

# Telomere length homeostasis

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**Abstract** The physical ends of chromosomes, known as telomeres, protect chromosome ends from nucleolytic degradation and DNA repair activities. Conventional DNA replication enzymes lack the ability to fully replicate telomere ends. In addition, nucleolytic activities contribute to telomere erosion. Short telomeres trigger DNA damage checkpoints, which mediate cellular senescence. Telomere length homeostasis requires telomerase, a cellular reverse transcriptase, which uses an internal RNA moiety as a template for the synthesis of telomere repeats. Telomerase elongates the 3' ends of chromosomes, whereas the complementary strand is filled in by conventional DNA polymerases. In humans, telomerase is ubiquitously expressed only during the first weeks of embryogenesis, and is subsequently downregulated in most cell types. Correct telomere length setting is crucial for long-term survival. The telomere length reserve must be sufficient to avoid premature cellular senescence and the acceleration of age-related disease. On the other side, telomere shortening suppresses tumor formation through limiting the replicative potential of cells. In recent years, novel insight into the regulation of telomerase at chromosome ends has increased our understanding on how telomere length homeostasis in telomerase-positive cells is achieved. Factors that recruit telomerase to telomeres in a cell cycle-dependent manner have been identified in *Saccharomyces cerevisiae*. In humans, telo-

merase assembles with telomeres during S phase of the cell cycle. Presumably through mediating formation of alternative telomere structures, telomere-binding proteins regulate telomerase activity in *cis* to favor preferential elongation of the shortest telomeres. Phosphoinositide 3-kinase related kinases are also required for telomerase activation at chromosome ends, at least in budding and fission yeast. In vivo analysis of telomere elongation kinetics shows that telomerase does not act on every telomere in each cell cycle but that it exhibits an increasing preference for telomeres as their lengths decline. This suggests a model in which telomeres switch between extendible and nonextendible states in a length-dependent manner. In this review we expand this model to incorporate the finding that telomerase levels also limit telomere length and we propose a second switch between a non-telomerase-associated “extendible” and a telomerase-associated “extending” state.

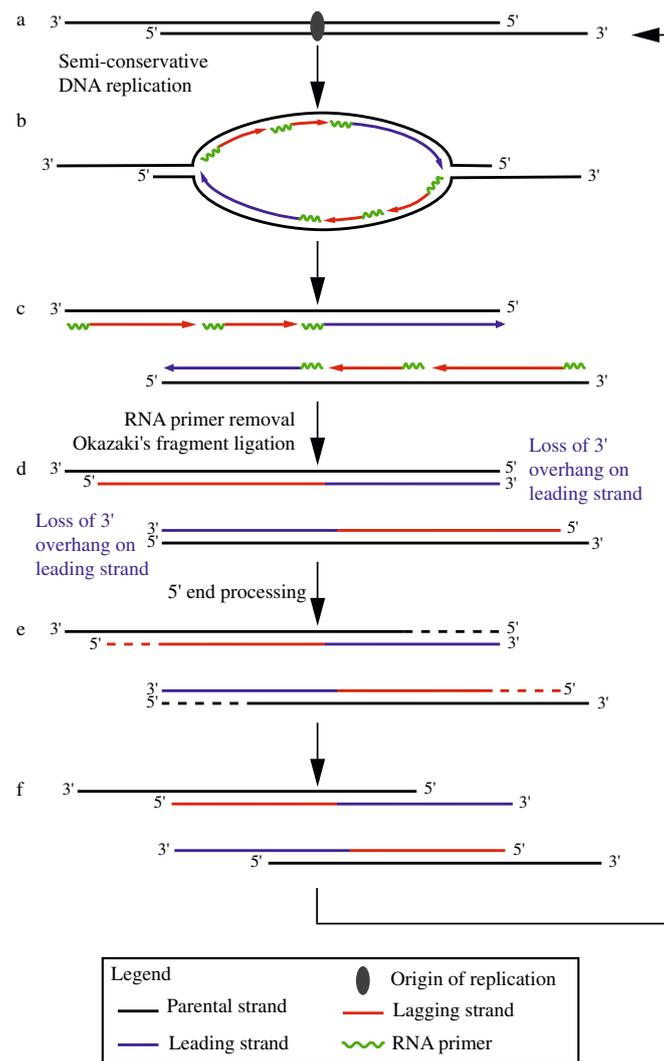
## Introduction

All eukaryotes, and a few prokaryotes, keep their genomes in the form of linear DNA molecules. This requires special mechanisms to fully replicate DNA ends because of the following reasons: First, DNA replication is semiconservative; DNA polymerases use a parental template strand to synthesize a complementary daughter molecule. Eukaryotic telomeres end with 3' protrusions at both chromosomal ends, as demonstrated for ciliated protozoa, budding yeast, and human telomeres (Klobutcher et al. 1981; Makarov et al. 1997; Chai et al. 2006a; Wellinger et al. 1993). Thus, the 5' parental strand is resected and it cannot provide a template for the synthesis of a 3' overhang [Fig. 1b,c, leading strand (Lingner et al. 1995)]. Semiconservative replication of the 5'-end-containing strand during leading

