

Telomere replication: poised but puzzling

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Abstract

Faithful replication of chromosomes is essential for maintaining genome stability. Telomeres, the chromosomal termini, pose quite a challenge to replication machinery due to the complexity in their structures and sequences. Efficient and complete replication of chromosomes is critical to prevent aberrant telomeres as well as to avoid unnecessary loss of telomere DNA. Compelling evidence supports the emerging picture of synergistic actions between DNA replication proteins and telomere protective components in telomere synthesis. This review discusses the actions of various replication and telomere-specific binding proteins that ensure accurate telomere replication and their roles in telomere maintenance and protection.

Keywords: telomere replication • telomerase • shelterin • C-strand fill-in • G-quadruplex • cancer

Introduction

Telomeres, telomerase and their roles in genome stability

Maintenance of stable chromosome ends, telomeres, is a key contributor of genome stability. Chromosomal abnormalities due to dysfunctional telomeres have been implicated in cancer and premature aging disorders. Telomeres are special nucleoprotein structures composed of double-stranded (TTAGGG)_n DNA repetitive sequence ranging from ~3 to 15 kb and a number of telomere associated proteins. The ds telomeric DNA terminates at a 3' single-stranded G-rich overhang of about 12–500 bases [1–3]. This protruding 3' end can invade the duplex DNA and form a lariat-like structure called 't-loop', establishing a protective cap that shields chromosome ends from being recognized as damaged DNA and prevents nucleolytic degradation and inappropriate fusions of telomeres. The t-loop is stabilized by a complex of ds and ss stranded telomere binding

proteins known as the 'shelterin' proteins (telomere repeat binding factor 1 and 2 [TRF1, TRF2], protection of telomeres 1 [POT1], TRF1 and TRF2 interacting nuclear protein 2 [TIN2], TIN1-TIN2 interacting protein, PIP1-POT1 interacting protein, PTOP-POT1 and TIN2 interacting protein [TPP1] and repressor activator protein 1 [Rap1]) [4]. The primary role of shelterin is to mark telomeres as the natural chromosome ends and suppress the DNA damage response pathways at telomeres [5]. Readers are referred to the excellent comprehensive reviews about the end-protection mechanisms conferred by the shelterin complex [5–6].

Due to the inability of the conventional DNA polymerases to completely duplicate linear chromosomal DNA and additional nuclease degradation of telomeric DNA, telomeres in human

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somatic cells progressively shorten as cells divide [7]. Critically short telomeres result in telomere deprotection and activate DNA damage response pathways, leading to cellular senescence or apoptosis. Such progressive loss of telomere DNA is visualized as a mechanism to limit the proliferative ability of the dividing cells [7]. Telomere damage response can also be triggered by disrupting the functions of shelterin proteins.

The majority of tumour cells acquire cellular immortality and stabilization of telomere length by activating a normally dormant enzyme, telomerase, which extends the 3' end of the chromosomes to replenish the telomeric repeats in a sequence-specific manner. Telomerase is a special reverse transcriptase consisting of an RNA subunit hTR that serves as the template and a catalytic protein subunit telomerase reverse transcriptase (hTERT). It is expressed during early embryogenesis and subsequently down-regulated in most somatic cells [8]. The activity of telomerase is tightly regulated *in vivo* at the level of its expression as well as its biogenesis [9–13].

The fact that the majority of cancer cells require telomerase activity to maintain telomeres makes telomerase an attractive target in clinical medicine. Telomerase inhibition therapies targeting hTERT, hTR or its associated proteins are picking up pace. Inhibition can be carried out by using telomerase inhibitor ligands such as Imetelstat (lipid modified 13 mer oligonucleotide N3'→P5' thiophosphoramidate [GRN163L]) [14–18] and antisense oligonucleotides [19–21]. Applying more than one strategy is also in place to enhance the anti-cancer effect. Strategies such as combining RNAi with hammerhead ribozymes [22] or using natural telomerase inhibitor with chemotherapeutic agents have been reported [23]. Telomerase immunotherapy has also been shown to have promising developments [24].

