Stop pulling my strings — what telomeres taught us about the DNA damage response

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Abstract | Mammalian cells have evolved specialized mechanisms to sense and repair double-strand breaks (DSBs) to maintain genomic stability. However, in certain cases, the activity of these pathways can lead to aberrant DNA repair, genomic instability and tumorigenesis. One such case is DNA repair at the natural ends of linear chromosomes, known as telomeres, which can lead to chromosome-end fusions. Here, we review data obtained over the past decade and discuss the mechanisms that protect mammalian chromosome ends from the DNA damage response. We also discuss how telomere research has helped to uncover key steps in DSB repair. Last, we summarize how dysfunctional telomeres and the ensuing genomic instability drive the progression of cancer.

Linear chromosomes pose a challenge to eukaryotic cells. This problem was first recognized by Barbara McClintock and Herman Muller1,2, who postulated that specialized structures named ‘telomeres’ distinguish chromosome ends from sites of DNA double-strand breaks (DSBs). Since then, extensive research has revealed the composition, structure and function of telomeres (FIG. 1). Mammalian telomeres consist of arrays of TTAGGG repeats that range from 5 kb in human cells to 100 kb in mice, which are polymerized by telomerase, a specialized reverse transcriptase3. Telomeres end with a single-stranded G-rich overhang4,5 that can invade the preceding double-stranded region to generate a special lariat-like structure called the telomere loop or t-loop6,7. Telomere DNA is transcribed by RNA polymerase II into a long non-coding telomeric repeat-containing RNA (TERRA)8. The function of TERRA is not fully understood, but the emerging view is that it functions as a molecular scaffold for proteins that assist in proper telomere function (for a review, see REF. 9).

Telomeres are bound by shelterin, a six-subunit protein complex that protects chromosome ends from aberrant activation of the DNA damage response (DDR)10 (FIG. 1). Shelterin recognizes TTAGGG repeats through the binding of its TRF1 (telomere repeat-binding factor 1; also known as TERF1)11 and TRF2 (REFS 12,13) subunits to duplex DNA. TRF1 and TRF2 co-interact with TEN1 (TRF1-interacting nuclear factor 2), which in turn binds the TPP1 (PTOP, PIP1 or TINT1)–POT1 (protection of telomere 1) heterodimer14,18. POT1 is the third DNA-binding component within shelterin. It is recruited to telomeres by interacting with TPP1 and coats the single-stranded part of the TTAGGG repeats with its oligonucleotide/oligosaccharide binding folds19,20. Rodents express two POT1 paralogues — POT1A and POT1B — that are structurally similar yet functionally divergent21,22. RAP1 (repressor activator protein 1) is the sixth and most conserved shelterin component; it is recruited to telomeres by interacting with TRF2 (REFS 23–25). The current view is that shelterin can form as a six-subunit complex as well as subcomplexes lacking TRF1 or TRF2–RAP1 (REFS 14,15,18,26). The telomere proteome comprises additional telomere-associated proteins27–30, including DNA damage factors (Ku, MRN (MRE11–RAD50–NBS1)), nucleases (structure-specific endonuclease subunit SLX4, Apollo), helicases (Bloom syndrome, RecQ helicase-like (BLM), Werner syndrome, RecQ helicase-like (WRN), regulator of telomere elongation helicase 1 (RTEL1)) and chromatin modifiers (a-thalassaemia/mental retardation syndrome X-linked (ATRX)). A key complex that is central for telomere function is the trimeric CST complex, which is composed of the DNA polymerase α (Pol α)–primase accessory factors CTC1, STN1 and TEN1 (REFS 31,32). Interestingly, recent data suggest that in mouse germ cells, a meiosis-specific telomere complex, composed of TERB1 (telomere repeats-binding bouquet formation protein 1), TERB2 and membrane-anchored junction protein (MAJIN), replaces the shelterin complex to facilitate the attachment of telomeres to the inner nuclear membrane33,34.
Overview of telomere composition and function. Mammalian telomeres are composed of long stretches of TTAGGG repeats that range from 5 kb in human cells to 100 kb in mice and end with a single-stranded 3' overhang of up to a few hundred nucleotides in length. Telomeric DNA is bound by the specialized shelterin complex, transcribed into a long non-coding telomeric repeat-containing RNA (TERRA) and packaged into a t-loop (telomere loop) configuration. Shelterin subunits include TRF1 (telomere repeat-binding factor 1), TRF2, TIN2 (TRF1-interaction factor 2), RAP1 (repressor activator protein 1), TPP1 and POT1 (protection of telomere 1; POT1A and POT1B in mice). The six-subunit complex protects chromosome ends from DNA damage signalling by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related), and from DNA repair by c-NHEJ (classical non-homologous end joining), RNF168, thereby preventing the accumulation of 53BP1 (REF. 36), which is a key effector of ATM. The two-step mechanism by which TRF2 inhibits ATM activation may be crucial for telomere protection during the S phase of the cell cycle. The progression of the replication fork through telomeric DNA triggers transient unwinding of t-loops and renders chromosome ends susceptible to the DDR. Direct inhibition of the ATM signalling pathway by TRF2 will therefore ensure end protection. Finally, biochemical analysis and atomic force microscopy has highlighted a topological mechanism of TRF2-mediated repression of ATM. Specifically, the wrapping of DNA around the TRFH domain of TRF2 was recently proposed to promote t-loop formation and inhibit ATM signalling.