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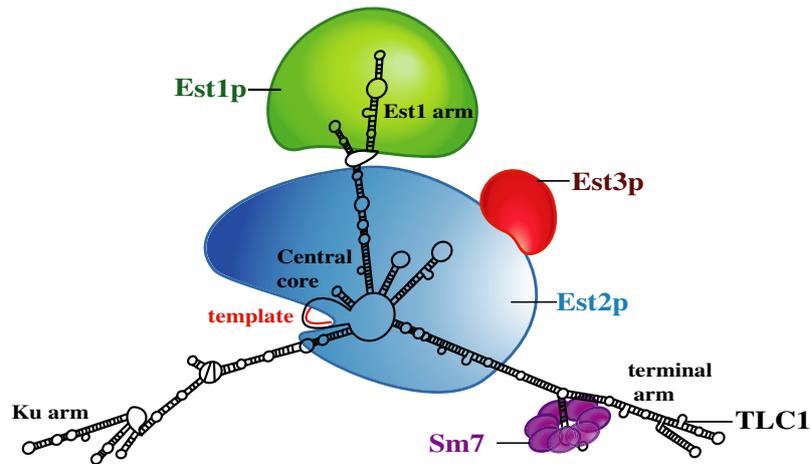
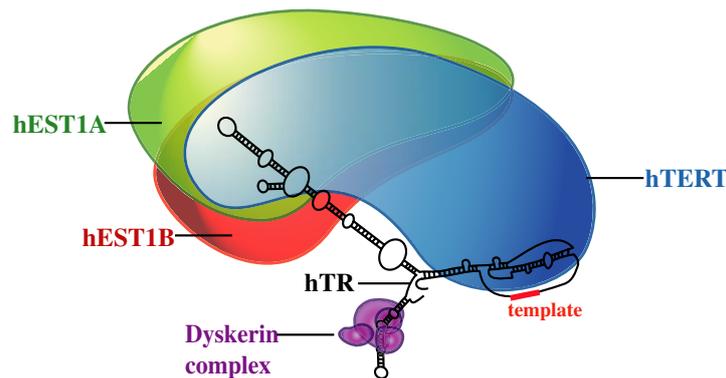
**Fig. 1** The DNA end replication problem. DNA replication starts at replication origins (a, gray oval). For simplicity, only one origin of replication is shown. The replication forks move in opposite directions (b). Because DNA polymerases only elongate in the 5' to 3' direction, each fork contains a leading (continuous) and a lagging (discontinuous) strand. Lagging strand synthesis is primed by short RNA

oligonucleotides (wavy line). Terminal DNA is lost at the leading strand end because the parental 5'-end-containing template is recessed (d). If RNA primers were laid down on the 3' overhang as depicted in (c), their removal would not lead to a net loss of sequence at the lagging strand. Finally, 5'-end processing occurs to regenerate a 3' overhang at the leading strand telomere (e, f)

strand synthesis should lead to loss of the 3' overhang. However, the presumed blunt end intermediate is not detected and the templating strand is probably quickly resected to regenerate the 3' overhang. Therefore, nucleolytic processing of telomere ends after semiconservative DNA replication contributes to telomere shortening [Fig. 1e,f (Chai et al. 2006b; Jacob et al. 2003; Larrivee et al. 2004)]. Chromosome end processing may occur also at the lagging strand telomere (Sfeir et al. 2005). Second, DNA polymerases cannot start DNA synthesis de novo. Instead, DNA polymerases extend short RNA primers, 8–12 nucleotides in length, which are synthesized by primases. Removal of RNA primers will leave a gap behind and therefore, DNA polymerases cannot synthesize

the extreme 5' end of a blunt end DNA molecule. Thus, upon replication of the 3'-end-containing strand during lagging strand synthesis, one would predict sequence loss that corresponds at least to the length of the RNA primer (Olovnikov 1973; Watson 1972). However, because the parental DNA strand is protruding at this end, the loss of DNA during semiconservative DNA replication depends on where the most distal RNA primer is laid down with respect to the overhang. If laid down on the 3' overhang only, no sequence will be lost upon primer removal (Fig. 1c,d, lagging strand).

The most common way out of the end replication problem occurs through the ribonucleoprotein (RNP) enzyme telomerase, discovered in 1985 in the holotrichous

A *Saccharomyces cerevisiae* telomeraseB *Homo sapiens* telomerase

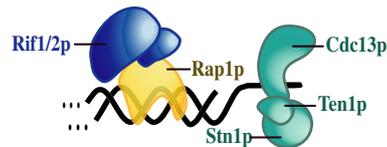
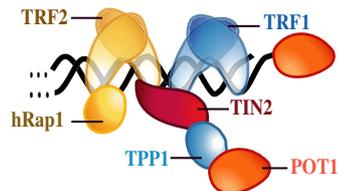
**Fig. 2** Telomerase models. **a** *Saccharomyces cerevisiae* telomerase. The telomerase RNA secondary structure is based on published models (Dandjinou et al. 2004; Zappulla and Cech 2004). The protein subunits are discussed in the text. **b** *Homo sapiens* telomerase. The

telomerase RNA secondary structure is based on the published model (Chen et al. 2000). The protein subunits are discussed in the text. A large set of additional proteins, which are not represented, has been reported to interact with human telomerase

