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Telomerase recruitment by the telomere end binding protein-β facilitates G-quadruplex DNA unfolding in ciliates

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The telomeric G-overhangs of the ciliate *Stylonychia lemnae* fold into a G-quadruplex DNA structure *in vivo*. Telomeric G-quadruplex formation requires the presence of two telomere end binding proteins, TEBPα and TEBPβ, and is regulated in a cell-cycle dependent manner. Unfolding of this structure in S phase is dependent on the phosphorylation of TEBPβ. Here we show that TEBPβ phosphorylation is necessary but not sufficient for a G-quadruplex unfolding rate compatible with telomere synthesis. The telomerase seems to be actively involved in telomeric G-quadruplex DNA structure unfolding *in vivo*. Significantly, the telomerase is recruited to telomeres by phosphorylated TEBPβ, and hence telomerase recruitment is cell-cycle regulated through phosphorylation. These observations allow us to propose a model for the regulation of G-quadruplex unfolding and telomere synthesis during the cell cycle.

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Telomeres are nucleoprotein complexes at the termini of linear eukaryotic chromosomes. Their main functions are to prevent chromosomal ends from being recognized as sites of DNA damage, to contribute to the overall nuclear architecture and to ensure correct replication of chromosomal ends[1]. Telomeric DNA consists of a tandem arrangement of a short sequence repeat containing a guanine-rich strand, oriented 5′ to 3′ toward the chromosome end. The protrusion of the guanine-rich strand (G-overhang) is a feature conserved from simple eukaryotes to vertebrates[2,3].

The macronuclear DNA of stichotrichous ciliates, such as *Stylonychia lemnae,* exists as short nanochromosomes, each capped by telomeres, terminating in a G-overhang consisting of 16 nucleotides $(T_4G_4T_4G_4)^2$. The G-overhangs recruit two OB-fold–containing telomere end binding proteins, TEBPα and TEBPβ, forming a protective capping structure[4–6]. Whereas TEBPα binds sequence specifically to the single-stranded form of the G-overhang, TEBPβ is able to bind to the telomere only via a G-overhang–dependent heterodimerization with TEBPα[7,8]. These ciliate telomere end binding proteins have homologs in higher eukaryotes, suggesting that G-overhang binding and telomere protection is more conserved than was previously thought. The *Schizosaccharomyces pombe* and vertebrate POT1 is a homolog of TEBPα[9–12], whereas the POT1-interacting factor, TPP1 (refs. 13,14), shares partial sequence and structural homology with TEBPβ, and, like TEBPα and TEBPβ, POT1 and TPP1 bind to the G-overhang as a heterodimer[15,16]. Notably, human TPP1 has been shown to have a role in recruiting the telomerase to telomeres[15,16].

Two different higher-order DNA structures have been proposed to provide telomere protection. For vertebrate telomeres, evidence suggests the presence of a lasso-like structure called the t-loop, which is formed by the telomeric single-stranded G-overhang invading the double-stranded region of telomeric DNA[17–19]. For ciliates, recent *in vivo* evidence showed that the telomeric G-overhangs of macronuclear DNA in *S. lemnae* fold into a G-quadruplex DNA structure[20,21]. Numerous *in vitro* studies have shown that sequences containing runs of guanines have the inherent propensity to fold intra- or intermolecularly to form G-quadruplexes at physiological ionic conditions[22,23]. The first *in vivo* evidence for the presence of G-quadruplexes at telomeres came from the use of high-affinity, single-chain antibodies selected to be specific for *S. lemnae* telomeric G-quadruplex DNA structures[20]. The unusual genome organization of the ciliate *S. lemnae,* in which about 2×10^8 telomeres are present in one macronucleus, permits the presence of G-quadruplexes to be observed by *in situ* immunostaining. Subsequently, we discovered that the two TEBPs have separate, but essential, roles in G-quadruplex formation[21]. TEBPα is involved in both attaching telomeres to a subnuclear structure and recruiting TEBPβ to telomeres. The presence of TEBPβ is essential for G-quadruplex formation, and this specifically involves the C-terminal domain of TEBPβ, as suggested by earlier *in vitro* studies[24]. This C-terminal domain contains two putative phosphorylation sites[25].

