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Journal
Nature Structural & Molecular Biology, 22(11)

ISSN
1545-9993

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Publication Date
2015-11-01

DOI
10.1038/nsmb.3083

Peer reviewed

HHS Public Access

Author manuscript

Nat Struct Mol Biol. Author manuscript; available in PMC 2016 February 24.

Published in final edited form as:

Nat Struct Mol Biol. 2015 November ; 22(11): 848–852. doi:10.1038/nsmb.3083.

Control of telomerase action at human telomeres

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Abstract

Recent progress has greatly increased the understanding of telomere-bound shelterin proteins and the telomerase holoenzyme, predominantly as separate complexes. Pioneering studies have begun to investigate the requirements for shelterin-telomerase interaction. From this vantage point, focusing on human cells, we review and discuss models for how telomerase and shelterin subunits coordinate to achieve balanced telomere-length homeostasis.

Telomeric DNA and proteins distinguish natural chromosome ends from double-strand DNA breaks[1]. Remarkably, the shelterin telomere proteins prevent chromosome 3′ ends from being accessed by the DNA-replication and DNA-repair machineries yet also solicit telomerase for chromosome 3′ -end elongation[2–4]. In the early human embryo and some adult stem cells, shelterin and telomerase collaborate to specify a range of telomere lengths that are maintained with cell proliferation. In contrast, differentiated, telomerase-negative somatic cells count down telomere length with each cell division until a critically short telomere length, which signals the limit of proliferative capacity, is reached[5,6]. Interestingly, shelterin-telomerase coordination appears to differ with cell type: the telomere lengths maintained in human embryonic stem cells (hESCs) are much longer than those typically maintained in most cancers and cancer cell lines[5,7]. Understanding the cross-talk between shelterin and telomerase requires knowledge of each complex alone and also of how the assemblies change with interaction. As a starting point, here we consider the biochemical and genetic pathways that underlie telomerase action at telomeres and telomerase regulation by shelterin in humans. Insights gained from this research illuminate numerous cellular processes that are fundamental for the preservation of genome stability and organism viability.

Shelterin, telomerase and telomere elongation

Proteins in the human shelterin network are anchored by TRF1 and TRF2, which bind to double-stranded telomeric repeats; these two proteins recruit the sequentially interacting TIN2, TPP1 and POT1 proteins[1,8] (Fig. 1). POT1 interacts with the 3′ overhang and/or

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

internal regions of single-stranded $(TTAGGG)_n$ (G strand) displaced from the complementary $(CCCTAA)_n$ (C strand) by t-loop formation[9,10]. Little is known about how shelterin complexes and other chromatin components distribute along telomeric-repeat arrays, except that there is assembly heterogeneity of the five telomerase-regulating shelterin proteins listed above and a sixth shelterin protein, RAP1, that is not involved in telomere-length regulation[11]. Important distinctions between the telomere structure in human cells and that in commonly studied single-celled model organisms include human telomeres' long, ~100-nt G-strand single-stranded overhang and the functional specialization of the two double-stranded DNA–binding proteins TRF1 and TRF2. Interestingly, although TRF1 and TRF2 bind separate domains of TIN2 and thus could assemble a homogeneous array of TRF1–TIN2–TRF2 complexes[12], the two double-stranded DNA–binding proteins have different abundance and exchange dynamics at telomeres[13,14]. Additionally, TIN2 recruits only substoichiometric TPP1–POT1 (ref. 14). Relative levels of the shelterin subunits have been quantified for only a few cell lines to date. Different ratios of shelterin proteins to each other and to telomeric DNA could be part of the mechanism determining telomere-length readout, as described below.