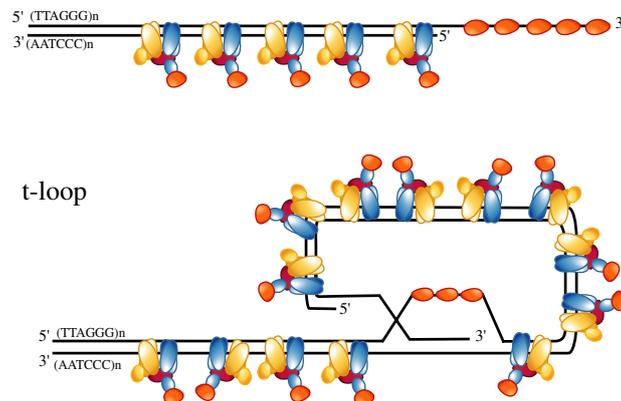
ciliate *Tetrahymena thermophila* by Carol Greider and Elizabeth Blackburn (Greider and Blackburn 1985). The telomerase enzyme contains an RNA moiety (Greider and Blackburn 1987) with a sequence complementary to the telomeric repeats (Greider and Blackburn 1989), which serves as a template for reverse transcription (Yu et al. 1990) (Fig. 2). The telomerase reverse transcriptase (TERT; Est2p in *Saccharomyces cerevisiae*) catalyzes reverse transcription of the RNA template into telomeric repeats (Lingner et al. 1997) while the telomerase subunit Est1p and the telomerase interacting protein Ku in *S. cerevisiae* are required for telomerase recruitment [(Lendvay et al. 1996; Lundblad and Szostak 1989; Stellwagen et al. 2003) discussed below]. Telomerase-associated proteins involved in biogenesis are shared with other small nuclear (sn) or

nucleolar (sno) RNPs and their identity is not conserved in different phyla (Fig. 2). The dyskerin complex, which is part of vertebrate telomerases and of snoRNPs is involved in RNP biogenesis and stability (Mitchell et al. 1999). Sm proteins, which associate also with snRNPs, form part of yeast telomerases (Seto et al. 1999; for reviews on telomerase and its biogenesis, see Chen and Greider 2006; Cristofari and Lingner 2006a). Telomerase activity at chromosome ends is regulated by telomere-binding proteins and phosphoinositide 3-kinase related kinases (PIKKs), and its action is coordinated with semiconservative DNA replication in a cell cycle-dependent manner.

In this review, we first describe the roles of telomere proteins to specify different telomeric states, which in turn regulate the telomerase enzyme. Then, we describe how

A *Saccharomyces cerevisiae*B *Homo sapiens*

## C Telomere structure models



**Fig. 3** Telomere proteins and structures. Only a subset of the proteins is depicted

telomerase activity assembles with chromosome ends in a cell cycle-dependent manner. Finally, we integrate the current knowledge of telomerase regulation into an expanded model for telomere length homeostasis. Through most parts of this review, we focus on knowledge gained in the yeast *S. cerevisiae* and in human cells.

### Telomeric states

Telomeric DNA in most eukaryotes consists of short tandem repeats, which are G-rich in the strand containing the 3' end. This strand is referred to the G-strand, whereas the 5'-end-containing complementary strand is referred to as C-strand. In humans the telomeric repeat sequence is 5'-TTAGGG-3', while in budding yeast telomeric DNA contains degenerate repeat sequences with the consensus 5'-(TG)<sub>0-6</sub>TGGGTGTG(G)-3'. In cells that express telomerase, telomere length is kept within a species- and cell type-specific narrow range. The length of the duplex telomeric tract ranges from <30 bp in some ciliates, to

200–300 bp in budding yeast, to 5–15 kb in humans, and up to ~50 kb in mice. The telomeric 3' overhang has a length of a few nucleotides in ciliates and roughly 30–110 nucleotides in humans (Chai et al. 2006a; Klobutcher et al. 1981). Yeast telomeres exhibit short 3' overhangs of 12–14 nucleotides throughout most of the cell cycle (Larrivee et al. 2004). Only in S phase, upon replication by the conventional DNA machinery, can longer overhangs be detected (Wellinger et al. 1993). The generation of the short overhangs outside of S phase depends on Mre11p, which is part of the Mre11–Rad50–Xrs2 complex (MRX). The overhangs during S phase are also reduced in *mre11*Δ strains (Larrivee et al. 2004). In human cells, down-regulation of the Mre11–Rad50–Nbs1 complex (MRN) resulted in transient shortening of the 3' overhangs in telomerase-positive but not in telomerase-negative cells (Chai et al. 2006b). This has been interpreted to suggest that MRN is involved in telomerase recruitment (Chai et al. 2006b). To conclude, although the MRN/MRX complexes seem to contribute to nucleolytic processing of chromosome ends, the identity of the nucleases that generate,

during S phase, upon semiconservative DNA replication, the 3' overhang at leading strand telomeres remains elusive.

The telomeric repeat sequences are associated with a group of specialized telomere-binding proteins to protect chromosome ends from degradation and from end-to-end joining events (Fig. 3). At the same time, telomere-binding proteins regulate telomerase activity to achieve telomere length homeostasis. Although telomere function is conserved to a large extent across species, the telomere protein organization varies significantly, as discussed in the next section.

#### Double-strand telomere-binding proteins

Budding yeast Rap1p is a major component of telomeric chromatin that binds the telomeric repeats with high affinity through a Myb-like DNA binding domain. Rap1p negatively affects telomere length (Conrad et al. 1990; Lustig et al. 1990). It recruits, through a C-terminal domain, two proteins, Rif1p and Rif2p, which mediate the telomere length control (Hardy et al. 1992; Wotton and Shore 1997) (Fig. 3). Deletion of either *RIF1* or *RIF2* leads to substantial elongation of telomeres establishing their function as negative regulators of telomere length. Telomere elongation analysis of single-chromosome end molecules in *rif1* and *rif2*-mutant cells reveals that these proteins downregulate the frequency of telomerase-mediated telomere elongation (Teixeira et al. 2004; discussed below). However, the exact mechanism by which Rif1 and Rif2 transmit their signal to telomerase remains unclear. Subtelomeric chromatin also contributes to telomere length control (Berthiau et al. 2006).

