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Shelterin-Mediated Telomere Protection

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Abstract

For more than a decade, it has been known that mammalian cells use shelterin to protect chromosome ends. Much progress has been made on the mechanism by which shelterin prevents telomeres from inadvertently activating DNA damage signaling and double-strand break (DSB) repair pathways. Shelterin averts activation of three DNA damage response enzymes [the ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases and poly(ADP-ribose) polymerase 1 (PARP1)], blocks three DSB repair pathways [classical nonhomologous end joining (c-NHEJ), alternative (alt)-NHEJ, and homology-directed repair (HDR)], and prevents hyper-resection at telomeres. For several of these functions, mechanistic insights have emerged. In addition, much has been learned about how shelterin maintains the telomeric 3′ overhang, forms and protects the t-loop structure, and promotes replication through telomeres. These studies revealed that shelterin is compartmentalized, with individual subunits dedicated to distinct aspects of the end-protection problem. This review focuses on the current knowledge of shelterin-mediated telomere protection, highlights differences between human and mouse shelterin, and discusses some of the questions that remain.

INTRODUCTION

DDR: DNA damage response

Initial work on the molecular aspects of telomere function emphasized the end-replication problem, which originates from the inability of the canonical DNA replication pathway to complete the duplication of the ends of linear DNA. Like most eukaryotes, mammals solve this problem using telomerase, which extends the 3′ ends of chromosomes with telomeric DNA (16).

Compared to the straightforward end-replication problem, the telomere end-protection problem is complicated because it arises from the myriad of cellular pathways that can sense and act on DNA ends. Early work by McClintock (94, 95) pointed to special features of telomeres that allowed them to escape the fusion reaction observed with broken chromosomes—the first hint that telomeres could avert nonhomologous end joining (NHEJ) and perhaps other double-strand break (DSB) repair pathways. In addition to inappropriate DNA repair reactions, telomeres need to prevent the activation of pathways that sense DNA damage. The early literature on eukaryotic DNA damage signaling pathways and checkpoints (reviewed in 15) did not address the obvious dilemma that cells need to distinguish DNA breaks from chromosome ends. Recent data indicate that telomeres are threatened by at least seven distinct DNA damage response (DDR) pathways (**Figure 1**).

The system that handles the many tasks related to the end-protection problem employs multiple mechanisms. Different threats to telomere integrity are dealt with using vastly different molecular strategies, each primarily targeting the initiation step of the DNA signaling or repair reaction. Remarkably, the various tricks to block the DDR are all performed by a single telomeric protein complex, shelterin (**Figure 1**). For some pathways, shelterin uses its own biochemical features to block the threat, and for others, it has co-opted proteins involved in genome maintenance. Although work in the past decade has brought many insights, we are far from understanding how this fascinating complex multitasks at telomeres.

This review focuses on how shelterin solves the end-protection problem. The role of shelterin in recruiting and regulating telomerase and the nontelomeric functions of some of the shelterin subunits are discussed elsewhere (61, 91).

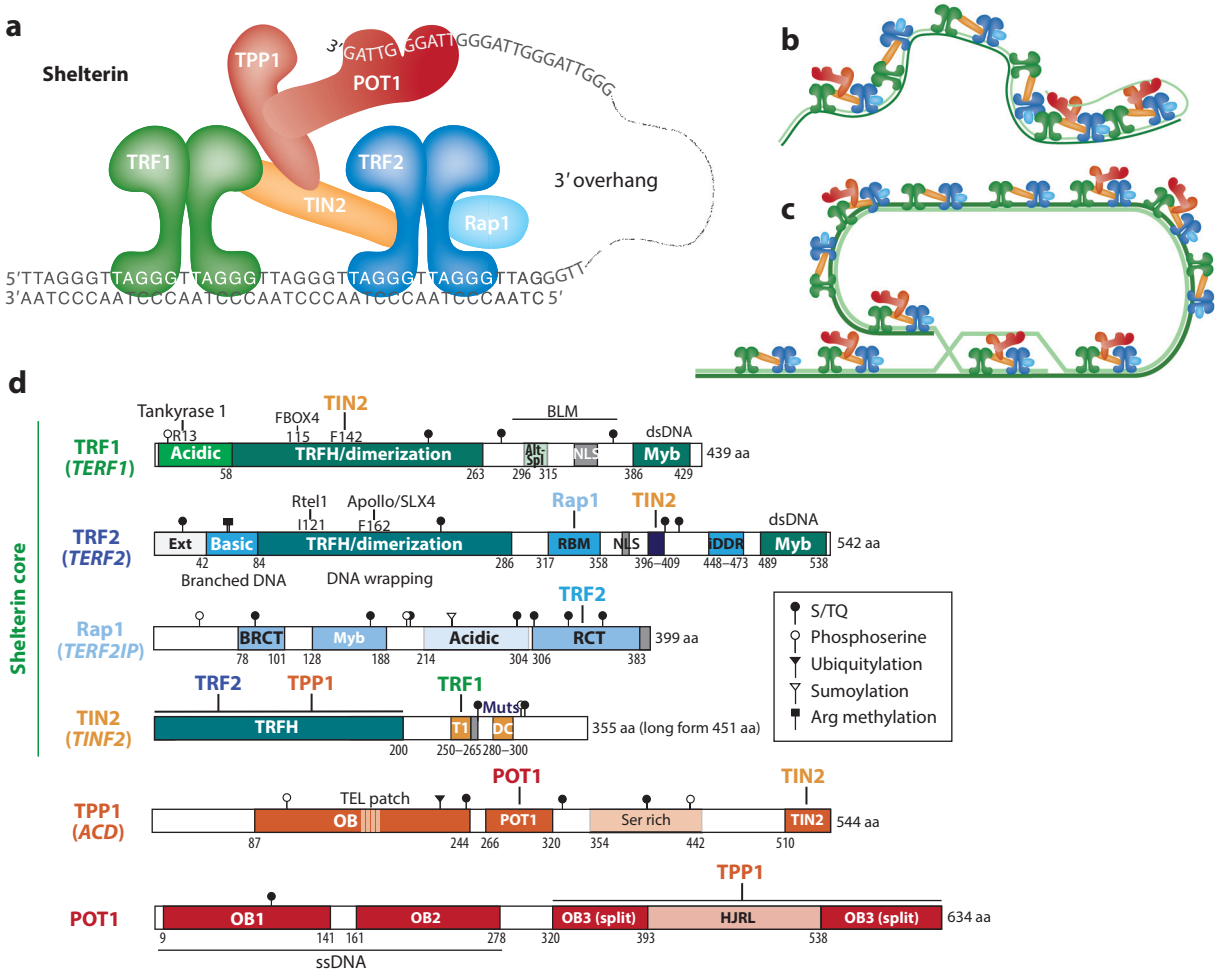
SHELTERIN STRUCTURE AND DNA BINDING FEATURES

Shelterin has evolved to bind specifically to the sequence and structure of mammalian telomeres (**Figure 1*a***). Mammalian telomeres contain many kilobase pairs (kb) of tandem double-stranded (ds) TTAGGG repeats terminating in a 50–400 nucleotide 3′ protrusion of single-stranded (ss) repeats of the G-rich strand. Shelterin interacts with both ds and ss telomeric DNA and is sufficiently abundant to bind all telomeric DNA (**Figure 1*a*–*c***) (128).

Human shelterin consists of six distinct proteins, TRF1, TRF2, Rap1, TIN2, TPP1, and a single version of POT1 (**Figure 1*a*,*d***) (32). In contrast, rodent shelterin contains two closely related POT1 proteins, POT1a and POT1b, which arose through gene duplication (62, 142). The main interaction interfaces of the shelterin subunits have been established based on co-immunoprecipitation experiments, yeast two-hybrid analysis, and structural studies (**Figure 1*d***). This work revealed that TRF1 and TRF2 bind to TIN2 using distinct interaction surfaces. TIN2 also binds to TPP1, which in turn binds to POT1 (or POT1a and POT1b in the mouse). Rap1 binds TRF2, completing the six-subunit complex. The binding interfaces between shelterin subunits are varied in structure and probably do not involve posttranslational modifications, despite the numerous reported modifications of the subunits (**Figure 1*d***). The formation of shelterin also does not require interactions with DNA (44).

