Replicating through telomeres: a means to an end

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Abstract

Proper replication of the telomeric DNA at chromosome ends is critical for preserving genome integrity. Yet, telomeres present challenges for the replication machinery, such as their repetitive and heterochromatic nature, their potential to form non-Watson-Crick structures as well as the fact that they are transcribed. A number of telomere-bound proteins are required to facilitate progression of the replication fork throughout telomeric DNA. In particular, shelterin plays crucial functions in telomere length regulation, protection of telomeres from nuclease degradation, control of DNA damage response at telomeres and the recruitment of associated factors required for telomere DNA processing and replication. In this review, we discuss the recently uncovered functions of mammalian telomere-specific and telomere-associated proteins that facilitate proper telomere replication.

Telomeres and their shelterin complex

The end of linear chromosomes is formed by special heterochromatic structure, known as the telomere, which protects chromosome ends from degradation and DNA repair and recombination activities. Therefore, telomeres are essential to ensure chromosome stability [1-8]. Mammalian telomeres comprise several kilobases (ie, 10-15 Kb in humans and 25-50 Kb in mice) of tandem TTAGGG DNA repeats [9]. Telomeres are characterized by the presence of a 30-400 nucleotide long 3'-overhang of the G-rich strand, known as the G-strand overhang [10, 11]. The G-strand overhang can fold back and invade the double stranded telomeric region forming the so-called T-loop and generating a displacement loop, or D-loop (**Figure 1a**). The T-loop structure has been proposed to protect chromosome ends from degradation and DNA repair activities as well as from telomerase activity [12, 13].

Telomeres are bound by a specialized complex known as shelterin. that plays crucial functions in telomere length regulation, protection of telomeres from the DNA damage response (through repression of the ATM and ATR signalling pathway), and in masking the chromosome ends from DNA repair machinery [8] (**Figure 1b**). The shelterin complex is composed of six core proteins: the telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), the TRF1-interacting protein 2 (TIN2), protection of telomeres protein 1 (POT1), TIN2 and POT1 interacting protein (TPP1) and the repressor/activator protein 1 (RAP1) (for a review see [7, 14, 15]. Homodimers of TRF1 and TRF2 bind double-stranded telomeric DNA and nucleate the assembly of the shelterin complex via TRF homology domains present on both factors, which bind to F/YxLxP motifs present on TIN2 [16].. POT1 possesses high specificity for the single-stranded telomeric DNA sequence 5'-TAGGGTTAG-3', thereby binding to the G-strand overhang and probably the displaced G-strand at the D-loop [17-19]. While human cells contain only one *POT1* gene, mouse cells have *Pot1a* and *Pot1b* [20-22]. POT1a primarily prevents ATR activation while POT1b regulates the 3'-overhang [21, 23-26]. TIN2 binds TRF1 and TRF2 through independent domains and recruits the TPP1-POT1 complex, bridging the different shelterin components [16, 27-31]. TPP1 binds TIN2 and POT1 through its C-terminal and central domains, respectively [28, 32]. TPP1 recruits POT1 to telomeres [33, 34]. In addition, TPP1 is required for the recruitment of telomerase to chromosome ends *in vivo* (**Figure 1b**)[35-37]. Finally, RAP1 binds to telomeres via association with TRF2 [2, 38, 39]. Human RAP1 is required for inhibition of the NHEJ pathway in the absence of TRF2 while mouse RAP1 is necessary to prevent telomere fragility and inhibits homologous recombination [40-42]. RAP1 also associates to non-telomeric genomic sites where it has been demonstrated to regulate gene expression, in particular of metabolic pathways, protecting from obesity and metabolic syndrome (**Figure 1c**)[41, 43-45].

Telomeres also associate with a growing number of accessory proteins that assist with proper chromatin structure, chromosome end protection, telomere length regulation, and telomere processing (reviewed in [8, 46, 47]. In this review, we discuss the recently uncovered functions of mammalian telomere-specific and telomere-associated proteins that facilitate proper telomere replication and length regulation and how these processes are relevant for genome instability and human disease.

Semiconservative telomeric DNA replication and telomerase-dependent telomere extension