In humans, six proteins, TRF1, TRF2, hRap1, TIN2, TPP1, and POT1, form the so-called shelterin complex, which is a constitutive component of human telomeres (reviewed by de Lange 2005). The related TRF1 and TRF2 proteins bind double-stranded telomeric repeats as preformed homodimers (Bianchi et al. 1997), whereas POT1 binds to the single-stranded telomeric 3' overhang (Fig. 3). TIN2 connects, through protein interactions, TRF1, TRF2, and TPP1. TPP1 binds, in addition, POT1, thus recruiting POT1 also to the double-stranded part of telomeres. Human Rap1, the ortholog of yeast Rap1, associates with telomeres through interaction with TRF2. POT1 has also been reported to interact directly with TRF2 (Houghtaling et al. 2004; Kim et al. 1999; Li et al. 2000; Liu et al. 2004a,b; Loayza and De Lange 2003; O'Connor et al. 2004; Yang et al. 2005; Ye et al. 2004a,b).

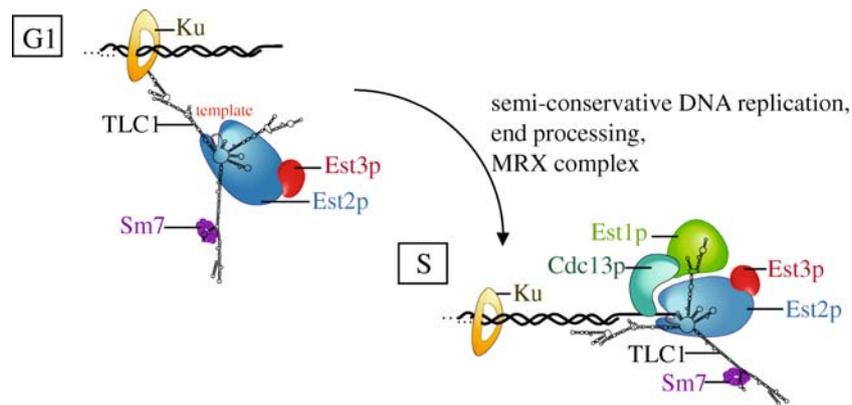
Shelterin is involved in telomere length control. Disturbing expression levels of shelterin components strongly impacts on telomere length (reviewed by Smogorzewska and De Lange 2004). For example, inhibition of TRF1 leads to telomere elongation in human tumor cells, while

overexpression of TRF1 causes telomere shortening without affecting telomerase activity in vitro (van Steensel and de Lange 1997). The binding of TRF1 to telomeres is controlled by tankyrase 1 and 2, poly[adenosine diphosphate (ADP)-ribose] polymerases, which modify TRF1 by the addition of ADP-ribosylation polymers, thus inhibiting the capability of TRF1 to bind telomeric DNA in vitro and in vivo (Cook et al. 2002; Kaminker et al. 2001; Sbodio et al. 2002; Seimiya and Smith 2002; Smith and de Lange 2000; Smith et al. 1998). Upon telomere dissociation, TRF1 is degraded by ubiquitin-mediated proteolysis (Chang et al. 2003). TIN2 protects TRF1 from tankyrase modification (Ye et al. 2004b). Reduction of TIN2 protein levels or the overexpression of mutant alleles that disrupt TIN2 interaction with TRF1 and TRF2 leads to telomere lengthening (Kim et al. 2004; Ye and de Lange 2004). Suppression of TPP1 by RNAi or the disruption of the TPP1–POT1 interaction also results in telomere lengthening, accompanied by loss of the POT1 signal at telomeres (Liu et al. 2004b; Ye et al. 2004b). Overexpression of TRF2 causes telomere shortening (Smogorzewska et al. 2000), but this shortening does not only occur through in vivo inhibition of telomerase, but also through an increased shortening rate (Ancelin et al. 2002). Finally, perturbation of Rap1 expression levels leads to mild telomere lengthening (Li and de Lange 2003; O'Connor et al. 2004).

How does shelterin mediate telomerase control at the 3' end of the telomere? Longer telomeres load more shelterin complexes and this may provide a length-sensing mechanism (Smogorzewska et al. 2000). As discussed below, the association of POT1 with shelterin may affect POT1 loading on the single-stranded telomeric DNA where it may act as an inhibitor of telomerase activity (Loayza and de Lange 2003). Alternatively or in addition, shelterin may inhibit telomerase by promoting the formation of the t-loop structure, in which the 3' overhang is inaccessible, being tucked into the double-stranded part of the telomere (Griffith et al. 1999) (Fig. 3c). In addition to shelterin, telomere length is also negatively regulated by histone methyltransferases in the mouse, which may be required to assemble telomeric heterochromatin (Garcia-Cao et al. 2004).

#### Single-strand telomere-binding proteins

Single-strand telomere-binding proteins regulate telomerase access to the telomeric 3' end. In *S. cerevisiae*, Cdc13p is the major single-strand DNA-binding protein. Cdc13p's functions include telomerase recruitment, telomerase repression, coordination of G- and C-strand synthesis, and protection of telomere ends from nucleolytic degradation. The latter task is immediately essential for viability. Loss of *CDC13* leads to degradation of the telomeric C-rich strand,