The high-affinity DNA binding domains within shelterin are well established (**Figure 1*d***). The Myb/SANT domains of TRF1 and TRF2 bind to duplex telomeric DNA with nanomolar affinity

(44). High-affinity binding by TRF1 and TRF2 is dependent on the formation of homodimers, mediated by the TRFH domain (11, 12). Together, the TRF1 and TRF2 homodimers contribute four ds 5′-TAGGGTT-3′ recognition modules to shelterin (**Figure 1*a***) (12, 99). Shelterin contains an additional Myb-like domain centrally located in Rap1. However, the surface charge of this Myb fold is unsuitable for binding to DNA (60), explaining its low affinity for DNA (1) and suggesting



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DDR pathway	Shelterin subunit(s)	Mechanism	General repressor
ATM kinase	TRF2 (TIN2)	t-loop	None
ATR kinase	POT1a (POT1b)	RPA exclusion	None
PARP1	TRF2, TIN2	Branched-DNA binding, ?	Ku70/80
c-NHEJ	TRF2	t-loop (iDDR, Rap1)	CYREN (S/G2)
alt-NHEJ	TRF2 (TIN2, POT1a/b)	t-loop, PARP1 repression, ?	Ku70/80
HDR	POT1a or POT1b + Rap1	?, ?	Ku70/80
Hyper-resection	POT1a/b, TRF2	Repression of ATM/ATR	53BP1/Rif1/Rev7

(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Shelterin structure and function. (*a*) Depiction of the six-subunit human shelterin complex associated with the double-stranded and single-stranded telomeric DNA. Note that mouse shelterin contains two POT1 proteins, POT1a and POT1b. TRF1 and TRF2 are shown as dimers, and the other shelterin subunits are each depicted once, although it is not known how many copies of each are present in the whole shelterin complex. (*b*) Depiction of shelterin complexes loaded onto telomeres in an open linear configuration and (*c*) in the t-loop configuration. Because TPP1 and POT1 are much less abundant than the other shelterin subunits, some complexes are depicted without TPP1 and POT1. (*d*) Domain structure of the six human shelterin subunits and their protein and DNA interactions. Official gene names are given in parentheses. TRF2 is expressed as two functionally equivalent isoforms (L. Timashev & T. de Lange, unpublished data) either containing or lacking the N-terminal extension (*gray box*). TIN2 has also been reported in a longer form. Splice variants of POT1 are not shown. The Myb/SANT domains of TRF1 and TRF2 are indicated with Myb. Reported and potential modification sites are indicated according to the key. (*e*) Summary of DDR pathways that are repressed by shelterin, the subunits dedicated to each pathway, and their proposed mechanism of action. For several pathways, general repressors are listed. In the repression of c-NHEJ, it is speculated that Rap1 and the iDDR domain in TRF2 contribute to preventing c-NHEJ when telomeres are in a linear state. Abbreviations: alt-NHEJ, alternative nonhomologous end joining; Alt-Spl, alternatively spliced exon; ATM, ataxia-telangiectasia-mutated; ATR, ataxia telangiectasia and Rad3-related; BRCT, BRCA1 C-terminal phosphopeptide interaction motif; c-NHEJ, classical nonhomologous end joining; CYREN, cell cycle regulator of nonhomologous end joining; DC, patch of mutations associated with dyskeratosis congenita; DDR, DNA damage response; dsDNA, double-stranded DNA; HDR, homology-directed repair; HJRL, Holliday junction resolvase-like domain; iDDR, inhibitor of the DNA damage response; NLS, nuclear locatization signal; OB, oligonucleotide/oligosaccharide binding fold; PARP1, poly(ADP-ribose) polymerase 1; RBM, Rap1 binding domain; RCT, Rap1 C-terminal domain; ssDNA, single-stranded DNA; T1, TRF1 binding; TEL, telomerase recruitment; TRFH, telomeric repeat binding factor homology domain; ?, unknown.

that it might interact with a protein rather than DNA. The POT1 proteins bind to ss telomeric DNA with two N-terminal oligonucleotide/oligosaccharide binding (OB) folds (9, 79). In vitro POT1 binds with subnanomolar affinity to ss 5′-TTAGGGTTAG-3′ sites either at a 3′ end or (with slightly lower affinity) when at an internal position (79, 84). TPP1 also contains an OB fold, but unlike its ciliate ortholog (TEBPβ), TPP1 does not interact with DNA (138).

The DNA binding proteins in shelterin do not show cooperativity when analyzed individually in vitro (11, 12, 44, 84). Similarly, the whole shelterin complex appears to lack strong cooperative interactions in vitro (44). Shelterin complexes may therefore bind as independent units to the telomeric DNA (**Figure 1*b*,*c***). In vitro experiments suggest that shelterin can find its binding sites through a diffusive 3-D search as well as by a 2-D search along the DNA (44).

Shelterin has two additional DNA interaction features, both involving TRF2. TRF2 has a short N-terminal region that is predominantly basic in nature (referred to as the Basic domain) (**Figure 1*d***). This domain can bind in a sequence-independent manner to branched DNAs (e.g., four- and three-way junctions) in vitro, including the structure at the base of the t-loop (see the section titled T-Loop Protection) (47, 105, 115). The affinity of the Basic domain for structured DNA is low (submicromolar), and its interaction with branched structures is likely to require anchoring to the dsDNA via the TRF2 Myb/SANT domains. The second DNA interaction involves the TRF2 TRFH domain, which has a number of exposed lysine residues that bind to nonspecific dsDNA with low affinity (10). Using these interactions TRF2 can wrap approximately 90 bp of DNA around its TRFH domain, which promotes the formation of t-loops (see the section titled T-Loop Formation) (10).

Rap1 has a subtle influence on the interaction of the TRF2 Basic domain with DNA. Rap1 diminishes the interaction of the Basic domain with branched DNA (56, 98) and also prevents it from engaging in sequence-independent interactions with DNA, thereby increasing the specificity of TRF2 for TTAGGG repeats (69).

Little is known about the in vivo stoichiometry of the shelterin complex. In human and mouse cells, TRF1, TRF2, TIN2, and Rap1 are about 10 times more abundant than TPP1 and POT1 (128). Therefore, the core of shelterin is likely to be a complex of the four most abundant subunits

(**Figure 1*b–d***) with a fraction of the complexes also containing TPP1 and POT1. Although the TIN2 bridge stabilizes TRF1 and TRF2 on telomeres (83, 148), it is not excluded that separate TRF1- or TRF2-based subcomplexes exist at telomeres. For instance, when TRF2 is deleted, a subcomplex composed of TRF1, TIN2, TPP1, and POT1 continues to be functional (33). The abundance of the core of shelterin is sufficient to bind all ds TTAGGG repeats, and there is a tenfold excess of TPP1/POT1 over its ss TTAGGG binding sites, suggesting that most of the telomeric DNA is associated with shelterin proteins.

Despite the (over)abundance of shelterin, the nucleosomal structure of telomeres appears unaffected by shelterin (116, 144). However, in vitro TRF1 and TRF2 binding to nucleosomal chromatin has discernable effects, and the interplay between shelterin and nucleosomes in vivo deserves further analysis (6, 7, 52–54).

Although the structure of the whole shelterin complex is not yet known, X-ray crystallography, small-angle X-ray scattering, and NMR studies have revealed the structure of many of the domains present in shelterin. The structures of the Myb/SANT domains of TRF1 and TRF2 and their TRFH domains have been solved (45, 59), as have the OB folds of POT1 and TPP1 (79, 138). Recently, the structure of the TPP1–POT1 interface was solved by X-ray crystallography (22, 110). This work revealed that the C-terminal half of POT1 contains a third OB fold as well as a Holliday junction resolvase-like fold and that TPP1 binds to multiple sites in these domains (**Figure 1*d***). The structure of a TIN2 peptide binding to the TRFH domain of TRF1 has also been solved as has the C-terminal TRF2-interaction domain (the RCT) of Rap1 (24, 25). Finally, an N-terminal domain of TIN2 and its interactions with peptides from TRF2 and TPP1 have been crystallized (66). Remarkably, this part of TIN2 revealed a distantly related TRFH domain that does not mediate dimerization but instead binds to TRF2 and TPP1 (66).

The finding of the TRFH domain in TIN2 and a similar TRFH domain in a telomeric protein (Poz1) in fission yeast (147) argues that the TRFH domain has played a central role in the evolution of telomeric proteins. Fission yeast telomeres contain a shelterin-like complex that includes a TRF1/2 homolog (Taz1) (29) with a Myb/SANT domain and the OB fold–containing Pot1, the ortholog of mammalian POT1 (9). Thus, the Myb/SANT domain, the TRFH domain, and OB folds are the common motifs in fission yeast and mammalian shelterin. These folds were likely to have been present in an ancestral shelterin complex.

Budding yeast has lost its TRFH domain protein from the telomeric complex and lacks the TPP1/POT1 homodimer. Instead, budding yeast telomeres contain Rap1 and an alternative OB fold protein (Cdc13), which is part of a trimeric replication protein A (RPA)-like CST (Ctc1, Stn1, Ten1) complex (55, 106). Unlike mammalian Rap1, budding yeast Rap1 binds telomeric DNA directly using two tandem Myb-like domains that are distinct from the Myb/SANT domains in TRF1, TRF2, and Taz1 (75). These changes in the telomeric proteins are consistent with the view that the ancestral budding yeast underwent a drastic alteration in the telomerase RNA component (81, 88), resulting in a change in the telomeric sequence, loss of the canonical shelterin-like complex, and emergence of a new set of telomeric proteins.