During each cell division cycle, telomeres shorten as a result of the incomplete replication of linear DNA molecules by conventional DNA polymerases, which is called the “end-replication problem” [48, 49]. The molecular basis for this DNA loss is the requirement by DNA polymerases of a 3’-OH group as the site for nucleotide addition and therefore they cannot initiate DNA synthesis *de novo*. Semiconservative replication relies on a coordinated action of DNA-dependent DNA-polymerase complexes to synthesize the continuous leading strand as well as the discontinuous lagging strand, which is assembled via the joining of Okazaki fragments (**Figure 2).** Initiation of DNA synthesis in eukaryotic cells relies on the Pol α- primase complex (PP complex), a multi-protein complex endowed with polymerase and primase activity [50]. During primer synthesis, the primase subunits of the PP complex synthesise *de novo* an oligomer of 7–12 ribonucleotides in length, followed by limited primer extension with deoxyribonucleotides by Pol α that assembles the RNA-DNA hybrid primer required by the processive Pol δ and Pol ε for bulk DNA synthesis on the lagging and leading strand, respectively [50]. The final lagging RNA primer has been shown to be randomly positioned at 70-100 nucleotides from the ends [51] (**Figure 2**). The RNA primers are subsequently degraded and the gap is filled with DNA by Pol δ except for the last RNA primer of the lagging strand that cannot be replaced [50]. Thus, lagging daughter strands have an almost mature overhang size very soon after replication of the duplex telomeric DNA and are capped by a terminal RNA primer during 1 hour following replication prior to its removal. In contrast, leading daughter strands are transiently blunt-ended and develop a maturely sized G-overhang 1-2 hours after duplex telomere replication through nuclease processing. Leading daughter strands do not acquire a specific C-strand terminal sequence until late S/G2 [51] (**Figure 2**). Thus, incomplete lagging strand synthesis and post-replicative resection of the C-rich strand to generate the 3'-overhangs in both newly synthesized telomeres contribute to telomere shortening [52, 53].

Telomerase compensates for telomere attrition through *de novo* addition of TTAGGG repeats onto chromosome ends in those cells where it is normally expressed, such pluripotent stem cells and adult stem cell compartments [54-56]. To do this, the telomerase reverse transcriptase (TERT) uses an associated RNA component (*Terc)* as a template [57] (**Figure 3a).** Although telomerase is expressed in embryonic stem cells (ES) and in most adult stem cell compartments, this is not sufficient to maintain telomere length associated with cell division throughout life and therefore telomere shortening occurs with age in most tissues [6, 54-56, 58]. This progressive telomere shortening has been proposed as one of the molecular hallmarks of aging [59].. After completion of replication, both complementary 5'- and 3'- telomere DNA strands (the G- and C-strands) are sequentially extended by the telomerase and the PP complex, respectively (**Figure 3a**). The shelterin components TIN2-TPP1 recruit telomerase to the telomeres and POT1-TPP1 promotes processive telomere elongation [31]. Telomerase lengthens the G-strand through reverse transcription of the *Terc* RNA template subunit, *Terc* [57]**.** The PP complex lengthens the C-strand by copying the elongated G-overhang [60]. Mammalian CST complex has recently emerged as a key player in C-strand fill-in after G-strand extension by telomerase [61]. In cultured human cancer cell lines extension of the telomeric G-strand is coupled to telomere replication throughout S-phase, whereas the C-strand fill-in is delayed until late S phase. C-strand fill-in occurs through a stepwise process that is distinct from conventional Okazaki fragment synthesis in lagging strand replication [62].

Previous works indicated that telomerase is preferentially recruited to the shorter telomeres, which adopt a transient uncapped structure [63-65]. Recent work has also suggested that the status of the heterochromatin structure at telomeres can modulate the access of telomerase to the chromosome ends, thus modulating telomere extension [66-68]. In particular, telomeres and subtelomeric regions are organized into nucleosomes enrinched with epigenetic modifications of “closed” or “silenced” chromatin states, including DNA hypermethylation, H3K9me3 and H4K20me3, hypoacetylated H3 and H4 and high HP1 density [67, 69, 70]. These heterochromatin marks serve as a regulators of telomere length control and structural integrity (**Figure 3b**). Indeed, it has been shown that the telomeric chromatin in pluripotent stem cells is characterized by a more “open” state, which allows the generation of hyper-long telomeres by telomerase. During differentiation the telomeric chromatin switches to a more repressive state, and telomeres shorten [54]. Conversely, the reprogramming of adult somatic cells into induced pluripotent stem cells (IPSC) results in the switch in telomeric chromatin from a “closed” state to a more “open” state concomitant with telomerase-dependent telomere elongation [56]. These findings reveal that the structure of the telomeric chromatin is dynamic and controlled by epigenetic programs associated with the differentiation potential of the cells.

In contrast to the preferential recruitment of telomerase to short telomeres in normal cells, in cancer cells telomerase elongates the majority of telomeres every replication cycle, indicating significant differences in telomere length regulation between normal and cancerous cells [62, 64, 65]. The differences observed between cancer and normal cells are probably the result of both changes in the epigenetic status as well as alterations in the regulation of the multiple factors involved in telomere length maintenance pathways in cancer cells .In the following sections we discuss the function of different factors involved in telomere length maintenance.