Biologically active human telomerase contains the human telomerase RNA (hTR); telomerase reverse transcriptase (TERT); two dyskerin–NHP2–NOP10–GAR1 complexes bound to the two hairpin stems of the hTR hairpin-hinge-hairpin–ACA (H/ACA) motif; and a WD40-domain protein, TCAB1 (also known as WDR79 or WRAP53β), bound to the 3′ hairpin loop[15–17] (Fig. 1). A plethora of additional interacting factors make less stable or substoichiometric associations, as reviewed in detail elsewhere[15,17–19]. Biogenesis of the human telomerase holoenzyme begins with the cotranscriptional assembly of a nascent hTR transcript with an initial H/ACA motif–binding complex of dyskerin, NHP2, NOP10 and NAF1 (refs. 20,21). These proteins bind to all H/ACA RNAs, which in human cells other than hTR are intron-encoded small nucleolar RNAs or small Cajal body (CB) RNAs that guide RNA modification[22]. After initial H/ACA ribonucleoprotein (RNP) assembly, hTR undergoes 5′ - and 3′ -end maturation accompanied by exchange of NAF1 for GAR1, thus generating the mature, biologically stable hTR H/ACA RNP[15,17]. The hTR 3′ -hairpin CAB-box motif recruits the multifunctional CB-localization factor TCAB1 (refs. 23–25). Separately from the TCAB1-hTR interaction, TERT binds two structurally independent hTR domains, thus generating the catalytically active telomerase RNP[26–29]. Important distinctions between telomerases of humans and of unicellular model organisms include human telomerase's highly chaperoned assembly as an H/ACA RNP and the complexity of subunit trafficking through nucleoli, CBs and other nuclear areas[16–18,30,31].

How does telomerase meet a telomere substrate? A generally accepted model is that telomerase can access a telomere only after a replication fork remodels end-protected telomeric chromatin[32]. In support of this model, hTR colocalization with telomeric DNA occurs predominantly in the cell-cycle S phase[30,33]. However, it has not been tested whether the S-phase enhancement of telomerase-telomere interaction arises solely from the need for replication-dependent shelterin remodeling.

Undifferentiated hESCs and most other human cell lines that grow indefinitely in culture support their proliferative immortality by maintaining telomere-length homeostasis[5,6]. In

these telomerase-positive cells, the average telomere length can be considered to be the set point for a dynamic equilibrium of terminal sequence loss by incomplete replication and nucleolytic processing versus sequence gain initiated by telomerase G-strand extension[34]. Telomere lengths vary among chromosomes and even among copies of the same chromosome in different cells of a population[35], but the extent and dynamics of length heterogeneity have not been well characterized. It remains speculative how individual telomere lengths are measured for feedback to telomerase. Previously proposed hypotheses for length-sensitive steps of regulation include shelterin-autonomous models, such as sequestration of the 3′ overhang, or models invoking differences in shelterin-telomerase communication that affect the amount of new repeat synthesis. Below, we consider the contributions of telomerase, shelterin and associated factors to telomere-length homeostasis in human cells.

Regulation via telomerase

Ectopic overexpression of both hTR and TERT can dramatically increase telomerase catalytic activity in a broad range of cell lines, thus indicating that no other holoenzyme components are limiting for active RNP assembly. An increase in active RNP level generally results in increased telomere length. Indeed, in cancer cell lines, overexpression of telomerase by approximately ten-fold or less may be sufficient to entirely defeat feedback regulation of telomerase by telomere length[36]. Thus, not only the telomere-length set point but also telomere-length homeostasis may require a finely tuned cap on the cellular level of active RNP. Telomere length in many telomerase-positive cell lines, humans and mice is limited by the level of telomerase RNA[37,38]. In hESC and HT1080 fibrosarcoma cells, overexpression of hTR alone but not TERT induces dramatic telomere elongation[36,39]. In comparison, HeLa-cell telomeres are slightly elongated by overexpression of TERT, but dramatic telomere elongation requires co-overexpression of TERT and hTR[36,40]. Once assembled, hTR and TERT remain stably associated throughout the cell cycle, as demonstrated by enzyme activity assays and subunit cross-linking *in vivo*[41,42].