Replication protein A (RPA): a single-stranded DNA sensor in the ataxia telangiectasia and Rad3-related pathway

Ctc1/Stn1/Ten1 (CST): a replication protein A-like complex and accessory factor of Polα/primase

SHELTERIN ACCESSORY FACTORS

Shelterin interacts with several proteins and protein complexes that contribute to its function (**Supplemental Table 1**, available online) (**Figure 1*d***) (reviewed in 38). Some of the interactions control the abundance of shelterin subunits but may not contribute to shelterin function per se (e.g., RNF6, Fbx4). However, many of the shelterin accessory factors have well-defined functions in DNA repair pathways, and it appears that shelterin has co-opted these functions to facilitate enzymatic processing of the telomeric DNA (e.g., nucleolytic degradation, helicase activity, DNA replication).

Supplemental Material >

Mre11/Rad50/Nbs1 (MRN): a double-strand break sensor in the ataxia-telangiectasia-mutated pathway

ATM: ataxia-telangiectasia-mutated

The first shelterin accessory factor to be identified was tankyrase 1, which binds to TRF1 and regulates telomere cohesion and telomere length homeostasis (43, 121, 122). Another example of a shelterin accessory factor is the Apollo/SMN1B nuclease, which helps to generate the correct overhang at telomeres (78, 80, 133, 145). Apollo interacts with TRF2 at a patch within the TRFH domain around F162 (F120 in the short form of TRF2) (**Figure 1*d***). This patch in TRF2 can bind numerous proteins that have an accessible H/YxLxP motif (25). Apollo contains an H/YxLxP motif, as does SLX4, a DNA repair protein that binds human TRF2 (25, 113). A similar site in the TRFH domain of TRF1 (around F142) interacts an FxLxP motif, which is how TRF1 binds to TIN2 (25). Additional shelterin accessory factors are discussed in the sections below.

It is noteworthy that the interactions of shelterin with its accessory proteins are not highly conserved between mouse and human. For instance, tankyrase 1 interacts with human TRF1, but mouse TRF1 has no tankyrase binding site, and no tankyrase is found at mouse telomeres (42). Human SLX4 carries an H/YxLxP site to allow interaction with TRF2, but this site is missing in mouse SLX4. Furthermore, the interaction of the shelterin accessory factor CST with TPP1/POT1 appears distinct in mouse and human shelterin (see the section titled Formation of the 3′ Overhang).

FORMATION OF THE 3′ OVERHANG

Following DNA replication, telomeres have to regenerate a 3′ overhang of the correct length. The formation of the 3′ overhang is critical for t-loop formation (see the section titled T-Loop Formation) and thus for telomere protection. Furthermore, the postreplicative processing of telomere ends determines the rate at which telomeres shorten during cell proliferation (64).

Formation of the 3′ overhang at mouse telomeres involves an intricate set of steps that are controlled by TRF2 and POT1b (**Figure 2**) (143). It involves nucleolytic attack by Apollo and Exonuclease 1 (Exo1) and fill-in DNA synthesis mediated by CST and Polα/primase. The processing of the two sister telomere ends differs slightly, likely due to their distinct structures after DNA replication.

TRF2-bound Apollo is needed to initiate the nuclease attack on telomeres synthesized by leading-strand DNA synthesis (leading-end telomeres). Presumably, leading-end telomeres are initially blunt. As is the case in DSB repair, such ends require an initial cleavage step before further nuclease attack can take place (96, 103, 150). In DSB repair, this step is performed by a complex containing Mre11/Rad50/Nbs1 (MRN) and C-terminal binding protein 1 (CtBP1) interacting protein (CtIP), which creates the 5′ recessed ends that are a substrate for further nucleolytic attack by Exo1 and DNA2. Similarly, at leading-end telomeres, Apollo appears to prepare the substrate for Exo1 (**Figure 2*a***). When Apollo is deleted or prevented from binding to TRF2, leading-end telomeres become dysfunctional, activating the ataxia-telangiectasia-mutated (ATM) kinase and fusing to other leading-end telomeres (78, 145). At lagging-end telomeres, Apollo is apparently dispensable, perhaps because DNA replication leaves 5′ recessed ends that can immediately be processed by Exo1. POT1b appears to limit the action of Apollo at both telomere ends, but the mechanism of this control is not known (**Figure 2*a***) (143).

In the second step of end processing, both newly replicated telomeres are resected by Exo1 (**Figure 2*b***). This resection appears to be unregulated and can be extensive, leading to a temporary increase in the telomeric overhang signal in S/G2. The setting of the 3′ overhang length is primarily determined in the third step, which involves CST-mediated fill-in by Polα/primase (**Figure 2*c*,*d***). In mouse shelterin, CST is recruited by POT1b, leading to more extensive telomeric overhangs and more rapid telomere shortening in POT1b-deficient mouse cells (64, 143).

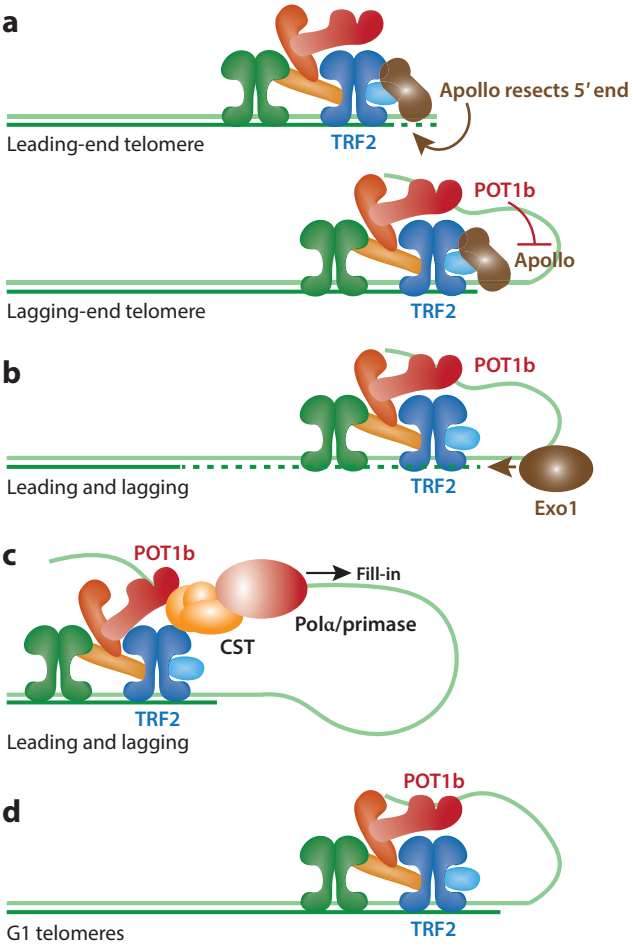

Figure 2

Shelterin-dependent generation of the 3′ overhang. (*a*) After replication of the telomeric DNA, the leading-end telomere is resected by the TRF2-bound Apollo nuclease to generate a short 3′ overhang. At the lagging-end telomere, DNA replication is assumed to lead to a short 3′ overhang. POT1b inhibits extensive resection by Apollo. (*b*) Leading- and lagging-end telomeres undergo extensive resection by Exonuclease 1 (Exo1), transiently generating long 3′ overhangs in the S/G2 phase of the cell cycle. (*c*) POT1b interacts directly with the Ctc1, Stn1, Ten1 (CST) complex to allow Polα/primase fill-in of the overhang. (*d*) The product (in the G1 phase of the cell cycle) of shelterin-dependent 3′ overhang generation.

Apollo, CST, and Polα/primase are likely to have similar processing roles at human and mouse telomeres (30, 37, 48, 67, 80, 133, 139), but the mechanism by which human shelterin regulates this processing is not fully understood. Although the interaction of Apollo with TRF2 is conserved, the human CST complex binds both POT1 and TPP1 rather than POT1b alone as in the mouse (23, 104, 137). However, the precise role of human TPP1 and POT1 in controlling overhang processing is still unclear. Even though TPP1 is involved in recruitment of CST, recent data indicate that an alternatively spliced form of POT1 (V5) is important for limiting the telomeric overhang, and work with a disease-associated mutation of POT1 indicates that POT1 promotes CST-mediated fill-in (74, 127).

Bloom's syndrome mutated (BLM): a RecQ-type helicase

In addition to questions about the mechanism by which human telomeric overhangs are generated, many other aspects of telomere end processing still need clarification. What limits the CST fill-in reaction so that a 3′ overhang of the desired length is generated, and is this process the same at lagging- and leading-end telomeres? What determines the end of the C-rich strand of human telomeres, which nearly always have the sequence ATC-5′ at their end (120)? Is the RNA primer used for fill-in synthesis removed or retained? What is the length of the noncanonical Okazaki fragment synthesized by Polα, and how is it ligated to the rest of the C-rich strand?

