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Function, replication and structure of the mammalian telomere

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Abstract

Telomeres are specialized structures at the ends of linear chromosomes that were originally defined functionally based on observations first by Muller (1938) and subsequently by McClintock (1941)that naturally occurring chromosome ends do not behave as double-stranded DNA breaks, in spite of the fact that they are the physical end of a linear, duplex DNA molecule. Double-stranded DNA breaks are highly unstable entities, being susceptible to nucleolytic attack and giving rise to chromosome rearrangements through end-to-end fusions and recombination events. In contrast, telomeres confer stability upon chromosome termini, as evidenced by the fact that chromosomes are extraordinarily stable through multiple cell divisions and even across evolutionary time. This protective function of telomeres is due to the formation of a nucleoprotein complex that sequesters the end of the DNA molecule, rendering it inaccessible to nucleases and recombinases as well as preventing the telomere from activating the DNA damage checkpoint pathways. The capacity of a functional end-protective complex to form is dependent upon maintenance of sufficient telomeric DNA. We have learned a great deal about telomere structure and how this specialized nucleoprotein complex confers stability on chromosome ends since the original observations that defined telomeres were made. This review summarizes our current understanding of mammalian telomere replication, structure and function.

Abbreviations: 53BP1 – TP53 binding protein 1; ATM – Ataxia telangiectasia mutated; BRCT – BRCA1 carboxy terminal; DAT – Dissociates activities of telomerase; DNA-PKcs – DNA-dependent protein kinase catalytic subunit; EST1/hEST1 – Ever shorter telomeres 1; MRN – MRE11/RAD50/NBS1 complex; PARP – Poly ADP-ribose polymerase; PINX1 – Pin2/TRF1 interacting protein; POT1 – Protection of telomeres 1; RAD54 – radiation senstitivity mutant 54; hRAP1 – human homologue of *S. cerevisiae* Repressor Activator Protein 1; hTERT/mTERT – human and mouse homologues of telomerase reverse transcriptase catalytic subunit; hTERTα – alternatively spliced inhibitory form of hTERT; TIN2 – TRF1 interacting protein 2; TPE – Telomeric position effect; hTR/mTR – Human and mouse homologues telomerase template RNA subunit; TRF1 – TTAGGG-repeat binding factor 1; TRF2 – TTAGGG-repeat binding factor 2; XPF-ERCC1 – Xeroderma pigmentosum complementation group F/Excision repair cross-complementing 1; XRCC4 – X-ray-complementing Chinese hamster gene 4.

Telomere function – providing chromosome stability

The primary function of the telomere is to provide stability to chromosome ends. In fulfilling this function the telomere acts to protect chromosome ends from a number of detrimental processes including degradation by nucleases and the action of recombinases. The telomere also permits continued cellular division in the presence of multiple naturally occurring double-stranded DNA ends by

sequestering the ends of the DNA molecule. Using electron microscopic analysis of purified telomeres, Griffith et al. (1999) demonstrated that telomeres exist *in vivo* as circular structures, called telomere loops or t-loops. The t-loop is formed by invasion of the double-stranded telomeric repeats by the G-strand overhang, thereby sequestering the chromosome end and providing a chromosome 'cap'. These observations were the first demonstration of a specific telomeric protective structure. The double-stranded telomeric binding protein TRF2 (see below) is located at the base of this structure, and can promote its formation *in vitro* (Stansel et al. 2001). However, the assembly of t-loops *in vivo* is likely to be dependent upon the involvement of additional telomere-associated proteins.

Telomeres were thrust into the spotlight a number of years ago due to their role in the opposing processes of tumorigenesis and induction of senescence. The status of the telomeric complex clearly is a central factor regulating the two processes. In some instances, the genome instability accompanying loss of telomere end–protection function promotes tumorigenesis (Chin et al. 1999; Harrington 2004). However, dysfunctional telomeres, which resemble double-stranded DNA breaks, usually activate signal transduction pathways resulting in the induction of replicative senescence or apoptosis. Telomeres that have lost capping function, either through forced uncapping or as a natural consequence of telomere attrition arising from cellular divisions in the absence of telomerase, directly engage components of the DNA damage surveillance machinery including γH2AX, 53BP1 and activated ATM (d'Adda di Fagagna et al. 2003; Takai et al. 2003). Thus, the primary role of telomeres is to protect chromosome ends by preventing them from being recognized and/or or behaving as double-stranded DNA breaks.

