Chromosome Research (2008) 16:721–728
DOI: 10.1007/s10577-008-1222-x

Cell cycle-dependent regulation of telomere tethering in the nucleus

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Received 5 March 2008. Received in revised form and accepted for publication by Dean Jackson 24 March 2008

Key words: cell, ciliate, G-quadruplex, macronucleus, nuclear matrix, telomere

Abstract

It is well established that telomeres are tethered in the eukaryotic nucleus, but a detailed analysis of the regulation of telomere attachment throughout the cell cycle is still lacking. We show here that the telomeres in the macronucleus of the ciliate *Stylonychia lemnae* are bound to a sub-nuclear structure by an interaction of the telomere end-binding protein TEBPα with three SNS proteins that are integral parts of this structure. In the course of replication, the interaction of TEBPα with the SNS proteins is resolved and this process is regulated by cell cycle-specific phosphorylation of the SNS proteins. Our data can be incorporated into a mechanistic model for the regulation of telomere conformation and localization throughout the cell cycle.

Abbreviations

ATP	adenosine triphosphate
CDK2	cyclin-dependent kinase 2
PVDF	polyvinylidene fluoride
SDS-PAGE	SDS–polyacrylamide gel electrophoresis
SNS	sub-nuclear structure
TEBPα	telomere end-binding protein alpha
TEBPβ	telomere end-binding protein beta
TTBS	Tween-supplemented Tris-buffered saline

Introduction

Telomeres are the terminal structures of eukaryotic chromosomes. Besides their important functions in providing genome stability and the correct replication of chromosomal ends, telomeres also contribute to functional nuclear architecture (Cech 2004). It is well established that in eukaryotes the telomeres are attached to the nuclear envelope and co-purify with components of the sub-nuclear structure, the nuclear matrix/scaffold, and such are thought to have an influence on global regulation of gene expression (de Lange 2002, Gasser 2002, Jonsson & Lipps 2002, Zakian 1995). Localization of telomeres to the nuclear periphery is capable of contributing to heterochromatin formation and has been reported for yeast (Gasser *et al*. 1998, Andrulis *et al*. 1998, Taddei & Gasser 2004), *Drosophila* (Mathog *et al*. 1984, Hochstrasser & Sedat 1987) and the pathogenic protozoa *Trypanosoma brucei* and *Plasmodium falciparum* (Perez-Morga *et al*. 2001, Scherf *et al*. 2001, Marty *et al*. 2006). In mammalian cells, telomeres are not found at the nuclear periphery but co-purify in a nuclear scaffold preparation (de Lange 1992).

In all organisms, nuclear tethering of telomeres presumably requires at least two components, a telomere-bound protein and at least one protein associated with a sub-nuclear structure, such as the nuclear envelope or nuclear matrix/scaffold. In *Saccharomyces cerevisiae*, telomeres can be anchored in the nucleus either by the Sir or the Ku complex. Telomere-bound Sir4p interacts with Esc1p at the

inner nuclear membrane to hold telomeres at the periphery (Palladino *et al*. 1993, Taddei & Gasser 2004). It is not known how the Ku heterodimer interacts with a sub-nuclear structure in eukaryotic cells. So far, the regulation of telomere tethering throughout the cell cycle is poorly understood.

We have recently reported that the telomeres of the ciliate macronucleus as well as the telomere end-binding proteins are attached to a sub-nuclear structure and that telomeric DNA adopts an antiparallel G-quadruplex conformation when bound to this structure. In the course of telomere replication, both, nuclear attachment and G-quadruplex DNA structure become resolved (Postberg *et al*. 2001, Schaffitzel *et al*. 2001, Jonsson *et al*. 2002).

One of the unique features of ciliated protozoa is the presence of two different kinds of nuclei, the generative micronucleus and the somatic macronucleus (Prescott 1994). Macronuclear DNA consists of short DNA molecules. The size of these DNA molecules (more recently called nanochromosomes; Cavalcanti *et al*. 2004) in *Stylonychia lemnae* varies between 0.4 and 40 kbp. Each nanochromosomes is terminated by telomeric DNA sequences of homogeneous length (Lipps & Steinbruck 1978, Klobutcher *et al*. 1980). A heterodimer of the two telomere end-binding proteins, TEBPα and TEBPβ, caps the 3′-telomeric overhang (Lipps *et al*. 1982, Gottschling & Zakian 1986). One macronucleus contains about 10^8 nanochromosomes. Owing to this high concentration of telomeres, ciliates are an attractive system for studying study telomere structure, function and behaviour.

