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Replication of Telomeres and the Regulation of Telomerase

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Telomeres are the physical ends of eukaryotic chromosomes. They protect chromosome ends from DNA degradation, recombination, and DNA end fusions, and they are important for nuclear architecture. Telomeres provide a mechanism for their replication by semiconservative DNA replication and length maintenance by telomerase. Through telomerase repression and induced telomere shortening, telomeres provide the means to regulate cellular life span. In this review, we introduce the current knowledge on telomere composition and structure. We then discuss in depth the current understanding of how telomere components mediate their function during semiconservative DNA replication and how telomerase is regulated at the end of the chromosome. We focus our discussion on the telomeres from mammals and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Herman Muller and Barbara McClintock were probably the first scientists to recognize that the ends of eukaryotic chromosomes have special properties and crucial functions (reviewed by Blackburn 2006). Herman Muller studied *Drosophila* chromosomes and found that X rays could induce chromosome rearrangements. However, he never observed chromosomes carrying terminal deletions (Muller 1938). These results led him to conclude that "the terminal gene must have a special function, that of sealing the end of the chromosome, so to speak," and that "for some reason, a chromosome cannot persist indefinitely without having its ends thus sealed." Muller coined the term "telomere" for this terminal gene (from the Greek words *telos* = end and *meros* = part). Barbara McClintock analyzed maize strains in which dicentric chromosomes were produced with high frequency. Dicentric chromosomes break when the two centromeres are pulled toward opposite poles of the mitotic spindle during anaphase of the cell cycle. The broken chromosomal ends were unstable and fused with other broken ends with which they came in contact. However, when dicentric chromosomes were present in embryonic cells, the broken ends were somehow "healed" (McClintock 1941).

The molecular sequence of telomeric DNA was first determined by Elizabeth Blackburn and Joseph Gall in the ciliated protozoan *Tetrahymena thermophila* (Blackburn and Gall 1978). These experiments revealed that the DNA ends

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in this organism consist of 5′-T_2G_4-3′/5′-C_4A_2-3′ telomeric repeats with the G-rich strand running toward the 3′ end of the chromosome. Cloning and sequencing of telomeres from other eukaryotes revealed that the repetitive nature of telomeric repeats and the presence of a G-rich strand are common features of nearly all eukaryotes. Furthermore, it has been established that the 3′-end-containing strand protrudes at both ends of the chromosome. However, notable exceptions do exist. The roundworm *Caenorhabditis elegans* contains both 3′ G-overhangs as well as 5′ C-overhangs (Raices et al. 2008). Several genera of insects and plants do contain long DNA repeats (Martinez et al. 2001). *Drosophila* contains, instead of short telomeric repeats, retrotransposons at chromosome ends that have overtaken telomere functions (Pardue and Debaryshe 2011).

The formulation of the DNA end-replication problem by Watson and Olovnikov inferred that specialized mechanisms must exist to maintain telomere length (Olovnikov 1971; Watson 1972). Greider and Blackburn discovered telomerase activity in extracts from *Tetrahymena* (Greider and Blackburn 1985). They also identified the telomerase RNA moiety in this organism, which contains a sequence complementary to *Tetrahymena* telomeric repeats (Greider and Blackburn 1989). Mutation of this sequence proved that the telomerase RNA moiety provides the template for DNA repeat synthesis and that the telomerase functions as reverse transcriptase (Yu et al. 1990). Around the same time, Lundblad and Szostak were the first to discover an essential telomere maintenance protein gene in *Saccharomyces cerevisiae*. Dysfunction of the identified gene gave rise to an ever shorter telomeres (*est*) phenotype culminating in cellular senescence (Lundblad and Szostak 1989). The discovery of *EST1* was followed by the identification of *EST2*, *EST3*, and *EST4* in the Lundblad laboratory (Lendvay et al. 1996). Est2 turned out to be orthologous with the p123 telomerase reverse transcriptase (Tert) protein subunit (Lingner et al. 1997) that was identified in the ciliate *Euplotes aediculatus* upon biochemical purification of the telomerase enzyme (Lingner and Cech 1996). The identification of the first Tert subunits opened the door to identify the corresponding genes in humans and other organisms, allowing the study of telomerase during development and in cancer. Significantly, it was shown that ectopic expression of human Tert in various primary human cells is sufficient to reconstitute telomerase, rendering them immortal (Bodnar et al. 1998).

Ciliated protozoa were also instrumental in purifying and cloning the first eukaryotic telomere-binding proteins (Gottschling and Zakian 1986; Hicke et al. 1990; Gray et al. 1991). However, orthologs of these proteins were at first not recognized in other eukaryotes, and independent approaches were undertaken to identify the first telomeric proteins in various eukaryotes including yeasts and humans. Only much later was it realized that the ciliate telomere-binding proteins contain counterparts in a broad range of eukaryotes (Baumann and Cech 2001).

In this review, we introduce known basic components of telomeres and review the mechanisms of semiconservative DNA replication of telomeric DNA and the regulation of telomerase. For the discussion of telomere protection and the three-dimensional structures of telomere components, the reader is referred to excellent recent reviews (de Lange 2009; Jain and Cooper 2010; Lewis and Wuttke 2012).

TELOMERE COMPONENTS

In the following section, the stable constituents of telomeres that are implicated in regulating telomere replication are discussed. Abundant telomere-binding proteins as well as telomerase subunits from *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Homo sapiens* described in detail in the text are summarized in Table 1.

S. cerevisiae

The telomeric DNA repeat consensus sequence for the 3′-end-containing strand from *S. cerevisiae* is 5′-$(TG)_{0-6}$TGGGTGTG(G)-3′ (Forstemann and Lingner 2001). The telomere length is around 300 bp. Native chromosome ends in *S. cerevisiae* have a number of subtelomeric

Table 1. Abundant telomere-binding proteins and telomerase proteins in *S. cerevisiae, S. pombe,* and *H. sapiens*

Organism	Subcomplex	Protein	Interaction partner	Ortholog	Function
S. cerevisiae		Rap1	Sir3, Sir4, or Rif1, Rif2	Rap1 (*S. p.*, *H. s.*)	Essential; major double-stranded telomere-binding protein; transcriptional regulation of telomeres and protein-coding genes; protection from NHEJ; negative regulator of telomere length
	Sirtuins	Sir2	Sir3, Sir4		NAD^+-dependent histone deacetylase; telomeric silencing
		Sir3	Sir2, Sir4, Rap1		Telomeric silencing
		Sir4	Sir2, Sir3, Rap1		Telomeric silencing
	Rifs	Rif1	Rif2, Rap1		Negative regulator of telomere length
		Rif2	Rif1, Rap1		Negative regulator of telomere length
	CST	Cdc13	Ten1, Stn1, Est1, Pol1	Ctc1 (*H. s.*; limited sequence similarity)	Essential G-strand single-stranded telomere-binding protein; protects from C-strand loss; recruits telomerase through interaction with Est1; essential for telomerase activity in vivo but not in vitro; interacts with Pol1, the catalytic subunit of DNA polymerase α-primase, promoting fill-in synthesis of telomerase-elongated telomeres
		Stn1	Ten1, Cdc13, Pol12	Stn1 (*S. p.*, *H. s.*)	Essential; protects from C-strand loss; negative regulator of telomere length; interacts with Pol12, the B subunit of DNA polymerase α-primase, promoting fill-in synthesis of telomerase-elongated telomeres
		Ten1	Stn1, Cdc13	Ten1 (*S. p.*, *H. s.*)	Essential; protects from C-strand loss; negative regulator of telomere length
	Telomerase	Est1	Tlc1, Est2, Cdc13	Est1 (*S. p.*, *H. s.* Est1A has limited sequence similarity)	*est* phenotype; binds Tlc1 and G-strand single-stranded telomeric DNA; recruits telomerase through interaction with Cdc13; essential for telomerase activity in vivo but not in vitro
		Est2	Tlc1, Est1	Tert (*H. s.*), Trt1 (*S. p.*)	*est* phenotype; telomerase reverse transcriptase; essential for telomerase activity in vivo and in vitro
		Est3	Est1, Est2		*est* phenotype; essential for telomerase activity in vivo but not in vitro