Recruitment of telomerase to telomeres is stimulated by the holoenzyme subunit TCAB1 (ref. 25). TCAB1 knockdown decreases telomerase colocalization with telomeres and decreases telomere length[25,43]. TCAB1 mutations underlie prematurely short telomeres in some people with dyskeratosis congenita (DC)[44], a human bone-marrow failure syndrome[38]. One consequence of TCAB1 interaction with telomerase RNPs is their redistribution to CBs from the H/ACA RNP default localization in nucleoli[25,45]. TCAB1 assembly with telomerase RNP increases in G1 (ref. 42), thus accounting for the G1 increase in the CB concentration of hTR[45]. CBs could provide important accessory factors for telomerase-telomere interaction, or they could indirectly promote telomerase association with telomeres by clustering active RNPs for multivalent association with shelterin. Consistently with the clustering hypothesis, telomerase RNAs of mice and yeast concentrate in nuclear foci that are not CBs[46,47]. However genetic knockout of coilin, a protein essential for CB formation, has been shown not to compromise telomere-length maintenance in a human tumor cell line[48]. In addition, overexpressed telomerase can elongate telomeres even if hTR-TCAB1 interaction is severely decreased, and telomerase overexpression is associated with loss of the S-phase restriction for telomere colocalization[40,49]. Perhaps TCAB1 affects telomerase

association at telomeres in ways that are limiting for net telomere synthesis in some conditions but not others. This model fits with the observation that many but not all endogenous foci of hTR at telomeres colocalize with CBs[45]. In future studies, it will be of interest to compare TCAB1- and coilin-knockout phenotypes in parallel, in a range of cell types, to distinguish direct TCAB1 functions from those dependent on CBs.

Regulation via shelterin

Shelterin is the key protein complex that orchestrates telomerase action at telomeres. Shelterin-telomerase communication defines the set point for telomere length and establishes telomere-length homeostasis by regulating telomerase at each telomere according to telomere length. The initial model for telomere-length regulation by shelterin proposed a *cis*-regulatory negative feedback loop in which shelterin subunits count telomeric repeats and impose inversely proportional telomerase inhibition[50]. This model is based on the finding that all three direct telomeric DNA–binding proteins—TRF1, TRF2 and POT1—are negative regulators of telomere length[51–54]. Thus, telomere length could be measured by the occupancy of TRF1 and TRF2 on the double-stranded part of the telomere, and this readout would then be transduced to POT1. POT1 binding to a single-stranded G strand could then restrict telomerase access to the 3′ end of long telomeres[54]. Telomere length–dependent, TRF2-directed t-loop formation remains a good model for the mechanism underlying telomere-length homeostasis: a length-dependent increase in the rate of t-loop formation could correspondingly decrease telomerase action[9,50]. Alternate models have been proposed, including shelterin control of chromatin-remodeling activities that alter telomeres' accessibility to telomerase[55,56].

Recent studies have indicated that shelterin's control of telomere length is more complex than just steric blocking of telomerase access to the chromosome 3′ end. Several lines of evidence have suggested that there are multiple stages of telomerase-telomere interaction progressing from physical association of telomerase with telomeric chromatin ('recruitment') to capture of the DNA 3′ end in the telomerase active site ('engagement'). Several requirements for telomerase recruitment have been elucidated by studies of colocalization of hTR or TERT with telomeres. One critical factor is TPP1 (refs. 3,4). Genetic and biochemical assays have indicated that telomerase recruitment to telomeres requires a protein surface of the TPP1 OB fold termed the TEL patch[57], in both immortalized human cancer cell lines and hESCs[57–60]. Active telomerase binds directly to the TEL patch[61], and cells deficient for this interaction show progressive telomere shortening at a rate equivalent to that in telomerase-negative cells[60]. Moreover, cells expressing hypomorphic TEL-patch substitutions show graded phenotypes that directly reflect the ability of the TPP1 variant to bind telomerase[60,62]. This suggests that TPP1 binding of telomerase can be limiting for telomerase action at telomeres and is thus a potential target step for *cis*-regulatory telomere-length feedback.