Apart from cancers, dysfunctional telomeres are associated with a number of disorders such as dyskeratosis congenita (DC). Mutations seen in DC patients involve genes such as hTR [25–26], hTERT [27], dyskeratosis congenita 1 (DKC1) (encoding dyskerin), components of H/ACA small nucleolar ribonucleoprotein complex (NOP10 and NHP2) [28–30]. The latter three genes are key components of telomerase ribonucleoprotein complex [31]. Defective dyskerin, NOP10, NHP2 impair the assembly of telomerase complex. Mutations in hTR impair the level and accumulation of telomerase RNA and mutations in hTERT leads to haploinsufficiency in telomerase activity [32–33]. In a recent report, mutations in a shelterin component TIN2 also lead to DC [34]. Taken together, it is suggested that DC is a telomere maintenance disorder. Patients with DC display bone marrow failure, pulmonary fibrosis, mucocutaneous abnormalities and defects in the highly replicating tissues such as the hematopoietic cells. Extensive chromosomal recombinations and greater susceptibility to acute myeloid leukaemia and other cancers are associated with DC patients as well [35–36]. Other dysfunctional telomeric disorders that are associated with inherited mutations in genes responsible for telomere maintenance include aplastic anaemia, acute myeloid leukaemia and idiopathic pulmonary fibrosis [35–36]. Therefore, telomere maintenance and telomerase are currently the promising targets in cancer and aging related therapeutics.

Telomere DNA replication: not a cake walk for the replication fork

Faithful replication of chromosomal DNA is critical for genome stability. Replication of telomeric DNA is thought to be initiated from origins located in the sub-telomeric region [37]. The TTAGGG (G-rich) strand is replicated by discontinuous lagging strand synthesis initiated by small RNA primers. After the last RNA primer is removed, there is no mechanism to fill in the gap, leaving the daughter telomeres shorter than the parental ones. Meanwhile, the CCCTAA (C-rich) strand is replicated by continuous leading strand synthesis, making them blunt-ended. The C-rich strand is further processed/resected by nucleases and helicases including mammalian Apollo to generate longer ss G-overhangs [38]. These ss G-overhangs are finally converted into the protective t-loops to stabilize telomeres.

Due to the unusually long G-rich repetitive tracts and the unique telomere chromatin structures at chromosome ends, replication fork faces a number of potential challenges while passaging through the telomere region. Examples of these challenges that are very stable *in vivo* and difficult to resolve include G-quadruplexes, heterochromatinized DNA and the special t-loop structure.

G-quadruplexes, also referred to as G-quartets or G₄, are stable inter- or intra-molecular structures held intact by four guanine residues through Hoogsteen base-pairing [39]. During replication, separation of ds telomeric DNA exposes the ss G-rich strand, triggering the formation of G-quadruplexes. Their resolution requires the actions of helicases such as Bloom syndrome protein (BLM) and Werner syndrome protein (WRN) to further the movement of replication fork. The activities of ss DNA binding protein replication protein A (RPA) and the shelterin component POT1 are also necessary towards this end [40–41]. Thus, various players contribute towards the reduction of this structural difficulty for the passage of the replication fork through the telomere region to ensure a faithful replication. On the other hand, strategies to promote and stabilize the G₄ structure with small molecules such as telomestatin and G₄ ligand 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4) induce defective telomere replication, and are effective cancer therapeutic approaches [23, 42–43].

Chromatin in the telomeric and sub-telomeric regions is highly repressed and termed as heterochromatin. Heterochromatinized environment is proposed to be a mechanism that negatively regulates telomere elongation, as the loss of chromatin modifiers leads to a substantial elongation of telomeres [44]. Furthermore, lack of DNA methylation deregulates telomere length homeostasis and leads to accelerated telomere recombination events [45]. It appears that a higher-order epigenetic regulation may control both telomere length homeostasis and telomeric protective function. Although this regulatory mechanism is poorly understood, a formal possibility is that the heterochromatinized environment poses a challenge for the replication fork to pass through the telomeric repeats. Efficient and accurate resolution of heterochromatin structure is necessary for the faithful replication of telomeric DNA and proper maintenance of chromosome ends.

In addition, t-loop itself poses a challenge to DNA replication. This special chromatin structure bound by various proteins is unlikely to rotate freely and is considered as a topological barrier. As the replication fork approaches telomere end, superhelical stress in the unreplicated DNA accumulates. Unwinding of t-loop is essential for resolving such topological stress and allowing efficient and complete replication of telomeric DNA.