Telomere replication

Replication of chromosome termini involves novel proteins and strategies in addition to those employed across the remainder of the chromosome. While most of the chromosome is replicated by the conventional DNA replication machinery, complete replication of chromosomal termini is hampered by the unidirectional nature and primer requirements of the DNA polymerases. Due to these features, a region of unreplicated DNA will remain on the parental DNA strand acting as the template for lagging strand synthesis following removal of the most terminal primer. Without some means of replicating chromosome ends, this process would eventually result in loss of essential genetic information. This has become known as the end replication problem (Olovnikov 1973). However, it was apparent that cells had evolved a mechanism for overcoming the end-replication problem because chromosomes are stable over multiple cellular divisions, across generations and through evolution.

Telomeric DNA

The DNA element of vertebrate telomeres is fairly simple, being composed of short, tandem repeats of the sequence 5'(TTAGGG)3'. The length of the telomeric repeat array is genetically regulated and is species specific, extending for approximately 15 kb in the human germline, and something shorter than that in somatic tissues depending on age and replicative history (Cooke and Smith 1986; Lindsey et al. 1991). In contrast, mice have considerably longer telomeric DNA arrays, which can extend in excess of 50 kb (Kipling and Cooke 1990). Telomeres are characterized by strand asymmetry due to the G-rich strand always being oriented towards the chromosome end. At the very end of the chromosome there is a single-stranded protrusion of the G-rich strand that extends for approximately 75–200 nucleotides in humans (Makarov et al. 1997; McElligott and Wellinger 1997; Wright et al. 1997). Some recent studies suggest that the maintenance of G-strand overhang in humans may rely at least in part upon telomerase (Masutomi et al. 2003), although this is inconsistent with reports of telomerase null mice in which the overhang is preserved with no detectable alterations (Hemann and Greider 1999). Following completion of conventional DNA synthesis in telomerase null cells, the daughter telomeres arising from leading strand synthesis would be predicted to be composed of blunt ended molecules. This would imply a window in the cell cycle, perhaps very short, in which the G-strand overhang would be absent from half the telomeres. To date this predicted decrease in the G-strand overhang has not been detected. However, it is clear

that maintenance of the telomere capping complex and the preservation of the G-strand overhang are tightly linked as removal of specific factors such as TRF2 (see below) can lead to the rapid loss of this feature of the telomere (van Steensel et al. 1998).

Telomeric chromatin is organized with shorter nucleosome spacing than is found in bulk chromatin (Tommerup et al. 1994). It has been suggested for many years that telomeres are heterochromatic. Telomeric heterochromatin has been documented extensively in yeast through demonstration of the ability of telomeres to silence the expression of adjacent genes, a phenomenon termed telomeric position effect (TPE) (Tham and Zakian 2002). Similarly, in human cells telomeres are able to silence expression of a nearby gene (Baur et al. 2001), although these effects are not as dramatic as those observed in yeast. Consistent with this, heterochromatin proteins HP1alpha and HP1beta are also associated with telomeres (Garcia-Cao et al. 2004) and over-expression of these proteins perturbs genetically determined telomerase-dependent telomere length regulation (Sharma et al. 2003). Recent work studying mice that are doubly deficient for the histone methyltransferases SuVar(39)H1 and SuVar(39)H2 also supports this notion (Garcia-Cao et al. 2004). The telomeres of the SuVar(39)H1/SuVar(39)H2 knockout mice rapidly increase in length without accompanying changes in telomerase activity and so likely reflect an alteration in chromatin structure that renders telomeric DNA more accessible to telomerase (see above). These data support the hypothesis that telomeric chromatin is organized as tightly compacted heterochromatin. The epigenetic regulation of telomere length has recently been reviewed (Blasco 2004).