Continued

Table 1. *Continued*

Organism	Subcomplex	Protein	Interaction partner	Ortholog	Function
			Sm proteins	Smb1, Smd1, Smd2, Smd3, Sme1, Smx3, Smx2	Form heteroheptameric Sm ring that binds and stabilizes Tlc1
S. pombe		Taz1	Rif1, Rap1	Trf1, Trf2 (*H. s.*, *S. c.* Tbf1 has limited sequence similarity)	Major double-stranded telomere-binding protein; protection from NHEJ; negative regulator of telomere length; promotes telomere replication; telomeric silencing and TERRA repression; meiosis
		Rif1	Taz1		Negative regulator of telomere length; recruited by Taz1
		Rap1	Taz1, Poz1	Rap1 (*S. c.*, *H. s.*)	Negative regulator of telomere length; recruited by Taz1; telomeric silencing and TERRA repression
		Poz1	Rap1, Tpz1	Pot1 (*H. s.*)	Negative regulator of telomere length
		Tpz1	Poz1, Pot1, Ccq1	Tpp1 (*H. s.*)	Protects telomeric 3′ overhang; telomerase recruitment through interaction with Poz1 and Ccq1
		Pot1	Tpz1	Pot1 (*H. s.*)	Essential; single-stranded G-strand telomere-binding protein; protects telomeres from nucleolytic degradation
		Ccq1	Tpz1, Est1		Telomerase recruitment through interaction with Est1; meiosis
		Ten1	Stn1	Ten1 (*S. c.*, *H. s.*)	Telomere-capping protein; protects from telomere loss
		Stn1	Ten1	Stn1 (*S. c.*, *H. s.*)	Telomere-capping protein; needed for telomere maintenance
	Telomerase	Est1	Ter1, Ccq1, Trt1	Est1 (*S. c.*)	*est* phenotype; binds Ter1, which has an Sm protein-binding motif; recruits telomerase through interaction with Ccq1; essential for telomerase activity in vivo but not in vitro
		Trt1	Ter1, Est1		*est* phenotype; telomerase reverse transcriptase; essential for telomerase activity in vivo and in vitro
		Sm proteins	SmB, SmD1, SmD2, SmD3, SmE, SmF, SmG		Form heteroheptameric Sm ring that binds Ter1 precursor and promotes spliceosomal cleavage; promotes hypermethylation of the Ter1 5′ cap by Tgs1
		Lsm proteins	Lsm2–8		Bind mature Ter1 and promote association with Trt1; protect Ter1 from nucleolytic degradation

H. sapiens	Shelterin	Trf1	Tin2	Taz1 (*S. p.*)	Double-stranded homodimeric telomere-binding protein; negative regulator of telomere length; promotes telomere replication
		Trf2	Rap1, Tin2	Trf1 (*H. s.*), Taz1 (*S. p.*)	Double-stranded homodimeric telomere-binding protein; protection from NHEJ; repression of Atm
		Rap1	Trf2	Rap1 (*S. c.*, *S. p.*)	Recruited by Trf2; repression of telomere recombination and homology-directed repair
		Tin2	Trf1, Trf2, Tpp1		Stabilization of shelterin complex; recruits telomerase through interaction with Tpp1
		Tpp1	Pot1, Tin2, Tert	Tpz1 (*S. p.*)	Increased affinity of Pot1 for single-stranded telomeric DNA; stimulation of telomerase processivity in conjunction with Pot1; recruits telomerase through interaction with Tin2 and telomerase
		Pot1	Tpp1	Pot1 (*S. p.*)	Binds single-stranded G-strand telomeric DNA; inhibits telomerase in absence of Tpp1; Pot1–Tpp1 promotes telomerase processivity; repression of Atr
	CST	Ctc1	Stn1, Ten1	Cdc13 (*S. c.*; limited sequence similarity)	When assembled as CST, it binds single-stranded DNA with preference for telomeric G-strand sequence; inhibits telomerase activity through direct interaction with Pot1/Tpp1 and primer sequestration; increased telomere binding upon telomerase-mediated telomere elongation; competes with Pot1/Tpp1 for binding of telomeric DNA; with Stn1, Ctc1 stimulates affinity of DNA polymerase α-primase for template DNA; semiconservative DNA replication of specialized DNA sequences
		Stn1	Ten1, Ctc1	Stn1 (*S. p.*, *S. c.*)	See Ctc1
		Ten1	Ctc1, Stn1	Ten1 (*S. p.*, *S. c.*)	See Ctc1

Continued

Table 1. *Continued*

Organism	Subcomplex	Protein	Interaction partner	Ortholog	Function
	Telomerase	Tert	Terc, Tpp1		Telomerase reverse transcriptase; essential for telomerase activity in vivo and in vitro
		Tcab1			Telomerase localization to Cajal bodies and to telomeres in S phase; essential for telomerase activity in vivo but not in vitro
		Dyskerin	Nop10, Nhp2, Gar1		Binds H/ACA RNA motif–containing RNAs including Terc; Terc stability
		Nop10	Dyskerin, Nhp2		See Dyskerin
		Nhp2	Dyskerin, Nop10		See Dyskerin
		Gar1	Dyskerin		See Dyskerin
		Est1a	Terc, Tert	Est1 (*S. p.* and *S. c.* Est1 have limited sequence similarity; no evidence for conserved functional role in telomerase recruitment)	Negative regulator of TERRA at telomeres; depletion leads to stochastic telomere loss; overexpression leads to telomere uncapping and telomere fusions; involved in nonsense-mediated mRNA decay

NHEJ, nonhomologous end joining; TERRA, telomeric repeat–containing RNA; CST, Cdc13–Stn1–Ten1.

repeat elements termed X and Y′, adjacent to the telomeric repeats, which vary between ends and strains (Louis and Borts 1995). The 473-bp core X is found at all chromosome ends. It contains an ACS (ARS consensus sequence, the binding site for the yeast origin recognition complex) and an Abf1p-binding site at 31 out of 32 ends. There are also binding sites for the telomere-binding protein Tbf1 (Bilaud et al. 1996). So-called Y′ elements with a length of 6.7 kb or 5.2 kb are present at approximately half of *S. cerevesiae* telomeres.