Other than the contributions of the TPP1 TEL patch, shelterin's contributions to telomerase recruitment and engagement are not well understood. TPP1 and its heterodimer partner POT1 are substantially less abundant on telomeres than are TRF1, TRF2 and TIN2 (ref. 14), thus raising the question of where along the double-stranded length of telomeric repeats the

substoichiometric TPP1–POT1 complexes bind and where along the TPP1–POT1 complexes telomerase is initially recruited. Specialized shelterin structures could affect the preference for telomerase recruitment to near the 3′ overhang rather than to internal telomeric repeats. However, telomerase may find the 3′ overhang while bound to internal repeats. After recruitment, telomerase action at telomeres would still be limited by the need for the active site to find a substrate 3′ -OH, possibly through translocation of TPP1–POT1 along single-stranded DNA[63].

The biochemical properties of POT1 have served as a basis for hypotheses for the regulation of telomerase engagement. *In vitro*, human POT1 has multiple competing influences on telomerase. Depending on the assay, interaction of POT1 with DNA can either facilitate or block primer elongation by telomerase[64]. Stimulation of telomerase by POT1-DNA interaction has been found to be the consequence of both decreased DNA guanosine-quadruplex formation[65] and increased telomerase repeat–addition processivity (RAP)[2]. Inhibition by POT1 is the consequence of DNA binding competition[64,66]. *In vivo*, evidence for POT1 competition with telomerase comes from overexpression studies of POT1 ΔOB, a truncated protein lacking the first POT1 OB fold and therefore severely compromised in DNA binding affinity[54]. Because POT1 ΔOB retains binding to TPP1, overexpressed POT1 ΔOB competes with endogenous POT1 for TPP1 association and then, through TPP1, assembles into shelterin complexes at telomeres[54]. Expression of POT1 ΔOB results in rapid telomerase-mediated telomere elongation[54,67]. In addition, depletion of POT1 in telomerase-positive tumor cells leads to rapid telomere elongation[68]. All of these findings are consistent with the model in which POT1 inhibits telomerase engagement of the chromosome 3′ end.

The biochemical properties of TPP1 also suggest multiple roles for this protein beyond telomerase recruitment. TPP1–POT1 stimulation of telomerase RAP is lost upon mutation of the TPP1 TEL patch[57,61] or of residue L104 in the OB-fold domain, distant from the TEL patch. Substitutions of L104 differentially affect TPP1–POT1 stimulation of telomerase RAP *in vitro* and TPP1-mediated telomerase recruitment to telomeres *in vivo*[57]. When expressed in hESCs, TPP1 L104A supports stable but short telomere lengths at homeostasis, an abrogation of set-point control[60]. Because TPP1 L104A still mediates telomerase recruitment[57], this phenotype suggests an opportunity to investigate steps of telomerase activation after recruitment that are responsive to telomere length. Alternatively, the L104A variant could eliminate the normal molecular pathway of telomerase-telomere interaction while supporting an alternate recruitment or activation mechanism dependent on very short telomeres. Intriguingly, L104 packs against an α-helix bridging the telomerase-binding TPP1 OB fold to the POT1-binding domain[2], which contains sites of TPP1 ubiquitination[69,70]. Therefore the TPP1 L104A variant may disrupt the direct biochemical coordination of POT1, TPP1 and telomerase or indirectly affect this coordination in a manner dependent on TPP1 post-translational modifications.