Telomerase

Telomeric DNA is maintained in the germline and self-renewal tissues by telomerase, a reverse transcriptase that uses an RNA moiety as a template for the addition of telomeric sequences onto the 3' end of an existing DNA molecule (Kelleher et al. 2002). In this way, terminal sequence loss is balanced by de novo addition of telomeric repeats. Because the majority of tumors activate telomerase (Shay and Bacchetti 1997), this enzyme is an attractive target for chemotherapeutic intervention. A number of accessory proteins influence the efficiency and regulation of telomerase activity *in vivo*. A 3' nuclease activity that removes non-telomeric sequences from the end of a molecule is associated with telomerase *in vitro* and may be involved in proofreading activity (Oulton and Harrington 2004). However, the *in vivo* requirement for this nuclease activity has not yet been tested. In addition to the proteins that directly interact with telomerase or are components of the holoenzyme, the status of the telomeric complex may also affect the ability of this enzyme to adequately extend telomeric arrays. Recent work suggests that the telomeric complex exists in at least two states, only one of which is permissive for addition of telomeric DNA repeats by telomerase (Vega et al. 2003; Blasco 2004).

Evidence from mice that are heterozygous for the mTERT protein indicates that the level of the catalytic subunit of telomerase is limiting, in that insufficient enzymatic activity is present in the mTERT+/- mice to maintain the longest telomeres, which can extend to 50 kb and beyond in length (Liu et al. 2000; Erdmann et al. 2004). Thus, telomeres become shorter in the mice heterozygous for mTERT. Interspecies crosses have demonstrated that haploinsufficiency of mTR also limits the ability of telomerase to extend telomeres (Hathcock et al. 2002). Although unable to maintain wild type telomere lengths, adequate telomerase is present to sustain short telomeres so that the phenotype associated with complete loss of telomere function is not observed in this background, although the mice do have increased genetic instability (Erdmann et al. 2004). The bias towards the maintenance of shorter telomeres in multiple systems (Zhu et al. 1998; Hemann et al. 2001; Teixeira et al. 2004), including human cells (Cerone et al. 2001; Perrem et al. 2001), may reflect increased accessibility of shorter telomeres to telomerase. Alternatively, telomerase may be differentially recruited to shorter telomeres.

Regulation of telomerase activity

Telomerase activity is limited in most human somatic tissues. However, telomerase is active in some somatic tissues, most notably self-renewal tissues such as the hematopoietic system (Broccoli et al.

1995; Counter et al. 1995). The restricted activity of telomerase is the consequence of regulatory mechanisms active at numerous levels (Stewart 2002). First, transcription of the catalytic subunit, hTERT, is absent or very low in most human somatic cells (Meyerson et al. 1997). The transcriptional silencing of hTERT was originally believed to be the defining feature of telomerase regulation. However, over the last few years it has become evident that the situation is considerably more complicated than was originally thought. For example, alternative splicing of the hTERT transcript leads to at least one inhibitory form, hTERTα, which might downregulate enzymatic activity (Colgin et al. 2000; Yi et al. 2000). The assembly of the holoenzyme may also be regulated at several levels. The chaperones p23, hsp70 and hsp90 are associated with hTERT and are implicated in the proper assembly of the holoenzyme (Holt et al. 1999; Forsythe et al. 2001). In addition, the telomerase RNA moiety, hTR, is associated with Cajal bodies, subnuclear organelles involved in the biogenesis of small ribonucleoproteins (Matera 2003), in cells with active telomerase but not in telomerase negative cells (Zhu et al. 2004). The association of hTR with Cajal bodies in telomerase negative primary cells can be induced by over-expression of hTERT and hTERT is also found in these subnuclear structures, suggesting that association of telomerase components with the Cajal body is important in the assembly of a functional holoenzyme. Furthermore, it has been demonstrated that mutant versions of telomerase can trans-complement to reconstitute activity (Beattie et al. 2001). Thus, multimerization of telomerase may also be regulated. Phosphorylation of hTERT has also been reported to directly affect enzymatic activity, although there have so far been reports of both activating (Liu et al. 2001) and inhibitory events (Kharbanda et al. 2000). The complex regulation surrounding the generation of active telomerase holoenzyme provides a multitude of targets to be tested for therapeutic intervention.