The telomeric repeat sequence is bound by the double-stranded DNA-binding protein Rap1 (repressor activator protein 1) (Fig. 1) (Lustig et al. 1990). Rap1 also binds chromosome internal sites acting as positive transcriptional regulator of genes involved in growth control. Rap1 has two central Myb domains responsible for DNA binding and a Rap1 carboxy-terminal domain (RCT). RCT recruits the Rif proteins, which repress telomerase (see below), and it recruits the Sir3 and Sir4 proteins, which in turn interact with the Sir2 NAD^+-dependent histone deacetylase involved in transcriptional repression. Rap1 is also important for protection from end fusions (Marcand et al. 2008).

The telomeric 3′ overhang is bound by a trimeric complex consisting of Cdc13, Stn1, and Ten1 (CST) (Fig. 1) (Gao et al. 2007). Despite binding to the G-strand, the CST complex protects the C-strand from 5′-3′ resection. In addition, CST regulates telomere length through telomerase (see below). CST has been proposed

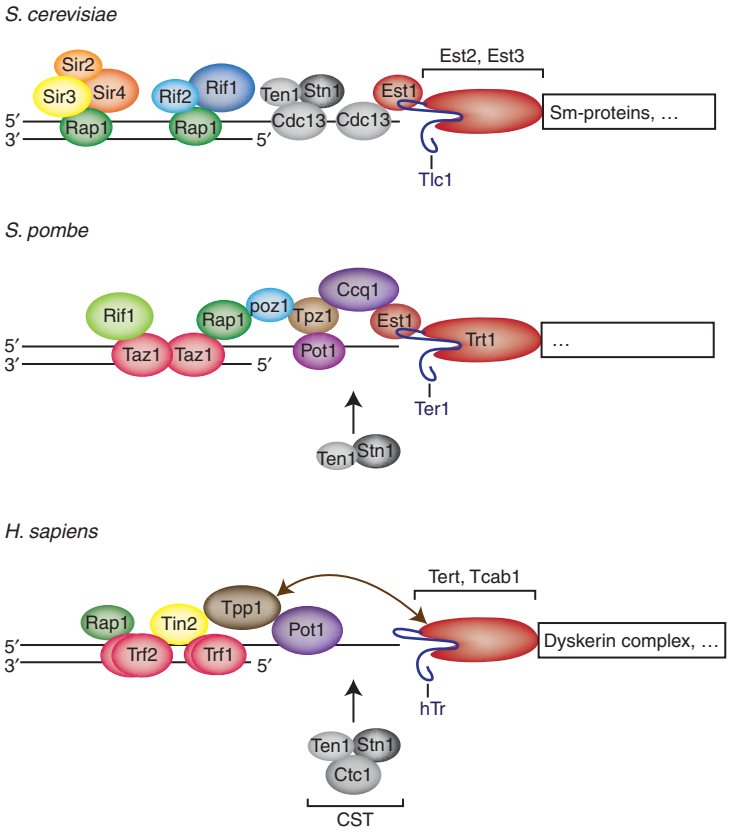

Figure 1. Telomeric proteins and telomerases at yeast and human telomeres. Only abundant telomere-binding proteins and telomerase components are indicated. Trf1 and Trf2 bind as homodimers to the double-stranded telomeric DNA repeats. Homologous proteins are shown in the same color.

to resemble in structure the replication protein A (RPA) complex. CST contains as RPA multiple oligosaccharide/oligonucleotide binding (OB)-folds. In particular, Stn1 and Ten1 proteins contain structural similarities with Rpa2 and Rpa3 (Gao et al. 2007).

Telomeres in *S. cerevisiae* are transcribed by RNA polymerase II into the long noncoding telomeric repeat-containing RNA (TERRA) (Luke et al. 2008). TERRA transcription starts in the subtelomeric DNA and proceeds approximately 100 nucleotides into the telomeric tract. *S. cerevisiae* TERRA carries a cap structure at the 5′ end and is polyadenylated. TERRA is degraded by the 5′-3′ exonuclease Rat1. TERRA transcription is negatively regulated by the Sir proteins at telomeres that contain only the X subtelomeric element at their chromosome ends. At Y′ elements containing telomeres, TERRA transcription is negatively regulated by the Rif proteins (Iglesias et al. 2011).

Telomeres in *S. cerevisiae* have been inferred to form fold-back or looped structures because Rap1 is associated with subtelomeric chromatin as well as with telomeric DNA (reviewed by Ottaviani et al. 2008). This fold-back structure has been proposed to be critical for the so-called telomere position effect, which refers to the repression of reporter genes inserted in the subtelomeric region (Gottschling et al. 1990). Silencing at telomeres is mediated by the silent information regulators Sir3, Sir4, and Sir2.

S. pombe

The telomeric DNA sequence from *S. pombe* is unusually heterogeneous with the consensus 5′-$C_{1-8}G_{0-1}T_{0-2}GTA_{1-3}$-3′ (Sugawara 1989). Telomere length, as in *S. cerevisiae*, is ~300 bp. The subtelomeric DNA in *S. pombe* contains specialized repeats (*dg* and *dh*), which are also found at other heterochromatic loci contributing to formation of heterochromatin by the RNA interference (RNAi)-induced transcriptional silencing machinery (Kanoh et al. 2005). The first identified telomere-binding protein, Taz1, was identified in a one-hybrid screen using the double-stranded telomeric DNA as bait (Cooper et al. 1997). Taz1 is orthologous with the mammalian telomere-binding proteins Trf1 and Trf2. Taz1 recruits Rap1 to telomeres (Fig. 1). Rap1 binds Poz1, Poz1 binds Tpz1, and Tpz1 interacts with Ccq1 and Pot1 (Miyoshi et al. 2008). Pot1 also binds the telomeric single-stranded 3′ overhang (Baumann and Cech 2001). Thus, multiple protein interactions bridge the double-stranded part of the telomeric DNA to the single-stranded 3′ overhang, which is reminiscent of the situation in mammals. *S. pombe* Pot1 also plays crucial roles in protecting chromosome ends from degradation, Ccq1 interacts with the telomerase subunit Est1, recruiting telomerase to telomeres (see below). In addition, Stn1 and Ten1 orthologs but not Cdc13 orthologs have been identified in fission yeast (Martin et al. 2007). They protect from rapid loss of telomeric DNA. TERRA and other telomeric transcripts have been recently characterized in fission yeast (Bah et al. 2012; Greenwood and Cooper 2012). Their expression is negatively regulated by Taz1 and Rap1.

Mammals

Vertebrate telomeric DNA consists of 5′-TTAGGG-3′ repeats. The telomere length in humans is considerably shorter (5–15 kb) than in *Mus musculus* (>40 kb), and the human subtelomeric sequences are not well annotated. They contain repetitive sequence variants of the 5′-TTAGGG-3′ repeat sequence and a patchwork of segmentally duplicated DNA tracts that are shared in some instances between different telomeres and are highly polymorphic (Riethman 2008). Biochemical purification identified the first human telomere-binding protein, Trf1 (Chong et al. 1995). Dimeric Trf1 binds to double-stranded telomeric DNA (Fig. 1). A second homologous protein termed Trf2 also binds directly to the double-stranded telomeric DNA as a dimer (Bilaud et al. 1997; Broccoli et al. 1997). Trf1 and Trf2 interact with Tin2, which in turn binds Tpp1. Tpp1 also binds the single telomere-binding protein Pot1 (de Lange 2005). Notably, whereas in humans there is only one Pot1 protein, the mouse contains two Pot1-like proteins termed Pot1a and Pot1b (Hockemeyer

et al. 2006; Wu et al. 2006). Trf2 also recruits Rap1 to telomeres. Thus, as in fission yeast, Rap1 is tethered to telomeres via protein interaction, whereas in budding yeast it directly binds the double-stranded telomeric DNA. No mammalian protein that is orthologous with *S. pombe* Ccq1 has yet been identified. However, a human CST complex was identified that is present at telomeres and at other regions in the genome (Miyake et al. 2009; Surovtseva et al. 2009).

