Figure 1B and C). Importantly, the shortening effect of (Reb1)₃ in *tel1Δ* cells is only slightly affected in *tbf1Δi* cells. These results establish the specificity of *TBF1* toward the effect of (Tbf1)₃ on telomere length regulation.

Chromatin immunoprecipitation (ChIP) experiments show that the Tbf1Δi protein binds *in vivo* to vertebrate repeats similar to Tbf1 (Figure 2B). Neither Tbf1 nor Tbf1Δi binds to the (Reb1)₃ insert used in Figure 1, which is consistent with

Figure 1 The role of Tbf1 or Reb1 in telomere length regulation. (A) Structure of Tel 7L^{tr} and the endogenous *ura3-1* locus. *B* = BamHI; *E* = EcoRV, *H* = HindIII. (B) Examples of Southern blots used to measure the length of Tel 7L^{tr} containing either the NR control sequence, (TG₁₋₃)₂₃ or (T₂AG₃)₁₀ insert in different genetic settings, as indicated. The genomic DNA was digested with HindIII and EcoRV and hybridized with the URA3 probe shown in panel A. The reference band of 753 bp emanates from the *ura3-1* locus (see panel A) and the smear corresponds to the terminal restriction fragment that contains the distal yeast repeats. The calculated length of the distal (TG₁₋₃)_n is indicated below each lane. In order to obtain the length of the distal telomeric repeats, 800 nt was subtracted from the size of the measured terminal fragment, as shown below each lane. (C) Schematic representation of the results. The mean length corresponding to the average of at least three independent cultures is indicated on the right, together with the standard deviation. For an identical genetic setting, the part of the telomere corresponding to the distal repeats of Tel 7L^{tr} containing an NR control insert is boxed. As the NR sequence is not taken into account for length regulation, the boxed sequence is named the 'regulated length'. Thus, in Tel 7L^{tr} containing the yeast- or vertebrate-type inserts, the part of the chromosome end that is not taken into account for length regulation appears outside the box of the 'regulated length'. (D) *Xho*I Southern analysis of Y' telomeres (left part) and PCR analysis of Tel 1L (right part) of humanized yeasts containing a plasmid copy of either *TBF1* (pTBF1) or *tbf1Δi* (ptbf1Δi).

the idea that the effect of this sequence on telomere length is Tbf1 independent.

In addition to its role in length regulation, the Tbf1 domain is involved in the transcriptional properties of Tbf1:

namely, (i) it is necessary for the full transactivation activity of Tbf1, as revealed by a reduced capacity of vertebrate repeats to transactivate the expression of a UAS-less *CYC1''LacZ* reporter gene in *tbf1Δi* cells as compared to

Figure 2 Characterization of the *tbf1Δi* allele. (A) Representation of Tbf1 and Tbf1Δi proteins. (B) The binding of Tbf1 was assayed by ChIP analysis followed by real-time PCR. The results are expressed as the enrichment of the immunoprecipitated Tel 7L^{tr} DNA to that of an ASF2 DNA (see Materials and methods). The average value for at least three experiments is shown as well as the estimated standard deviation. (C) β-Galactosidase assay performed by a X-gal coloration of patches of cells corresponding to the indicated genetic setting and containing the plasmids pSX178, pSXHuTel2 and pSXYTel carrying the CYC1-lacZ reporter construct shown on the right (see Koering *et al*, 2000). (D) Transactivation assays for several hybrid proteins containing different parts of *TBF1*. The threshold of 3-AT concentration required to inhibit the *HIS3* reporter gene is indicated for each protein. (E) Antisilencing assay using the tethering of Gbd-Tbf1I between a telomere and a *URA3* reporter gene. As the FOA compound is toxic for cells expressing *URA*, the percentage of FOA-resistant cells reflects *URA3* repression.