In addition to regulation of the hTERT protein and assembly of a catalytically active holoenzyme, telomerase activity in vivo is clearly regulated through the ability of telomerase to access the chromosome end (Vega et al. 2003). Telomerase is sequestered in the nucleolus in primary cells ectopically expressing GFP-tagged protein, being released at the time of DNA replication (Wong et al. 2002). In contrast, in tumor cells in which telomerase is activated, hTERT is always nucleoplasmic. An amino terminal domain of telomerase, called Dissociates Activities of Telomerase (DAT domain), is critical for the ability of catalytically active telomerase to elongate telomeres *in vivo* (Armbruster et al. 2001). The evidence from tethering experiments is consistent with the suggestion that mutations in the DAT domain perturb interactions of telomerase with some other component(s) of the telomeric complex and prevent localization of the enzyme to its site of action, namely chromosome termini (Armbruster et al. 2003; Armbruster et al. 2004). The recent identification of human homologs of yeast EST1 (Reichenbach et al. 2003; Snow et al. 2003), a protein that interacts directly with telomerase and is involved in either recruitment or activation of the enzyme to allow telomere extension (Evans and Lundblad, 1999; Evans and Lundblad 2002; Taggart et al. 2002), suggests that the processes underlying the ability of telomerase to access telomeres are, at least in part, conserved. Over-expression of one homolog, hEST1A, causes telomere elongation (Snow et al. 2003), consistent with this protein enhancing the ability of telomerase to access chromosome ends. Over-expression of EST1A also generates chromosome end-to-end fusions, indicative of loss of capping function, with telomeric DNA remaining at the fusion point (Reichenbach et al. 2003). On the surface, one might predict longer telomere arrays to be associated with increased chromosome end stability. However, it is possible that the telomere extension that accompanies over-expression of hEST1A alters telomeric chromatin and/or titrates telomeric factors essential for chromosome stability (see below) thereby perturbing capping function.

Telomere structure

A great deal has been learned about mammalian telomere structure over the last decade (Figure 1A). In particular, a number of the protein components of the telomere have been identified and the contributions these proteins make to the end-capping function of telomeres have been elucidated. In addition to essential structural roles at the telomere, these proteins affect telomere