T-LOOP FORMATION

Telomeres in mammals and many other organisms form t-loops (31). T-loops are large lariat structures that are generated through the invasion of the 3′ overhang into the ds telomeric DNA (**Figure 3*a***) (58). Although the exact nature of the structure at the base of the t-loop is not known, evidence suggests that the 3′ overhang pairs with the C-rich strand and displaces the G-rich strand into a D loop (for discussion, see 39). T-loops can have large loops and short tails or vice versa, indicating that the position where the 3′ overhang invades the dsDNA is not specified.

TRF2 is both necessary and sufficient for t-loop configuration (41; L. Timashev & T. de Lange, unpublished data). In vitro TRF2 has the ability to remodel telomeric substrates into structures resembling t-loops (58, 123), suggesting that TRF2 has some inherent ability to change the structure of telomeric DNA. A feature of TRF2 that can explain this attribute is the ability of the TRFH domain to wrap DNA around itself (10). This is a low-affinity interaction that presumably only takes place once the Myb/SANT domains of TRF2 are bound to dsTTAGGG repeats. The wrapping of the telomeric DNA is thought to induce local unwinding and invasion by the 3′ overhang (**Figure 3*a***) (10). Indeed, a mutant of TRF2 (called Top-less) that lacks this DNA wrapping activity in vitro has a diminished ability to form t-loops in vivo (10).

Although the branched-DNA binding domain of TRF2 (the Basic domain) might be expected to help stabilize t-loops, its removal from TRF2 does not impede t-loop formation in vivo (115). Similarly, the POT1 proteins, which could engage the D loop with their OB folds and could perhaps bind the three-way junction with their Holliday junction resolvase-like domain, are not required for t-loop formation (41).

Many questions regarding the t-loop configuration remain. The exact structure at the base of the t-loop has not been established, and the minimal length of telomeres required for t-loop formation is not known. Although t-loops can be observed in all stages of the cell cycle (L. Timashev & T. de Lange, unpublished data), it is not known what percentage of telomeres is in this structure. A final conundrum is that the 3′ end of a telomere in the t-loop configuration is potentially a substrate for telomere extension by canonical DNA polymerases. Is such t-loop extension blocked, and if so, how?

T-LOOP PROTECTION

Whereas t-loops represent a remarkable architectural solution to the end-protection problem, they also come with challenges. If branch migration occurs at the base of the t-loop, a double Holliday junction (dHJ) can be formed (**Figure 3*b***). Branch migration of the dHJs could be mediated by the Bloom's syndrome mutated (BLM) helicase and restore the original t-loop structure (13). However, cleavage of the dHJ by HJ resolvases, such as the Mus81, SLX4, SLX1, and Emi1 complex or Gen1 (reviewed in 146), can result in removal of the loop, leading to large telomere deletions (111) (**Figure 3*b***). Such deleterious t-loop cleavage is prevented by the branched-DNA binding (Basic) domain of TRF2 (111, 140).

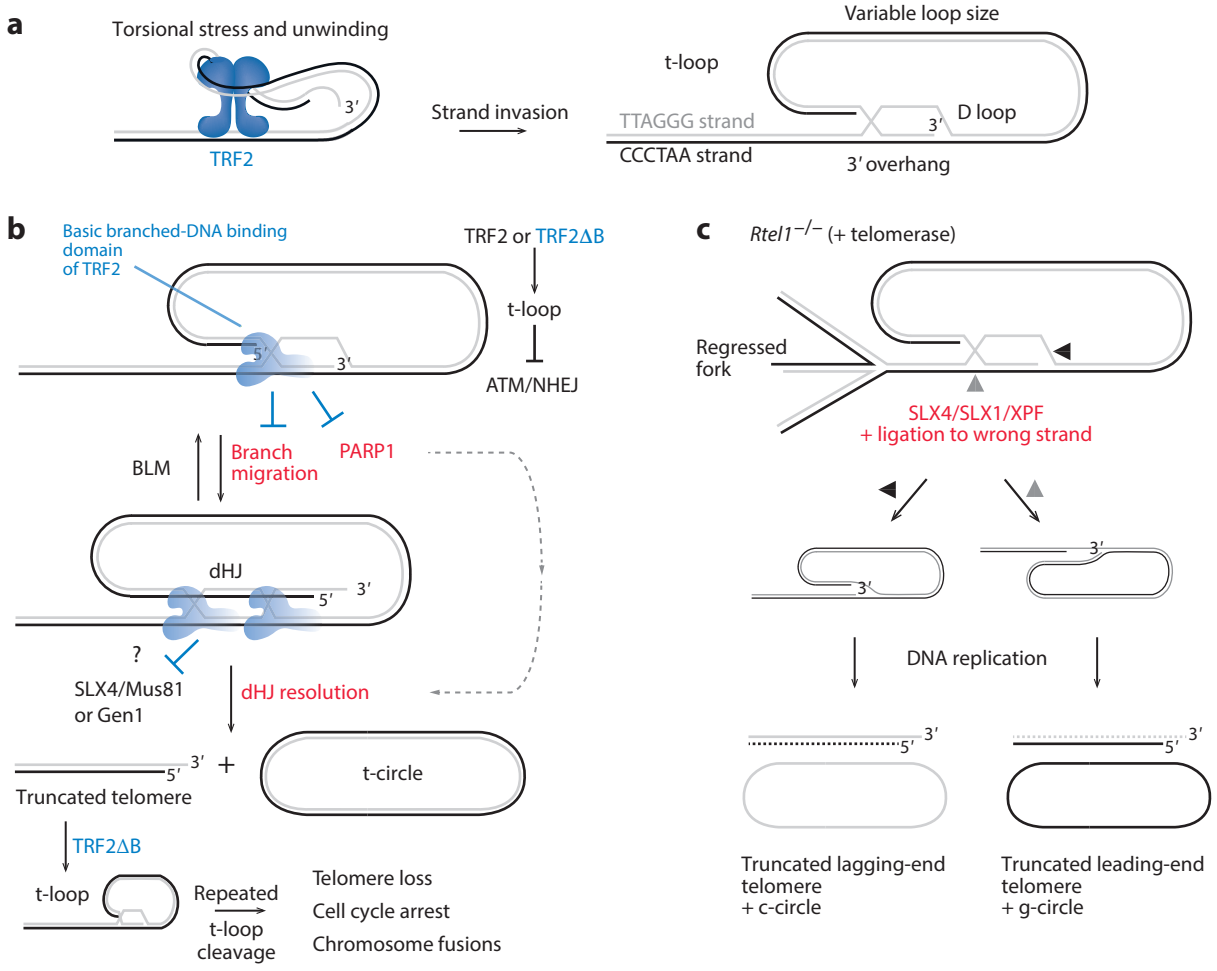

Figure 3

Formation and protection of t-loops. (*a*) Model for t-loop formation by TRF2. TRF2 is depicted with 90 bp of ds telomeric DNA wrapped around the telomeric repeat binding factor homology domains. Wrapping and the associated topological stress are thought to induce invasion of the 3′ overhang in underwound ds telomeric DNA, thereby promoting t-loop formation. The presumed structure of the t-loop is shown on the right. (*b*) Repression of t-loop cleavage by the Basic domain of TRF2. The Basic domain is depicted as a branched-DNA binding domain at the base of the t-loop. Its engagement is proposed to mask the 5′ end of the telomere from PARP1 and to block branch migration, thereby preventing formation of a dHJ. PARP1 activation promotes t-loop cleavage by nucleases that resolve HJs. The BLM helicase can dissolve dHJs formed in the absence of the Basic domain and thus prevent t-loop cleavage. If a dHJ is formed in the presence of the Basic domain, its binding to the HJs can block HJ resolvases. T-loop cleavage in absence of the TRF2 Basic domain results in stochastic truncation of telomeres (leading- or lagging-end telomeres) and formation of circular telomeric DNA. Because the Basic domain is not required for t-loop formation, repression of the ATM kinase, or preventing NHEJ, the truncated telomeres remain protected and can undergo multiple rounds of t-loop cleavage. Ultimately, critically shortened telomeres form that lead to fusions and cell cycle arrest. (*c*) A second type of t-loop cleavage is repressed by the Rtel1 helicase. Rtel1 deficiency leads to replication fork arrest at the t-loop base. The arrested fork can regress and be stabilized in the chicken foot structure shown when telomerase is present. Two nucleases can then attack the t-loop leading to telomere truncations and circular telomeric DNA. Note that the nucleases involved in panels *b* and *c* are distinct, suggesting that HJs are not formed in the scenario in panel *c*. As depicted in panel *b*, the Basic domain of TRF2 prevents branch migration in the absence of Rtel1. Abbreviations: ATM, ataxia-telangiectasia-mutated; BLM, Bloom's syndrome mutated; c-circle, circular form of the C-rich telomeric DNA; dHJ, double Holliday junction; ds, double-stranded; g-circle, circular form of the G-rich telomeric DNA; NHEJ, nonhomologous end joining; PARP1, poly-ADP ribose polymerase 1; Rtel1, regulator of telomere elongation helicase 1; t-circle, circular telomeric DNA.