TBF1 cells (Figure 2C); (ii) it exhibits a potent transactivation activity when fused to the Gal4 DNA-binding domain (amino acids 1–147, named Gbd) in a *HIS3*-based reporter assay (Figure 2D); (iii) it prevents silencing when tethered at an immediate subtelomeric region, in both *TEL1* and *tel1Δ* cells (Figure 2E).

We then asked whether the terminally located Tbf1 proteins present at humanized yeast telomeres can also play a role in length regulation. Although we have been unable to replace *TBF1* by *tbf1Δi* in humanized yeast, the presence of both *TBF1* and *tbf1Δi* reveals a dominant effect of the

mutant allele. This leads to lengthening of Y'-type, Tel 7L^{tr} and 1L telomeres (Figure 1D and data not shown). These results indicate that Tbf1 is a negative regulator of telomere length in the context of the humanized yeast and confirm the important role of the Tbf1I domain in this regulation.

Telomere length is decreased proportionally to the number of Gbd-Tbf1 and Gbd-Reb1 tethered proteins

We then asked whether the number of Tbf1 or Reb1 proteins assembled at a single subtelomere determines the length of the terminal repeats, as in the Rap1-dependent counting

mechanism of telomere length regulation. For this, we used a tethering procedure previously described to assay the counting effect of Rap1 (Marcand *et al*, 1997). Specifically, Gbd-Tbf1N, Gbd-Tbf1I and Gbd-Reb1N hybrids are expressed in a series of strains with increasing numbers of binding sites for Gal4 (two, four and six UAS_G) inserted at Tel 7L^{tr} (Figure 3A). As control, we used the Gbd-Rap1 hybrid containing the C-terminus of Rap1 (amino acids 653–827) (Marcand *et al*, 1997). The length of Tel 7L^{tr} in cells expressing these various hybrids was normalized to that in cells expressing Gbd alone. Because we noticed some telomere length variations in different clones derived from the transformation with the plasmid encoding the fusion proteins, we measured telomere length in at least five independent transformants grown for more than 50 generations. Importantly, we systematically determined the number of UAS_G sequences inserted at Tel 7L^{tr} by PCR analysis in all the clones tested for telomere length. We never observed a modification in the number of UAS_G in any of the genetic settings used in these experiments (data not shown).

As expected, in cells expressing a Gbd-Rap1 hybrid, the length of the targeted telomere is reduced proportionally to the number of UAS_G (Figure 3B). In *tel1Δ* cells expressing Gbd-Rap1, this reduction is much less pronounced, confirming that the counting effect of the C-terminal part of Rap1 is partially *TEL1* dependent (Brevet *et al*, 2003). In *TEL1* cells, the expression of Gbd hybrids with Tbf1N, Tbf1I or Reb1N does not significantly change the length of the terminal repeats, indicating that Reb1 and Tbf1 do not influence telomere length in *TEL1* cells (Figure 3B). By contrast, the expression of Gbd-Tbf1N and Gbd-Reb1N in *tel1Δ* cells leads to an incremental shortening of the terminal repeats proportional to the number of UAS_G, to the same extent as the shortening induced by Gbd-Rap1 in *TEL1* cells (Figure 3B). Importantly, the expression of Gbd-Tbf1I does not lead to

telomere shortening (Figure 3B). This result, together with the impaired capacity of Tbf1 DNA binding site to shorten telomere in *tbf1Δi* cells (Figure 1), shows that Tbf1I is necessary but not sufficient to downregulate telomere length. The length of the Y' telomeres in *tel1Δ* cells remains unchanged upon expression of the various Gbd hybrid proteins, showing that the effect of the hybrids is specific of the targeted Tel 7L^{tr} and does not correspond to a *trans* effect such as the decreased expression of the telomerase (data not shown). Overall, in *tel1Δ* cells, telomere length regulation becomes sensitive to the number of Tbf1 and Reb1 proteins bound at a subtelomeric location.