The above events may result in replication fork stalling which then activates ataxia telangiectasia mutated-/ataxia telangiectasia and Rad3 related (ATM-/ATR)-mediated DNA damage response, leading to cell growth arrest or creating a situation that may lead to accelerated telomere attrition. In fact, telomeres resemble fragile sites resulting from defects in replication and are prone to deletions and chromosome rearrangements [46]. In higher eukaryotes these issues seem to be tackled by mainly two protein consortiums:

- (1) Actions of replication proteins at telomeres: To deal with the difficulties in telomere replication, replication proteins ought to have robust activities. In addition, they perform multiple functions and participate in multiple pathways including DNA repair to ensure genome stability. The mechanism of the actions of replication proteins at telomeres is still poorly understood, and whether it is the same as general replication or enhanced at telomeres is yet to be explored.
- (2) Actions of telomeric proteins in resolving the challenges faced by the replication fork: Passage of replication fork through telomeric tracts requires coordinated actions of telomeric proteins with replication factors. This may involve either the resolution of structural difficulties or just aiding in the assembly of the higher-order replication complex (RC).

Functions of replication proteins in telomere maintenance

Studies of the activities of replication proteins from lower eukaryotes such as yeast and ciliates indicate that telomerase extension of telomeres is in close collaboration with replication machinery. However, the complexity of telomere structure in higher eukaryotes requires more enhanced mechanisms to be functional. Here, we review a number of proteins playing multiple roles in replication, repair and other processes, and discuss their roles specific to telomere DNA replication.

DNA polymerase α -primase (pol α /primase)

The pol α /primase complex consists of four subunits responsible for initiating the lagging strand DNA synthesis *de novo* [47]. The p48 and p58 subunits form the catalytic primase that synthesizes a short RNA primer. The 180kD subunit of the complex, p180 or pol α , synthesizes short stretches of DNA following the RNA primer, while another subunit, p70 or the B subunit or PolA2, is thought to regulate the p180 activity [47]. The pivotal role of pol α in telomere maintenance was initially reported in

lower eukaryotes. In budding yeast pol α is essential for telomerase extension of telomere ends [48]. Mutations in pol α lead to a telomerase-independent increase in the amount of G-overhangs specifically in S phase [49–50], and show telomerase-dependent telomere elongation [51]. Furthermore, pol α interacts with Cdc13p, a telomere binding protein that regulates telomerase-mediated telomere extension [52]. Analogous physical interaction between lagging strand replication machinery and telomerase has also been detected in the ciliate *Euplotes crassus* [53] and fission yeast [54]. In fission yeast, mutation in pol α leads to telomere length abnormalities and decrease in telomerase protein stability [54]. Taken together, it is likely that in lower eukaryotes, replication apparatus and telomeric complexes aid in the fine tuning of telomerase action to generate the homeostatic telomere length.

Pol α has been implicated in telomere maintenance in higher eukaryotes as well. In a mouse mutant cell line harbouring a temperature sensitive allele of pol α , inhibition of pol α induces an increase in the amount of G-overhangs and increases POT1 and TRF1 bound to telomeres [55]. Such alteration in telomere structure contributes to genome instability as revealed by an increase in Robertsonian chromosome fusions. In human cells, pol α localizes to telomeres in both S phase and G2 phase [56], presumably for DNA synthesis and the delayed C-strand synthesis (see below) [57]. Due to the complexity of the genome in higher eukaryotes, homeostatic telomere length may be achieved by unique mechanisms. This may involve additional roles of replication proteins in close collaboration with telomeric factors. At present, the function of pol α in telomere maintenance in higher eukaryotes remains poorly understood.

Replication protein A

RPA binds to and stabilizes the ssDNA intermediates encountered during various DNA metabolic processes by using the evolutionarily conserved oligonucleotide/oligosaccharide (OB)-fold domains [58–59]. Initially thought as a DNA replication component, RPA has emerged as an important player in DNA repair, checkpoint activation, and cell cycle regulation. It is now known to be important for telomere maintenance and participates in multiple activities at telomeres, thus validating its role as a genome guardian [60].

RPA is a heterotrimeric complex consisting of three subunits – RPA70, RPA32 and RPA14 [61]. During DNA replication, RPA coats ssDNA and acts as a molecular platform for the assembly and activation of the pre-RC, thus recruiting the pol α /primase complex to the origin of replication [62]. During DNA repair, RPA interacts and activates components like PCNA, RFC and pol δ , perhaps to stabilize the ssDNA structures arising during repair [59]. RPA also has a role in checkpoint activation by mediating an ATR-mediated DNA damage response [63].

In budding yeast, a double mutant for RFA1 (hRPA70 homologue) and yKu70 showed a synergistic shortening in telomere length, indicating a role of RPA in telomere length regulation [64]. In fission yeast, a mutant similar to the above double mutant displays both DNA damage sensitivity and shortened telomeres [65]. Both Rfa1 and Rfa2 (hRPA34 homologue) localize to telomeres

during the S phase, and a mutant Rfa2 lacking the N-terminus showed impaired binding by Est1p, suggesting a role of RPA in telomerase action [66].