PARP1: poly(ADP-ribose) polymerase 1

Rtel1: regulator of telomere elongation helicase 1

T-loop cleavage is promoted by poly(ADP-ribose) polymerase (PARP1) activation at telomeres, possibly because PARP1 promotes the recruitment of HJ resolvases (107, 115) (**Figure 3*b***). The base of the t-loop contains a PARP1 activation site in the form of a 5′ ds–ssDNA transition. The branched-DNA binding domain of TRF2 prevents PARP1 from recognizing this structure. However, repression of PARP1 is not the only mechanism by which TRF2 prevents t-loop cleavage; if PARP1 is activated at telomeres containing TRF2, no t-loop cleavage takes place (115).

In vivo and in vitro data suggest that TRF2 blocks branch migration at the base of the t-loop, thereby preventing dHJ formation (98, 115). When the Basic domain is absent, the BLM helicase, which can dissolve dHJs, mitigates t-loop cleavage, presumably by branch migrating the dHJ back to the t-loop structure. The Basic domain of TRF2 can also block HJ resolvases and the WRN helicase from acting on HJs in vitro (100, 105), possibly acting as a fail-safe in case a dHJ is formed. The Basic domain can be functionally replaced by branched-DNA binding domains from bacterial proteins (RuvA, RuvC) (115), indicating that the branched-DNA binding feature of this domain is indeed relevant to telomere protection. The Basic domain also resembles the viral LANA protein, which is known to bind the nucleosome acidic patch (77). A LANA-type interaction of the Basic domain with nucleosomes could provide an additional mechanism to block branch migration and dHJ formation (77).

Although Rap1 can diminish the ability of the Basic domain to interact with branched DNA in vitro (56, 98), in vivo studies have yielded conflicting results with regard to the role of Rap1 in t-loop cleavage (107, 115). Regardless, it is clear that TRF2 has evolved both to generate t-loops and to protect this structure.

A potentially different type of t-loop cleavage can take place during replication where the t-loop has been proposed to impede replication fork progression (**Figure 3*c***). The replicative helicase (CMG) is thought to be loaded on the leading-strand template (50). If this is correct, the CMG and the replisome should not have a problem progressing through the base of the t-loop. Nonetheless, it appears that active unwinding of the t-loop by the TRF2-bound regulator of telomere elongation helicase 1 (Rtel1) is needed for replication to proceed (90, 112, 135). When Rtel1 is absent from telomeres, formation of circular telomeric DNA is observed, as well as loss of either the leading- or lagging-end telomere. The structure that is cleaved under these conditions may be distinct from the dHJ that is processed when the Basic domain of TRF2 is absent. Whereas SLX4, Mus81, and Gen1 contribute to t-loop cleavage in the absence of the TRF2 Basic domain, t-loop cleavage in response to Rtel1 absence is dependent upon SLX4, SLX1, and XPF but not Mus81 (135). This suggests processing of a three-way junction, e.g., the three-way junction at the base of the t-loop, when Rtel1 is absent. Under these conditions, TRF2 would still be present to prevent branch migration. The outcome would be formation of a telomeric circle and, depending on which strands are cleaved and ligated, a truncation of the leading- or lagging-strand telomere (**Figure 3*c***). Remarkably, t-loop cleavage in Rtel1-deficient cells is completely dependent on the presence of telomerase, which appears to affect the regressed fork in such a way that cleavage is promoted (90).

REPRESSION OF ATAXIA-TELANGIECTASIA-MUTATED (ATM) SIGNALING

The ATM kinase is a potent threat to telomeres (**Figure 4**). If ATM is activated at telomeres, it induces a DDR that enforces cell cycle arrest and possibly senescence or apoptosis. The ATM kinase is activated upon the interaction of its DSB sensor, the MRN complex, with a DNA end (124). The exact structure that is recognized by MRN is unknown, but work with telomeres has

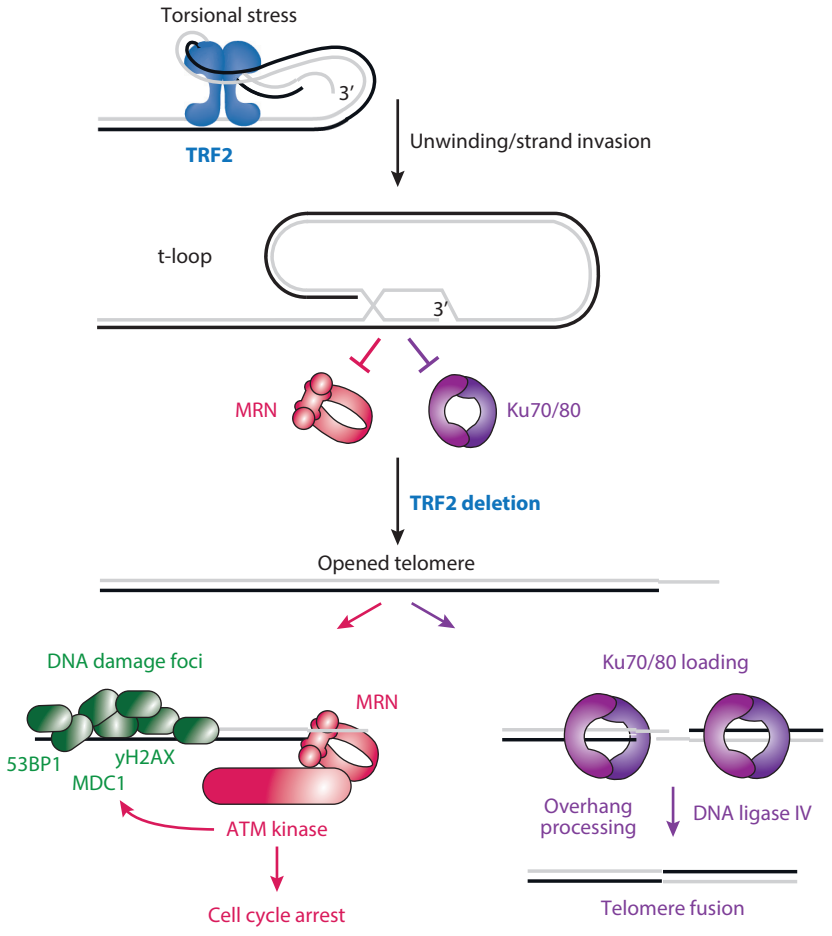

Figure 4

Repression of ataxia-telangiectasia-mutated (ATM) signaling and classical nonhomologous end joining (c-NHEJ) by TRF2. TRF2 prevents end-loading of the Mre11/Rad50/Nbs1 (MRN) complex and the Ku70/80 heterodimer by sequestering the telomere end in the t-loop, thereby blocking ATM signaling and c-NHEJ at their initiation steps. When TRF2 is absent, MRN can associate with the telomere end and activate the ATM kinase. ATM signaling leads to cell cycle arrest and accumulation of DNA damage factors (*green*) at telomeres. In addition, Ku70/80 can load onto the open telomere end and initiate c-NHEJ by DNA ligase IV.

shown that the presence of the 3′ overhang is no deterrent for MRN-dependent ATM activation (3, 17, 34, 36).

TRF2 is required to prevent MRN-dependent ATM activation at telomeres. The ATM kinase becomes active at telomeres lacking TRF2, but not when TRF1, POT1a or POT1b, Rap1, or TPP1 is absent (17, 33, 117, 118). TIN2 deletion results in ATM kinase activation, but this is in part due to the loss of TRF2, which is stabilized on telomeres by the TIN2–TRF1 bridge (129).

The ability of TRF2 to promote the formation of t-loops can explain how telomeres avert ATM kinase signaling. Although MRN can recognize telomere ends, it is unlikely to do so when the telomere terminus is sequestered in the t-loop. The TRF2/t-loop model is consistent with most

of the available data, but its ultimate proof requires replacement of TRF2 with another protein that can form t-loops.

ATR: ataxia telangiectasia and Rad3-related

ATM kinase activation occurs within hours after inactivation of a temperature-sensitive allele of TRF2, suggesting that t-loops can open spontaneously when TRF2 is not present (76). Rapid ATM activation also occurs when TRF2 is inactivated in G1, indicating that telomere replication is not required to create a telomere conformation that is recognized by MRN.