Using a combination of *in vivo* and *in vitro* studies and RNA interference to silence expression of the two TEBPs, we were able to elucidate the biological function of both proteins (Paeschke *et al*. 2005). TEBPα binds in a sequence-specific manner to the 3′-telomeric overhang and recruits TEBPβ to the telomere complex. TEBPβ promotes the formation of the telomeric, antiparallel G-quadruplex DNA structure (Fang & Cech 1993, Paeschke *et al*. 2005). The binding of telomeres to a sub-nuclear structure during G_1- and G_2-phases of the cell cycle is mediated by TEBPα. In the course of replication, which takes place in a morphologically distinct region (the replication band), telomeres as well as both TEBPs are no longer attached to the sub-nuclear structure and the telomeric G-quadruplex DNA structure is resolved (Postberg *et al*. 2001, Jonsson *et al*. 2002, Paeschke *et al*. 2005). These two events are likely to be involved in telomerase activation during S-phase. One major event during S-phase is the phosphorylation of TEBPβ. Phosphorylated TEBPβ can no longer form a heterodimer with TEBPα, and as a consequence no G-quadruplex DNA structure can be maintained during replication, which makes the telomeres accessible for telomerase (Paeschke *et al*. 2005).

To understand the regulation of telomere attachment during the cell cycle, we identified the TEBPα interaction partner that tethers the telomeric complex to a sub-nuclear structure and analysed how this interaction is resolved during replication. Results obtained in this study together with data from previous analyses allow us to propose a mechanistic model for the regulation of telomere conformation throughout the cell cycle.

Material and methods

Isolation and electroelution of macronuclei

Stylonychia lemnae macronuclei were isolated and electroeluted essentially according to established protocols (Ammermann *et al*. 1974, Jackson *et al*. 1988, Postberg *et al*. 2001).

In situ antibody staining and western analysis

In situ antibody staining was performed as described (Postberg *et al*. 2001). For the modified western analysis proteins were separated by 15% SDS-PAGE, blotted onto nitrocellulose membranes and incubated overnight with TEBPα or TEBPβ (1 μg/ml) at 4°C. After thorough washing with TTBS (100 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% (v/v) Tween 20), the membranes were probed with specific antibodies as described (Paeschke *et al*. 2005).

In vitro and in vivo protein phosphorylation

Macronuclei were electroeluted and phosphorylated by incubation with [γ-^{32}P]ATP (800 Ci/mmol) or 200 nM ATP and 200 units of Cdk2-cyclin A for 60 min at 30°C. Phosphorylated nuclear proteins were separated by 15% SDS-PAGE and autoradiographed or used for the modified western analyses. For analysis of *in vivo* protein phosphorylation, cells

were synchronized (Juranek *et al*. 2000) and pulse-labelled for 2 h with [γ-^{32}P]ATP (800 Ci/mmol). Phosphorylated nuclear proteins were separated by 15% SDS-PAGE (Schagger & von Jagow 1987) and autoradiographed. For *in situ* antibody staining, macronuclei were phosphorylated using 200 nM ATP and 200 units of Cdc2 kinase and electroeluted.

Mass spectroscopy of SNS proteins

For mass-spectroscopic analyses the three SNS proteins were separated by SDS-PAGE following electroelution, transferred onto PVDF membrane (Amersham), analysed by mass spectroscopy (Cristea *et al*. 2004) and analysed for their homology to other known proteins.

Results

TEBPα tethers telomeres through interactions with three SNS proteins

We have previously shown that TEBPα attaches the telomeres to a sub-nuclear structure and that this interaction is resolved during replication, which may be a requirement for telomere replication to take place (Paeschke *et al*. 2005).

In order to identify TEBPα interaction partners, we performed a modified western analysis. Macronuclei were isolated and total macronuclear proteins were separated by 15% SDS-PAGE and blotted onto nylon membranes. The membranes were incubated with recombinant TEBPβ (Figure 1a, lane 2) or TEBPα (Figure 1a, lane 3) and after extensive washing the membranes were probed with a TEBPα-specific antibody. In addition to the expected detection of TEBPα, membranes that had been incubated with TEBPα exhibited specific binding to three proteins with molecular weights ranging from about 20 to 30 kDa (Figure 1a). These three proteins could not be detected when the membranes were incubated with TEBPβ (Figure 1a, lane 2) or with the TEBPα antibody on its own (Figure 1a, lane 1). They were not observed when membranes were incubated with TEBPβ and probed with a TEBPβ-specific antibody (data not shown), indicating that binding of TEBPα to these three proteins is not the unspecific binding of a protein to three prominent nuclear proteins.