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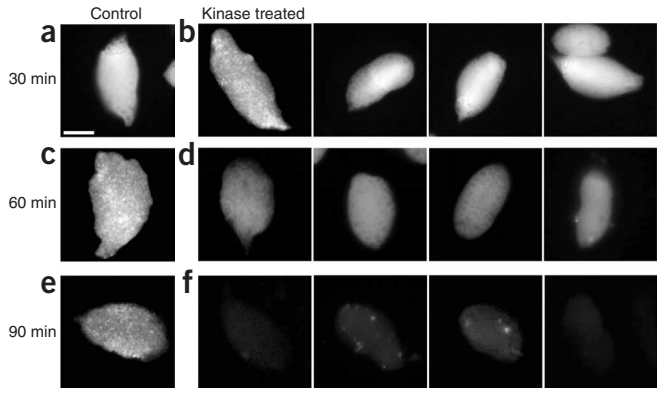

Figure 1 Phosphorylation of TEBPβ is not sufficient for resolving telomeric G-quadruplexes fast enough for telomere synthesis. (**a**,**c**,**e**) *S. lemnae* control unphosphorylated macronuclei. (**b**,**d**,**f**) Macronuclei phosphorylated with Cdk2 after 30 min (**b**), after 60 min (**d**) and after 90 min (**f**) of phosphorylation. Immunostaining was done using the Sty49 anti–G-quadruplex antibody. Scale bar represents 10 μm.

During DNA replication and telomere synthesis, which in the ciliate macronucleus takes place in a morphologically distinct region known as the replication band[26,27], G-quadruplexes are lost. G-quadruplex unfolding correlates with phosphorylation of TEBPβ in S phase[21].

Whereas the conventional DNA-replication machinery replicates the main part of telomeric DNA, the distal part is synthesized by the telomerase in S phase[28–30]. The telomerase enzyme consists of an RNA subunit (TR) that encodes the template for the telomere repeat sequence and a protein subunit (TERT) that contains the reverse-transcriptase catalytic activity[31]. Synthesis of telomeric DNA by the telomerase requires that the 3′ end of the telomeric G-overhang be single stranded to be available for base-pairing with a segment of the TR template region. *In vitro* experiments have shown that G-quadruplexes inhibit telomerase-dependent telomere elongation[32,33]. Therefore, the unfolding of G-quadruplexes would be expected to be a prerequisite for the telomerase to gain access to the telomeric end. Single-stranded DNA binding proteins such as POT1[11,34] OK in combination with helicases[23] have been implicated in disrupting G-quadruplexes during telomerase-dependent telomere elongation[35–37]. Our recent results have shown that telomeric G-quadruplexes are resolved during S phase, indicating that this is an essential event for telomere synthesis to proceed *in vivo*[21]. However, a detailed *in vivo* analysis of the cell cycle–dependent regulation of telomere conformation is still lacking.

Here we aimed to provide an understanding of how the G-quadruplex DNA structure present at *S. lemnae* telomeres is resolved *in vivo*. We find that the cell cycle–dependent phosphorylation of TEBPβ is necessary, but not sufficient, to unfold telomeric G-quadruplexes fast enough for end replication to proceed. We provide new evidence that the telomerase is recruited to telomeres by phosphorylated TEBPβ in S phase and that telomerase recruitment results in G-quadruplex unfolding.

RESULTS

TEBPβ phosphorylation is insufficient for G-quadruplex unfolding

In vitro, G-quadruplex DNA structures exist in a slow equilibrium between the exceedingly stable G-quadruplex and the unfolded, single-stranded form; therefore, spontaneous unfolding is slow[22]. The important question for biology is: how do telomeric G-quadruplexes unfold fast enough to permit telomere synthesis? Calculations indicate that on the order of 2.5×10^3 nanochromosomes need to be replicated per second in *S. lemnae* macronuclei, and hence the G-quadruplex unfolding rate must be rapid. Because G-quadruplex unfolding correlates with the phosphorylation of TEBPβ[21], we first investigated whether phosphorylation affects the unfolding rate of telomeric G-quadruplexes *in vivo*.

Isolated *S. lemnae* macronuclei were phosphorylated *in vitro* by the cyclin-dependent kinase Cdk2 for increasing lengths of time, with phosphorylation being complete at 30 min, as described[21]. Cdk2-treated macronuclei were fixed and immunostained with an antibody specific for the antiparallel G-quadruplex DNA structure[20,21]. About 50 macronuclei were analyzed for each time point. No difference in immunostaining was observed between control nuclei (**Fig. 1a**) and nuclei that had been incubated with Cdk2 for 30 min (**Fig. 1b**). After 60 min, a slight decrease in the intensity of immunostaining was observed (**Fig. 1c**,**d**). Only after 90 min of phosphorylation was there a substantial loss of G-quadruplex immunostaining, and hence G-quadruplex unfolding is slow (**Fig. 1e**,**f**). These results show that phosphorylation of TEBPβ *per se* does not result in a G-quadruplex unfolding rate that would be compatible with telomere synthesis, strongly suggesting that G-quadruplex unfolding *in vivo* must be aided by additional factors.