Human TERRA is transcribed from several or possibly all chromosome ends (Azzalin et al. 2007; Schoeftner and Blasco 2008). Transcription starts in the subtelomeric region and proceeds up to ~400 nucleotides into the pure TTAGGG tract (Porro et al. 2010). Poly(A) tails containing and lacking TERRA fractions can be distinguished. Approximately half of the poly(A)$^-$ TERRA fraction is chromatin-associated, colocalizing with telomeres. The less abundant poly(A)$^+$ TERRA is all nucleoplasmic (Porro et al. 2010).

Mammalian telomeric DNA has been found to fold into so-called T-loop structures in which the 3′ overhang of telomeres invades through strand displacement the double-stranded part of telomeric repeats (Griffith et al. 1999). T-loops have been proposed to provide a mechanism for telomere capping, but this awaits experimental testing.

TELOMERASE

The catalytic core of telomerase consists of Tert and the telomerase RNA. The Tert subunit reverse transcribes the template sequence of telomerase RNA in an iterative fashion at the ends of chromosomes. When the 3′ end of the telomere is extended to the 5′ end of the RNA template, telomerase may reposition the substrate to the other side of the template and add the next telomere repeat, or it may dissociate from the substrate. The propensity of some telomerases to add several repeats without dissociation is referred to as repeat addition processivity. The Tert subunit is well conserved throughout evolution. It contains canonical reverse transcriptase motifs also found in retroelements (Lingner et al. 1997; Nakamura and Cech 1998). In addition, Tert contains a telomerase essential amino-terminal domain (TEN), a telomerase RNA-binding domain (TRBD), and a carboxy-terminal extension (reviewed by Lewis and Wuttke 2012). Other telomerase subunits are less well conserved. These subunits are not involved per se in catalysis, but they are important for telomerase assembly or the recruitment to telomeres. The *S. cerevisiae* Est1, which recruits telomerase to telomeres (discussed below), has a clear counterpart in *S. pombe* (Beernink et al. 2003; Webb and Zakian 2012), but the orthologous relationship to human Est1-like proteins (referred to as Est1A/Smg6, Est1B/Smg5, and Est1C/Smg7) is uncertain (Reichenbach et al. 2003; Snow et al. 2003). Est1A interacts as the budding and fission yeast Est1 proteins with telomerase, but a role in telomerase recruitment has not been shown (Redon et al. 2007). Est1A depletion has a strong effect on TERRA abundance at telomeres (Azzalin et al. 2007). Furthermore, Est1A to -C are also involved in the nonsense-mediated mRNA decay in the cytoplasm (Isken and Maquat 2008).

S. cerevisiae Est3 is essential for telomerase action at chromosome ends, but its contribution is not clear. Est3 contains an OB-fold, which is similar to an OB-fold present in the mammalian Tpp1 protein (Lee et al. 2008). Sm proteins associate with *S. cerevisiae* telomerase RNA, contributing to telomerase maturation and stability (Seto et al. 1999). *S. pombe* telomerase RNA (Ter1) also contains an Sm consensus site (Leonardi et al. 2008; Webb and Zakian 2008). The canonical Sm ring and the related Lsm2–8 complex sequentially associate with *S. pombe* Ter1 (Tang et al. 2012). The Sm ring binds the Ter1 precursor, stimulating its maturation by spliceosomal cleavage. Subsequently, the Lsm2–8 complex associates with Ter1, promoting the association with catalytic moiety Trt1 (Tang et al. 2012). In mammals, the dyskerin protein complex may fulfill similar functions in telomerase assembly (Mitchell et al. 1999; Pogacic et al. 2000). Another crucial component of human telomerase is Tcab1, which is discussed further below (Venteicher et al. 2009).

TELOMERE REPLICATION

Semiconservative Replication of Telomeric DNA

The largest part of the telomeric DNA is replicated by semiconservative DNA replication. The G-strand serves as a template for lagging-strand synthesis and the C-strand for leading-strand synthesis (Fig. 2). Only in recent years did it become apparent that even the semiconservative replication of telomeric DNA is difficult, requiring specialized helicases and telomere-binding proteins. In *S. cerevisiae*, the Rrm3 5′-3′ helicase prevents replication fork stalling at telomeres (Ivessa et al. 2002; Makovets et al. 2004). In fission yeast, it was noted that efficient replication of telomeric DNA requires the telomere-binding protein Taz1 (Miller et al. 2006). In its absence, replication forks frequently stall and collapse, leading to rapid loss of entire telomeric tracts. Taz1-deleted cells can only survive because telomerase very efficiently reextends the transiently truncated telomeric sequences. Evidence for truncated and by telomerase reextended telomeres was also obtained in wild-type budding yeast cells, supporting the notion that the semiconservative replication of telomeric DNA is error-prone (Chang et al. 2007).

In mouse cells, it was shown that efficient replication requires the Taz1 ortholog Trf1 (Martinez et al. 2009; Sfeir et al. 2009). Upon Trf1 deletion in mouse cells, replication forks stall and telomeric DNA splits in multiple signals at metaphase chromosomes, indicative of telomeric DNA breakage or lack of condensation. The mechanism by which Trf1 promotes telomere replication involves recruitment of the Blm or Rtel1 helicases (Sfeir et al. 2009; Vannier et al. 2012). Both of these helicases belong to the highly conserved RecQ 3′-5′ helicase family,