Key factors in telomeric DNA replication

The CST complex

The CST complex is composed of proteins CTC1, STN1 and TEN1, all of which contain putative OB-fold domains involved in binding single-stranded DNA and promoting protein interactions [71, 72]. Human CTC1 and STN1 were initially purified as Polα-associated stimulatory factors (AAF) that stimulate template binding and enzyme processivity [73, 74]. The CST complex has structural and functional similarities to Replication Protein A (RPA) and has a role in DNA replication both at telomeres and elsewhere in the genome [75-78]. However, CST is not a conventional replication factor because it does not colocalize with replication foci in S phase but rather plays a key role in rescuing stalled replication forks under conditions of replication stress or at natural replication barriers [71, 77]. At mammalian telomeres, CST functions independently in two distinct aspects of telomere replication: telomere duplex replication and C-strand fill-in synthesis (**Figure 3a**) [23, 61, 78-81]. Depletion of individual mouse and human CST components results in increased loss of leading C-strand telomeres, accumulation of excessive G-strand overhang, telomere shortening and a high incidence of multitelomeric signals (MTS), a hallmark of telomere replication defects [61, 77, 78, 81]. The CST complex has been proposed to facilitate telomere replication by promoting efficient restart of stalled replication forks by assisting Polα to efficiently duplicate lagging-strand telomeres [78, 80].(**Figure 3a**). Formation of a trimeric CST complex at telomeric 3' overhangs limits telomerase action at individual telomeres while mediating a physical interaction with DNA polymerase-α to facilitate fill-in synthesis of the C-strand after G-strand extension by telomerase and ExoI-dependent resection [23, 61, 79-82]. The mechanism by which CST complex inhibits telomerase activity is through primer sequestration and physical interaction with the telomerase-stimulator POT1-TPP1 [82, 83]. In addition, CST competes with POT1-TPP1 for telomeric DNA substrate binding (**Figure 3a**)[82].

TRF1

Telomeres present challenges for the replication machinery, including topological interference by the t-loop and the formation of non-Watson-Crick DNA secondary structures such as G-quadruplexes [84, 85].. Work in yeasts was first to reveal fork pausing and stalling at telomeric DNA and to demonstrate the essential role of telomere binding proteins in facilitating telomere replication (for a review see [86];[87]). Later, it was shown that replication forks also stall naturally at mammalian telomeres and require an ATR dependent restart for replication to complete [88]. Mammalian telomeres resemble fragile sites and shelterin proteins, including TRF1, are essential to prevent telomere breakage associated with replication fork stalling at telomeres and are visualized by the presence of MTS at single chromosome ends [36, 89, 90:{Martinez, 2010 #854]. Deletion of *TRF1* results in telomere fragility, ATR activation, end-to end fusions, high incidence of sister telomere associations and the formation of ultra fine bridges (UFB) during mitosis (**Figure 4**) [89-91]. Recently it was demonstrated that TRF1 ablation in liver under chronic replicative stress leads to increased ploidy of hepatocytes due to endoreduplication, highlighting the role of TRF1 in protecting from replicative stress [92].

Recent work has shed light on the molecular mechanism underlying TRF1 functions [89, 90]. Similar to TRF1 deficient cells, knockdown of either BLM or RTEL1 results in telomere fragility. The severity of telomere fragility in *RTEL1*$^{-/-}$ *BLM*$^{-/-}$ double mutants is similar to the level observed in TRF1-deficient cells, indicating that both BLM and RTEL1 act in the same pathway as TRF1 [90, 93]. However, cells doubly deficient for BLM and RTEL1 show an additive effect on the incidence of MTS, suggesting that BLM and RTEL1 function in genetically distinct pathways to suppress telomere fragility [93]. The role of BLM in telomere fragility is likely directly regulated by TRF1. This is supported by the observation that human TRF1 binds directly to BLM through the TRF1 hinge domain (**Figure 4**) [94, 95]. In terms of function, the loss of BLM increases the number of MTS, with a strong bias toward lagging telomeres, suggesting that TRF1-BLM interaction is specifically required to suppress lagging telomere fragility [95]. However, interaction between TRF1 and BLM is dispensable for TRF1-dependent repression of DNA damage signaling and sister telomere association, arguing for a BLM-independent mechanism [95]. In contrast to BLM-deficient cells, TRF1-deficient cells do not show any bias toward fragility at leading or at lagging telomeres.