Phosphorylated TEBPβ is evicted from telomeres during S phase

To investigate the mechanism of telomeric G-quadruplex unfolding and telomere synthesis, we asked which proteins are bound to telomeres during replication and whether the composition of the telomeric complex is affected by phosphorylation of TEBPβ. We have previously established that both TEBPs, telomeric DNA and the telomerase are present in the replication band of *S. lemnae* macronuclei[21,27]. We have also shown that telomeric DNA and both TEBPs can be electroeluted from the replication band, and hence they have to become detached from a subnuclear structure during replication[21,26]. Synchronized cells in S phase that were embedded in agarose were electroeluted as previously described[21,38], and the eluant was analyzed by gel filtration (Methods). In this fractionation, DNA and DNA-bound proteins elute in the void volume, whereas unbound proteins are retained in the column and elute in later fractions[26]. Analysis of the column fractions by denaturing gel electrophoresis and western blot showed clearly that TEBPα comigrated with the DNA peak (**Fig. 2a**,**b**, fraction 3), whereas TEBPβ was retained on the column (**Fig. 2b**, fraction 12). This result shows that during telomere synthesis TEBPβ becomes detached from the G-overhang, whereas TEBPα remains bound. The number of TEBPα molecules bound per G-overhang during replication, calculated from the concentration of TEBPα and the optical density OD at 260 nm (OD_{260}) of the DNA of the column fractions, was found to be close to one (**Supplementary Fig. 1** online), in agreement with previous conclusions[39].

As phosphorylation of TEBPβ coincides with the loss of telomeric G-quadruplexes during DNA replication and telomere synthesis[21] and detachment of TEBPβ from telomeres *in vivo* (**Fig. 2a**,**b**), we next investigated whether phosphorylation is sufficient to cause the detachment of TEBPβ from the telomeric complex. Both phosphorylation sites in the C-terminal domain of TEBPβ are indeed phosphorylated *in vivo* in S phase (**Supplementary Fig. 2** online). To test the effect of phosphorylation on the stability of the telomeric complex, recombinant TEBPα and TEBPβ were assembled on a model telomere (consisting of a 20-nucleotide, double-stranded region and a 16-nucleotide, single-stranded G-overhang)[21] and, subsequently, the

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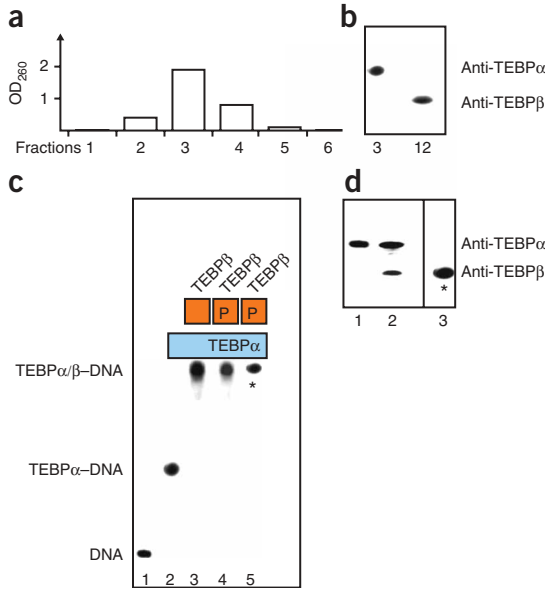

Figure 2 Telomerase displaces phosphorylated TEBPβ from telomeres during replication. (**a**,**b**) Telomeres undergoing synthesis were electroeluted from the replication band in S phase and subsequently fractionated on a Sepharose 2B column. (**a**) The DNA concentration (OD_{260}) of each fraction is shown. (**b**) Western blot of the column fractions using anti-TEBPα and anti-TEBPβ antibodies. For an estimate of the number of bound TEBPα molecules per telomere, see **Supplementary Figure 1**. (**c**) Phosphorylation of the already preformed heterodimer does not disrupt the telomeric complex *in vitro*: lane 1, naked model telomere; lane 2, telomere incubated with TEBPα; lane 3, telomere incubated with TEBPα and TEBPβ lanes 4 and 5, telomere incubated with TEBPα and TEBPβ and phosphorylated with Cdk2 after assembly of the telomeric complex. In lanes 1–4, the telomeric DNA (1 μM) was labeled with [$\gamma-^{32}$P]ATP. In lane 5, TEBPβ was phosphorylated with [$\gamma-^{32}$P]ATP (indicated by a star), and the DNA was unlabeled. (**d**) Telomerase is required to displace phosphorylated TEBPβ from the telomeres. TERT expression was knocked down by RNAi, and cells were synchronized and pulse labeled in S phase with [$\gamma-^{32}$P]ATP. The telomeric complex was electroeluted, fractionated and analyzed as described in **a**. Western blots of column fractions using anti-TEBPα and anti-TEBPβ antibodies: lane 1, control cells; lane 2, protein content after silencing TERT expression; lane 3, autoradiograph of lane 2. The star denotes phosphorylated TEBPβ.

complex was fully phosphorylated with Cdk2 *in vitro* (**Supplementary Fig. 2**). **Figure 2c** (lanes 4 and 5) shows that, once the TEBPβ has bound to the TEBPα–G-overhang complex, subsequent phosphorylation is not sufficient to cause the disruption of the telomeric complex. Only after 3 d did we observe that a small amount of phosphorylated TEBPβ had become detached from the complex (data not shown). In contrast, prephosphorylation of either of the two phosphorylation sites present in TEBPβ prevents complex formation (**Supplementary Fig. 3** online).