Our understanding on human RPA primarily comes from biochemical assays with purified proteins. *In vitro*, human RPA modulates telomerase activity, stimulates WRN's activity in disrupting D-loops, and is able to unfold telomeric G-quadruplexes [40, 67–69]. During replication of genomic DNA, RPA is thought to coat ss DNA as replication fork passes. However, biochemical analysis shows that its affinity to ss G-rich DNA is about two times weaker compared to the shelterin protein POT1 [70], raising the question of how the ss telomeric DNA is protected during replication at the telomeric region. It will be interesting to know how RPA interplays with shelterin components such as POT1 and other ss DNA binding proteins (e.g. Cdc13-Stn1-Ten1 [CST]) during the replication of telomeres.

RecQ helicases

RecQ is a conserved family of helicases including RecQ1, RecQ4, RecQ5, BLM and WRN. These enzymes are essential for maintaining genomic integrity, as mutations in these proteins are associated with hereditary cancer predisposition syndromes [71–73].

Cumulative evidence suggests the importance of RecQ helicases in telomere replication. In budding yeast, RecQ helicase Sgs1 helps in efficient resolution of telomeric recombination. Failure to do so leads to premature aging in yeast mother cells [74–76]. Studies on cells derived from Werner syndrome patients show that WRN-deficient cells specifically lose telomeres replicated by lagging strand synthesis, and a dominant-negative mutation in WRN leads to increased telomere loss and aberrant chromosomal recombination [70, 77–78]. Comparable telomere-associated phenotype has been observed in mice null for both WRN and *mTerc* [79–80]. Given that WRN localizes to telomeres during the S phase [78], and the helicase activity of WRN unwinds G-quadruplexes [81–82], it is highly possible that WRN is an important player in resolving structural barriers generated during replication. In agreement with this view, the amount of ss G-rich DNA significantly increases during S phase upon the removal of WRN, suggesting replication fork stalling at lagging telomeres [70]. Notably, both TRF2 and POT1 are able to stimulate WRN's helicase activity *in vitro* [81, 83–84]. Taken together, it appears that the WRN helicase activity is enhanced by telomere specific proteins for efficient telomere replication.

Another mammalian RecQ helicase, BLM, may play an overlapping function alongside WRN. BLM gene is mutated in Bloom's syndrome, an autosomal recessive disorder associated with cancer predisposition and premature aging [73]. Bloom's syndrome cells show increased genomic instability, sister chromatid exchanges and elevated levels of chromosomal aberrations including translocations [85–87]. Like WRN, BLM is able to unwind G-quadruplexes [88–89]. It binds to TRF1 and its helicase activity can be stimulated by certain shelterin proteins on a variety of DNA structures *in vitro* [81, 83, 90]. Thus, its helicase activity is likely to be needed for efficient replication of telomeric DNA as well. Indeed, BLM-deficient cells show a high frequency of fragile telomeres [46].

Flap endonuclease 1 (FEN1)

FEN1 is a conserved, structure-specific endonuclease involved in various DNA metabolic pathways [91–92]. It recognizes a dsDNA with a 5' displaced flap structure, a common intermediate arising during lagging strand replication and long patch base excision repair [92]. FEN1 possesses three distinct nuclease activities: 5'→3' FEN, 5'→3' exonuclease and gap-dependent endonuclease (GEN) [93]. The FEN activity is used in the removal of RNA primers during Okazaki fragment processing either alone on short flaps, or with Dna2 on longer flaps [94–96]. FEN1 is also needed in DNA repair, particularly in the removal of damaged base [93].

Studies from budding yeast suggest that the FEN1 homologue Rad27 is likely to be responsible for the generation of G-overhangs at the lagging telomere ends [97–98]. Possibly due to the role of Rad27 in G-overhang generation, deletion of *rad27* causes an accelerated senescence phenotype in strains also lacking components required for telomerase activity [98].

In human cells, FEN1 localizes at telomeres during the S and G2 phases of cell cycle and associates with at least one shelterin protein, TRF2 [56, 99]. In telomerase-expressing cells, FEN1 forms a complex with the catalytic component of telomerase, hTERT [100]. Although the functional importance of this association is yet to be explored, it has been shown that FEN1 depletion leads to progressive telomere shortening in cancer cells [100], suggesting that FEN1 may assist telomerase in elongating telomeres. In addition, FEN1-deficient mouse embryonic fibroblasts showed increased telomere end-to-end fusions [100].