The *cis*-shortening due to Tbf1 can be uncoupled from telomeric position effect, short mean telomere length and MEC1-dependent function

We investigated the possibility that the *cis*-shortening induced by Tbf1 results from the derepression of the subtelomeric *URA3* gene. In *sir3Δ* cells, there is a complete loss of telomeric repression (Aparicio *et al*, 1991) and consequently a normal expression of *URA3* at Tel7L^{tr}. Cells carrying a deletion of *yku70* display a partial derepression albeit far more than that seen in *tel1Δ* cells (Boulton and Jackson, 1998; Gravel *et al*, 1998; Laroche *et al*, 1998; Nugent *et al*, 1998). In *sir3Δ* and *yku70Δ* cells, we observed that the insertion at Tel7L^{tr} of a stretch of vertebrate repeats, containing several Tbf1 binding sites, leads to a shortening of 19 and 12 nt, respectively (Figure 4B). This is consistent with the mild effect of (Tbf1)₃ in *TEL1* cells (see Figure 1). Importantly, this shortening effect is significantly less as compared to the potent effect of the vertebrate repeats in *tel1Δ* cells (–76 bp; Figure 4B). These results indicate that the role of Tbf1 in telomere length regulation can be uncoupled from a global telomere derepression.

As *TEL1* exhibits overlapping functions with its paralog *MEC1* in DNA-damage checkpoint and telomere regulation (Morrow *et al*, 1995), we asked whether *mec1Δ* could behave as *tel1Δ*, reinforcing the ability of vertebrate repeats to regulate telomere length. As the lethality owing to *mec1Δ* can be rescued by the deletion of *SML1*, we compared the effect of the vertebrate repeats in *sml1Δ* and *sml1Δ mec1Δ* cells (Figure 4B). In both cases, the vertebrate repeats are partially taken into account for length regulation but not at the level observed in *tel1Δ* cells (Figure 4B). Hence, *MEC1*-dependent functions do not counteract the ability of Tbf1 binding sites to act as telomere length regulators.

In conclusion, neither silencing alleviation (*sir3Δ* and *yku70Δ* cells) nor short mean telomere length (*yku70Δ* cells) nor checkpoint alteration (*mec1Δ sml1Δ* cells) is sufficient to recapitulate the effect of the *tel1Δ* genetic background regarding the ability of the vertebrate repeats to be taken into account for telomere length regulation.

The absence of *TEL1* leads to a more accessible conformation of the telomeric end-complex

We next looked for changes in chromatin and telomere structure that could account for the telomere length regulatory role of Tbf1. Using DNase I and micrococcal nuclease (MNase) sensitivity analyses, we failed to detect any major subtelomeric chromatin change associated with the presence of subtelomeric Tbf1 binding sites, in both *TEL1* and *tel1Δ* cells (Supplementary Figure 1). Then, we investigated

Figure 3 Tethering at an immediate subtelomeric location of Tbf1 and Reb1 N-terminal domains reduces telomere length. (A) Schematic representation of Tel 7L^{tr} and UAS_G sites. (B) The graphs represent, as a function of the number of UAS_G, the ratio of the mean length calculated from cells expressing the hybrid indicated above each graph to that from cells expressing the pGbd plasmid. The gray line represents experiments performed in *TEL1* cells, and the black line, in *tel1Δ* cells.

Figure 4 Absence of relationships between *SIR3*, *yku70* and *MEC1* and the *cis*-shortening induced by a subtelomeric stretch of vertebrate telomeric repeats. (**A**, **B**) Southern blots and schematic representation of the results as described in Figure 1.

whether the absence of *TEL1* modifies the structure of the end-complex. DNA was isolated from DNase I- and MNase-treated cell lysates and hybridized with a TG₁₋₃ or a Y' probe (Figure 5). The digestion rate of the TG₁₋₃ DNA is similar in *TEL1* and *tel1Δ* lysates, as indicated by the similar decrease of total TG₁₋₃ signal during the time course of the nuclease digestion (the measurements of total signals are not shown), indicating that the comparison was performed within a similar linear range for the degradation of total and TG₁₋₃ DNA.