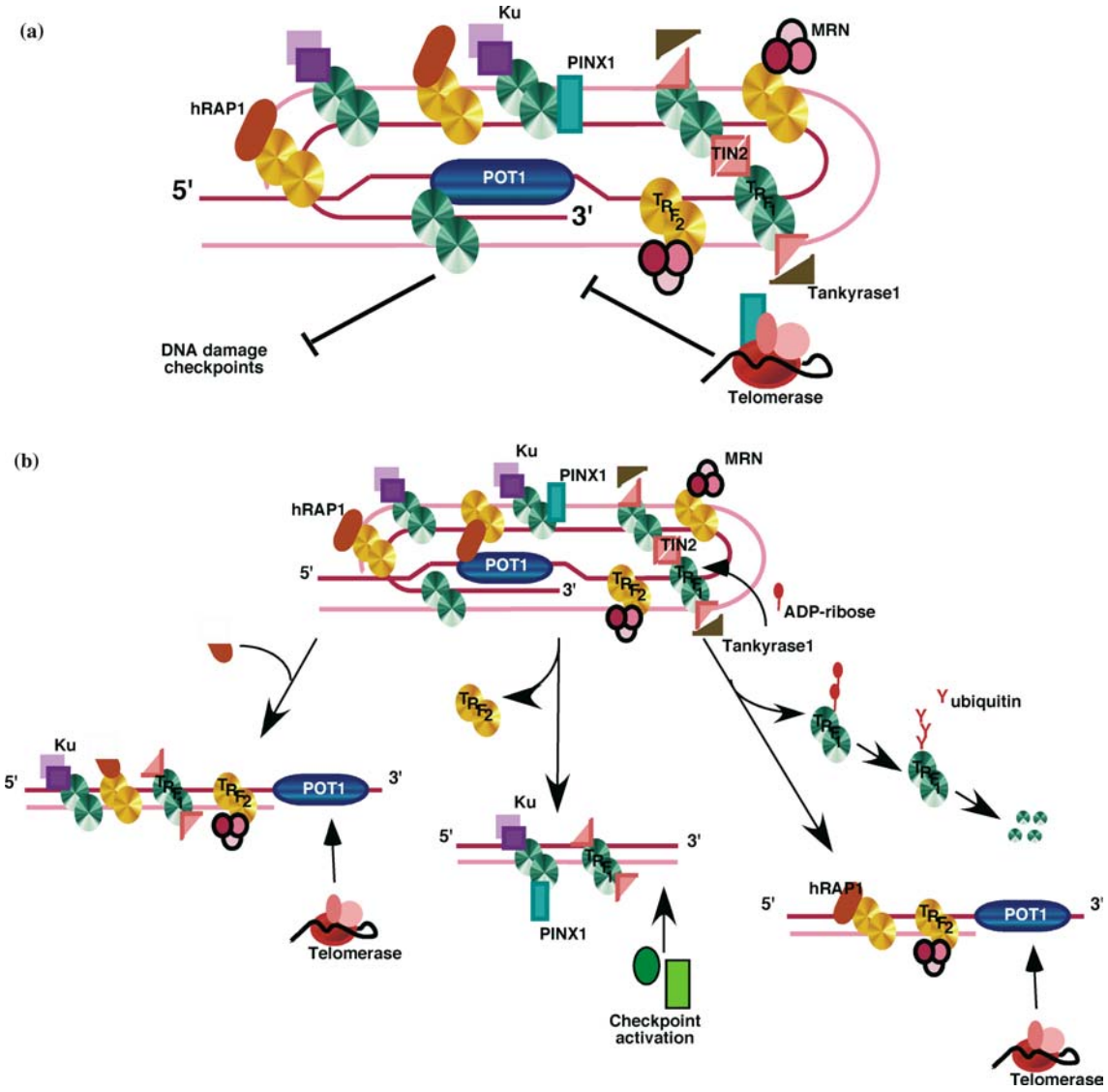

Figure 1. Schematic of the mammalian telomere complex. (A) Telomeres are organized into telomere loops (t-loops) in which the 3′ single-stranded overhang invades the duplex telomeric repeats. This structure protects chromosome ends, making them inaccessible to DNA damage checkpoint machinery and to telomerase. A number of protein factors have also been identified and the association of these factors is also depicted. (B) The function(s) of a number of telomere binding proteins has been elucidated. Over-expression of N-terminal truncations of hRAP1 renders telomeres more accessible to telomerase without perturbing association of other factors. Absence of TRF2 abrogates telomere capping function and the chromosome ends now are accessible to the DNA damage checkpoint machinery. TRF1 acts as a negative regulator of telomere length. The association of TRF1 with telomeric DNA is regulated by two associated factors, TIN2 and Tankyrase 1 (see text for details).

length regulation through interactions with telomerase or by remodeling the telomeric chromatin. Proteins involved in other aspects of DNA metabolism, most notably those involved in repair of damaged DNA, have also been implicated in telomere maintenance.

Telomeric DNA binding Proteins

A number of the protein components of mammalian telomeres have been identified in recent years. Telomere specific factors include the double-stranded telomeric DNA binding proteins