**Fig. 4** Recruitment pathways for *S. cerevisiae* telomerase. The Ku70/80-hetero-dimer recruits telomerase in G1 through binding the Ku-stem loop in the TLC1 telomerase RNA. This recruitment pathway

does not enable telomerase-mediated telomere elongation, but increases the elongation's efficiency. In S phase, Cdc13p recruits telomerase through binding telomerase-associated Est1p

causing a Rad9-mediated cell-cycle arrest (Garvik et al. 1995). Cdc13p is present at telomeres throughout the cell cycle (Bourns et al. 1998; Schramke et al. 2004; Taggart et al. 2002), but the levels of Cdc13p at telomeres peak in late S phase, consistent with the idea that Cdc13p assembles along the extended telomeric 3' overhang present at that time. The *cdc13-2* and *cdc13-5* mutant alleles uncovered two separate functions for Cdc13p in telomere replication (Chandra et al. 2001; Nugent et al. 1996). The *cdc13-2* allele shows an *in vivo* telomerase defect, which results from a disturbed interaction with the Est1p subunit of telomerase (Bianchi et al. 2004; Evans and Lundblad 1999; Lendvay et al. 1996; Nugent et al. 1996; Pennock et al. 2001; discussed further below). The *cdc13-5* allele gives rise to overelongated telomeres and overly long 3' overhangs, pointing to a defect in C-strand synthesis by the lagging strand DNA replication machinery (Chandra et al. 2001). Indeed, Cdc13p limits telomerase-mediated telomere elongation in conjunction with Stn1p and Ten1p, two essential Cdc13-associated proteins (Grandin et al. 1997, 2001).

The presumed major single-stranded telomere-binding protein in humans, POT1 (Baumann and Cech 2001), has been proposed to act as the terminal transducer of telomere length control from TRF1 (Loayza and De Lange 2003). POT1 binds single-stranded telomeric DNA with high sequence-specificity using two oligonucleotide/oligosaccharide-binding (OB) folds (Lei et al. 2004). Overexpression of a mutant POT1 allele that lacks the first OB fold resulted in rapid telomere elongation (Loayza and de Lange 2003). Similarly, siRNA-mediated depletion of hPOT1 resulted in telomere elongation (Ye et al. 2004b). In other studies, however, expression of the full-length protein caused telomere elongation (Armbruster et al. 2004; Colgin et al. 2003). The crystal structure of the N-terminal half of human POT1 containing the two OB folds with telomeric

DNA oligonucleotides shows that the 3' terminal G is buried in the protein, which should render it inaccessible to telomerase (Lei et al. 2004). Indeed, association of recombinant POT1 with telomeric oligonucleotide ends inhibits binding of telomerase (Kelleher et al. 2005; Lei et al. 2005). On the other hand, POT1 is able to disrupt G-quadruplex structures, which form through Hoogsteen base pairing in telomeric DNA *in vitro*. This could also explain how POT1 positively contributes to telomerase-dependent elongation, because G-quadruplex structures represent very poor substrates for telomerase (Zahler et al. 1991; Zaug et al. 2005). The disruption of G-quadruplex structures could also be facilitated by the recruitment of WRN or BLM helicases, both of which were shown to physically interact with POT1 (Opresko et al. 2005).

### Regulation of the telomerase enzyme in a cell cycle-dependent manner

In *S. cerevisiae*, telomere elongation of an artificially shortened telomere coincides with semiconservative telomere replication, which occurs late in S phase, suggesting coupling between the two processes. No elongation occurs in G1 of the cell cycle or in nocodazole-arrested cells (Marcand et al. 2000). The latter finding contrasts telomere addition to a double-stranded DNA break adjacent to a short telomeric DNA tract, which can occur in nocodazole-arrested cells in M phase (Diede and Gottschling 1999). Genetic evidence further supports the coupling of telomerase action with semiconservative DNA replication. Telomere addition to the double strand breaks in nocodazole-arrested cells depends on functional DNA polymerases  $\alpha$  and  $\delta$  (Diede and Gottschling 1999). Mutations in DNA polymerase  $\alpha$  cause telomere lengthening in *S. cerevisiae* and in mouse cells (Adams and Holm 1996; Nakamura et

al. 2005). The B subunit of DNA polymerase  $\alpha$  physically and genetically interacts with the Cdc13p-interacting protein Stn1 (Grossi et al. 2004). In addition, Cdc13p interacts with the catalytic subunit of DNA polymerase  $\alpha$  (Qi and Zakian 2000). Thus, the end protein complex not only regulates telomerase recruitment and activity but may also orchestrate telomerase action with lagging strand DNA synthesis.

Telomerase assembles with telomeres in *S. cerevisiae* and in humans in a cell cycle-dependent manner. In *S. cerevisiae*, two separate pathways of telomerase recruitment appear to exist (Fig. 4). Cdc13p binds the telomerase-associated Est1p, and this interaction is essential for telomerase activity at chromosome ends (Pennock et al. 2001). The recruitment function of Cdc13 is reduced by a point mutation (*cdc13-2*) at amino acid 252 (Glu→Lys), which can be suppressed by a missense mutation at residue 444 of Est1p (Lys→Glu). This reciprocal suppression depends on oppositely charged residues in Cdc13 and Est1, providing excellent evidence for a direct physical interaction between these two proteins (Pennock et al. 2001). Studies with fusion proteins are consistent with a model in which the interaction between Est1p and Cdc13p mediates the recruitment of telomerase (Evans and Lundblad 1999). Strikingly, a fusion between Cdc13p and Est2p allows telomere maintenance even in the absence of Est1p. Recruitment assays to DNA breaks containing telomere repeat sequences at the break site further support the telomerase recruitment functions of Cdc13 and Est1 (Bianchi et al. 2004). Chromatin immunoprecipitation (ChIP) experiments demonstrate preferential association of Est1p with telomeres during S phase, thus correlating well with the time of telomerase action (Schramke et al. 2004; Taggart et al. 2002).