Additional steps of telomerase regulation by shelterin remain to be elucidated. In the near future, at least three additional mechanisms should be clarified. First, TIN2 may have a telomerase-stimulatory role abrogated by heterozygous expression of mutations associated with severe DC[71,72]. When modeled in mice, a TIN2 DC mutation exacerbates telomere shortening by a telomerase-independent pathway[73]. However, this finding does not exclude

the hypothesis that TIN2 DC mutations compromise telomerase-mediated telomere elongation as well[74]. Another tentative mechanism of telomerase regulation by shelterin is through the CTC1–STN1–TEN1 (CST) complex[75], a stimulator of DNA polymerase α-primase (PαP) activity[76,77]. CST is recruited to the chromosome 3′ overhang at least in part by POT1 (Fig. 1). Telomere-associated CST and PαP have been proposed to limit G-strand synthesis by telomerase and/or to affect the nature of C-strand synthesis by PαP[78,79]. A third tentative connection between shelterin and telomerase is the TRF2-recruited 5′ -3′ exonuclease Apollo (Fig. 1), which mediates C-strand resection after leading-strand synthesis[79–81]. The association of Apollo gene mutation with the DC-related disease Hoyeraal-Hreidarsson syndrome[82] suggests the possibility of an unrecognized influence of Apollo on telomerase action at telomeres. For example, the timing and extent of Apollo-mediated 3′ -overhang generation could affect the availability of 3′ overhangs to telomerase. Details of how shelterin coordinates telomerase extension of the G strand with C-strand synthesis and 3′ -overhang processing will be of high interest to approach with methods that can resolve the complexity of shelterin interactions and subunit functions.

Summary

To date, human shelterin and telomerase have been characterized predominantly in isolation from each other. As described above, the focus of research in the field is shifting to bridge this gap at the cellular, molecular and biochemical levels. One outstanding question concerns the potentially different telomerase–shelterin complexes that mediate recruitment, engagement and other yet-unknown states of interaction. A priori, it seems likely that conformational transitions or subunit exchanges are necessary to drive a unidirectional progression of telomerase from physical recruitment to functional engagement to release. Recent studies in hESCs and fission yeast have suggested a genetic separation of function in TPP1 (Tpz1) sequence requirements for telomerase recruitment and engagement[60,83], highlighting the utility of allele replacements as a genetic approach. Another outstanding question is how human-cell telomeres count a net length of repeats at each telomere for feedback to telomerase. This presumably involves higher-order architecture of shelterin interactions along the array of telomeric repeats, which would topologically and spatially constrain DNA accessibility. Multiple pathways of communication between and within shelterin and telomerase complexes may be a necessary feature of the regulation robustness required for telomere-length homeostasis.

Acknowledgments

We thank A. Wu and J. Boyle for comments and the US National Institutes of Health (RCA196884A (D.H.) and HL0795985 (K.C.)) for funding.