Other factors such as Timeless and Topoisomerase IIα (TopoIIα) have been suggested to interact with TRF1 and repress telomere fragility [91, 96]. Timeless is a component of the fork protection complex (FPC) that is recruited to replication origins. The FPC functions as a replication fork stabilizer that couples DNA replication with the sister chromatid cohesion that is established at replication forks [97]. Timeless associates with TRF1 and its depletion abrogates the ability of TRF1 to accumulate replisome components at telomeres and significantly delays telomeric DNA replication [96]. TopoIIα is essential for the resolution of DNA replication intermediates and has been shown to bind to telomeres in a TRF1-dependent manner. TopoIIα depletion leads to similar phenotypes as TRF1 abrogation; that is, telomere fragility and UFB, suggesting that both proteins act in the same pathway [91]. It has been suggested that TopoIIα is engaged in the resolution of MTS and that MTS originate from incomplete resolution of catenated DNA, leading to UFB in anaphase [91]. In addition, TopoIIα has also been proposed to cooperate with Apollo and TRF2 to relieve topological stress during telomere replication [98].

Finally, it is of interest to point out that TRF1 is highly expressed in pluripotent stem cells and in adult stem cells [99-101]. TRF1 is essential for both induction and maintenance of pluripotency in a telomere length independent manner [99]. Thus, TPP1 deficient IPSC that do not elongate the telomeres during reprogramming also present increased TRF1 levels similar to wild type IPSC [99]. TRF1 is a direct transcriptional target of the pluripotency factor OCT3/4 which binds the TRF1 promoter to upregulate TRF1 expression, providing a mechanistic link between TRF1 and pluripotency [99]. Although TRF1 is clearly essential for IPSC generation and in the maintenance of pluripotency, the mechanism by which TRF1 impacts stemness has yet to be elucidated and warrants future work.

WRN

Efficient and complete G-rich telomeric DNA replication by lagging strand synthesis in human cells requires the helicase activity, but not on the nuclease activity, of the Werner's syndrome RecQ helicase (WRN) [102]. WRN associates with telomeres in an S-phase specific manner and has been proposed to resolve the G-quadruplexes that are potentially formed in the G-rich telomeric strand and act as a roadblock to replication (**Figure 5**). Thus, a lack of WRN helicase activity leads to stalling of telomere lagging strand synthesis due to unresolved G-quadruplexes on the G-strand and consequently to partial or complete loss of the affected telomeres [102]. In addition, WRN has recently been shown to facilitate telomeric recombination processes [103]. The telomeric protein TRF2 physically associates with WRN, and enhances its strand exchange activity on telomeric DNA [103, 104]. This TRF2-dependent strand exchange activity of WRN is proposed to function in several telomere recombination processes such as T-loop formation, T-loop resolution needed for telomere replication, suppression of sister telomeric exchange, as well as to facilitate break-induced replication (BIR) to restart collapsed replication forks at telomeres (**Figure 5**) [103]. The BIR mechanism at telomeres might require WRN association to TRF2 for proper invasion of the detached arm of the replication fork into the homologous duplex. This would be consistent with the observation that WRN deficient cells show telomere loss during replication. The lost telomere is often in the chromatid derived from lagging strand synthesis, which is more prone to collapse due to G-quadruplex formation [102, 103, 105]. Further work is needed to elucidate whether the role of WRN in proper telomere replication is mediated either by its potential ability to resolve G-quadruplexes in the G-rich strand or by its speculative role in telomeric BIR [102, 103].

BLM

The Bloom's syndrome RecQ helicase (BLM) has also been implicated in mammalian telomere maintenance by facilitating the dissolution of late-replicating structures that occur throughout the entire genome including telomeres (**Figure 4 and 5**) [90, 95, 106]. The presence of BLM at telomeres is enhanced in a genetic background of telomere dysfunction, like WRN deficient cells or TRF1 knockdown cells [106]. In contrast to WRN, which localizes to telomeres in late S-phase and facilitates lagging-strand replication, BLM localizes to telomeres in G2-M and is required for post-replicative processing of both leading and lagging telomeres. Interestingly, the absence of both WRN and BLM exacerbates telomere dysfunction in human cells, arguing towards a non-redundant function of WRN and BLM in chromosome end maintenance [106].

FEN1

The Flap endonuclease 1, FEN1, is a structure-specific endonuclease that participates in Okazaki fragment processing during lagging strand synthesis and DNA repair mechanisms [107]. FEN1 co-localizes to stalled replication forks, and is proposed to aid in restarting them genome-wide [108, 109]. FEN1 is also important for preventing telomere fragility and lagging strand loss [110, 111]. FEN1 function in Okazaki fragment processing is dependent upon its interaction with Proliferating Cell Nuclear Antigen (PCNA), therefore the observation that its PCNA interaction domain is dispensable for its telomeric function indicates that the two roles of FEN1 are independent [110, 111]. The extreme C terminus of FEN1 interacts with both RecQ helicases,WRN and BLM [112]. FEN1 localizes to telomeres through interactions with TRF2 during S and G2/M phases of the cell cycle (**Figure 5**)[88, 110]. The gap endonuclease activity (GEN) and the TRF2- and RecQ helicase-interacting domains of FEN1 are essential for its activity at telomeres, where it facilitates replication through the G-rich lagging strand telomere by re-initiating stalled replication forks (**Figure 5**)[110, 111]. Similar to BLM or WRN, FEN1 depletion also leads to a fragile telomere phenotype and to lagging strand sister telomere loss [111].