As phosphorylation *per se* of TEBPβ did not result in its dissociation from the preformed telomeric complex *in vitro* in a timely manner (**Fig. 2c**), and as TEBPβ was not bound to the telomeric complex during telomere synthesis (**Fig. 2a**,**b**, and **Supplementary Fig. 1**), we next investigated whether a component of the telomerase could be involved in displacing TEBPβ from telomeres *in vivo*. The expression of TERT or TR was silenced by RNA inhibition (RNAi)[21,40]; inhibition of expression was observed in more than 90% of the analyzed cells (**Supplementary Fig. 4** online), with maximal silencing obtained by feeding *S. lemnae* cells *Escherichia coli* expressing double-stranded RNA (dsRNA) targeted against TERT or TR for 4 d. At that time, there was no detectable loss of telomeric DNA, as determined by fluorescence *in situ* hybridization (FISH) or Southern analyses of total macronuclear DNA using telomeric DNA as a probe (data not shown).

To visualize the phosphorylation state of TEBPβ *in vivo*, *S. lemnae* cells were synchronized and pulse labeled with [$\gamma-^{32}$P]ATP during S phase[21], and telomeric complexes were purified as described above. Western blot analysis of the column fractions showed that, in cells in which TERT expression had been silenced, both TEBPβ and TEBPα copurified with telomeric DNA (**Fig. 2d**, lanes 1 and 2), in contrast with control cells, where only TEBPα copurified with telomeric DNA (**Fig. 2a**,**b**). We obtained the same result after silencing of TR (data not shown). Thus, inhibition of an active telomerase prevented the displacement of TEBPβ from telomeres. Autoradiography of the gel confirmed that the telomere-bound TEBPβ is indeed phosphorylated (**Fig. 2d**, lane 3). These results, together with the *in vitro* observation that phosphorylation *per se* is not sufficient for disruption of the telomeric complex, lead us to conclude that the telomerase, or a telomerase-associated factor, is required to displace TEBPβ from the telomeric complex during telomere synthesis.

Telomerase is involved in G-quadruplex unfolding *in vivo*

An obvious question that arises is whether the telomerase could be involved in telomeric G-quadruplex unfolding. We found that, in >95% of more than 50 macronuclei analyzed, silencing of either TR or TERT expression resulted in an accumulation of G-quadruplexes in the replication band (**Fig. 3**). This observation, made after 4 d of RNAi silencing, at which time there is no detectable loss of telomeric DNA (see above), was confirmed by the visible accumulation of telomeric G-quadruplexes during replication. As a control, the expression of proliferating cell nuclear antigen (PCNA), an important component of the replication machinery[41], was also knocked down using RNAi (**Fig. 3c** and **Supplementary Fig. 4f**): the total absence of G-quadruplexes in the replication band showed that the DNA-replication machinery *per se* was not involved in G-quadruplex unfolding. Thus, removal of either of the telomerase subunits apparently prevents the unfolding of telomeric G-quadruplexes, suggesting that the telomerase, or a telomerase-associated protein, is involved in the unfolding of telomeric G-quadruplexes *in vivo*.

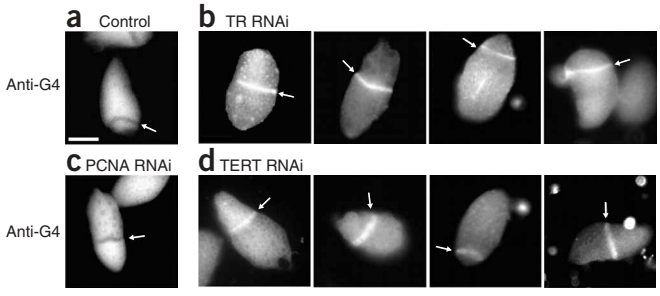

Figure 3 Telomerase is involved in telomeric G-quadruplex unfolding *in vivo*. *In situ* immunostaining was done using the Sty49 anti–G-quadruplex antibody (anti-G4). (**a**) Control macronuclei. (**b**) Macronuclei in which TR expression was knocked down by RNAi. (**c**) Macronuclei in which PCNA expression was knocked down by RNAi. (**d**) Macronuclei in which TERT expression was silenced by RNAi. Arrows point to the replication band. Scale bar represents 10 μm.