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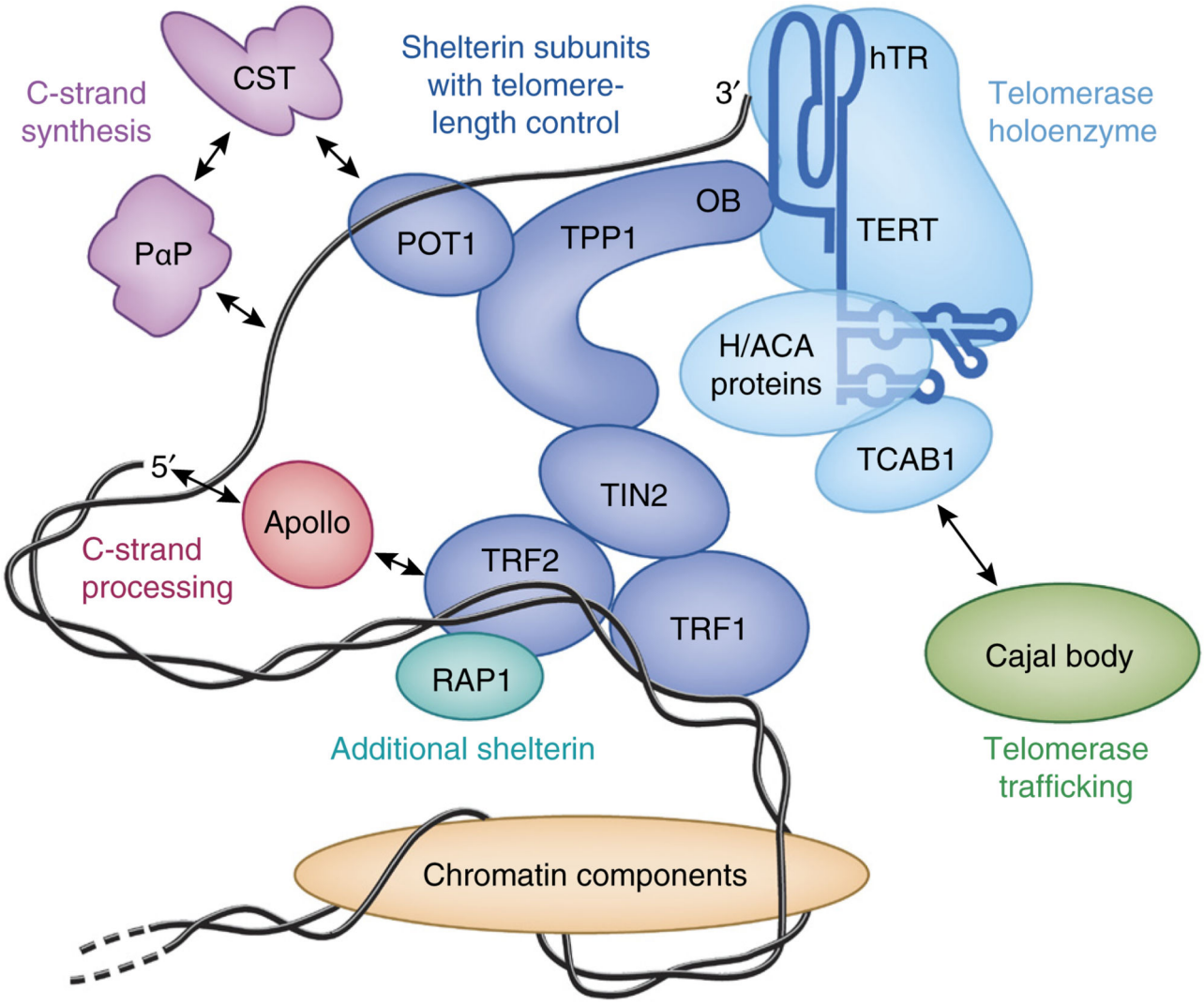

Figure 1.

Human shelterin and telomerase-subunit interactions. The human shelterin protein complex is anchored by binding of the proteins TRF1 and TRF2 to double-stranded telomeric repeats. TRF1 and TRF2 are bridged to the single-stranded telomeric-repeat G-strand DNA–binding protein POT1 through TIN2 and TPP1. Additionally, shelterin RAP1 binds directly to TRF2. A catalytically active human telomerase holoenzyme has the integral RNA subunit hTR, TERT and the H/ACA proteins dyskerin, NHP2, NOP10 and GAR1. TERT and the H/ACA proteins interact with nonoverlapping regions of hTR. TCAB1 assembles with telomerase holoenzyme at least in part through interaction with the loop of an hTR H/ACA-motif hairpin, and it mediates RNP cellular concentration in Cajal bodies. Telomere maintenance involves numerous additional activities of DNA synthesis and processing that occur at telomeres and are at least partly dependent on shelterin. TRF2 binds Apollo, a C-strand 5′ -3′ exonuclease. POT1 and potentially other shelterin subunits interact with the CST complex, which has been proposed to stimulate synthesis of the C-strand by DNA

polymerase α-primase (PαP). Telomere structure and dynamics depend on general chromatin proteins not discussed in this perspective.