In telomerase negative human fibroblasts, depletion of FEN1 leads to the loss of sister telomeres replicated by lagging strand synthesis, and as a consequence, resulting in an increase of dysfunctional telomeres as revealed by γ -H2AX precipitated telomere DNA [101]. This is attributed to FEN1's GEN activity, which has been implicated in replication fork re-initiation [102–103]. Mutational analyses indicate that both FEN1's GEN activity and its ability to interact with WRN helicase are required for its role at telomeres, suggesting that stimulation of FEN1 activity by WRN is essential for replication fork re-initiation. This is reminiscent of the observation that WRN stimulates FEN1 through direct interaction [104], and that FEN1's GEN activity is shown to be required for resolving stalled replication forks [102]. Consistent with its role in lagging strand replication of telomeres, FEN1 appears to be able to efficiently process 5' DNA flap substrates bearing G4 quartets [105]. Taken together, FEN1's collaborative role with other factors to re-initiate stalled replication forks may be one of its functions, yet the functional significance of its interactions with telomerase and TRF2 remain to be determined.

Dna2

Dna2 is a helicase and nuclease whose function is implicated in the maturation of Okazaki fragments alongside FEN1 on longer flap intermediates [96]. In *Saccharomyces cerevisiae*, Dna2 localizes at telomeres in a cell cycle regulated manner and has a role in both telomere replication and perhaps capping as well [106]. In *S. pombe* Dna2 is also involved in the generation of

telomeric overhangs [107]. However, its telomeric function in higher eukaryotes remains largely unknown.

Functions of telomere binding proteins in telomere replication

Shelterin proteins that tightly bind to telomeric DNA were once considered as blockades for telomere replication. It was thought that they might be displaced from telomeres as replication fork moves along the telomere region until recent studies suggest that efficient and faithful replication of the repetitive telomeric DNA requires the participation of these telomere protective proteins.

TRF1

TRF1 is an abundant shelterin protein that binds to the duplex telomeric repeats with a role in telomere length regulation. Recently, its role has emerged to be predominant in efficient telomere replication. Loss of TRF1 leads to aberrant telomere structural changes resembling common fragile sites, which activate the ATR kinase in S phase [46]. Single-molecule analysis of replicated DNA reveals that the absence of TRF1 greatly weakens replication efficiency, suggesting that TRF1 promotes efficient replication of telomeric DNA by preventing fork stalling [46]. This study further stresses the notion that the telomeric region poses a great challenge to replication machinery.

Consistently, deletion of Taz1 (TRF1 homologue) in fission yeast causes a block in telomere replication, resulting in aberrant telomeric fragments [108]. As a consequence, Taz1 depleted cells show rapid telomere loss in the absence of telomerase [108]. Collectively, it is obvious that TRF1 not only helps in telomere capping and telomere length control, but also promotes efficient telomere replication, perhaps by recruiting helicases such as BLM and RTEL1 and other factors to the stalled replication forks [46].

TRF2

Like TRF1, TRF2 is also a duplex DNA binding shelterin protein essential for telomere end protection and length regulation. It plays a monumental role in the formation of the protective t-loop structure. Recent studies have discovered TRF2 as an essential player in assisting efficient replication of telomere DNA, but likely with a mechanism different from TRF1. First, TRF2 preferentially binds to positively supercoiled DNA *in vitro* and is enriched at telomeres upon the loss of topoisomerase 2 (Top2 α) activity [109]. TRF2 also induces positive supercoiling of DNA, suggesting that its binding to positive supercoils may be energetically favoured [110]. Such activities are proposed to be used for sensing the topological stress created by fork progression and then recruit proteins such as Apollo and Top2 α to release the topological strain [109]. Second, TRF2 is potentially involved in the dissolution of t-loops and holliday junctions [110]. This event is speculated to be aided by local telomere unwinding factors such

as BLM and WRN. Furthermore, TRF2 is likely to be involved in the formation of pre-RC in order to initiate replication at telomeres, as TRF2 interacts with the origin recognition complex (ORC) and reduction of TRF2 leads to reduced ORC binding and pre-RC assembly at telomeres [111–112]. In agreement with this view, a recent *in vitro* study using *Xenopus* cell free extract shows that TRF2 binds to the telomeric DNA substrates to initiate replication in an origin-dependent manner [113]. Notably, pre-RC proteins such as ORC2, minichromosome maintenance (MCM)6, Cdc6 also assembled following the binding of TRF2 [113]. How TRF2 could promote all these activities probably relies on the formation of different protein complexes through its wide array of protein–protein associations [114]. Some of these associations in the context of replication are Apollo, WRN, BLM and FEN1 [84, 90, 99, 109]. It has been proposed that TRF2 essentially acts as a protein hub and coordinates the associations with various proteins by recruiting them to telomeres at different stages during the cell cycle for efficient telomere maintenance [115].