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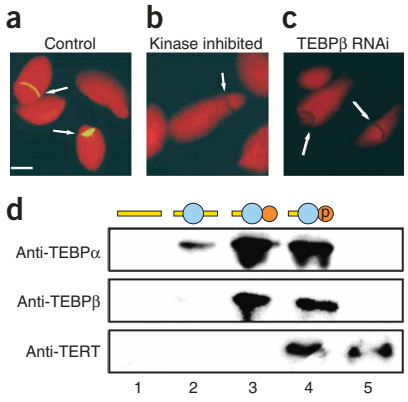

Figure 4 Phosphorylated TEBPβ recruits the telomerase to the telomeres. (**a**–**c**) Visualization of the telomerase by FISH analyses using TR as a probe (FISH-TR). (**a**) Control macronuclei. (**b**) Macronuclei in which phosphorylation was inhibited with 10 μM butyrolactone. (**c**) Macronuclei in which TEBPβ expression was knocked down by RNAi. Arrows point to the replication band. The macronuclear DNA was stained with DAPI. Scale bar represents 10 μm. (**d**) Co-immunoprecipitation experiments show that the telomerase interacts with telomeres via phosphorylated TEBP β. Telomeric complexes of different compositions (shown above as cartoons) were incubated with macronuclear extract containing telomerase and co-immunoprecipitated with the anti-TEBPα antibody. Lane 1, naked telomeric DNA; lane 2, telomeric DNA bound by TEBPα; lane 3, telomeric DNA bound by TEBPα–TEBPβ; lane 4, phosphorylated telomeric TEBPα–TEBPβ complex; lane 5, macronuclear telomerase extract used as a loading control. The precipitates were analyzed by 12.5% (w/v) SDS-PAGE and probed with anti-TEBPα, anti-TEBPβ and anti-TERT antibodies.

Phosphorylated TEBPβ recruits telomerase to telomeres

As telomeric G-quadruplex structure unfolding requires both phosphorylation of TEBPβ[21] and telomerase (**Fig. 3**), one possible model is that phosphorylated TEBPβ is involved in the recruitment of the telomerase to telomeres.

First, we addressed the question of whether phosphorylation of TEBPβ affects the presence of the telomerase in the replication band. Phosphorylation was inhibited by treating *S. lemnae* cells with butyrolactone, a selective inhibitor of Cdk kinases[21,42]. FISH analysis using TR as a probe shows that no telomerase can be detected in the replication band after inhibition of phosphorylation, in contrast to untreated control cells, where telomerase is highly enriched in the replication band (**Fig. 4a**,**b**)[27]. This suggests that phosphorylation is indeed connected with telomerase recruitment. Because butyrolactone inhibits protein phosphorylation by Cdks globally, we asked whether TEBPβ is required for telomerase recruitment by knocking down its expression using RNAi[21] and subsequently testing whether the telomerase is present in the replication band. The FISH analysis using TR as a probe (**Fig. 4c**) showed that silencing of TEBPβ expression resulted in the absence of TR from the replication band, providing strong evidence that TEBPβ in the phosphorylated form is involved in recruiting the telomerase to the replication band.

Finally, to investigate whether the telomerase and phosphorylated TEBPβ interact, we generated chicken anti-TERT antibodies using peptides derived from the reverse-transcriptase domain of the *S. lemnae* TERT subunit (**Supplementary Fig. 5** online). Various telomeric complexes were assembled and phosphorylated *in vitro*: naked model telomere (a 20-nucleotide, double-stranded region and a 16-nucleotide, single-stranded G-overhang[21]); telomere bound by TEBPα; telomere bound by the TEBPα–TEBPβ heterodimer; and telomere bound by the TEBPα–TEBPβ heterodimer and subsequently phosphorylated by Cdk2. The assembled telomeric complexes were incubated with macronuclear telomerase extract and immunoprecipitated with the anti-TEBPα antibody. Western blotting with anti-TEBPα, anti-TEBPβ or anti-TERT antibodies (**Fig. 4d**) showed that, whereas TEBPα and TEBPβ are present in the appropriate complexes, the TERT subunit co-immunoprecipitates with only the telomeric complex containing phosphorylated TEBPβ (**Fig. 4d**, lane 4). The same result was obtained when the co-immunoprecipitation was performed using the anti-TEBPβ antibody (data not shown). As a control, we incubated phosphorylated TEBPβ alone with the macronuclear telomerase extract and performed the same co-immunoprecipition using the anti-TEBPβ antibody. In this case, the TERT subunit was not detected in the co-immunoprecipitate (data not shown). These results provide strong evidence for a physical interaction between telomerase and phosphorylated TEBPβ, suggesting that the telomerase is recruited to telomeres by phosphorylated TEBPβ. Furthermore, as TEPBβ is phosphorylated in S phase[21] (**Supplementary Fig. 2**), this observation indicates that telomerase recruitment is regulated in a cell cycle–dependent manner.