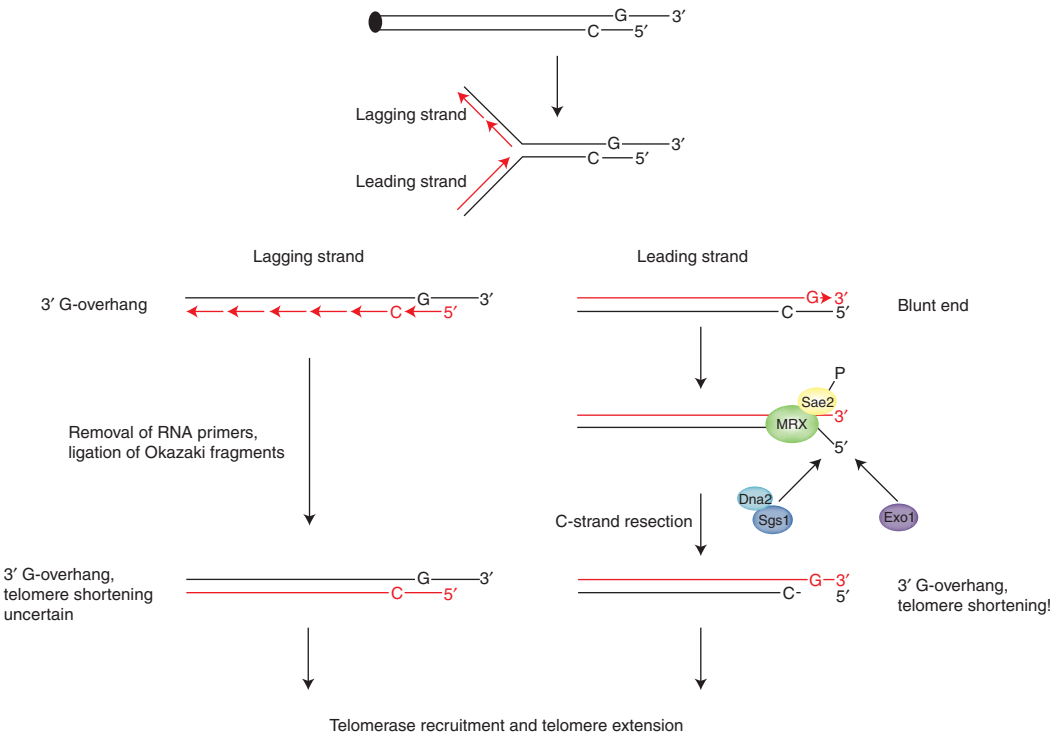

Figure 2. The end-replication problem and DNA end resection. The G-rich strand serves as a template for lagging-strand synthesis and the C-rich strand for leading-strand synthesis. The presumed blunt end intermediate at the leading-strand telomere is processed by 5′ end resection in order to recreate a 3′ overhang (*right*). Therefore, the leading-strand telomere shortens.

which use ATP hydrolysis to drive the unwinding of RNA and DNA. Another member of this family, the Werner helicase (Wrn), is required for efficient telomere lagging-strand synthesis (Crabbe et al. 2004; Arnoult et al. 2009). Interestingly, the stochastic telomere loss in Wrn-deficient cells and mice can be healed by the expression of telomerase (Chang et al. 2004; Crabbe et al. 2004). Indeed, in mice, the Wrn phenotype, which is characterized by multiple signs of premature aging, only manifests itself upon concomitant deletion of telomerase (Chang et al. 2004). *S. cerevisiae* Pif1 is another DNA helicase important for replication of G-rich sequences being enriched at telomeres and other G-rich sequences throughout the genome that may form G-quadruplex structures (Paeschke et al. 2011).

The Upf1 DNA- or RNA-dependent ATPase and 5′-to-3′ helicase is best known for its roles in cytoplasmic RNA quality control. However, human Upf1 binds to telomeres in vivo (Azzalin et al. 2007), interacting with the shelterin component Tpp1 (Chawla et al. 2011). Upf1 depletion leads to frequent loss of the telomeres replicated by leading-strand synthesis (Chawla et al. 2011). Upf1 depletion also leads to an increase of the TERRA signal at telomeres, suggesting a link between this RNA and telomere loss.

The crucial functions of helicases during replication of telomeric DNA appear to be multiple. First, helicases may unfold G-quadruplex DNA structures (Vannier et al. 2012) that may form during the lagging-strand replication of telomeric DNA, during which single-stranded G-rich DNA forms transiently. The structures involve guanine tetrads that associate through Hoogsteen hydrogen bonding to form a square planar structure, and several tetrads stack on top of each other to form a G-quadruplex (Parkinson et al. 2002). Second, in the T-loop structures, the 3′ overhang of telomeres invades through strand displacement the double-stranded part of telomeric repeats (Griffith et al. 1999). It is conceivable that helicases are necessary to unwind T-loops during semiconservative DNA replication (Vannier et al. 2012). Third, TERRA is associated with telomeres. If TERRA and telomeric DNA form R-loops, which are characterized by RNA–DNA hybrids and the displacement of single-stranded DNA, they may need to be resolved by helicases in order to avoid formation of double-stranded DNA breaks (Huertas and Aguilera 2003; Chawla et al. 2011; Wahba et al. 2011).

Telomere End Resection

Telomere leading-strand synthesis is predicted to yield a double-stranded blunt end intermediate (Fig. 2, right panel). This presumed intermediate is not detectable, as it will be quickly processed to regenerate a 3′ overhang. Thus, the 5′-end-containing parental C-rich strand is resected (Fig. 2, right panel). The generated G-rich 3′ overhang is important in order to generate a substrate for telomerase in addition to providing a platform for the binding of telomeric proteins and the ability to invade the double-stranded part of the telomere in the T-loop configuration. Thus, the telomere will shorten at the leading-strand end because the parental C-rich strand is resected during each round of replication (Lingner et al. 1995). During telomere lagging-strand synthesis, short RNA primers of 8–14 nucleotides are synthesized by primase and extended (Fig. 2, left panel). It is unknown where on the G-rich strand the most telomere-proximal primer is laid down. Because the parental G-rich strand forms a 3′ overhang that is longer than the RNA primer, the end-replication problem may not manifest itself at this end of the chromosome. However, telomerase binds leading- and lagging-strand telomeres (see below). Telomere lagging-strand replication also generates a 3′ overhang. Whether this involves simply RNA primer removal or in addition nucleolytic processing is currently unknown.

The 5′ end resection of leading-strand telomeres involves redundant activities consisting of nucleases, DNA helicases, and associated proteins that also resect 5′ ends from DNA double-strand breaks, preparing them for homologous recombination (Mimitou and Symington 2009). The Mre11–Rad50–Xrs2 (MRX) complex plays a role in end processing, as in its absence the G-overhangs at *S. cerevisiae* telomeres that have a length of 12–14 bases are shorter

(Larrivee et al. 2004). Furthermore, the longer G-overhangs that accumulate in yeast in late S phase are also diminished. However, Mre11 has 3′-5′ exonuclease activity as well as endonuclease nicking activity but not the 5′-3′ exonuclease activity that should be required to generate a telomeric 3′ overhang. Furthermore, although MRX is important for wild-type telomere length maintenance, this function may at least partially relate to its activity as a Tel1 recruiter (see below). Indeed, a nuclease-deficient Mre11 mutant was proficient in telomere maintenance (Tsukamoto et al. 2001). This is in contrast to results obtained with an endonuclease-induced cut at telomere repeats at which resection and telomerase-mediated healing depends on MRX (Diede and Gottschling 2001; Bonetti et al. 2009). Sae2 and Sgs1 define parallel pathways required for processing of telomere ends (Bonetti et al. 2009). The Sae2 nuclease interacts with MRX and may start telomere end resection (Fig. 2). The Sgs1 helicase functions in conjunction with the Dna2 Flap endonuclease/helicase. Concomitant absence of Sae2 and Sgs1 triggered shorter telomeres but not cellular senescence, indicating that telomerase is still able to act (Bonetti et al. 2009). Indeed, exonuclease 1 (Exo1) can also contribute to telomere end resection, and telomere shortening in *sae2*Δ/*sgs1*Δ cells is partially suppressed by Exo1 overexpression (Bonetti et al. 2009). Cdk1 activity is required to promote 3′ overhang formation at *S. cevevisiae* telomeres (Frank et al. 2006; Vodenicharov and Wellinger 2006). This involves phosphorylation of Sae2 and possibly additional substrates. How phosphorylation activates Sae2 is not known.