The Southern blot analysis with a TG₁₋₃ probe is shown in Figure 5A. In *TEL1* cells, both DNase I and MNase produce a broad peak (between 550 and 300 bp) of TG₁₋₃ interacting DNA (Figure 5A, lanes 7–12 and 19–24). Under our experimental conditions, this DNA is resistant to nuclease treatment for about 10 min, which likely corresponds to the telosome structure described in earlier studies (Wright *et al*, 1992). In *tel1Δ* cells, both nucleases produce TG₁₋₃ DNA peaks, which have higher mobility (corresponding to the overall shorter telomeres in this strain) and are broader (between 500 and 200 bp) as compared to the *TEL1* samples (Figure 5A, compare lanes 1–6 to 7–12 and 13–18 to 19–24). Importantly, the signals in the TG₁₋₃ peaks of the *tel1Δ* samples are weaker despite the approximately equal intensity of the non-degraded TG₁₋₃ DNA at the top of the corresponding lanes. For example, after 5 min of DNase I treatment of *tel1Δ* lysate (Figure 5A, lane 3), 43% of the TG₁₋₃ signal is in

the end-complex peak, whereas in the *TEL1* lysate this signal represents 74% (Figure 5A, lane 9). A graphical comparison of the differences in the TG₁₋₃ signals at each time point is presented in Figure 5C. The images extend the notion of a broader and lower intensity TG₁₋₃ DNA peaks in the *tel1Δ* versus *TEL1* samples. Together, these results show a higher sensitivity of telomere DNA to nucleases in *tel1Δ* cells and suggest a more accessible conformation of the end-complex. The higher sensitivity of telomeres to nuclease digestion in *tel1Δ* is not a mere consequence of the shorter mean telomere length in this strain. In *yku70Δ* cells, the mean telomere length is comparable to *tel1Δ*, yet the *yku70Δ* telomeres have a nuclease resistance comparable to that of WT, rather than *tel1Δ* cells (Supplementary Figure 2). We also compared the patterns of degradation of the Y' subtelomeric DNA in *tel1Δ* and *TEL1* cells. The data are shown in Figure 5B. In contrast to telomere DNA, the Y' regions in both cells display little differences in sensitivity to both nucleases (Figure 5B, compare lanes 1–6 to 7–12 and 13–18 to 19–24), arguing that the difference in the end-complex between *tel1Δ* and *TEL1* cells does not extend into the subtelomeric regions of the chromosomes. This digestion pattern further supports the notion that the nucleosomal structure of subtelomeric DNA in *tel1Δ* cells is intact, as revealed by the analysis of Tel 7L^{tr} (Supplementary Figure 1).

Overall, the results with MNase and DNase I indicate a more accessible conformation of the end-complex in the

Figure 5 Structure of the telomeric end-complex and subtelomeric chromatin in *TEL1* and *tel1Δ* cells. (A) DNA was isolated from fresh cell lysates treated with DNase I or MNase and then analyzed by Southern blot with a TG_{1-3} probe. (B) DNA was isolated from fresh cell lysates treated with DNase I or MNase and then analyzed by Southern blot with a Y' probe. (C) Analysis of the profiles of the TG_{1-3} hybridizing DNA. The thick line represents the *tel1Δ* samples. The thin line represents the *TEL1* samples.

absence of *TEL1*. This altered organization of telomeric structure is likely to be related to the ability of Tbf1 to affect telomere length regulation.