DISCUSSION

Telomeric G-quadruplex formation in the ciliate *S. lemnae* requires the cooperation of the two G-overhang binding proteins TEBPα and TEBPβ. During replication, the G-quadruplex DNA structure is resolved correlating with phosphorylation of TEBPβ[21]. Here we show that TEBP β phosphorylation is necessary, but not sufficient, for a G-quadruplex unfolding rate compatible with telomere synthesis. We find that the telomerase contributes to the unfolding of G-quadruplexes *in vivo* and is recruited to telomeres by phosphorylated TEBPβ. A scheme that incorporates our findings and provides a possible explanation for the regulation of G-quadruplex formation and telomere synthesis during the cell cycle is presented in **Figure 5**.

Because G-quadruplex formation at *S. lemnae* telomeres is induced by the binding of TEBPβ to the TEBPα–G-overhang complex[21], eviction of TEBPβ from telomeres is likely to be a prerequisite for G-quadruplex unfolding. Whereas immunostaining shows that G-quadruplexes are absent from the replication band of macronuclei[20,21] (**Fig. 3a**), analysis of the composition of the telomeric complex in the replication band indeed shows that during replication TEBPβ has been evicted and only TEBPα stays bound (**Fig. 2a**,**b**). The binding of one TEBPα (**Supplementary Fig. 1**) would render the G-overhang accessible for the telomerase[39], consistent with the suggestion that the closely related human POT1 could trap the telomeric G-overhangs in an unfolded form that allows elongation by the telomerase[35,37]. The finding that phosphorylation of TEBPβ *per se* does not lead to the disassembly of the telomeric complex *in vitro* (**Fig. 2c**) raised the question of what causes the eviction of TEBPβ from telomeres during replication. Our *in vivo* observations provide evidence that the telomerase, or a telomerase-associated protein, is involved in the dissociation of TEBPβ from the telomeric complex. These results present a dilemma because the TEBPα–TEBPβ heterodimer has two apparently opposite functions: first, it provides a capping structure that inhibits telomerase activity; and second, the telomerase is somehow involved in the disassembly of the capping structure to permit telomere synthesis. These functions are mimicked by the human POT1–TPP1 telomeric capping complex[13,14], which has been shown to regulate telomerase activity *in vivo* both positively[43,44] and negatively[45,46].

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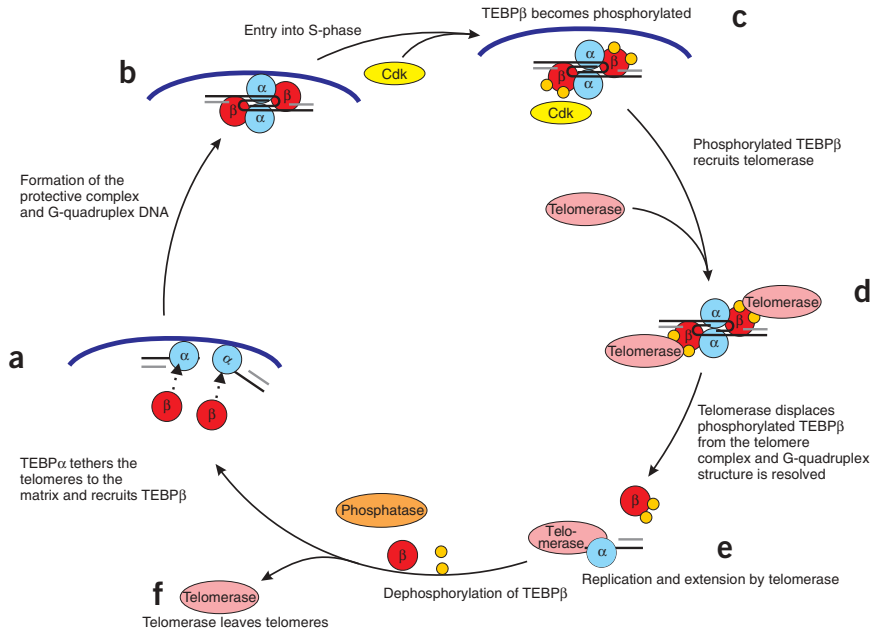

Figure 5 Proposed scheme for the regulation of the telomeric G-quadruplex DNA structure during the cell cycle. (**a**) TEBPα (blue) attaches telomeres to a subnuclear structure and recruits TEBPβ (red) to the G-overhang. (**b**) This leads to G-quadruplex structure (black) formation[17]. (**c**) In S phase, attachment to the subnuclear structure is lost, the G-quadruplexes unfold[17,22] and TEBPβ becomes phosphorylated[17]. (**d**) Here we show that phosphorylation of TEBPβ is essential for telomerase recruitment (green). (**e**) Telomerase recruitment results in the eviction of phosphorylated TEBPβ from the telomeric complex during replication. Upon TEBPβ eviction, one TEBPα molecule stays bound to the replicating telomere and traps the telomeric G-overhang in its single-stranded form, permitting telomere synthesis. (**f**) Following replication, to reform the telomeric capping structure, TEBPβ would need to become dephosphorylated to rebind to TEBPα and promote G-quadruplex structure formation[21].