RTEL1

T-loops must be disassembled in S-phase to avoid fork collision during telomeric DNA replication and to allow telomerase access to the telomeric 3'- end. The regulator of telomere length 1, RTEL1, is a helicase with D-loop-disrupting activity that has been proposed to facilitate T-loop unwinding and suppress telomere fragility by counteracting the formation of telomeric G-quadruplexes during telomere replication (**Figure 5**)[93, 113, 114]. Interactions with either PCNA or TRF2 through distinct RTEL1 domains endow RTEL1 with a dual function in suppressing telomere fragility and disassembling T-loops, respectively [93, 115, 116].

RTEL1 interacts with the replication clamp PCNA through its PIP (PCNA interacting protein) domain [116]. PCNA is a processivity factor for DNA polymerase and an integral component of the replisome during S-phase [117]. Loss of the RTEL1-PCNA interaction causes replication forks to progress more slowly, increases fork stalling and/or collapse, and increases origin firing in a genome-wide manner [116]. The role of RTEL in suppressing telomere fragility is mediated by its G-quadruplex unwinding activity, which in turn is dependent on RTEL1-PCNA interactions (**Figure 5**) [90, 93, 116].

A role for RTEL1 in T-loop disassembly *in vivo* is supported by the observation that RTEL1 loss causes telomere length heterogeneity and the accumulation of telomere circles (TC) [93]. In the absence of RTEL1, TC formation is dependent on the structure especific endonuclease complex (SLX-MUS) composed of the scaffold protein SLX4 and the three nucleases SLX1, MUS81 and XPF [118], .The SLX-MUS complex possesses Holliday junction resolvase activity and is capable of cleaving persistent T-loop structures [118]. Knockdown of either SLX4, SLX1 or XPF in RTEL1-deficient cells suppresses TC formation observed in the absence of RTEL1 [93]. RTEL1 interacts with TRF2 via its C4C4 metal binding motif and the TRFH dimerization domain of TRF2. Mutations in either motif disrupt TRF2-RTEL1 interaction and abolish RTEL1 telomere localization (**Figure 5**). The RTEL1 C4C4 motif is essential for suppression of TC and telomere loss but fully dispensable to prevent telomere fragility arising for telomeric DNA replicative defects [115].. These observations clearly reflect two distinct activities of RTEL1 at telomeres; i.e. T-loop disassembly and prevention of telomere fragility.

G-strand overhang generation

A conserved feature of telomeres is the 3' overhang composed of G-rich repeats that protrudes beyond the complementary C-rich telomeric repeat strand. The lengths of G-strand overhangs in mammalian cells vary between 30-400 nucleotides and they are present at both ends of each chromosome [10, 11]. DNA processing to generate the G-overhangs occurs regardless of whether a cell expresses telomerase and takes places in telomeres replicated by both leading and lagging strand synthesis. The terminal nucleotides on the C-rich strand are remarkably precise, ending in 3'-CCAATC-5' in more than 80% of the leading and lagging telomeres, indicating that 5'-resection in both strands must be highly regulated [119].

The generation of the telomeric overhang in mammalian cells is a complex highly regulated process that involves at least two shelterin proteins, POT1 (POT1b in mouse) and TRF2, as well as three DNA processing factors, the Apollo/SNM1B nuclease, the CST complex and Exonuclease 1 (Exo1) (**Figure 6**) [21, 23, 25, 26, 71, 72, 120-122]. Apollo/SNM1B is a member of the SMN1/PSO2 nuclease family that localizes to telomeres through its interaction with TRF2. Cells lacking Apollo activate the ATM kinase at their telomeres in S phase and show leading-end telomere fusions. The 5'-3' exonuclease function of Apollo is implicated in the generation of 3' single-stranded overhangs at newly replicated leading-strand telomeres [120, 122] (**Figure 6**). POT1b is a negative regulator of Apollo when bound to ssTTAGGG repeats; however, POT1b is inactive at blunted telomeres. Therefore, at newly synthesized leading-ends telomeres, resection by Apollo may first generate a POT1b binding site before it can further block resection. By contrast, on the lagging-strand POT1b can bind to the overhang resulting from incomplete synthesis and inhibit Apollo immediately (**Figure 6**)[23]. Interestingly, the overhang is approximately two-fold longer in S/G2 than in G1 and this transient elongation of the telomeric overhang after DNA replication has been shown to be dependent on Exo1 and independent of telomerase (**Figure 6**) [23, 120]. After Exo1-dependent generation of G-overhangs in S/G2, additional events take place to decrease the overhang to the length observed in G1. In mouse, POT1b and the CST complex have been shown to restore the overhang signals to their G1 size by fill-in synthesis (see the section on the CST complex) (**Figure 6**) [23]. Depletion of either individual CST components or *Pot1b* deletion leads to a significant increase in the overhang at both ends, abolishing the difference between overhang length in S/G2 and G1 cells [23]. Interaction with POT1b is required for CST telomeric localization (**Figure 6**) [23]. Thus, POT1b has a dual role in overhang regulation: it recruits CST to correct the overhangs generated by Exo1, but also inhibits hyperresection of the leading and lagging telomeres by Apollo.