The *cis*-shortening effect of Tbf1 requires telomerase expression

To determine whether the effects of Tbf1 were dependent on telomerase, we constructed diploid strains (*EST2/est2Δ TEL1/tel1Δ*) containing a Tel 7L^{tr} telomere with or without Tbf1 sites ($(T_2AG_3)_{10}$). The diploid strains were sporulated and the rate of telomere shortening at the Tel 7L^{tr} telomere was measured in cell cultures established from different spores. As expected, in cultures from spores lacking *EST2*, the median telomere length decreased linearly (Figure 6A). The degradation rates were similar in cultures from spores lacking *EST2* and from spores lacking *EST2* and *TEL1*, at Tel7L^{tr} or Tel7L^{tr}-(T_2AG_3)₁₀ (Figure 6B). Therefore, *tel1Δ* does not exacerbate the rate of telomeric DNA degradation caused by the loss of telomerase, even in the presence of subtelomeric

Tbf1 sites. It is noteworthy that the presence of Tbf1 sites appears to reduce telomere length heterogeneity with increasing generations (Figure 6A), as well as slightly reducing the degradation rate of the Tel 7L^{tr} (Figure 6B). A decrease in the degradation rate should lead to telomere lengthening in telomerase-positive cells and thus cannot account for the telomere shortening induced by Tbf1 in telomerase-positive cells. Overall, these data are consistent with the physical presence of Tbf1 at telomeres impairing the telomerase-mediated telomere elongation pathway. As the tethering of Tbf1 acts only on its proximal telomere but not on the other telomeres, we conclude that Tbf1 *cis*-inhibits telomerase without affecting the overall level of telomerase expression.

Discussion

In this work, we demonstrate that the subtelomeric proteins Tbf1 and Reb1 act as negative regulators of telomere length

Figure 6 Tbf1 shortening effect requires telomerase expression. (A) Representative Southern blots used to calculate the degradation rates shown in panel B. Spores were grown in a rich liquid medium. Genomic DNAs were digested with *EcoRV* and *HindIII* and probed with *URA3*. (B) Telomere length was plotted versus generations and telomeric degradation rates were calculated from linear regression curves. The values represent the average of at least two independent experiments.

(Figure 1). This activity can be revealed at engineered telomeres containing subtelomeric Tbf1 sites and at telomeres of humanized yeast, which contain terminally located Tbf1 binding sites. In particular, we show that the number of Tbf1 or Reb1 N-terminal domains present in a subtelomeric region is 'counted' to determine the overall length of the telomere (Figure 3). The ability of Tbf1 and Reb1 to regulate telomere length appears maximal in the absence of the *TEL1* gene, which encodes a kinase involved in the Rap1-dependent telomere length regulation pathway. We were unable to detect any change in the phosphorylation status of Tbf1 between *TEL1* and *tel1Δ* cells (A-S Berthiau and E Gilson, unpublished data). Confirming these results in another telomere setting, the tethering of Tbf1 and Reb1 at a remote subtelomeric location (> 23 kb) of Tel 6R triggers telomere shortening of the proximal telomere in *tel1Δ* but not *TEL1* cells (Hediger *et al*, 2006). Importantly, we found that the presence of subtelomeric Tbf1 sites in telomerase-negative cells does not exacerbate telomere shortening, showing that the *cis*-shortening effect of Tbf1 requires telomerase expression. Based on these results, we conclude that Tbf1 and Reb1 negatively regulate telomere length by *cis*-inhibiting telomerase in a Tel1- and Rap1-independent manner. As telomerase activity is linked to conventional replication (Chakhparonian and Wellinger, 2003), Tbf1 and Reb1 could interfere with replication. However, the telomeric replication fork pause

appears to be independent of these proteins (Makovets *et al*, 2004).

The *cis*-shortening effect of Tbf1 and Reb1 does not result from transcription activation