Other models of how TRF2 prevents ATM kinase signaling have been proposed. One recent suggestion is that TRF2 (and TRF1) mediates compaction of the telomeric chromatin, thereby denying the ATM kinase access to the telomere end (8). However, the reported expansion of the telomeric chromatin upon shelterin inhibition has not been observed by others (132, 134). One explanation for these disparate results is that dysfunctional telomeres can become clustered after extensive periods of time, perhaps leading to the impression of expanded chromatin domains (132). A second model suggested that TRF2 has the inherent ability to prevent ATM activation by directly associating with the enzyme (71). Neither the chromatin compaction model nor the TRF2-dependent ATM inhibition model accounts for the finding that the ATM kinase can become activated when a DSB is created inside the telomeric repeat array (40, 130). Both compaction and TRF2-mediated ATM inhibition should have prevented the DDR at a telomere-internal break as well as at the telomere end.

REPRESSION OF ATAXIA TELANGIECTASIA AND RAD3-RELATED (ATR) SIGNALING

The ataxia telangiectasia and Rad3-related (ATR) kinase is activated through two distinct pathways (reviewed in 28). The first described pathway involves the binding of RPA to ssDNA and the loading of the 9–1–1 clamp on the neighboring 5′ ds–ss transition by the Rad17 clamploader. ATR is recruited by the interaction of its binding partner ATRIP with RPA and is activated when TopBP1, a protein with multiple phosphopeptide binding domains, interacts with the 9–1–1 clamp (**Figure 5**). The ATR kinase can also be activated by ETAA1, which binds both ATR/ATRIP and RPA. This activation pathway is independent of TopBP1, Rad17, and 9–1–1 and does not require a 5′ ds–ss transition. The constitutive 3′ overhang of mammalian telomeres is of sufficient length to bind RPA, and either TopBP1- or ETAA1-dependent ATR activation could occur if shelterin failed to protect the telomeres. This holds for telomeres in the t-loop configuration as well as for linear telomeres (**Figure 5**).

Shelterin uses POT1 to repress ATR signaling. In human shelterin, this task is delegated to the single POT1 protein, whereas mouse shelterin has two ATR repressors, POT1a and POT1b. When POT1a and POT1b are both deleted, ATR is activated at telomeres throughout the cell cycle (33, 57). At genome-wide DSBs, ATR activation is primarily observed in S phase, when resection creates the ssDNA needed for RPA loading. However, at telomeres, resection is not required to generate an ATR activation site, explaining the telomeric ATR response in G1 as well as in S and G2. As expected, this activation is dependent on ATRIP and RPA (57). ATR signaling at telomeres is abrogated in the absence of TopBP1 (57), suggesting that the ETAA1 pathway plays a minor role (if any).

RPA can be detected at telomeres lacking POT1a and POT1b throughout the cell cycle, although the intensity of RPA at telomeres is much greater in S and G2 than in G1 (57). Based on the localization of RPA, a simple competition model has been proposed of how the POT1 proteins prevent ATR activation (27, 33, 57). The model proposes that POT1 binding to the ssDNA blocks RPA from gaining access to the telomeric overhang, thereby averting the threat of ATR activation (**Figure 5**).

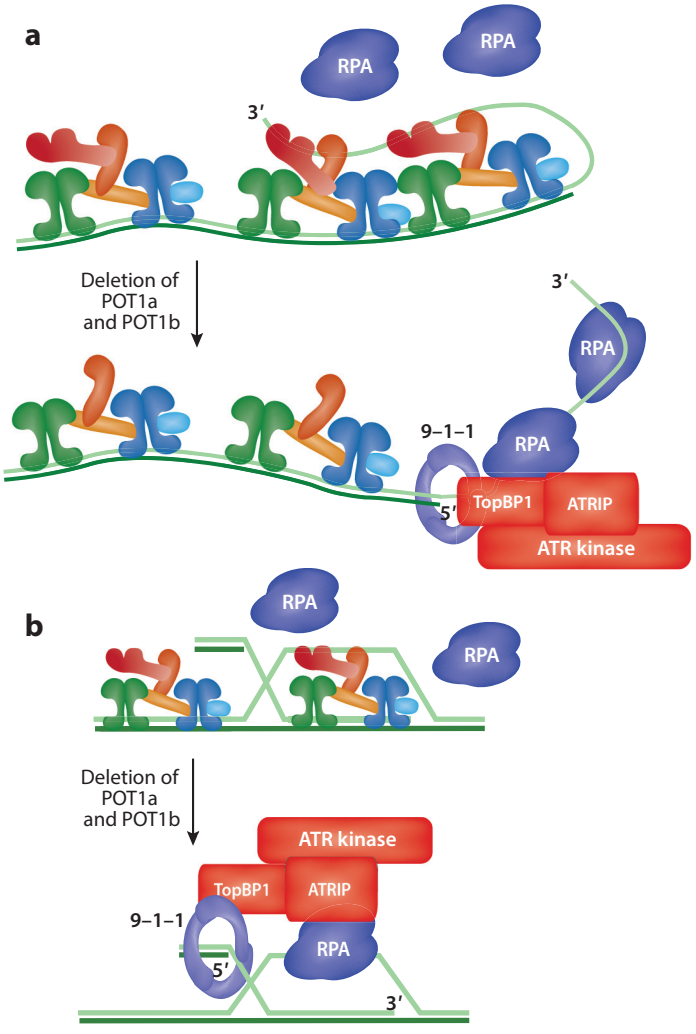

Figure 5

Repression of ATR signaling. Upon deletion of POT1a (or POT1a and POT1b) from telomeres, ATR becomes active in a manner that depends on the ssDNA binding protein RPA, the 9–1–1 clamp interacting protein TopBP1, and the ATR and RPA binding protein ATRIP. (*a*) This activation occurs at telomeres in a linear state and (*b*) when telomeres are in the t-loop configuration (only the base of the t-loop is shown in panel *b*) because the requirements for ATR activation (ssDNA to bind RPA and a 5′ ds–ss transition for the loading of the 9–1–1 clamp and its interaction with TopBP1) are present in each telomere configuration. The POT1 proteins are thought to block activation of ATR by excluding RPA from the ss telomeric DNA in panels *a* and *b*. RPA exclusion requires the TPP1-mediated tethering of POT1 to the shelterin core. Abbreviations: ATR, ataxia telangiectasia and Rad3-related; ds, double-stranded; RPA, replication protein A; ss, single-stranded.

POT1a and POT1b have no (or very limited) ability to repress ATR if they are incapable of binding to TPP1, or if TPP1 does not interact with TIN2 (49, 63, 72, 129). The tethering to the shelterin core allows POT1 to outcompete RPA, despite RPA and POT1 having the same affinity for ss telomeric DNA, and despite RPA being much more abundant than the POT1 proteins (129).

alt-NHEJ: alternative nonhomologous end joining

c-NHEJ: classical nonhomologous end joining

Whereas POT1a can repress ATR signaling throughout the cell cycle, POT1b can only effectively avert ATR activation in G1 (57, 62). This is a conundrum because both proteins are present at telomeres throughout the cell cycle and have the same affinity for telomeric DNA (62, 129). The critical difference between POT1a and POT1b is that POT1b binds to CST/Polα/primase, whereas POT1a does not. It is the binding to CST that interferes with the ability of POT1b to repress ATR signaling in S and G2 (77a). The mechanism by which CST has this effect is not clear. Perhaps the distinct manner in which human POT1 interacts with CST allows human POT1 to be an effective ATR repressor throughout the cell cycle.

An elaboration of the RPA exclusion model has been proposed wherein RPA is first removed by heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and then hnRNPA1 is replaced by POT1 (46). An additional suggestion is that G4 structures formed in the ss telomeric DNA could provide a binding advantage to POT1 because POT1 is better than RPA at binding to sites adjacent to some of these structures (109).

REPRESSION OF POLY(ADP-RIBOSE) POLYMERASE 1 (PARP1)

As discussed in the section titled T-Loop Protection, PARP1 is a potential threat to telomeres because they contain a PARP1 activation site (**Figure 3*b***). PARP1-mediated PARsylation of telomeric proteins, including shelterin subunits, is likely to be detrimental to telomere function because PAR adds negative charges that could interfere with DNA binding and other functions. Furthermore, PARP1 promotes t-loop cleavage (see the section titled T-Loop Protection) and alternative (alt)-NHEJ [see the section titled Repression of Alternative Nonhomologous End Joining (Alt-NHEJ)]. The binding of PARP1 to telomeres is prevented by TRF2 and TIN2 (115). TRF2 is thought to mask the PARP1 activation site using its Basic branched-DNA binding domain, but how TIN2 blocks PARP1 accumulation at telomeres is not clear. TIN2 acts independently of TRF2 such that maximal PARP1 activation only takes place upon their combined loss (115).

REPRESSION OF CLASSICAL NONHOMOLOGOUS END JOINING (C-NHEJ)

The formation of dicentric chromosomes through telomere-telomere fusion is a great threat to genome integrity. Dicentric chromosomes can give rise to many genome rearrangements (reviewed in 89). Telomeres avert formation of dicentric chromosomes by inhibiting both classical (c-) and alt-NHEJ (also referred to as microhomology-mediated end joining).