The MRN complex (MRE11-RAD50-NBS1) has been shown to have a protective role in preventing NHEJ-mediated fusion of leading strand telomeres after replication by promoting 5’ leading strand resection to generate 3’-overhangs [123]. However, the molecular mechanism underlying MRN role in G-overhang generation is still unclear.

Concluding remarks

Telomere maintenance is a multi-step, highly regulated process in which many different factors are involved, including epigenetic events. Replication of telomeric DNA involves passage of a replication fork along the telomeric DNA duplex, where the conventional replication machinery encounters challenges arising from topological interference by the T-loop and the formation of DNA secondary structures such as G-quadruplexes. After replication, generation of the G-overhang takes place through 5’-end resection, extension of the G-rich strand by telomerase and fill-in of the complementary C-rich strand. The G-overhang is essential for telomerase action, shelterin assembly and T-loop formation in order to recap the telomere in late S-G2 phase of the cell cycle. Telomeres have evolved complex mechanisms in which shelterin components and several telomere-associated proteins cooperate to facilitate fork progression, control G-overhang formation and to reestablish a closed telomere conformation. As discussed in this review, defective telomere replication induces telomere loss and telomeric DNA damage that may cause genome instability as well as a decrease in the proliferative potential of the cells and thereby affecting organismal homeostasis.

The relationship between telomere biology and disease is becoming evident (Text box 1). In particular, mutations in telomere maintenance genes can result in premature loss of the regenerative capacity of tissues and disease. Improper telomere homeostasis can arise from telomere replication defects. Indeed, apart from the known mutations in telomerase components, novel mutations in genes encoding for shelterin and telomeric accessory proteins are emerging as the cause of several human diseases characterized by shortened telomeres and/or cancer predisposition. These findings underscore the plethora of different components and pathways that control telomeric functions. Future research might uncover yet unknown factors that participate in telomere regulation that will extend our understanding of the mechanistic underpinnings of cancer and telomere shortening syndrome phenotypes (outstanding question box). Hopefully, this knowledge will be translated into the clinics for disease diagnosis and treatment.

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Glosary

Telomeric loop (t-loop): A higher order structure that is generated by invasion of the telomeric G-strand into an upstream duplex telomere region to form a D-loop. As the 3'-end becomes hidden into the T-loop, this structure provides protection against nucleolytic attack and DNA repair activities (for review see [7, 46]). Shelterin proteins are thought to aid in T-loop formation. In particular, TRF2 has been shown to be essential in stabilizing T-loops [12, 124]

Displacement loop (D-loop): DNA structure where the two strands of a double-stranded DNA molecule are separated for a stretch and held apart by a third strand of DNA that is complementary to one of the main strands and pairs with it inducing a displacement of the other complementary main strand [125].

G-quadruplex (G4): Four stranded DNA structures that occur in guanine rich regions of the genome such as telomeres. Four guanine nucleotides pair via Hoogsten hydrogen bonding to form a G-quartet, a square planar structure stabilized by a monovalent cation in its center. Stacking of these planar structures leads to G4 formation. During replication, failure to unwind G4s can lead to replication fork stalling [125]

Telomere circles (TC): Extrachromosomal duplex or single stranded circular DNA molecule composed of telomeric repeats. TCs can arise from intrachromosomal recombination within telomeric arrays or from a endonuclease-mediated excision from a T-loop that results in rapid telomere loss [126].

Multitelomeric signals (MTS): Telomere aberration in which multiple telomeric fluorescence in situ hybridization signals are visualized at a single chromatid end, giving telomeres a broken or incompletely condensed appearance. MTS are observed when replication forks stall during telomeric DNA replication and thereby constitute a sign of telomere fragility [89, 90].

Fill-in synthesis: DNA synthesis by the polymerase alpha (polα)-primase complex to fill in the terminal telomeric C-strand gap that remains after G-strand extension by telomerase and after exonucleolytic processing during G-strand generation. Fill-in synthesis is stimulated by the CST complex [23, 61, 82].