In normal human fibroblasts, 3′ overhang length at leading-stand telomeres is around 60 nucleotides, versus 105 nucleotides at lagging-strand telomeres (Chai et al. 2006). Eighty percent of the C-rich strands end with the sequence 3′-CCAATC-5′, whereas the sequence at the 3′ terminus is less precise (Sfeir et al. 2005). The enzymatic activities that are involved in telomere and double-strand break end resection in *S. cerevisiae* are conserved throughout evolution. The MRN complex (Mre11–Rad50–Nbs1) interacts with Trf2 and binds to telomeres (Zhu et al. 2000). The role of MRN and the other processing activities discussed above in the processing of mammalian telomeres is not established. However, the Apollo 5′-3′ exonuclease is a member of the Snm1/Pso2 family of nucleases, binding to telomeres through its interaction with Trf2 (Lenain et al. 2006; van Overbeek and de Lange 2006). Apollo$^{-/-}$ cells have reduced 3′ overhangs at telomeres and they lose telomere protection at the leading-strand telomeres (Lam et al. 2010; Wu et al. 2010). Whether other nucleases in addition to Apollo contribute to telomere end processing at mammalian telomeres, as in yeast, is unclear. Notably also, Apollo has been proposed to facilitate semiconservative replication of telomeric DNA, relieving topological stress in conjunction with topoisomerase 2α (Ye et al. 2010).

TELOMERASE RECRUITMENT, TELOMERE EXTENSION, AND LENGTH HOMEOSTASIS

S. cerevisiae

S. cerevisiae telomerase extends telomeres in late S phase, presumably after semiconservative DNA replication and telomere end processing. Single-telomere extension analysis (STEX) revealed that telomerase extends only a small fraction (~7%) of telomeres in a given cell cycle and that extension occurs most frequently at the shortest telomeres (Teixeira et al. 2004). Recognition of the shortest telomeres is mediated on one side by Tel1 (ortholog of mammalian Atm), which is recruited specifically to the shortest telomeres in an MRX complex-dependent manner (Fig. 3) (Bianchi and Shore 2007b; Hector et al. 2007; Sabourin et al. 2007). Tel1 function can be partially compensated for by Mec1 (ortholog of mammalian Atr) as *tel1*Δ/*mec1*Δ double-mutant cells but not the single mutants senesce (Ritchie et al. 1999). A second pathway that mediates recognition of the shortest telomeres requires Tbf1, a protein that carries sequence similarity with fission yeast Taz1 and mammalian Trf proteins (Arneric and Lingner 2007). Tbf1 binds to 5′-TTAGGG-3′ repeats that are present in the subtelomeric region of yeast telomeres (Koering et al. 2000).

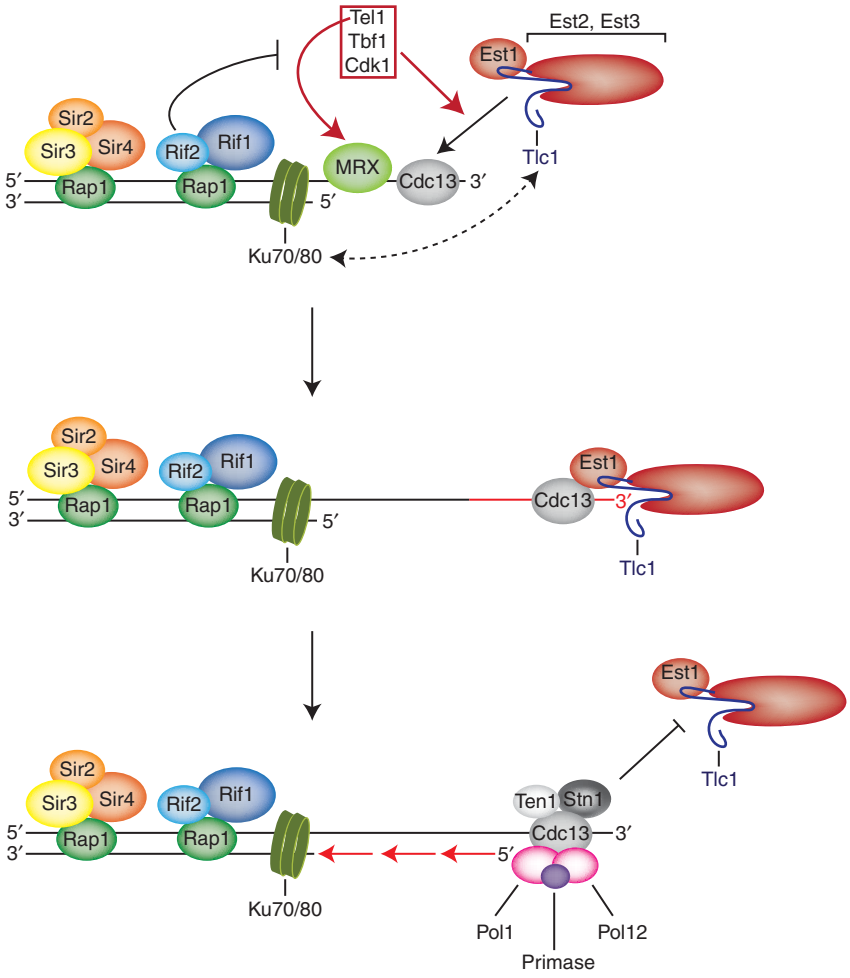

Figure 3. Telomerase recruitment and telomere extension in *S. cerevisiae*. MRX recruits Tel1 to the shortest telomeres, which promotes telomerase recruitment through the interaction of Cdc13 with Est1. Tbf1 can also promote preferential extension of the shortest telomeres in *tel1*Δ cells. Cdk1 contributes to the cell-cycle-dependent recruitment of telomerase through phosphorylation of Cdc13. Telomerase extends telomeres in a nonprocessive manner in *S. cerevisiae*. Human telomerase, on the other hand, adds ~60 nucleotides per telomere in a single binding and extension event (not shown; see text). Telomerase extension is terminated upon formation of the CST complex, which promotes recruitment of DNA Pol α-primase for fill-in synthesis.

Third, short telomeres replicate earlier in S phase. This therefore might provide more time for telomerase to mediate extension of the shortest telomeres (Bianchi and Shore 2007a).