We identify a 90 amino-acid long sequence within the N-terminal domain of Tbf1, which is necessary but not sufficient for the length regulation properties of the protein. This portion of Tbf1, named the Tbf1I domain, exhibits both antisilencing and transactivation activities (Figure 2). This, together with the fact that the N-terminal domain of Reb1 also acts as a transcriptional activator and as an inhibitor of telomere elongation, raises the possibility that the subtelomeric factors couple transcription to telomere length regulation, as previously reported for a forced transcription into the telomeric tract that slightly shortens telomere length (Sandell *et al*, 1994). However, the telomere *cis*-shortening induced by Tbf1 and Reb1 cannot be explained by transcription or derepression of the telomeric region: (i) the Tbf1I domain fused to the DNA binding domain of Gal4 acts as a transcriptional insulator and as a much more potent transactivator than a similar construct containing the whole N-terminal domain of Tbf1 (Figure 2), whereas it is unable to down-regulate telomere length in *tel1Δ* cells by itself (Figure 3); (ii) a deletion of *SIR3*, leading to a total derepression of the subtelomeric region, neither recapitulates nor favors the *cis*-shortening effect of Tbf1 or Reb1 (Figure 4); (iii) Tbf1 binding does not alleviate silencing initiation but prevents the spreading of the silent state away from the telomere (Fourrel *et al*, 1999), suggesting that the telomere is still in a silent state; (iv) by sequence inspection, the DNA binding sites for Tbf1 and Reb1 are not associated with TATA boxes; (v) the remote tethering (> 23 kb) of Tbf1 or Reb1 at Tel 6R does not induce the derepression of a Tel 6R-associated reporter gene but leads to telomere shortening in *tel1Δ* cells (Hediger *et al*, 2006). Therefore, we conclude that the effects of Tbf1 and Reb1 on telomere length regulation do not simply result from transcription activation.

Tbf1 and Reb1 alter the nuclear organization of the telomere: a link to telomerase regulation

We noticed that in *tel1Δ* cells, the telomere end-complex appears less structured as demonstrated by its higher sensitivity to nuclease digestion (Figure 5). This structural alteration seems to be restricted at the very terminal part of the chromosome, as the chromatin structure of the immediate subtelomeric regions is not modified by the absence of *TEL1* (Figure 5 and Supplementary Figure 1). It does not seem that the shortening of the telomere *per se* is the cause, as similarly short telomeres in *yku70Δ* cells do not show this characteristic alteration of the telomere structure (Supplementary Figure 2). The fact that Tel1 has a direct bearing on the structure of the end-complex is in agreement with its direct association with yeast telomeres (Takata *et al*, 2004) and with the fact that the *TEL1* ortholog *ATM* is involved in the higher-order structure of mammalian telomeres (Pandita, 2001).

The less structured end-complexes present in *tel1Δ* yeasts could render the telomeres more sensitive to the action of additional factors involved in length regulation or to alterations in the subnuclear telomere organization. In agreement with the latter possibility, Tbf1 and Reb1 act as antianchoring factors in *tel1Δ* but not *TEL1* cells, pulling the telomere away

from the nuclear periphery (Hediger *et al*, 2006). Consequently, a tempting model is that Tbf1 and Reb1 counteract telomerase by moving the telomere away from a telomerase-competent compartment (Hediger *et al*, 2006).

Subtelomeric proteins as telomere length regulators

Our results suggest that length regulation at natural telomeres depends on the array of Tbf1 and Reb1 sites adjacent to the terminal repeats of many yeast telomeres. This could account for the telomere-specific sensitivity toward *TEL1* shown in yeast (Craven and Petes, 1999) and, if similar mechanisms exist in mammalian cells, for the chromosome-specific length profile of human telomeres (Graakjaer *et al*, 2004). The antagonism between *TEL1* and the telomeric role of subtelomeric factors suggests a new link between DNA damage checkpoint and telomere length control. For instance, *TEL1* activation upon DNA damage might modulate the contribution of subtelomeric regions to telomere length regulation. In both *TEL1* and *tel1Δ* backgrounds, we did not detect any significant difference between *TBF1* and *tbf1Δ* cells in the mean length of Y' telomeres or of individual X-linked telomeres (A-S Berthiau and E Gilson, unpublished observations). However, the capacity of subtelomeric factors to regulate telomere length is likely to have been underestimated in the *tbf1Δ* setting, as this allele leads to only a partial loss of function and does not interfere with the activity of Reb1, leaving open the possibility that redundant effects exist between Tbf1, Reb1 and possibly other subtelomeric factors.