Text Box 1: Telomeres and human disease

Syndromes characterized by impaired telomere maintenance, known as "telomere syndromes" or "telomeropathies", were originally described in patients harboring mutations in telomerase components [127]. Their manifestation encompasses different disease states, such as pulmonary fibrosis and bone marrow failure, which are characterized by cells with very short telomeres [9, 128, 129]. Interestingly, mutations in some of the shelterin components have been found in various telomere disorders highlighting their importance in maintaining a proper telomere structure. In particular, TIN2 mutations have been found in patients with dyskeratosis congenita, Hoyeraal-Hreiderasson syndrome, and Revesz syndrome (reviewed in [128]). Mutations in telomerase and shelterin components have also been found in cancer. Mutations in the telomerase gene promoter that conferee increased telomerase expression have been found in melanoma, gliomas, thyroid cancer, bladder cancer and hepatocellular carcinoma [130]. Similarly, POT1 variants that disrupt proper telomere capping and result in telomere aberrations have recently been linked to familial melanoma, familial glioma, and to chronic lymphocytic leukemia [131-134]. Importantly, all the identified POT1 variants conferred a telomere lengthening effect, suggesting that telomere elongation in combination with the telomere aberrations induced by these mutations may favor tumor incidence.

Outstanding questions

What are the differences in the regulation of telomere length-maintenance pathways between normal and cancer cells?

What is the threshold in telomere length shortening that defines the pathological onset of telomere related syndromes? Does this threshold vary among individuals and/or among different tissues?

What are the detailed mechanisms underlying the epigenetic-mediated regulation of telomere maintenance?

Telomere accessory factors such as RTEL1, WRN, BLM, FEN1 and the CST complex, are not telomere specific proteins and play also a role in genomewide repair. How and when is the recruitment and function of these accessory factors to telomeres regulated?

What is the molecular mechanism underlying TRF1 essential role in pluripotency?

What are the molecular mechanisms that determine the specificity of certain specific disease-associated mutations/variants in shelterin and in telomere associated components? For example, specific POT1 variants give rise to chronic lymphocytic leukemia while other amino acid substitutions predispose to familial melanoma and others to gliomas. In addition, specific mutations in RTEL1 give rise to Hoyeraal-Hreidarsson syndrome while other specific RTEL1 variants have been linked to several distinct brain cancers.

How our knowledge on telomere biology and its connection with human disease can be translated into the clinics to improve human health?

Could we bypass telomere replication problems and associated disease states by therapeutic strategies involving telomerase activation or epigenetic modulation of telomeres?

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Figure legends

Figure 1: ***Telomere structure and the shelterin. a.*** Schematic representation of telomere bound proteins, the shelterin complex, and telomerase. The shelterin complex is composed by TRF1, TRF2, RAP1, TPP1, TIN2 and POT1. TRF1, TRF2 and POT1 bind directly to telomeric DNA repeats, with TRF1 and TRF2 binding to telomeric double-stranded DNA and POT1 to the 3'-singled-stranded G-overhang. TIN2 binds TRF1 and TRF2 through independent domains and recruits the TPP1-POT1 complex. TPP1 recruits telomerase to telomeres. Telomerase is a two-partner enzyme: it comprises a catalytic subunit (TERT) and an RNA template (*Terc*), which recognizes the 3'-OH at the end of the G-strand overhang and elongates the telomere. ***b.*** Schematic model of shelterin complex bound to a telomere in a T-loop configuration. Telomeres contain a double strand region of TTAGGG repeats and a 30-400 nucleotide long single-stranded overhang of the G-rich strand. The G-strand overhang (black) invades the doubled-stranded DNA region of the telomere to form a protective telomere T-loop with a displacement D-loop at the invasion site.***c.*** RAP1 binds to both telomeric and non-telomeric chromatin. At telomeres RAP1 binds through interactions with TRF2. At non-telomeric sites containing ATTGGG repeats, RAP1 likely binds through TRF2 interactions, whereas at genomic sites lacking telomeric repeats, RAP1 must bind via interactions with yet unknown factors. RAP1 exerts a transcriptional regulatory function controlling metabolic pathways. At subtelomere, RAP1 is involved in subtelomeric silencing. TRF1 and TRF2, the telomeric repeat binding factor 1 and 2; TIN2, the TRF1-interacting protein 2; POT1, protection of telomeres protein 1; TPP1, TIN2 and POT1 interacting protein; RAP1, the repressor/activator protein 1; TERT, telomerase catalytic subunit; TERC; telomerase RNA component