Apollo

Apollo is a 5 \rightarrow 3' exonuclease acting in a TRF2-dependent manner in protecting telomere ends from DNA damage response during the S phase [116–117]. Its nucleolytic activity is implicated in the protection of leading strand telomeres and efficient G-overhang maintenance in mammalian cells [38], but appears to be dispensable for G-overhang generation in human cells [109]. Expression of the nuclease-inactive Apollo results in S-phase specific defective telomeres, leading to increased telomeric fusions and accelerated onset of senescence [109, 117]. Apollo's nuclease activity is also crucial for protecting interstitial telomere repeats [109], strongly suggesting that Apollo could be involved in the progression of replication fork through telomeric chromatin. Such protection is achieved by the cooperation between TRF2, the nuclease activity of Apollo, together with Top2 α to effectively resolve the positive superhelical stress generated during the replication of telomeric DNA [109]. However, the molecular mechanism of this coordination remains to be elucidated.

POT1

POT1 binds to ss G-overhang and plays an essential role in telomere capping and chromosome end-protection [12, 118–123]. It represses the activation of ATR-mediated DNA damage response induced by dysfunctional telomeres [123–124]. Moreover, biomedical analysis shows that human POT1 protein stimulates the activities of the helicases WRN and BLM in unwinding G-quadruplexes *in vitro* [81]. In the absence of WRN, POT1 is required for normal replication of leading daughter telomeres [70]. It is further demonstrated that purified POT1 possesses a higher affinity to ss G-rich DNA than RPA, which accumulates at telomeres when WRN is deficient [70]. It is possible that POT1, together or in competition with RPA, binds to ss G-strand during replication and prevents a full replication fork stalling and activating the ATR kinase. Alternatively, POT1 may have additional functions in compensating