TTAGGG Repeat binding Factor 1 (TRF1) (Chong et al. 1995) and the related protein TRF2 (Broccoli et al. 1997). TRF1 and TRF2 bind to telomeric DNA as homodimers using a myb-type DNA binding domain located in the carboxy terminus of each protein (Broccoli et al. 1997). Although related, TRF1 and TRF2 play distinct roles at the telomere. Initial experiments probing the roles of TRF1 and TRF2 at telomeres took advantage of the binding characteristics of these proteins by utilizing dominant negative versions of each protein. These studies indicated that TRF1 acts as a negative regulator of telomere length in cells that contain telomerase (van Steensel and de Lange 1997), presumably by altering telomeric chromatin and thereby affecting the accessibility of the chromosome end to telomerase. TRF1 may also play a role in higher order telomeric structure as it has been noted that TRF1 can promote pairing between telomeric tracts *in vitro* (Griffith et al. 1998). Initial studies perturbing TRF1 association with telomeric DNA by overexpression of a dominant negative allele did not observe effects on cellular viability (van Steensel and de Lange 1997; Karlseder et al. 1999). However, recently it has been found that deletion of the TRF1 locus is lethal in the mouse (Karlseder et al. 2003; Iwano et al. 2004). Thus, TRF1 plays a critical function at the telomere, possibly by contributing to the overall stability of the telomeric complex since it has been noted that disruption of TRF1 leads to a decrease in the amount of TRF2 associated with telomeres (Iwano et al. 2004).

TRF2 is essential for telomere capping function. Forced removal of TRF2 (Figure 1B) leads to rapid loss of the G-strand overhang and subsequent end-to-end chromosome fusions via the non-homologous end joining pathway, with telomeric DNA being maintained at the fusion point (van Steensel et al. 1998; Smogorzewska et al. 2002). Degradation of the G-strand overhang under these conditions is dependent upon the XPF/XRCC2 nuclease (Zhu et al. 2003). Similar to TRF1, TRF2 can negatively regulate telomere length when overexpressed (Smogorzewska et al. 2000). However, in this case, telomere shortening occurs in all cells regardless of telomerase status. Finally, overexpression of TRF2 can delay the onset of senescence (Karlseder et al. 2002), presumably by shifting the equilibrium of telomeric complex formation to allow shorter DNA tracts to recruit sufficient protein to form a functional cap. Consistent with this model, the telomeres are significantly shorter in this background at senescence (Karlseder et al. 2002).

A third protein that directly binds to telomeric DNA has also been characterized. This is the G-strand overhang binding protein Protection Of Telomeres 1 (POT1) (Baumann and Cech 2001). POT1 has been found to associate with TRF1 and its associated factors (see below) and the ability of POT1 to be targeted to telomeres is dependent upon the TRF1 complex being present at the telomere (Loayza and De Lange 2003). Perhaps not surprising for an end-binding factor, POT1 also regulates telomere length. Expression of a mutant version of POT1 unable to bind to DNA results in telomere elongation (Loayza and De Lange 2003). Contradictorily, over expression of wild type POT1 also results in telomere elongation, with telomere extension being dependent upon telomerase activity (Colgin et al. 2003; Armbruster et al. 2004). These two observations may be reconciled as we gain a better understanding of the multiple interactions between the various components of the telomeric complex.

Telomere-associated factors

A number of interacting proteins, such as tankyrase 1 and 2, TIN2, PINX1 and hRAP1 are also components of mammalian telomeres. Tankyrase 1 was identified from a two-hybrid screen for proteins that interact with TRF1 (Smith et al. 1998). Tankyrase 2 was identified as a closely related homolog of tankyrase 1. Tankyrase 1 and tankyrase 2 are poly(ADP-ribose) polymerases (PARP) that ADP-ribosylate TRF1 and cause its dissociation from telomeres (Cook et al. 2002). Over-expression of tankyrase 1 leads to telomere elongation in telomerase-positive, but not primary telomerase-negative, cells suggesting that the telomere length alterations occur via a mechanism that affects the ability of telomerase to access the chromosome ends (Smith and de Lange 2000)(Figure 1B). Once ADP-ribosylated, TRF1 can no longer associate with telomeric DNA and the unbound TRF1 becomes ubiquitinated and targeted for degradation by the proteosome (Chang et al. 2003). Thus, tankyrase 1 regulates telomere

length by altering the levels of TRF1 associated with the telomere. Recently the PARP activity of tankyrase 1 has been shown to be required for dissolution of sister chromatid cohesion at telomeres (Dynek and Smith 2004), identifying a novel role for a telomeric protein.