At DSBs, c-NHEJ is mediated by the Ku70/80 heterodimer, which is a ring-shaped complex that can load onto accessible DNA ends and bring them together (**Figure 4**) (see 26 for a review). The ligation step in c-NHEJ is performed by DNA ligase IV (Lig4) in complex with XRCC4 and other factors. The DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which binds to Ku70/80, can promote the reaction through phosphorylation of target proteins. This pathway is active throughout the cell cycle and constitutes a constant threat to telomere and genome integrity.

Similar to the repression of ATM signaling, the main factor in shelterin that prevents c-NHEJ is TRF2. No telomere fusions occur upon deletion of Rap1 (117), and telomere fusions are rare when POT1a and POT1b, TPP1, or TRF1 are removed (62, 72, 92, 118, 142). The fusions in POT1a/b or TPP1-null cells are often between sister telomeres and are dependent on alt-NHEJ factors (108). TIN2 deletion results in frequent telomere fusions, but these fusions are due to destabilization of TRF2 (129). Reconstitution of telomeres that carry TRF2 but lack all other shelterin components showed that they are protected from c-NHEJ (L. Timashev & T. de Lange, unpublished data), indicating that TRF2 is both necessary and sufficient for the repression of c-NHEJ.

The main model of how TRF2 prevents telomere fusions is based on t-loop formation (**Figure 4**). In the t-loop, the telomere end is simply not available for the loading of the Ku70/80 ring. Loss of TRF2 (but not Rap1, POT1, TRF1, or TPP1) leads to loss of the t-loop structure, thereby providing the substrate for Ku70/80 loading and c-NHEJ (41). Furthermore, telomeres containing only TRF2 form t-loops, consistent with t-loop dependent protection from c-NHEJ (L. Timashev & T. de Lange, unpublished data).

Interestingly, the presence of the natural telomeric 3′ overhang per se does not appear to deter the c-NHEJ pathway. The 3′ overhang is normally removed during the process of c-NHEJ, in part by the XPF/ERCC1 flap endonuclease (149). However, the 3′ overhang at telomeres can diminish telomere fusion in S and G2 due to the effect of CYREN (2). CYREN is a small Ku70/80 binding protein that inhibits c-NHEJ substrates with a 3′ or 5′ overhang (2). As CYREN does not act in G1, it may explain why TRF2 depletion predominantly leads to chromosome-type fusions (which reflect telomere fusion before DNA replication) rather than chromatid-type ones, as well as the higher frequency of telomere fusions upon inactivation of a temperature-sensitive allele of TRF2 in G1 versus S and G2 (76).

Unlike the repair of DSBs by c-NHEJ, fusion of telomeres through c-NHEJ requires activation of the ATM kinase (33). The key component of the ATM pathway in this regard is 53BP1. In absence of 53BP1, c-NHEJ of telomeres lacking TRF2 is exceedingly rare (35). The contribution of 53BP1 to telomere fusion is based on the repression of hyper-resection (14, 85, 152) (see the section titled Repression of 5′ End Hyper-Resection) as well as on its ability to promote the dynamic movement of sites of DNA damage in the nucleus (86). In a 53BP1-dependent process that involves cytoplasmic microtubules and the LINC (linker of nucleus and cytoplasm) complex, deprotected telomeres and other sites of DNA damage become more mobile and roam larger domains in the nucleus (86). Because telomeres are physically separated in G1, their 53BP1-mediated mobility promotes telomere fusions by increasing the chance that one telomere is close to another. The presence of 53BP1 also leads to the clustering of deprotected telomeres, which may also facilitate telomere fusions (132).

In addition to blocking c-NHEJ by forming t-loops, TRF2 appears to have evolved the ability to minimize c-NHEJ when telomeres occur in a linear state. When linear telomeres activate ATM kinase signaling, TRF2 can minimize c-NHEJ by modulating the presence of 53BP1 at dysfunctional telomeres (101). This effect is due to a small domain in TRF2, referred to as inhibitor of DDR (iDDR) (**Figure 1*e***). When transposed into TRF1, the iDDR can sever the cascade of events that normally lead to the accumulation of 53BP1 upon activation of the ATM kinase. The presence of 53BP1 at sites of DNA damage, including at dysfunctional telomeres, depends on the ubiquitin ligase RNF168, which modifies histone H2A. RNF168 in turn binds to histone H1 when it is modified by the ubiquitin ligase RNF8. The iDDR of TRF2 can prevent 53BP1 accumulation through binding to MRN. MRN in turn binds to the BRCC3 deubiquitinylase, which can remove the ubiquitin mark from H1. Without H1 ubiquitylation, RNF168 and 53BP1 do not accumulate, and telomere fusions are minimized.

TRF2 may also be able to limit c-NHEJ at linear telomeres through an undefined attribute of the TRF2 interacting factor Rap1 (**Figure 1**). Rap1 was proposed to be involved in the repression of NHEJ based on a tethering experiment wherein artificial localization of Rap1 at telomeres could limit the fusion of telomeres after TRF2 inhibition (114). Consistent with this result, TRF2/Rap1, but not TRF2 alone, can inhibit c-NHEJ of telomeric substrates in vitro (4). Deletion of Rap1 from human or mouse cells does not give rise to telomere fusions (70, 117), indicating that the role of Rap1 in repressing c-NHEJ is irrelevant as long as t-loops are present. However, in cells expressing the TRF2 Top-less allele, which is incapable of making t-loops, Rap1 was found to limit telomere fusions (10). The idea that Rap1 can minimize c-NHEJ at linear telomeres is also

consistent with the observation that tethering of TRF2 (and therefore Rap1) to a DSB can inhibit c-NHEJ but not ATM signaling (51). The ability of TRF2 to repress c-NHEJ at linear telomeres has been invoked in models for telomere dysfunction resulting from telomere shortening (19, 20).

REPRESSION OF ALTERNATIVE NONHOMOLOGOUS END JOINING (ALT-NHEJ)

The role of alt-NHEJ in normal genome maintenance is elusive (see 119 for a review) because it only surfaces as a major DSB repair pathway in certain circumstances (e.g., when c-NHEJ is impaired). Although alt-NHEJ is a minimal threat in cells that are c-NHEJ proficient, shelterin has the ability to avert alt-NHEJ as well as c-NHEJ.

Alt-NHEJ is initiated when a 5′ ds–ss transition activates PARP1. PARsylation of nearby proteins (including histones) results in a binding platform for DNA Lig3 and a DNA polymerase. If two DNA ends have 3′ overhangs with slight homology, PARP1 activation, DNA polymerase-mediated fill-in, and ligation of the ends by Lig3 (or the replicative ligase, Lig1) lead to alt-NHEJ. As this pathway relies on the same factors that mediate base excision repair, it may be that the base excision repair pathway acts at DSBs if the substrate resembles a DNA duplex with a nick or a gap. The homology required for alt-NHEJ is minimal (one or more base pairs). In the case of two dysfunctional telomeres, the homology between the 3′ overhangs (2 bp per telomeric repeat) is more than sufficient for alt-NHEJ. Alt-NHEJ of telomeres is observed in mouse cells that lack Ku70/80, which normally inhibits the pathway by competing for PARP1 at the ends. The polymerase involved in the fill-in reaction is the error-prone translesion polymerase θ, leading to altered sequences at the fusion point (93).

When shelterin is completely removed from telomeres in cells that lack Ku70/80, nearly all telomeres fuse through alt-NHEJ (116). Within shelterin, TRF2 represses alt-NHEJ, presumably by hiding the telomere end in the t-loop. However, deletion of TRF2 from Ku70/80-deficient cells only leads to a mild telomere fusion phenotype (18), indicating other shelterin components repress alt-NHEJ. TIN2 is the most likely candidate for a second repressor of alt-NHEJ because, like TRF2, TIN2 can limit the accumulation of PARP1 at telomeres (115). Thus, t-loop formation and blocking PARP1 may be the two main mechanisms by which telomeres avoid this mysterious pathway. Alt-NHEJ at telomeres also occurs at low levels in cells lacking TPP1 or POT1a/b (62, 72, 108, 131). Through what mechanism(s) these fusions are averted is not yet clear.

REPRESSION OF HOMOLOGY-DIRECTED REPAIR (HDR)

One of the most vexing questions concerning shelterin is how it represses homology-directed repair (HDR). HDR between sister telomeres results in telomere sister chromatid exchanges (T-SCEs), which can be detected by differential labeling of the leading-strand and lagging-strand DNA synthesis products (5). These events are innocuous if they take place in register because telomere length is not altered by an equal exchange. But if the exchange is unequal, one daughter cell could inherit a severely shortened telomere, which, in the absence of telomerase, would consign this cell to a shorter replicative life span. With approximately 200 sister telomeres at risk for unequal exchange, it seems important to repress HDR at telomeres.