The recruitment to telomeres in late S phase and telomere extension depend on a physical interaction between Cdc13 that binds to the telomeric 3′ overhang and the telomerase subunit Est1 (Fig. 3). The *cdc13-2* allele gives an *est* phenotype (Evans and Lundblad 1999). Cdc13-2 carries a single point mutation (E252L). Intriguingly, this mutation can be suppressed by a single point mutation in Est1 (L444E) (Pennock et al. 2001). Because the suppression involves the restoration of opposite charges in Cdc13 and Est1, this provides excellent genetic evidence that Cdc13 and Est1 interact directly and that the interaction involves these two amino acids. A second telomerase recruitment pathway in *S. cerevisiae* is mediated by the

Ku70/80 heterodimer, which binds telomeres and other DNA ends via a preformed channel (Fig. 3). In addition, Ku80 binds the telomerase RNA subunit Tlc1 (Peterson et al. 2001; Fisher et al. 2004). Ku enriches telomerase near telomeres throughout the cell cycle as assessed by chromatin immunoprecipitation and positively contributes to telomere length maintenance. Disruption of the Tlc1–Ku80 interaction reduces telomere length while not eliciting an *est1* phenotype. When assessing telomere association of telomerase by live-cell imaging, it seemed that the Tlc1–Ku80 interaction might only promote a transient interaction with telomeres, whereas the stable interaction in late S phase is mediated by Cdc13–Est1 (Gallardo et al. 2011). The Ku-mediated stimulation of telomerase may also involve its role in nuclear accumulation of Tlc1, which is reduced in Ku-deficient cells (Gallardo et al. 2008; Pfingsten et al. 2012). How Tel1 and Tbf1 promote the interaction of Cdc13 and Est1 and the preferential extension of the shortest telomeres is unclear. Cdc13 contains several consensus S/TQ sites for Tel1, but mutation of all these sites did not substantially impact on telomere length when the *cdc13* mutants were tested in their chromosomal context (Gao et al. 2010). However, opposite results were reported in a previous study by another group, in which the mutant *cdc13* alleles were expressed from a plasmid (Tseng et al. 2006). However, it seems that crucial Tel1 targets remain to be discovered. Cdk1 contributes to the cell-cycle-dependent recruitment of telomerase. The phosphorylation of Cdc13 at T308 by Cdk1 in late S phase and G_2 favors the interaction of Cdc13 with Est1 rather than the competing interaction of Cdc13 with Stn1–Ten1 (Li et al. 2009). Another modification, Cdc13 SUMOylation, peaks in early to mid S phase (Hang et al. 2011). Mutation of the SUMO site reduced the interaction with Stn1, promoting telomere elongation (Hang et al. 2011). Ku70/80 and Sir4 are other telomeric proteins modified by Siz2 E3 ligase-dependent SUMOylation, promoting perinuclear position of budding yeast telomeres (Ferreira et al. 2011). Because telomeres shift away from the nuclear envelope when elongating, this modification possibly links nuclear position with telomerase regulation.

STEX analysis of yeast cells that expressed two RNA subunits that differed in the telomeric sequence revealed that *S. cerevisiae* telomerase acts mostly in a nonprocessive manner in terms of telomere repeat addition (Chang et al. 2007). However, when very short telomeres, <125 nucleotides, are elongated, yeast telomerase gains in repeat addition processivity. The increased processivity requires Tel1. The telomere-shortening rate in the absence of telomerase is length-independent (Marcand et al. 1999). The observed increased frequency of telomere elongation by telomerase with telomere shortening is sufficient to explain telomere length homeostasis. The steady-state telomere length corresponds to the point at which the product of frequency of elongation and average extension length equals the shortening rate. The frequency of elongation is regulated by the number of Rap1/Rif1/Rif2 proteins that are bound to telomeres in dependence of their length (Marcand et al. 1997). Mechanistically, this may involve the competition of Rif1/2 with Tel1 for binding of the carboxyl terminus of Xrs2 (Hirano et al. 2009). Thus, at short telomeres that bind less Rap1/Rif/Rif2, Tel1 recruitment to the MRX complex may be favored, leading to preferential recruitment and activation of telomerase at short telomeres (Fig. 3).

Telomerase-mediated telomere extension must be followed by fill-in synthesis of the C-strand, presumably by classical replication enzymes involved in lagging-strand synthesis. Importantly, the CST complex may play also an important role here, as physical interactions have been detected between Cdc13 and Pol1, the catalytic subunit of DNA Pol α-primase (Qi and Zakian 2000); and between Stn1 and Pol12, the B subunit of DNA Pol α-primase (Fig. 3) (Grossi et al. 2004). Whether CST substitutes the related RPA stimulating the fill-in reaction is unknown.

S. pombe

Telomerase recruitment to telomeres in *S. pombe* occurs through the interaction of the telomerase subunit Est1 with Ccq1, which is

tethered to telomeres through the interaction with Tpz1, which in turn interacts with Pot1 and Poz1 (Miyoshi et al. 2008; Tomita and Cooper 2008; Webb and Zakian 2012). Importantly, *S. pombe* Tel1 (ortholog of mammalian Atm) and Rad3 (ortholog of mammalian Atr) promote the direct interaction between Ccq1 and the Est1 subunit of telomerase (Moser et al. 2011; Yamazaki et al 2012). Ccq1 is phosphorylated on T93 by Tel1 and Rad3, and mutation of this site abolishes telomerase recruitment. A Cdc13 homolog has not been identified in *S. pombe*, and there is no known Ccq1 counterpart outside of fission yeast. Thus, telomerase recruitment seems to depend on different factors in fission and budding yeast. Telomere length regulation in *S. pombe* involves Taz1, Rap1, and Poz1, whose deletions lead to very substantial telomere elongation.

Mammals

Telomerase in humans is enriched in so-called Cajal bodies (CBs), dynamic subnuclear structures that are characterized by the coilin protein and that contain a variety of proteins and RNAs involved in the assembly and modification of small nuclear and nucleolar ribonucleoproteins (snRNPs and snoRNPs). CBs also harbor a class of CB-specific RNAs (scaRNAs) that are involved in methylation and pseudouridylation of small nuclear RNAs. In S phase of the cell cycle, a subset of CBs together with telomerase RNA is seen in association with telomeres in human cells (Jady et al. 2006; Tomlinson et al. 2006). As with other scaRNAs, human telomerase RNA contains a CAB-box motif that mediates association with CBs (Jady et al. 2004). Mutation of this motif does not prevent association of Tert with telomerase RNA (hTR), as catalytic telomerase activity is not impaired (Cristofari et al. 2007). However, CAB-box mutant telomerase does not efficiently associate with telomeres, and telomere extension is reduced (Cristofari et al. 2007). Tcab1 (Venteicher et al. 2009), also referred to as Wdr79 (Tycowski et al. 2009), was identified as a CAB-box binding protein and telomerase subunit. Tcab1 depletion from human cells leads to continuous telomere shortening (Venteicher et al. 2009). These results underline the importance of the CAB box and its interaction with Tcab1 for telomerase recruitment to telomeres. The CB association of telomerase has, in contrast to human cells, not been seen in mouse cells (Tomlinson et al. 2010). Thus, it will be interesting to determine if Tcab1 is also important for telomerase recruitment to telomeres in mice.

At the telomere, Tpp1 and Tin2 are important for telomerase recruitment or its retention, as depletion of either of these factors reduces (but does not abolish) telomerase association with telomeres (Abreu et al. 2010). Depletion and rescue studies further identified the amino-terminal OB-fold of Tpp1 as important for telomerase recruitment (Abreu et al. 2010), a domain that was found to also interact with telomerase in extracts (Xin et al. 2007). Whether telomerase recruitment is regulated in mammals by Atm or Atr, as in fission and budding yeast, is unknown, nor is it clear if in addition to Tpp1 other factors contribute to recruitment. The single-stranded telomere-binding protein Pot1 is an inhibitor of telomerase per se when binding to the 3′ end of the substrate (Kelleher et al. 2005; Lei et al. 2005). When associated with Tpp1, however, Pot1–Tpp1 decreases the dissociation of primer from telomerase and increases the efficiency of translocation (Wang et al. 2007; Latrick and Cech 2010). Therefore, the Pot1–Tpp1 complex increases repeat addition processivity of human telomerase severalfold. Thus, with the assistance of Pot1–Tpp1, telomerase can synthesize in vitro roughly with one or two binding and extension events the ~60 nucleotides that are typically added to a human telomere by telomerase in vivo (see below).