Figure 2: Semiconservative telomeric DNA replication and G-overhang processing in telomerase-negative cells. Telomere replication is a multistep process that requires

dynamic opening of the telomeric DNA. First, semiconservative replication of the telomere duplex occurs via the conventional replication machinery that synthesizes the continuous leading strand and the discontinuous lagging strand by copying the telomeric C-strand and the G-strand, respectively. Initiation of DNA synthesis relies on the Polα-primase complex. The primase subunit synthesizes the RNA primers that are then extended by Polα, originating the RNA-DNA hybrid required for processive polymerases for bulk DNA synthesis on the leading and lagging strands. The RNA primers on the lagging strand are subsequently degraded except for the last RNA primer that cannot be replaced. The gaps are filled and the Okazaki fragments ligated to ultimately assemble the lagging strand. The final RNA primer on the lagging strand is not terminal but randomly positioned 70-100 nucleotides (nt) from the ends and therefore lagging overhangs present nearly mature overhang size immediately following replication. Lagging C-strands present specific ends upon RNA primer removal in S-phase. Leading strand synthesis renders blunt ends or 1 or 2 nt short overhangs consistent with the concept that the replication machinery runs off and dissociates from the extreme chromosome termini prior to incorporation of the final 1-2 nt. The leading daughter strands are then processed 1-2 hours after duplex telomere replication by nucleases to generate mature overhangs and the C-rich strands do not acquire the ATC-5' specification until S/G2 phase. Adapted from [51].

Figure 3: Telomerase-dependent telomere lengthening and CST-dependent C-strand fill-in. ***a.*** In cells positive for telomerase, the G-strands of newly replicated leading and lagging telomeres are extended by reverse transcription of the Terc RNA template subunit of telomerase. The shelterin components TIN2-TPP1 recruit telomerase to the telomeres and POT1-TPP1 promotes processive telomere elongation. The CST complex, composed of CTC1-TEN1-STN1 subunits, binds to the telomerase-extended telomere 3' end. CST binding suppresses telomerase access and inhibits the telomerase-stimulatory function of POT1-TPP1. The CST complex promotes fill-in synthesis of the C-strand by

stimulating DNA polymerase α-primase (PP complex). CST binding at telomeres increases steadily during S phase, with a peak at S/G2, and is strictly dependent on telomerase. Adapted from [82]. ***b.*** Regulation of telomere length by epigenetic modifications. Mammalian telomeres and subtelomeres are enriched in trimethylated H3K9 and H4K20, and HP1 isoforms. Subtelomeric DNA is heavily methylated and inhibits telomere recombination. Both histone trimethylation and DNA methylation have been shown to independently act as negative regulators of telomere length. The structure of the telomeric chromatin is dynamic and is controlled by epigenetic programs associated with the differentiation potential of the cells. The heterochromatic marks on of the telomeric chromatin affects its structural conformation towards a more "closed" state that restricts telomerase access to the telomeres making.

Figure 4: Model for TRF1-mediated role in telomeric DNA replication. TRF1 recruits the BLM helicase to resolve G quadruplexes formed in the G-strand facilitating replication fork progression and preventing lagging strand fragility. Prevention of leading telomere fragility by TRF1 is BLM independent and suggests the requirement of additional factors. Presumably, TRF1 connects to TRF2/RAP1 through TIN2 interaction and therefore the recruitment of additional factors like WRN, FEN1 and RTEL could be mediated by TRF2 (see figure 5). TRF1 loss results in leading and lagging telomere fragility, ATR activation, end-to-end fusions, sister telomere associations and a high incidence of ultra fine bridges in mitosis. The current model proposes that deletion of *TRF1* results in single strand (ss) gaps at sites of fork stalling that are coated by RPA to further induce ATR signaling. Adapted from [95].

Figure 5: TRF2 assists in the recruitment of different factors required for telomere replication and recombination processes. TRF2 physically interacts with the helicase RTEL1 to disassemble T-loops during S-phase. RTEL1 also facilitates G-quadruplex unwinding at telomeres in a PCNA interaction dependent manner, preventing telomere

fragility. The RecQ helicase WRN associates to telomeres in a TRF2-dependent manner during S-phase and has been proposed to resolve G-quadruplexes and to facilitate telomeric recombination processes such as T-loop formation and resolution and break induced replication (BIR). The Flap endonuclease FEN1 localizes to telomeres through interactions with TRF2. FEN1 also binds BLM and WRN and has been proposed to prevent telomere fragility by facilitating G-quadruplex unwinding and to reinitiating stalled replication forks during lagging telomere synthesis.

Figure 6: Model for the regulation by TRF2/POT1b of G-overhang generation in mouse. TRF2 recruits the exonuclease Apollo at blunt leading telomeres and initiates G-overhang generation by 5'-nucleolytic resection. The initial G-overhang at leading telomeres and the G-overhangs of lagging telomeres (see Figure 2) are bound by POT1b, which inhibits hyperresection by Apollo. Exo1 then acts on both ends to generate transiently elongated overhangs in S/G2 phase. The CST complex is recruited by POT1b to the extended overhangs and facilitates fill-in synthesis of the C-strand by the DNA polymerase α-primase (PP) complex to restore G1 overhang size. Adapted from [23].