Implications for telomere evolution

It is worth noting that Tbf1 belongs to a family of proteins possessing a very similar and evolutionarily related Myb-like DNA binding domain, including the authentic telomeric proteins TRF1 and TRF2 (Bilaud *et al*, 1996; Teixeira and Gilson, 2005). Interestingly, TRF1 and TRF2 also alter the telomerase pathway, in a seemingly hRAP1-independent manner for TRF1 (Ancelin *et al*, 2002; Loayza and De Lange, 2003) and through the binding of hRAP1 for TRF2 (Li and de Lange, 2003). What determines the relative contribution of each of these pathways at individual telomeres or between different cell types is not known. Interestingly, *ATM*, the human ortholog of *TEL1*, is defective in the cancer-prone disorder ataxia telangiectasia (A-T) and influences telomere length as well as higher-order organization of chromosome ends (Pandita, 2001). Furthermore, *ATM* is known to interact with both TRF1 (Kishi *et al*, 2001) and TRF2 (Karslender *et al*, 2004). As the deletion of *TEL1* converts yeast telomeres from a Rap1-dependent to a Rap1-independent mechanism of length regulation, telomere length defects in A-T patients might result from a modified balance between the TRF1 and TRF2 pathways.

In the light of the work presented here, Tbf1 in yeast should be considered not only as an evolutionary remnant

of an earlier telomere-binding protein but also as a functional telomeric factor. This, together with the involvement in telomere length regulation of the subtelomeric protein Reb1 and with the role of the subtelomeric regions in modulating telomeric silencing (Fourel *et al*, 1999) and nuclear telomere positioning (Hediger *et al*, 2006), led us to propose that the subtelomeric regions are a functional part of telomeres. Given that Tbf1 and Reb1 behave as essential regulatory factors for gene expression at non-telomeric loci and that their transactivation domains are also involved in length regulation, these subtelomeric proteins might have been conserved through essential non-telomeric functions, while preserving their capacity to regulate telomere structure.

Materials and methods

Strains and constructs

The engineer Tel 7L^{tr} telomeres were previously described (Marcand *et al*, 1997; Fourel *et al*, 1999; Brevet *et al*, 2003). The pRS314-*tbf1Δ* plasmid results from the replacement of an *EcoRI*-*SacI* fragment of pRS314-TBF1 (Brigati *et al*, 1993) by a PCR fragment generated by Tbf1a (5'-AGTATCGAATTCAGAAAACCAAGCG) and Tbf1b (5'-AAAATTGAGCTCTGTTCATTTAAGCG) digested with *EcoRI* and *SacI*. In order to shuffle to *tbf1Δ* allele, first the genomic *TBF1* gene was disrupted by a *HIS3* insertion in W303-1A derivatives containing pRS316-TBF1 and then the pRS314-*tbf1Δ* plasmid was introduced and cells having lost pRS316-TBF1 were screened.

Assays for telomeric structure and composition

Telomere blotting and telomere length measurement were performed as described by Brevet *et al* (2003). The humanized telomere from Tel1L was PCR amplified according to a previously described method (Forstemann *et al*, 2000).

ChIP was performed, as described previously (Schramke *et al*, 2001), with a polyclonal antibody designed against the N-terminal domain of *TBF1* (Bilaud *et al*, 1996). Real-time quantitative PCR was realized, as described by Brevet *et al* (2003).

Analysis of chromatin structure

Spheroplasts were suspended in nuclease buffer (30 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM CaCl₂, 100 mM NaCl, 0.1 mg/ml BSA, 2 μg/ml pepstatin, 2 μg/ml aprotinin, 2 μg/ml leupeptin). MNase (Worthington) or DNase I (Sigma) was added at 50 or 12.5 U/ml final concentration, respectively. Aliquots were removed after 0, 1, 2.5, 5, 10 and 20 min and immediately mixed with STOP solution (3.5 M NaCl, 1.4% SDS, 100 mM EDTA, 100 mM EGTA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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