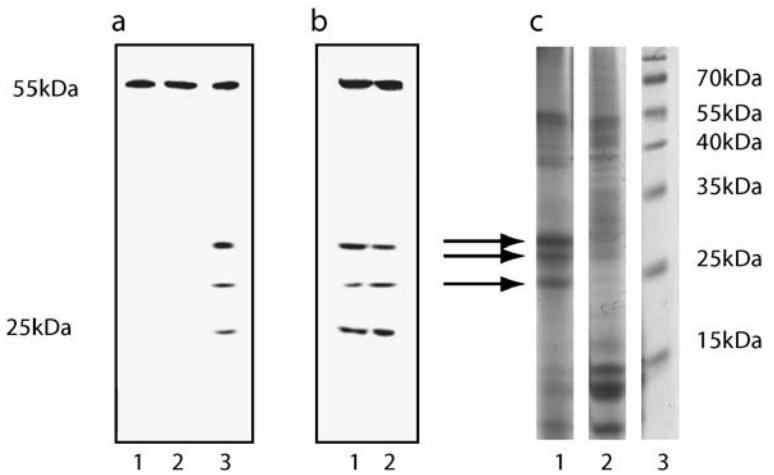

Figure 1. TEBPα attaches telomeres to a sub-nuclear structure through specific interactions with three SNS proteins. **(a)** Total nuclear proteins were separated by 15% SDS-PAGE and blotted onto nitrocellulose filters: lane 1, filter incubated with a TEBPα specific antibody; lane 2, filter incubated with TEBPβ followed by a TEBPα-specific antibody; lane 3, filter incubated with TEBPα followed by a TEBPα-specific antibody. **(b)** Total nuclear proteins, and nuclear attached proteins after electroelution, were separated by SDS-PAGE and blotted onto filters: lane 1, total nuclear proteins were blotted onto a nitrocellulose filter and incubated with TEBPα followed by a TEBPα-specific antibody; lane 2, after electroelution, nuclear proteins, which are anchored in the nucleus (possibly nuclear matrix proteins), were blotted onto a nitrocellulose filter and incubated with TEBPα followed by a TEBPα-specific antibody. **(c)** Coomassie blue staining of a SDS-PAGE gel: lane 1, total bound nuclear proteins after electroelution, lane 2, total nuclear proteins, lane 3, marker (PageRuler; Fermentas). Arrows point to the three SNS proteins.

To investigate, whether the three proteins are members of a sub-nuclear structure, we used the nuclear electroelution technique, which allows us to distinguish proteins and nucleic acid bound to sub-nuclear structures *in vivo* from unbound, free proteins and nucleic acids (Jackson *et al*. 1988, Paeschke *et al*. 2005). After electroelution, proteins that were not electroeluted and still attached in the nucleus were analysed by SDS-PAGE and the modified western protocol described above. These experiments