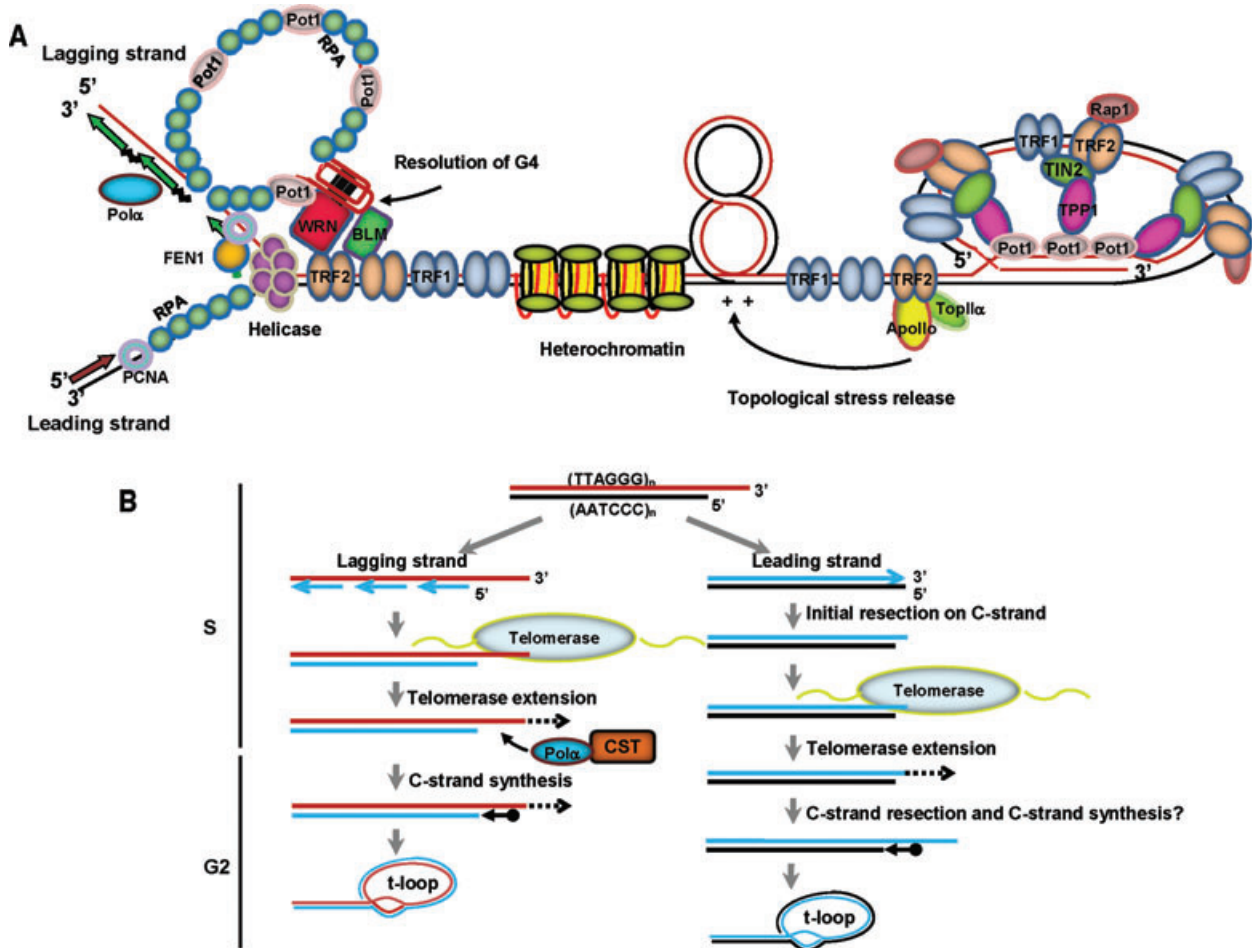


Fig. 1 (A) Activities of shelterin and replication factors during telomere replication. Challenges encountered while replication fork passes through telomere repeats are G-quadruplexes, heterochromatin, topological barriers and t-loops. The roles of various proteins in resolving these structural barriers that hamper replication fork progression is illustrated. WRN and BLM helicases unwind G4 and help in dissolving t-loops. TRF2, POT1 and RPA may also participate in unfolding G4 by stimulating the activities of WRN and BLM. TRF2 is essential for both forming and dissolving the t-loop. Progression of replication fork toward telomere ends causes local unwinding and subsequent accumulation of positive supercoils in the unreplicated DNA, which are efficiently resolved by TRF2 along with Apollo and Top2 α . TRF2 and FEN1 may possibly resolve the branched structures. (B) Diagram showing the possible events occurring at telomeres during and after telomere replication. Telomeres are replicated throughout the S phase. Replication of lagging strands produces ss G-overhangs, which are bound and extended by telomerase. During the late S/G2 phase, Pol α is recruited back to telomeres, most likely by CST, to carry out C-strand synthesis. In order for the blunt ended leading telomeres to be accessible to telomerase, they undergo initial resection to generate ss overhangs immediately after replication. It is unclear whether leading telomeres experience further end resection and C-strand synthesis during the late S/G2 phase. The processed telomere ends are converted into the protective t-loops.

for the absence of necessary factors like WRN to carry out telomere replication, such as preventing the formation of G-quadruplex structures in telomeric DNA [41].

The above findings have revealed that shelterin proteins are required for solving telomere replication problems in addition to their protective functions in telomere capping, telomere length regulation and repressing DNA damage response. It will be interesting to know the roles of other shelterin components such as TPP1, TIN2 and Rap1 in telomere replication. A model summarizing the roles of various proteins in telomere DNA replication is provided in Fig. 1A.

The delayed telomeric C-strand synthesis at late S/G2 phase – an additional regulatory step of telomere maintenance

Elongation of telomeres requires extension of both strands of telomeric DNA. While telomerase elongates the G-strand, extension of C-strand is thought to be accomplished by a DNA polymerization step similar to lagging strand synthesis. Lack of this synthesis