Several observations indicate that telomere-bound phosphorylated TEBPβ recruits the telomerase to telomeres: first, inhibition of *in vivo* phosphorylation results in the telomerase no longer being localized to the replication band of macronuclei; second, silencing of TEBPβ gene expression results in the loss of telomerase recruitment to the replication band (**Fig. 4a–c**); third, co-immunoprecipitation experiments demonstrate that the telomerase is bound to the telomeric complex only when TEBPβ is phosphorylated (**Fig. 4d**). The role of TEBPβ in telomerase recruitment is in agreement with and strengthened by recent findings demonstrating that the TEPBβ mammalian homolog TPP1 is directly involved in the recruitment of the telomerase to telomeres[15,16]. The domain of TPP1 involved in interacting with the telomerase was mapped to the OB-fold–containing, N-terminal half. This domain is conserved between TPP1 and TEBPβ[16] (data not shown). Our results from *S. lemnae* macronuclear telomeres go further, showing that telomerase recruitment is regulated in a cell cycle–dependent manner by phosphorylation of TEBPβ in S phase. Although information on the role of post-translational modifications in telomerase recruitment is scant, the *S. cerevisiae* G-overhang binding protein Cdc13, which is involved in telomerase recruitment[47,48], has been shown to be phoshorylated in a cell cycle–dependent manner[49]. The recent observations that telomere length is regulated by the cyclin-dependent kinase Cdk1 in yeast[50,51] and that cell-cycle progression is important for telomere length regulation in vertebrates[52] are consistent with our findings. Further to these observations, our results show that phosphorylation of TEBPβ and telomerase recruitment are intimately involved in G-quadruplex unfolding.

During replication, G-quadruplexes unfold and are absent from the replication band[20,21], which is in turn coupled to the eviction of TEBPβ from telomeres (**Fig. 2a**,**b**). As phosphorylation of TEBPβ is not sufficient for the disruption of the telomeric complex *in vitro* (**Fig. 2c**), *in vivo* TEBPβ eviction must involve another protein. We show here that silencing of either TR or TERT expression results in an accumulation of G-quadruplexes in the replication band, providing evidence that the telomerase is involved in promoting G-quadruplex unfolding. G-quadruplex unfolding could be facilitated by the binding of the telomerase to the G-quadruplex DNA structure[32,53]; however, because the recruitment of the telomerase by phosphorylated TEBPβ (**Fig. 4b**,**c**) leads to dissociation of TEBPβ from telomeres *in vivo* (**Fig. 2a**,**d**), we favor the explanation that the eviction of TEBPβ is an essential step for G-quadruplex unfolding. The rate of G-quadruplex unfolding could also be accelerated by helicases[23], but such helicases have not been found in ciliated protozoa.

The findings that ciliate macronuclear telomeres exist in the G-quadruplex conformation[20,21] and that vertebrate telomeres exist in the t-loops conformation[17,18] provide different models for telomere protection. Despite t-loops being a generally accepted telomere structure, key information on how the t-loop structure is regulated during the cell cycle to permit telomere replication is lacking[18]. Yet, despite the different telomere capping structures, it has emerged that the two ciliate telomere end binding proteins TEBPα and TEBPβ and the POT1 and TPP1 of vertebrates are evolutionarily related, and they function similarly in G-overhang binding[13,14] and telomerase recruitment[15,16]. However, the ciliate TEBPβ has the additional function of promoting G-quadruplex formation at telomeres. In this context, it is significant that the sequence and structural conservation between TEBPβ and TPP1 (data not shown) is restricted to their N-terminal halves, which contain the telomerase-recruitment domain in TPP1 (refs. 15,16). In contrast, the C terminus of TEBPβ, which is essential for G-quadruplex formation[21,24] and contains the sites for the cell cycle–regulated phosphorylation events, is not conserved in TPP1. This might reflect different requirements of macronuclear and vertebrate telomeres. If G-quadruplexes have a role in vertebrate telomere biology, their long G-overhangs are likely to have the ability to fold spontaneously into G-quadruplexes without the aid of a G-quadruplex–promoting protein(s)[22]. It remains to be established whether G-quadruplexes have a role in vertebrate telomere biology, or whether vertebrate telomere end binding proteins have evolved to prevent their formation and to facilitate telomere replication.

METHODS

Protein and oligonucleotide production and purification. We generated the TEBPβ mutants using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). Wild-type TEBPα and TEBPβ, and the three TEBPβ

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mutants, were expressed in *E. coli* and purified as described[21]. Oligonucleotides were synthesized, purified and annealed as described[21]. Oligonucleotide concentrations were estimated from the OD_{260} and sequence-specific extinction coefficients.