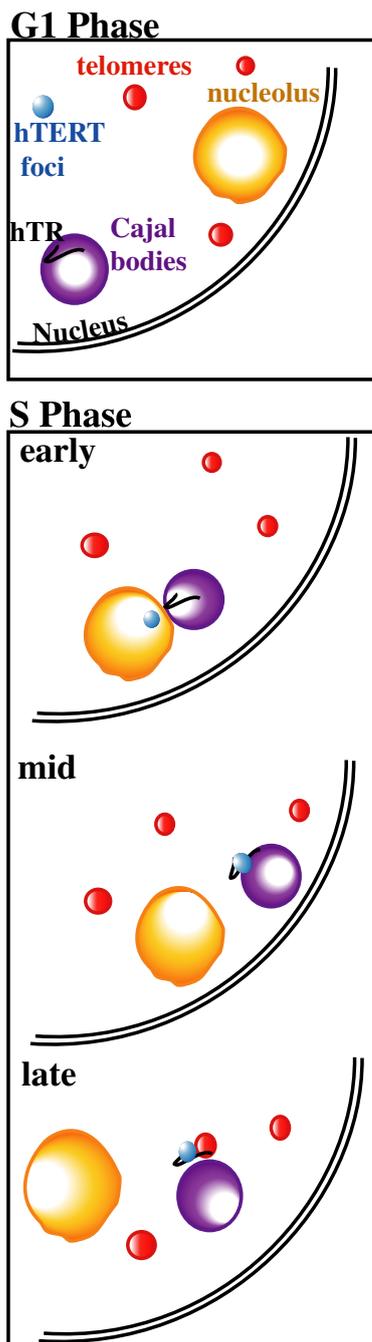
In budding yeast, the MRX complex and the ATM and ATR-related PIKKs, Tel1 and Mec1, have also been implicated in the hierarchical assembly of telomerase at the 3' end during S phase. Simultaneous deletion of these two PIKKs in fission or budding yeast gives an *EST* phenotype (Naito et al. 1998; Ritchie et al. 1999). Mec1 is recruited to telomeres during late S phase, whereas Tel1 associates with telomeres in G1 (Takata et al. 2004). MRX, which binds to telomeres also in late S phase, is required for the efficient recruitment of Mec1, Cdc13p, and Est1 at the telomere (Takata et al. 2005). However, because mutations in MRX or *MEC1* per se do not trigger an *EST* phenotype, Cdc13p- and Est1p-recruitment must still occur even in the absence of these factors. As mentioned above, the MRN complex has also been implicated in telomerase recruitment in human cells (Chai et al. 2006b). Association of the MRN complex at telomeres depends on TRF2 (Zhu et al. 2000). MRE11 and RAD50 are present at human telomeres throughout the cell cycle, whereas NBS1 asso-

ciates with telomeres in S phase (Zhu et al. 2000; Loayza and de Lange 2003).

A second telomerase recruitment mechanism in *S. cerevisiae* is provided in G1 through the interaction of the telomere-binding protein Ku with telomerase (Fisher et al. 2004). Ku recruits telomerase through its specific binding of a stem-loop in TLC1 telomerase RNA (Stellwagen et al. 2003). In the absence of Ku, telomeres are stable, albeit very short, demonstrating that the Ku-mediated recruitment of telomerase is important but not essential for telomerase activity (Boulton and Jackson 1998). Furthermore, the Ku-mediated recruitment is not sufficient for telomerase recruitment, as evidenced by the dependence of telomere-extension on the Cdc13p–Est1p interaction. The Ku-mediated recruitment of telomerase in G1 may increase the local concentration of telomerase near its substrate, thus favoring the assembly of telomerase with Cdc13p and the telomere 3' end in S phase. Recently, Pif1p helicase overexpression has been demonstrated to remove telomerase from chromosome ends, while its depletion increases telomerase association (Boule et al. 2005). In vitro, Pif1 releases telomerase from telomeric oligonucleotide substrates. Pif1 also interacts genetically with the Dna2 helicase/nuclease, which is required for Okazaki processing (Budd et al. 2006). It has been proposed that Dna2 and Pif1 may be required for processing of the last Okazaki fragment at the telomere, as there is no pol  $\delta$ /PCNA present to strand displace and to recruit Fen1 (Budd et al. 2006). It will be interesting to determine how Pif1 regulates the frequency and extent of telomere elongation in vivo.

In humans, semiconservative DNA replication of telomeres occurs throughout S phase (Ten Hagen et al. 1990; Wright et al. 1999). Direct proof that telomerase action is restricted to this part of the cell cycle is missing. However, based on in situ hybridization studies with oligonucleotide probes, human telomeres are more accessible in S phase (Jady et al. 2006) and they seem to lose part of their protective structure at the end of S phase, becoming recognized as DNA damage in G2 (Verdun et al. 2005). The identity of human telomerase recruitment factors is elusive, though human proteins with sequence homology to yeast Est1p have been identified (Reichenbach et al. 2003; Snow et al. 2003). Yeast Est1p and human EST1A and EST1B associate with telomerase in extracts, but their role for telomerase action remains to be elucidated (reviewed by Azzalin et al. 2005).