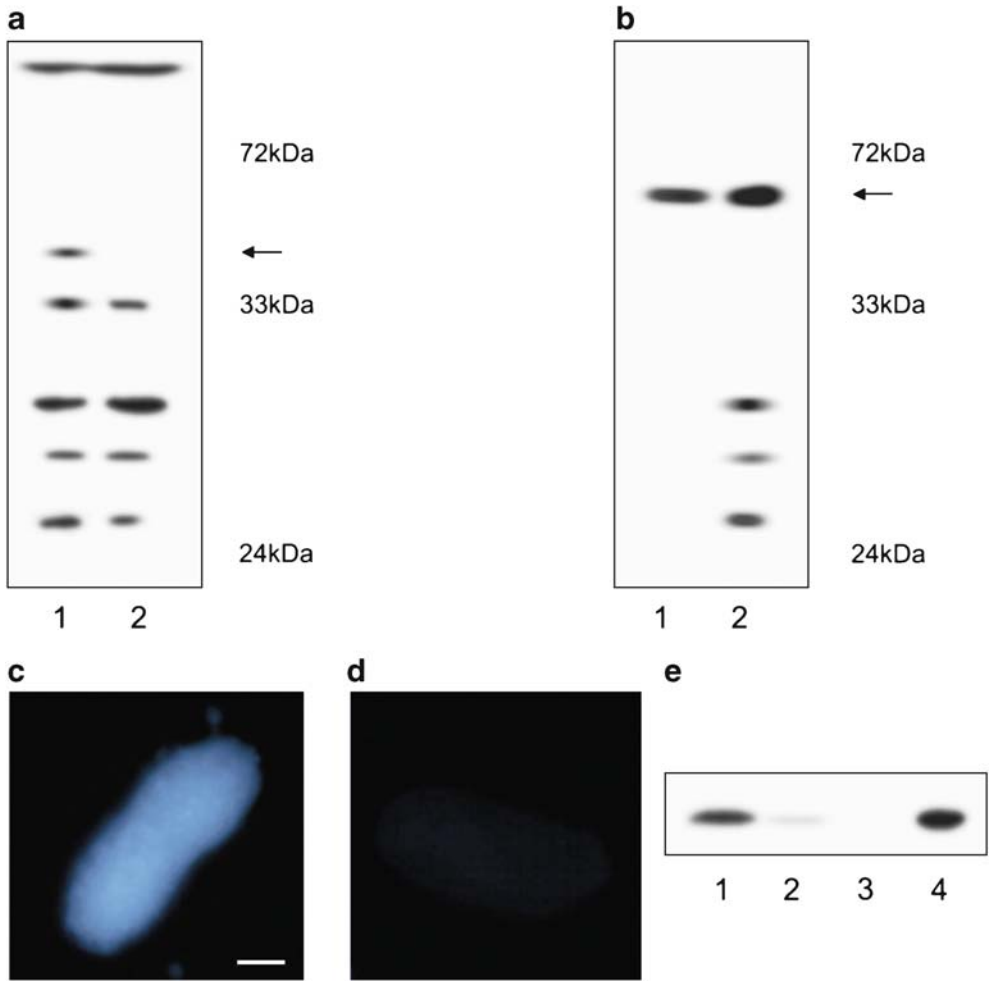

Figure 2. Cell cycle-dependent phosphorylation of the three SNS proteins prevents TEBPα from attaching telomeres to a sub-nuclear structure during S-phase. **(a)** The three SNS proteins are phosphorylated in S-phase: lane 1, attached macronuclear proteins that cannot be electroeluted were phosphorylated *in vitro* and separated by 15% SDS-PAGE; arrow points to TEBPβ; lane 2, cells were synchronized and pulse-labelled with [γ-^{32}P]ATP during S-phase, and attached macronuclear proteins that cannot be electroeluted were separated by SDS-PAGE. **(b)** TEBPα does not interact with phosphorylated SNS proteins: lane 1, *in vitro* phosphorylated total macronuclear proteins were separated by SDS-PAGE and blotted onto a nitrocellulose filter that had been incubated with TEBPα and a TEBPα-specific antibody; lane 2, control total unphosphorylated macronuclear proteins. Arrow points to TEBPα. **(c)** Macronuclei were electroeluted and TEBPα was immunolocalized. TEBPα remains bound to a sub-nuclear structure and cannot be electroeluted. Scale bar represents 10 μm. **(d)** Macronuclei were phosphorylated *in vitro* and electroeluted, and remaining nucleus materials were stained with a TEBPα-specific antibody. TEBPα does not bind to phosphorylated SNS proteins and is no longer attached in the nucleus. **(e)** *In vitro* phosphorylation of macronuclei prevents TEBPα from interacting with a sub-nuclear structure: lane 1, amount of TEBPα that remains bound to a sub-nuclear structure following electroelution; lane 2, amount of unbound TEBPα that can be electroeluted; lane 3, amount of TEBPα that remains bound to a sub-nuclear structure in *in vitro* phosphorylated macronuclei following electroelution; lane 4, amount of unbound TEBPα that can be electroeluted from *in vitro* phosphorylated macronuclei.

confirmed that these three proteins are integral components of a sub-nuclear structure and they were therefore called SNS proteins I–III (Figure 1b). Coomassie staining of bound nuclear proteins after electroelution showed that these three proteins are prominent members of the non-electroelutable nuclear proteins (Figure 1c). Mass-spectrometric analysis of the three SNS proteins revealed no obvious homology to known proteins.