Electrophoretic mobility shift assays (EMSA). The model telomeric DNA was radioactively labeled using [$\gamma-^{32}$P]ATP (GE Healthcare) and T4 polynucleotide kinase (New England Biolabs). Labeled DNA (1 μM) was incubated with TEBPα and TEBPβ in binding buffer (50 mM Tris-HCl, pH 8.0, 125 mM KCl, 5 mM DTT, 10% (v/v) glycerol) containing 100 μg ml^{-1} BSA (New England Biolabs) and 100 μg ml^{-1} sheared *E. coli* DNA) for 1–2 h at room temperature. Reaction mixtures were analyzed by native agarose gel electrophoresis (0.4% BioGene 'HiPure' Low EEO agarose, 0.25× TBE) at 7.5 V cm^{-1} for up to 120 min at 4 °C. Gels were dried onto DE81 paper (Whatman) and imaged using a Typhoon 8600 imaging system (GE Healthcare).

Isolation and electroelution of macronuclei. *Stylonychia lemnae* macronuclei were isolated and electroeluted according to established protocols[26,54].

***In situ* immunostaining, western and Southern blotting, and fluorescence *in situ* hybridization analyses.** We carried out *in situ* immunostaining and FISH analyses[20,27], and western and Southern analyses[26], as described. The antibody directed against *S. lemnae* telomeric G-quadruplexes (Sty49) was generated by ribosome display from the Human Combinatorial Antibody Library[55]. Antibodies directed against TEBPα were raised in rabbits and against TEBPβ in guinea pigs. The anti-TERT antibodies were obtained by immunizing chickens with three peptides derived from different regions of the reverse-transcriptase domain of *S. lemnae* TERT. The antibodies were affinity purified before use.

Inhibition of gene expression by RNAi. Knockdown of TEBP expression was carried out using RNAi as described[21,40]. For knockdowns of TR and TERT expression, the TR and TERT genes were amplified from total macronuclear DNA by PCR using primer combinations described for *Oxytricha*[56,57]. PCR products were verified by DNA sequencing and cloned into the L4440 vector[58]. The vector construct was transfected into RNase III–deficient *E. coli* HT115 cells, and dsRNAi expression induced by addition of IPTG to a final concentration of 0.4 mM when the cell culture reached an OD_{600} of about 0.4 (ref. 58). Specific inhibition of TR and TERT expression was achieved by feeding *S. lemnae* cells heat-inactivated *E. coli* containing the appropriate L4440 vector mixed with the food source *Chlorogonium elongatum*[21]. A strong RNAi-silencing effect was normally observed after about 4 d of feeding. The same result was obtained when different regions of the TERT gene were targeted by RNAi. As controls, *S. lemnae* cells were fed *E. coli* cells containing the L4440 vector without an insert or with a PCNA insert.

***In vitro* and *in vivo* protein phosphorylation.** Purified TEBPβ or TEBPβ mutants (4 μM) were incubated with 20 units of Cdk2-cyclin A (GE Healthcare) for 30 min at 30 °C in phosphorylation buffer (50 mM Tris, pH 7.4, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 10 mM γ-glycerol phosphate, 100 μM ATP). Total nuclear proteins were electroeluted and phosphorylated by incubation with [$\gamma-^{32}$P]ATP (800 Ci $mmol^{-1}$) and 200 units of Cdk2-cyclin A for 60 min at 30 °C. Phosphorylated nuclear proteins were separated on 15% (w/v) SDS-PAGE and visualized by autoradiography. For analysis of *in vivo* protein phosphorylation, cells were synchronized and pulse-labeled for 2 h with [$\gamma-^{32}$P]ATP (800 Ci $mmol^{-1}$). Nuclear proteins were electroeluted and digested with *Staphylococcus aureus* Strain V8 protease (Sigma-Aldrich) for 15 h at 37 °C. Proteolysis fragments were analyzed by 20% (w/v) SDS-PAGE and visualized by autoradiography.

Co-immunoprecipitation of telomeric complexes. Various telomeric complexes (telomeric DNA, telomeric–TEBPα complex, telomeric–TEBPα–TEBPβ complex, phosphorylated telomeric TEBPα–TEBPβ complex) were incubated for 20 min at 4 °C with macronuclear telomerase extract and cross-linked with 0.1% formaldehyde for 2 min, and the reaction was stopped with 125 mM glycine. Macronuclear telomerase extracts were isolated from synchronized cells using a modified version of two previously described protocols[39,57], snap frozen in liquid nitrogen and stored at –80 °C. Co-immunoprecipitations were performed using the anti-TEBPα antibody, incubated for 2 h at 4 °C with the different telomeric complexes. Protein G Dynabeads (Invitrogen) were added, followed by incubation for 1 h at 4 °C, and the precipitates were collected by centrifugation and washed according the manufacturer's protocol.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

K.P., S.J., T.S., A.H. and H.J.L. performed experiments; K.P., D.R. and H.J.L. designed the experiments; and K.P., D.R. and H.J.L. wrote the manuscript.

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