Recent cytological analyses carried out by two laboratories indicate S-phase-specific assembly of human telomerase with telomeres (Fig. 5) (Jady et al. 2006; Tomlinson et al. 2006). Subcellular localization of endogenous human telomerase RNA was assessed by in situ hybridization, and telomerase reverse transcriptase was detected by indirect immunofluorescence. Human telomerase RNA (hTR) was



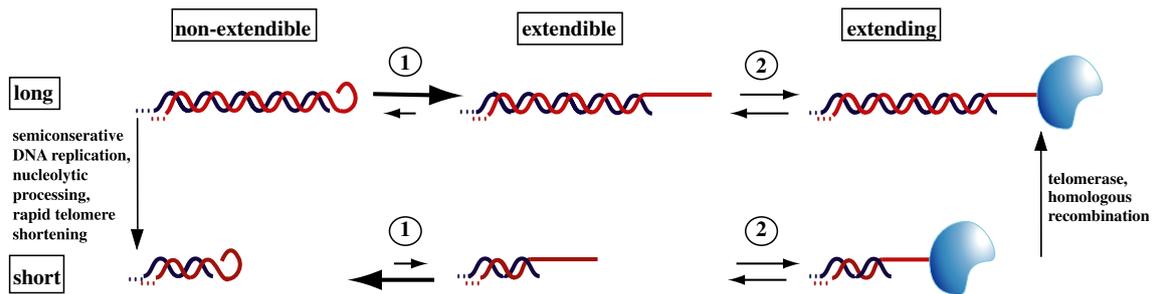
**Fig. 5** Cell cycle-dependent assembly of human telomerase and association with telomeres. In G1-cells, hTR is present in Cajal bodies and hTERT is present in nucleoplasmic foci. Early in S phase, Cajal bodies containing hTR are present at the periphery of nucleoli, while hTERT is nucleolar. During S phase, hTERT and hTR are both localized to foci adjacent to Cajal bodies. Some Cajal bodies, hTERT, and hTR are also found in association with telomeres

detected during interphase in Cajal-bodies, dynamic structures involved in the biogenesis of small RNPs. Human TERT (hTERT), on the other hand, was present in nucleoplasmic foci of unknown composition (Tomlinson et al. 2006). Early in S phase, Cajal bodies containing hTR were present at the periphery of nucleoli while hTERT

became nucleolar. During S phase, hTERT and hTR both localized to foci adjacent to Cajal bodies. Some Cajal bodies, hTERT and hTR were also found in association with telomeres during S phase. Live imaging indicated that the association between Cajal bodies and telomeres lasted about 30 min (Jady et al. 2006). These experiments suggest cell cycle-dependent assembly of active telomerase and cell cycle-dependent association of telomerase with telomeres. Whether hTERT and hTR assemble adjacent to Cajal bodies or at telomeres is not clear yet. The signal intensity of hTERT and hTR at telomeres may suggest that a large number of telomerase molecules associate with single chromosome ends in S phase. Thus, telomerase may not solely interact with the telomeric 3' overhangs through base-pairing of the RNA template with telomeric DNA, but recruitment pathways may involve, as in *S. cerevisiae*, interactions with telomeric proteins. Measurements of telomerase in vitro activity did not reveal cell cycle-dependent changes in the amount of extracted telomerase activity (Holt et al. 1997). It is conceivable that telomerase assembly occurred in extracts upon disruption of subnuclear structures or that a considerable fraction of, perhaps, less focally concentrated but assembled telomerase complexes was not detected in the cytological analyses. The Rb protein family is required for proper telomere length control in the mouse and it will be interesting to see if and how these cell cycle regulators impinge on telomerase assembly or telomere structure (Garcia-Cao et al. 2002).

### Telomere length homeostasis

The above data indicate that telomerase activity at chromosome ends is only permitted during S phase. This restriction may be enabled through cell-dependent changes in telomere structure and the cell cycle-restricted assembly of active telomerase. Telomere length homeostasis requires, in addition, mechanisms to measure telomere length in *cis*, which either promote more efficient elongation of short telomeres by telomerase or which promote activities to shorten preferentially overly long telomeres. Evidence for telomere length regulation in *cis* became apparent as early as 1982 in experiments in which a linear plasmid containing terminal telomeric repeat seeding sequences from *Tetrahymena* was transformed and maintained in yeast cells (Szostak and Blackburn 1982). Approximately 200 bp of new yeast telomeric sequence was added de novo to the end of the linear molecule, thus giving a telomere length resembling that of natural yeast chromosomes (Shampay et al. 1984). In yeast, the involvement of the Rap1-telomere-binding protein for *cis*-regulation of telomere length was demonstrated by targeting different numbers of Rap1p carboxyl termini via a heterologous DNA-binding



**Fig. 6** A three-state model for telomere length homeostasis. Telomeres are proposed to switch between nonextendible (*curly end, left*), extendible (*straight, middle*), and extending states (*straight end associated with telomerase, right*). The equilibrium between non-

extendible and extendible states (1) is a function of telomere length, while the equilibrium between extendible and extending state (2) is a function of telomerase concentration

domain to a model telomere (Marcand et al. 1997). Telomere length depended on the number of targeted molecules consistent with a feedback mechanism of telomere length regulation. This so-called protein counting mechanism was also recapitulated in human cells with TRF1 and TRF2 (Ancelin et al. 2002).