Phosphorylation of the SNS proteins prevents telomere attachment in S-phase

Previous experiments have shown that the binding of TEBPα to a sub-nuclear structure is resolved during replication (Paeschke *et al*. 2005). In addition, TEBPβ becomes phosphorylated during S-phase. We therefore considered the possibility that nuclear tethering of telomeres is regulated by a cell cycle-dependent phosphorylation of the three SNS proteins. To test this possibility, macronuclei from asynchronous cells were electroeluted to remove all non-attached components. Residual proteins were then phosphorylated with a cyclin dependent kinase (cdk2) and [γ-^{32}P]ATP as a label and analysed by SDS-PAGE. Figure 2a shows that a number of proteins are phosphorylated, including as expected TEBPβ (Paeschke *et al*. 2005) and also the three SNS proteins (Figure 2a, lane 1). In this figure only the prominent phosphorylated proteins are shown; after longer incubation many more phosphorylated proteins become visible.

After establishing that the three SNS proteins can be phosphorylated *in vitro*, we tested whether these proteins become phosphorylated in a cell cycle-dependent manner *in vivo*. For this purpose, cells were synchronized and pulse-labelled with [γ-^{32}P]ATP at different stages of the cell cycle. Macronuclei were isolated and electroeluted to remove all non-attached proteins and the remaining proteins were analysed as before by SDS-PAGE. Coomassie staining of proteins from different stages of the cell cycle revealed that the SNS proteins are present throughout the entire cell cycle (data not shown) but phosphorylation of the three SNS proteins is restricted to S-phase (Figure 2a, lane 2). In contrast to phosphorylated TEBPβ, which can be electroeluted during S-phase (Paeschke *et al*. 2005), the three phosphorylated SNS proteins can not be electroeluted. This provides further evidence that the three SNS proteins are integral components of a sub-nuclear structure and that their localization to the sub-nuclear structure is independent of the cell cycle stage and phosphorylation status.

The next question was whether there is a direct link between phosphorylation of the three SNS proteins and the dissociation of TEBPα from the sub-nuclear structure. Macronuclei were isolated and *in vitro* phosphorylated by cdk2, and nuclear proteins were subjected to the modified western analysis described above. An interaction between TEBPα and the SNS proteins could not be detected following phosphorylation of macronuclear proteins (Figure 2b). This observation was confirmed both by *in situ* antibody staining and western analysis. Macronuclei were isolated, phosphorylated *in vitro*, electroeluted and stained with the TEBPα specific antibody. Whereas strong antibody staining was observed in control non-phosphorylated macronuclei (Figure 2c), antibody staining was not detectable following *in vitro* phosphorylation of macronuclei (Figure 2d). Western analysis of bound versus electroeluted TEBPα from control macronuclei verified that TEBPα remains bound to the sub-nuclear structure and cannot be detected in the electroeluted fraction (Figure 2e, lanes 1, 2). In contrast, western analysis of bound versus electroeluted TEBPα from phosphorylated macronuclei revealed that TEBPα loses its attachment to the sub-nuclear structure and is instead found in the electroeluted fraction (Figure 2e, lanes 3, 4) (Paeschke *et al*. 2005). This result provides strong evidence for a direct link between the cell cycle-dependent phosphorylation of the three SNS proteins and the dissociation of TEBPα from the sub-nuclear structure during S-phase.

Discussion

It is well known that the efficiency and precision of transcription and replication depend on the positioning of nuclear domains in the eukaryotic nucleus (Gasser *et al*. 1998, Cremer & Cremer 2001, Gasser & Cockell 2001, Cremer *et al*. 2006). One of the best-characterized examples of long-range organization in the nucleus concerns telomeres, which are best studied in lower eukaryotes. In budding yeast most of the 32 telomeres are positioned near the nuclear periphery in three to eight foci that persist throughout mid S-phase (Gotta & Gasser 1996, Tham