HDR at telomeres is repressed by Ku70/80, as it is at DSBs. Telomere HDR is therefore best studied in Ku70/80-deficient cells, although very low levels of T-SCEs can be detected in Ku70/80-proficient cells lacking certain shelterin subunits (e.g., 142). Human cells are not viable in the absence of Ku70/80 (141), so relatively little is known about how human shelterin represses

HDR. In Ku70/80-deficient mouse cells, repression of HDR requires both Rap1 and one of the two POT1 proteins (102, 117). In absence of both POT1a and POT1b or when Rap1 is deleted, approximately 10% of chromosome ends show T-SCEs in metaphase, signifying recombination events in the preceding S/G2. This is a high frequency of HDR, equal to one recombination event per 300–500 kb of telomeric DNA. What initiates HDR between telomeres is not known. Are DSBs inherently frequent in the telomeric DNA, or are these exchanges due to a postreplicative processing event at telomeres that has yet to be discovered? Both the mechanism by which Rap1 and the POT1 proteins act and the nature of the HDR reaction they are blocking need to be clarified. These issues are critical for the understanding of the alternative lengthening of telomeres pathway, which uses HDR for telomere maintenance despite the presence of shelterin (21, 87).

REPRESSION OF 5′ END HYPER-RESECTION

Although 5′ end resection is needed to re-establish the correct telomeric overhang structure after DNA replication, excessive resection is a threat to telomere integrity. If the 5′ end of a telomere is permanently shortened, DNA replication generates a shortened leading-end product in the next S phase.

When shelterin is removed from telomeres, hyper-resection of the 5′ strand occurs (116). The C-rich strands appear to become vulnerable to the nucleolytic factors that act on DSBs (Exo1, BLM, CtIP). The nucleolytic attack is driven by a DNA damage signal transduced by either the ATM or ATR kinase (73, 85). Activation of these kinases also leads to accumulation of 53BP1 at telomeres, which diminishes resection. For this reason, the most extensive resection at dysfunctional telomeres occurs in cells lacking 53BP1 (73, 85, 116). 53BP1 diminishes resection by recruiting a series of 53BP1 dependent DDR factors, including Rif1 and the associated Shieldin complex. Recently, it has become clear that the mechanism by which 53BP1/Rif1/Shieldin act involves fill-in of the resected ends by CST and Polα/primase (97).

The hyper-resection of the telomeric 5′ end is primarily repressed by averting the DDR. As long as TRF2 and the POT1 proteins ensure that ATM and ATR remain inactive, resection is not initiated. When the POT1 proteins are removed (or when TPP1 is deleted), ATR signaling leads to hyper-resection activity that is exacerbated when 53BP1 (or its downstream factors) are absent (73). Under these conditions, TRF2 still dampens the resection. Most likely, formation of the t-loop protects the 5′ end to some extent. This protection from resection is analogous to what happens during homologous recombination in yeast, where Rad51-mediated strand invasion prevents further resection of the 5′ end (125). Conversely, when TRF2 is deleted from 53BP1-deficient cells, hyper-resection occurs but is dampened by the POT1 proteins (85). Presumably, it is in part the ability of POT1b to recruit CST that counteracts the resection.

REPLICATION THROUGH TELOMERES

The repetitive G-rich sequences of telomeres pose a challenge for the replisome. The replication fork has a propensity to stall in the telomeric repeat region, even when shelterin is intact. This stalling becomes very frequent when TRF1 is removed (**Figure 6**) (92, 118). Replication fork stalling can be observed by single telomere labeling techniques and is also inferred from the structure of telomeres in metaphase chromosomes. Upon TRF1 deletion, telomeric fluorescent in situ hybridization (FISH) shows an altered pattern, with multiple signals at single chromatid ends. In some cases, these signals appear to be connected by thin stretches of telomeric DNA. Because these structures resemble the common fragile sites where replication problems are

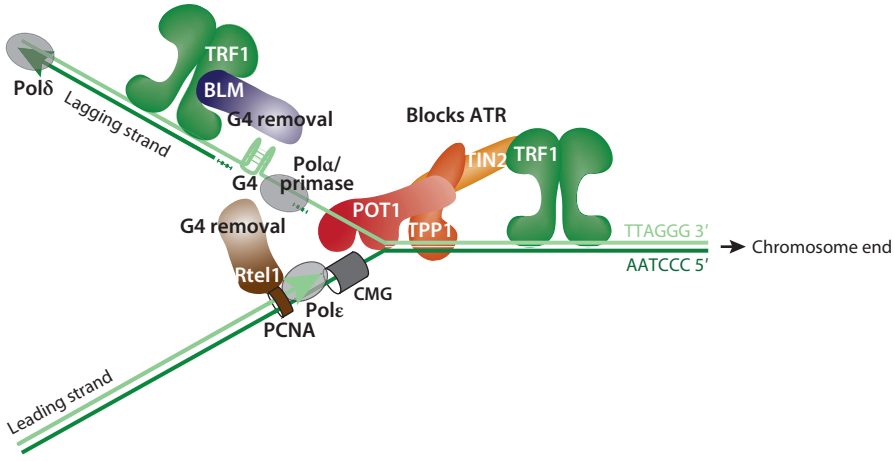

Figure 6

Replication through telomeres. Telomeres are replicated from a subtelomeric origin toward the chromosome end by the canonical replication machinery (the CMG helicase, Polδ, Polε, etc.). TRF1-bound BLM helicase removes G4 structures shown formed by the [TTAGGG]*n* template of lagging-strand DNA replication, thereby preventing replication problems. G4 structures are also removed by regulator of telomere elongation helicase 1 (Rtel1), which is associated with proliferating cell nuclear antigen (PCNA, only shown on the leading strand). TRF1 also represses ataxia telangiectasia and Rad3-related (ATR) signaling upon replication fork stalling using POT1 to prevent accumulation of replication protein A (RPA).

frequent, they were called fragile telomeres (92, 118). Fragile telomere formation is now used as a readout of replication problems. The nature of the defect that gives rise to the structure observed in metaphase is not known for telomeres or for common fragile sites. It is unlikely that bona fide DSBs are responsible for the apparent broken nature of the chromatin. More likely, fragile telomeres represent regions of incomplete chromatinization and/or condensation (118), possibly due to the presence of ssDNA or an altered mode of replication that fails to fully reconstitute chromatin.

The mechanism by which TRF1 promotes telomere replication in part involves the BLM helicase (**Figure 6**) (118, 151). BLM has the ability to unwind G4 structures (68, 126), which can form in the TTAGGG template for lagging-strand DNA synthesis of telomeres. By recruiting BLM to telomeres, TRF1 is thought to remove these obstacles. Consistent with this idea, loss of BLM from telomeres (e.g., using a TRF1 mutant that does not bind BLM) leads to a lagging-strand specific fragile telomere phenotype (151).

A second prominent player in telomere replication is the Rtel1 helicase (118, 135). Rtel1 depletion induces a fragile telomere phenotype that is epistatic with TRF1 deletion, suggesting that it acts in the same pathway (118). Rtel1 does not interact with TRF1 but is brought to the replication fork by an interaction of its PIP box with proliferating cell nuclear antigen (136), reflecting the general role for Rtel1 in replication. The Rtel1 deletion-induced fragile telomere phenotype is exacerbated by G4-stabilizing agents (135), indicating a role for Rtel1, as well as BLM, in removing G4 structures.

Supplemental Material >

Additional shelterin accessory factors that promote the replication through telomeres are AKTIP, TopIIα, Timeless, SA1, SLX4, and UPF1 (see **Supplemental Table 1** online). The myriad of proteins shelterin uses to manage telomere replication likely reflects the dangers of fork stalling in telomeres and the complexity of promoting fork restart events. Unlike elsewhere in the

genome, stalled forks at telomeres usually cannot be rescued by a fork arriving from the other direction.

In addition to mediating G4 resolution, the presence of TRF1 at telomeres dampens the ATR signaling in response to replication stress (151). For instance, when BLM is absent or when replication stress is induced with aphidicolin, telomeres show evidence of replication problems, but no DNA damage signal is observed. In contrast, ATR is activated when TRF1 is removed. This repression of ATR signaling involves TPP1 and POT1, which presumably exclude RPA from the ss G-rich telomeric DNA (**Figure 6**).

PERSPECTIVE

The first telomeric proteins were discovered in ciliates (82), where the macronucleus contains gene-sized chromosomes with tiny telomeres that are enveloped by a dimeric protein complex (65). These telomeres inspired the hackneyed aglet analogy, wherein chromosome ends are protected by a cap of tightly bound proteins. Work on shelterin has revealed the drastically different mechanism by which mammalian telomeres solve the end-protection problem. Shelterin is neither tightly bound nor simple. It uses a variety of molecular strategies to block the DDR, often involving different shelterin subunits. Shelterin also governs the structure of telomeres, forming the t-loop and modifying the telomere terminus. It is expected that many additional mechanistic insights will emerge from future work on this remarkable multifunctional complex.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Contents

Errata

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