Observation of the elongation rate of an abnormally shortened telomere in yeast demonstrated length-dependent re-elongation kinetics (Marcand et al. 1999). In mice, crosses between strains with short and long telomeres result in a preferential elongation of short telomeres (Hemann et al. 2001; Samper et al. 2001; Zhu et al. 1998). Similarly, the expression of limiting amounts of telomerase activity in human fibroblasts or in mice leads to preferential elongation of short telomeres (Liu et al. 2002; Ouellette et al. 2000). In the above studies, it was unclear whether the frequency with which telomerase accessed a telomere or the extent of elongation was regulated in a length-dependent manner. A recently developed assay that allows elongation analysis of single telomere molecules in yeast demonstrates that telomerase does not act on every telomere in each cell cycle but that it exhibits an increasing preference for telomeres as their lengths decline (Teixeira et al. 2004). At wild-type length (300 nucleotides), the frequency of telomere elongation was only around 6–8%, while it increased steadily to nearly 50% upon shortening of telomeres to 100 nucleotides. On the other hand, the number of nucleotides added to a telomere in a single cell cycle varies substantially between a few to more than 100 nucleotides and does not correlate well with telomere length. Thus, the extent of telomere elongation is not regulated in a length-dependent manner. This analysis therefore suggests that telomere length homeostasis is achieved via a switch between telomerase extendible and nonextendible states.

In human cells, it has not yet been demonstrated if the frequency of telomere elongation, the extent of elongation, or both, are regulated in a length-dependent manner. Nevertheless, the current models to explain the cell cycle

dependence and the effects of telomere-binding proteins on telomerase activity in vitro invoke the existence of nonextendible states. The t-loop with a telomeric 3' overhang invading the double-strand telomeric tract should be nonextendible due to the base-paired telomeric 3' end (Griffith et al. 1999). POT1-bound single-stranded telomeric DNA molecules also inhibit telomerase activity, at least in vitro (Kelleher et al. 2005; Lei et al. 2005).

How is the equilibrium between the putative extendible and nonextendible telomeric states regulated? The nature of these states and the molecular mechanisms of their transition remain speculative. However, it has become apparent that telomerase concentration limits telomere length in mammalian cells and, as argued below, this allows certain conclusions to be drawn about the function and dynamics of telomeric states. In dyskeratosis congenita patients, the decreased expression of telomerase due to mutations in the dyskerin, the telomerase RNA, or the TERT genes causes short telomeres and bone marrow failure (Mitchell et al. 1999; Vulliamy et al. 2001, 2005; Yamaguchi et al. 2005). Haploinsufficiency has also been reported in heterozygous  $mTR^{+/-}$  and  $mTERT^{+/-}$  mice (Hathcock et al. 2002; Liu et al. 2002). Interestingly, the length of very short telomeres present in  $mTR^{+/-}$  mice is not fully reset even in wild-type offspring derived from heterozygous parents, giving rise to a so-called occult form of genetic disease (Hao et al. 2005). On the other hand, an increasing of telomerase activity in human cells over a wide range demonstrates that telomerase association with telomeres as measured by ChIP depends on telomerase concentration (Cristofari and Lingner 2006b). The overexpression of telomerase increased its association with telomeric DNA, and this was sufficient to elongate telomeres in primary or cancer cells in a length-independent manner far beyond physiological size. Thus, even long telomeres are extendible, indicating that they do not permanently adopt a structural nonextendible state. In addition, we predict the existence of a distinct third telomeric state with extendible telomeres switching to an

extending state, upon productive association with telomerase (Fig. 6). In this model, the transition between the nonextendible and extendible states is length-dependent, whereas the transition between extendible and extending states depends on the concentration of telomerase. Thus, a low cellular concentration of telomerase is critical to achieve preferential elongation of short telomeres and telomere length homeostasis.

The current data suggest that telomerase activity at chromosome ends is regulated by telomere structure and telomerase assembly. Although not proven, the telomere structure may change in a cell cycle-dependent manner to restrict telomerase activity to S phase. It is conceivable that semiconservative DNA replication through the telomere triggers a structural change that is required to allow telomerase-mediated telomere extension. With limiting concentrations of telomerase, this extension elongates short telomeres more efficiently, but the structural changes at the telomere that mediate this preference are not clear. Perhaps all telomeres are accessible to telomerase for a short period of time after the semiconservative replication of the DNA end, but long telomeres switch back more quickly to a nonextendible state (e.g., a t-loop or 3' end covered with POT1) than short telomeres, and therefore, short telomeres may have a higher probability to bind telomerase (Fig. 6). PIKKs, telomere-binding proteins, and DNA polymerases involved in lagging strand synthesis may all contribute to this regulation.

### Prospects

Elucidation of the identities of the different cell cycle-dependent and length-dependent telomeric states remains a difficult challenge. ChIP experiments may contribute to unraveling the molecular composition of telomeres but they do not map the exact binding sites of the crosslinked proteins (Fisher et al. 2004; Takata et al. 2005; Verdun et al. 2005; Zhu et al. 2000). Measuring the fraction of t-loop molecules during the cell cycle may substantiate a putative role of this structure to restrict telomerase activity. The role of PIKKs for telomere maintenance and how they activate telomerase activity is also unclear. The identification of the critical kinase substrates at the telomere or in the telomerase complex and the elucidation of the downstream events of phosphorylation will be an important step. A long-term challenge will be the establishment of *in vitro* systems in which telomere substrate elongation *in vitro* recapitulates the *in vivo* requirements for telomerase recruitment factors, PIKKs, and other factors.

The cell cycle-dependent regulation of telomerase assembly has become apparent recently. The expression of Est1 in yeast is cell cycle-regulated (Taggart et al. 2002),

and this restriction may contribute to preventing the unscheduled association of the Est1–Est2–Est3–TLC1 complex with Cdc13p outside of S phase. The mechanisms that regulate cell cycle-dependent assembly of human telomerase and human telomerase's association with telomeres are unexplored. The recent establishment of methods to cytologically detect telomerase components at telomeres will allow the identification of the factors that mediate and regulate these events.

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