Using sophisticated molecular biological methods, the frequency of telomerase-mediated telomere elongation was measured to be 70%–100% at lagging-strand telomeres in HeLa and H1299 lung adenocarcinoma cells, and similar conclusions were drawn for the leading-strand telomeres (Zhao et al. 2009). This result was unexpected, as at lagging-strand telomeres the G-rich strand is parental and thus this strand is not predicted to shorten from incomplete end replication (Fig. 2, left panel). However, because

this end is extended by telomerase, this finding implies that the parental G-rich strand must be shortened again subsequent to its elongation by telomerase, if not in every cell cycle at least in every couple of cell cycles. If not, the parental G-strand should suffer from continuous telomere elongation. The telomerase-mediated extension length was determined to be ~60 nucleotides, and experimental inhibition or depletion of telomerase reduced the frequency of extension events but not extension length (Zhao et al. 2011). This supports the notion that one single telomerase molecule acts at every telomere in every cell cycle.

The restriction of human telomerase to a single telomere binding and extension event may involve the human CST complex, which was identified recently (Miyake et al. 2009; Surovtseva et al. 2009). Human Stn1 and Ten1 carry clear sequence similarity with their budding yeast counterparts. Ctc1 (conserved telomere maintenance components 1), also referred to as AAF-132 (α-accessory factor) (Casteel et al. 2009), which assembles with Stn1 and Ten1 in the trimeric CST complex, bears little sequence similarity with *S. cerevisiae* Cdc13. Like budding yeast CST components, those of human CST contain putative OB-fold domains involved in binding single-stranded DNA and promoting protein interactions. Human CST inhibits telomerase activity through primer sequestration and physical interaction with the Pot1–Tpp1 telomerase processivity factor (Chen et al. 2012). CST binding at telomeres increases during late S/G_2 phase only upon telomerase action, coinciding with telomerase shutoff. CST depletion unleashes excessive telomerase activity, promoting telomere elongation. Increased binding of CST upon telomerase-mediated telomere elongation coincides with a switch from Pot1–Tpp1 binding with concomitant telomerase stimulation to CST binding and concomitant telomerase repression. Through binding of the telomerase-extended telomere, CST may limit telomerase action at individual telomeres to approximately one binding and extension event per cell cycle (Chen et al. 2012).

C-strand fill-in synthesis in human cells follows telomerase extension at the end of S phase (Zhao et al. 2009). Whether Pot1–Tpp1 or RPA stimulates this process is unclear. Possibly more relevant for this is the CST complex, which binds to the telomerase-extended telomeric 3′ end (Chen et al. 2012). Indeed, human Ctc1 and Stn1 stimulate DNA polymerase α-primase, increasing its affinity for template DNA (Casteel et al. 2009), and CST has been implicated in assisting lagging-strand synthesis at telomeres and other special DNA sequences throughout the genome (Miyake et al. 2009; Price et al. 2010; Gu et al. 2012). How the binding of single-stranded telomeric DNA by telomere-binding proteins, which include Pot1–Tpp1, CST, RPA, and possibly hnRNPA1, is regulated is unclear, nor is it known how T-loop formation may be influenced by these factors, possibly in a cell-cycle-dependent manner. It has been proposed that a switch from RPA binding in S phase to Pot1–Tpp1 after S phase is triggered by hnRNPA1 and TERRA (Flynn et al. 2011). In vitro, hnRNPA1 can compete with RPA for the binding of the single-stranded G-strand (Flynn et al. 2011). An increase of TERRA levels after S phase may sequester hnRNPA1 from telomeric DNA, as it has higher binding affinity for the 5′-UUAGGG-3′ repeats in TERRA than for the same 5′-TTAGGG-3′ telomeric DNA sequence. Binding of hnRNPA1 to TERRA may in turn allow binding of the telomeric 3′ overhang by Pot1–Tpp1. On the other hand, it has been argued that the higher local concentration of Pot1–Tpp1 near telomeres is sufficient to trigger Pot1–Tpp1 binding once semiconservative telomere replication is completed (Takai et al. 2011).

The above results on telomere extension in vivo cannot on their own explain telomere length homeostasis. Telomere length homeostasis requires that the frequency of telomere elongation, the extension length, or the shortening of telomeres occurs in a length-dependent manner. Telomerase preferentially elongated critically short telomeres in the offspring of mice to which telomerase was reintroduced after their ancestors grew in the absence of the telomerase RNA gene for several generations (Hemann et al. 2001). Thus, under nonhomeostatic conditions, telomerase is regulated in a telomere

length-dependent manner, at least in mice. Telomere length-dependent regulation of telomerase in mammals is also supported by studies on the shelterin components, whose abundance at telomeres is length-dependent and whose experimental manipulation leads to telomere length changes (Smogorzewska and de Lange 2004; Hug and Lingner 2006). Generally, it has been observed that depletion of shelterin components by RNAi promotes telomerase-mediated telomere elongation, whereas their overexpression reduces telomere length. This may be at odds when considering the above-discussed positive roles of at least Tpp1 for telomerase recruitment and stimulation of processivity. However, it is important to consider that many telomere-binding proteins function like Swiss army knives. They may have multiple tasks in several processes at the chromosome end, such as telomere replication, regulation of telomerase, protection from nucleases, recombination and repair, and suppression of checkpoint signaling. Thus, depletion of a factor may highlight one function but may not allow detection of the multiple tools it may be equipped with to contribute to the correct functioning of chromosome ends.

CONCLUDING REMARKS

Elucidation of the complexity of telomere replication and the regulation of telomerase is a fascinating basic problem of biological research. It is clear that a better understanding of telomere replication and the regulation of telomerase in the future will come from studies in biochemistry, structural biology, genetics, and cell biology. Single-celled eukaryotes as well as animal model systems will continue to be of important value. For human telomere replication, the studies in induced pluripotent stem cells may become particularly revealing, as this system may allow learning about telomerase regulation and telomere replication in stem cells and during cellular differentiation. It is hoped that a much more thorough understanding of telomere maintenance may identify new targets that may become useful to inhibit telomerase action in cancer. After all these years (Kim et al. 1994), it has still not been possible to test if telomerase inhibition in human cancer is a valuable strategy to inhibit tumor growth. In addition, an increasing number of diseases have been linked to short telomeres (reviewed by Lansdorp 2009). Short-telomere diseases include dyskeratosis congenita (DC), idiopathic pulmonary fibrosis, the Werner syndrome, and the ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome. DC patients die prematurely of bone marrow failure. ICF syndrome patients suffer from reduced immunoglobulin levels. Werner patients age prematurely and are predisposed to cancer. Although in some cases defective telomerase could be linked to short-telomere diseases (particularly in DC), in other cases the molecular mechanisms that underlie abnormal telomere shortening remain to be defined. In conclusion, it seems safe to assume that telomeres will not cease to unveil many hidden treasures.

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