would give rise to excessive long ss G-rich DNA, which could harm telomere protection. Indeed, this additional DNA synthesis process termed C-strand fill-in or C-strand synthesis has been observed in *S. cerevisiae*. Remarkably, C-strand synthesis occurs at the same time as telomerase extension of telomeres and appears to regulate telomerase activity in yeast [48, 125–126]. Recently, two independent studies have revealed that human telomeres undergo C-strand synthesis as well [57, 127]. However, in human cells, this synthesis step is delayed until the late S/G2 phase, following global DNA replication and telomerase extension of telomeres [127], suggesting that it is independent of telomerase action (Fig. 1B). Therefore, the delayed C-strand synthesis could be more divergent in evolution. The current prevailing model of telomerase extension following telomere replication and being separate from C-strand synthesis may be an enhanced mechanism to avoid the persistence of excessive ss G-rich DNA generated during replication and further counteract accelerated telomere shortening in mammals. Regardless of the purpose of C-strand fill-in, this delayed synthesis may be unique to telomeres and mechanistically different from general lagging strand replication, thus providing a new strategy for cancer therapeutics. Inhibition of C-strand synthesis in tumour cells is expected to disrupt telomere maintenance without actually affecting the overall telomere replication. This opens up a new arena of research toward understanding the precise mechanistic details of C-strand synthesis and determining the factors responsible for regulating this event.

Our lab has recently shown that inhibition of pol α activity during the late S/G2 phase completely blocks C-strand synthesis, suggesting that C-strand synthesis requires the activity of pol α [57]. Furthermore, inhibition of the activity of one of the major kinases regulating the cell cycle progression, cyclin-dependent kinase 1 (CDK1), accumulates ss G-overhangs at late S/G2 phase, indicating that C-strand synthesis may be under the control of CDK1 [57]. These observations provide molecular insight into the mechanism regulating C-strand synthesis in human cells.

The CST complex

While the molecular targets of CDK1 at telomeres and how it controls pol α -mediated C-strand synthesis still remain to be determined, an evolutionarily conserved RPA-like heterotrimeric complex, the CST complex, has emerged as a key player in regulating C-strand synthesis [57, 128–129]. In budding yeast, telomerase action and C-strand fill-in is excellently coordinated by the CST complex. CST specifically binds to telomeric ssDNA *via* the putative OB-fold domains and protects telomere ends in multiple ways by repressing telomerase activity, restricting extensive nuclease degradation of C-strand and mediating C-strand fill-in [52, 126, 130–136]. Cdc13 has a dual role in both positively and negatively regulating telomerase. It binds to G-rich strand DNA and recruits telomerase (Est2 and Est1) to telomeres [130]. Dysfunction of Cdc13 leads to extensive C-strand degradation and G-overhang elongation [126, 137]. Yeast Stn1 negatively regulates telomerase action and coordinates DNA replication of the opposing telomeric C strand [133–134, 136–137]. Based on the associations of both Cdc13 and Stn1 with pol α , it is proposed that this complex could mediate the C-strand fill-in [52, 138].

The presence of the CST homologues in higher eukaryotes has recently been validated [128–129, 139–140]. The mammalian CST is formed by Ctc1-Stn1-Ten1 [129]. This complex binds to ssDNA in a sequence-independent manner and localizes at telomeres [129]. Depletion of components of CST leads to a significant increase of G-overhangs in human telomeres and induces telomere dysfunction [57, 128–129]. Interestingly, hCtc1 and hStn1 is also known as α accessory factors AAF132 and AAF44 due to their ability to stimulate pol α activity *in vitro* [141]. Thus, it is highly likely that mammalian CST may promote the recruitment of pol α /primase complex to the telomere DNA to carry out C-strand synthesis (Fig. 1B).

Given the multifunctional roles of yeast CST at telomeres, it is likely that mammalian CST may play additional roles such as telomerase regulation, telomere capping and perhaps restricting C-strand resection. Consistent with this notion, hStn1 associates with one telomere capping protein TPP1 and C-terminal deletion of hStn1 results in telomere elongation [142]. Furthermore, recent genome-wide association analysis has linked the genetic variation of hStn1 with alteration in telomere length [143]. These observations underscore the necessity to further our understanding of the telomeric function of mammalian CST.

Conclusions and perspectives

The last couple of decades have seen a surge of complex and dynamics world at telomeres. With the discovery of the growing number of proteins involved in telomere maintenance, it becomes evident that coordination between their actions is necessary for fulfilling the protective function of telomeres. Efficient strategies for cancer therapeutics can be developed only when a detailed mechanistic knowledge prevails on all the events taking place at telomeres. Examples of important areas that remain to be investigated are: t-loop structure formation and resolution before and after replication, the effects of telomeric heterochromatin on telomere maintenance, the concerted action of replication proteins and telomeric factors in safeguarding telomere integrity, the interplay between CST and shelterin components for regulating telomerase activity and telomere stability and regulation of C-strand synthesis. With the increasing wealth of knowledge and the advancement of technology, we expect rapid progress in unravelling the enigmatic events of telomere maintenance in the years to come.

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Conflict of interest

We declare no conflict of interest.

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