et al. 2001) and appear to facilitate the formation of repressed chromatin. Telomere anchoring in this organism depends on two parallel and partially redundant pathways. One requires the silencing factor Sir4 and a second the yKu heterodimer (Laroche *et al*. 1998, Taddei & Gasser 2004). Both proteins, Sir4 and yKu, bind telomeres *in vivo*, yet neither contains a transmembrane domain. Thus integral components of the nuclear envelope must also be involved. For Sir4, anchoring is achieved by binding Esc1, a large acidic protein that is found on the inner face of the nuclear envelope (Andrulis *et al*. 1998, Gartenberg *et al*. 2004, Taddei & Gasser 2004). The second yKu anchorage site remains unknown. It has also been reported that subtelomeric repeat elements and associated protein are involved in the regulation of telomere anchoring to the nuclear envelope and it is assumed that the factors that bind subtelomeric repeat elements can antagonize nuclear envelope interactions (Hediger *et al*. 2006). This antagonized effect alters higher-order folding of telomeres, which could account for both the insulator function of subtelomeric factors and their effect on anchoring efficiency. The folding of the telomeres and the attachment of the telomeres to the nuclear envelope has also been implicated in the switch from an accessible to a non-accessible state for telomerase action (Teixeira *et al*. 2004). In mammals, telomeres are not clustered at the nuclear periphery but co-purify with the nuclear scaffold or matrix (de Lange 1992) and telomere binding to this structure is probably mediated by the Ku heterodimer (Hsu *et al*. 1999).

In the present study we attempted to analyse the cell cycle-dependent regulation of telomere localization in the macronucleus of the ciliate *Stylonychia lemnae*. These analyses were facilitated by the unique genome organization of the ciliate macronucleus and the availability of very specific antibodies against both TEBPs.

Using a modified western protocol we were able to identify three SNS proteins to which TEBPα binds. Such a binding was not observed with TEBPβ or antibodies against TEBPα or TEBPβ on its own, demonstrating the specificity of this approach. These proteins cannot be electroeluted and can be considered as components of a sub-nuclear structure. To identify any homology to other proteins, mass-spectroscopic analyses of all three SNS proteins were done but no homologies to other known proteins were found. These analyses were further complicated by the fact that to date no complete genome sequence of the *Stylonychia* macronuclear genome is available. Owing to the insolubility of the three SNS proteins, all binding experiments had to be done under denaturing conditions and it was not possible to determine the interaction of these proteins with another or the interaction of TEBPα with them under physiological conditions. These proteins seem to occur in almost stoichiometric amounts and it may well be that they are organized in a complex interacting with TEBPα.

In the course of replication, nuclear attachment as well as telomeric G-quadruplex DNA structure is resolved and we have recently demonstrated that phosphorylation of TEBPβ during replication is a necessary prerequisite for resolving telomeric G-quadruplex DNA structures and preventing their re-formation (Paeschke *et al*. 2005, 2008). Both processes, resolving G-quadruplex DNA structure and the interaction of telomeres with the sub-nuclear structure, may be required for telomerase to gain access to telomeres during replication and therefore both processes could be linked, although stabilization of G-quadruplex DNA structure itself does not depend on nuclear attachment (Lipps *et al*. 1982). This assumption was tested *in vivo* and *in vitro*. These experiments clearly demonstrated that the SNS proteins can only be phosphorylated during S-phase and that TEBPα cannot bind to these phosphorylated SNS proteins *in vivo*. As a consequence of phosphorylation of the three SNS proteins, binding of telomeres to a sub-nuclear structure becomes resolved during replication.

These data, together with the results obtained in earlier studies (Postberg *et al*. 2001, Schaffitzel *et al*. 2001, Paeschke *et al*. 2005, 2008), can now be incorporated into a mechanistic model of the regulation of telomere conformation and localization during the cell cycle. During G_1-phase of the cell cycle, TEBPα interacts with three SNS proteins, so tethering the telomeres to a sub-nuclear structure. TEBPβ is recruited to the attachment sites and telomeres adopt the antiparallel G-quadruplex DNA structure when bound to this structure (Jonsson *et al*. 2002). In early S-phase, TEBPβ and the three SNS proteins become phosphorylated by a cyclin-dependent kinase. Phosphorylation is a necessary prerequisite for the unfolding of G-quadruplex DNA structure, and at the same time phosphorylation of the SNS proteins dissociates TEBPα and consequently the telomeres

from the sub-nuclear structure, making them accessible to the binding of telomerase. Upon entry into G_2-phase of the cell cycle, TEBPβ and the SNS proteins are dephosphorylated. Dephosphorylation leads to the reattachment of telomeres to the SNS proteins and TEBPβ is again recruited to these sites to promote folding of the extended telomeric G-overhang into the antiparallel G-quadruplex DNA structure so that the quiescent telomere state is restored.

Acknowledgement

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to H.J.L. and D.R.

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