TIN2 was also originally identified following a yeast two-hybrid screen for proteins that interact with TRF1 (Kim et al. 1999). Similar to what was observed for TRF1, TIN2 promotes the formation of higher order complexes between telomeric DNA tracts *in vitro* (Kim et al. 2003), possibly by promoting or stabilizing TRF1-TRF1 interactions. Inhibition of TIN2 leads to reduced TRF1 at the telomere and to telomere elongation, via a mechanism that is dependent upon the PARP activity of tankyrase 1 (Ye and de Lange 2004). Furthermore, TIN2 is able to prevent (ADP)-ribosylation of TRF1 by tankyrase 1 *in vitro*, suggesting that regulation of TRF1 association with the telomere is balanced between the activities of TIN2 and tankyrase 1 (Ye and de Lange 2004). The data are consistent with a model in which TRF1 negatively regulates access of telomerase to chromosome termini by modulating telomeric chromatin, while tankyrase 1 and TIN2 affect telomere length by controlling the levels of TRF1 associated with the telomere (Figure 1B).

PINX1, is the final TRF1 interacting factor that has been identified through a two-hybrid screen to date (Zhou and Lu 2001). PINX1 also interacts with and inhibits telomerase. Over-expression of PINX1 leads to telomere shortening and depletion of PINX1 leads to telomere elongation (Zhou and Lu 2001). In this instance, regulation of telomere length by PINX1 occurs by affecting telomerase activity directly, in contrast to the proteins discussed above which regulate telomere length without affecting telomerase activity.

The human homolog of the S. cerevisiae telomere binding protein RAP1 was identified through a two-hybrid screen using TRF2 as the bait (Li et al. 2000). In contrast to its yeast homolog, hRAP1 does not bind directly to telomeric DNA even though it has a myb type DNA binding domain similar to those in TRF1 and TRF2. Instead hRAP1 requires TRF2 to be targeted to the telomere, through an interaction with the C terminus of hRAP1 (Li and de Lange 2003). The NMR solution structure of the hRAP1 myb domain indicates that it lacks the positive surface charge found on canonical myb domains, perhaps explaining the inability of this protein to bind to negatively charged DNA (Hanaoka et al. 2001). Over-expression of hRAP1 leads to a modest increase in telomere length, which can be further increased by over-expression of constructs deleted for either the N-terminal BRCT domain (Figure 1B) or the centrally located myb domain (Li and de Lange 2003). Expression of a construct deleted for both the BRCT and myb domains did not act in an additive manner to increase telomere length relative to each single deletion, suggesting that the disrupted protein–protein interactions are not independent. Because all these deletions constructs are targeted to the telomere through C-terminal interactions with TRF2, the effects on length are likely mediated through as yet unidentified protein–protein interactions. Intriguingly, deletion of the BRCT domain of hRAP1 also decreases the heterogeneity of telomere lengths suggesting that hRAP1 and its interacting partners act to regulate telomere length distribution as well as affecting access of telomerase.

DNA damage response and repair factors

Intriguingly, factors involved in DNA repair are also present at telomeres. These include the Ku70/86 heterodimer (Hsu et al. 1999) and the MRE11/RAD50/NBS1 (MRN) complex (Zhu et al. 2000). Recent evidence suggests that RAD50, MRE11 and Ku86 may be recruited to hRAP1, independently of the interaction of hRAP1 with TRF2 (O'Connor et al. 2004). Mouse models have indicated that some of these proteins play a role in telomere maintenance. For example, Ku86 knockout mice have an increased incidence of end-to-end fusions indicative of loss of telomere capping function (Hsu et al. 2000). Other factors involved in cellular responses to DNA damage such as DNA-PKcs, XRCC4, RAD54 and ATM have been implicated in telomere metabolism based on phenotypes, telomere length changes and/or increased end-to-end fusions observed in their respective knockout mice (Blasco 2003). The role of these factors in telomere metabolism has been recently reviewed (Reaper et al. 2004).

Summary

We have come a long way towards understanding how the chromosome end-stability first observed in the 1930s is achieved. However, it is unlikely that all the proteins associated with this essential chromosomal element have been identified. In addition, we still have much to understand about how the dynamics that occur at telomeres are regulated and about how the interactions that occur between telomeric components, both between the DNA and the protein and between the protein elements themselves, affect telomere function.

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