TRF2, the bouncer at the gate. DNA damage signalling by ATM is primarily repressed at telomeres by TRF2 (REFS 36, 37). TRF2 inhibition activates ATM, which phosphorylates the downstream effectors Ser/Thr protein kinase CHK2 and p53 (REF. 24), thereby inducing the formation of telomere dysfunction-induced foci (TIFs). These are marked domains of telomere-associated DNA damage factors, including 53BP1 (p53-binding protein 1) and the histone variant H2AX. The underlying mechanism by which TRF2 inhibits ATM-dependent repair pathways is complex and not fully understood. The current view emphasizes two levels of control; the first involves the t-loop configuration, and the second consists of direct inhibition of the ATM signalling cascade (FIG. 2a). TRF2 binding to DNA in vitro stimulates strand invasion, forming structures that resemble t-loops. Furthermore, the frequency of t-loops in vivo is significantly reduced in cells lacking TRF2, implicating this shelterin subunit in their formation and/or stabilization 40. When telomere ends are engaged in a t-loop configuration, they are unlikely to be detected by the MRN complex, which is essential for ATM activation. In addition, TRF2 inhibits ATM signalling directly by inhibiting the kinase itself, as well as crucial downstream effectors of the ATM pathway (FIG. 2a). A motif within TRF2, termed the iDDR (inhibitor of the DDR pathway), inhibits the activity of the E3 ubiquitin ligase RNF168, thereby preventing the accumulation of 53BP1, which is a key effector of ATM. The two-step mechanism by which TRF2 inhibits ATM activation may be crucial for telomere protection during the S phase of the cell cycle. The progression of the replication fork through telomeric DNA triggers transient unwinding of t-loops and renders chromosome ends susceptible to the DDR. Direct inhibition of the ATM signalling pathway by TRF2 will therefore ensure end protection. Finally, biochemical analysis and atomic force microscopy has highlighted a topological mechanism of TRF2-mediated repression of ATM. Specifically, the wrapping of DNA around the TRFH domain of TRF2 was recently proposed to promote t-loop formation and inhibit ATM signalling.

The struggle to keep ends apart. The most deleterious outcome of telomere dysfunction is the formation of chromosome end-to-end fusions, resulting in dicentric chromosomes, which can lead to breakage–fusion–bridge cycles and induce extensive chromosomal instability. Mammalian cells have evolved sophisticated mechanisms to prevent such events from occurring. The major factor suppressing chromosome end-to-end fusions is TRF2. When telomeres are depleted of TRF2, they become substrates for c-NHEJ (BOX 1). Artificial tethering of TRF2 to non-telomere loci inhibits break repair, suggesting that TRF2 is both necessary and sufficient to suppress...
c-NHEJ). TRF2 forms a stable 1:1 complex with its interacting partner RAP1 (REFS 44, 45), and RAP1 protein is rapidly destabilized upon deletion of TRF2 (REF 24). Interestingly, tethering of RAP1 to telomere DNA in cells lacking TRF2 was reported to reduce the frequency of telomere fusions46. However, deletion of RAP1 from human and mouse telomeres is not sufficient to trigger c-NHEJ. A recent study provides a plausible explanation for the contrasting data by suggesting that RAP1 provides a redundant mechanism to block c-NHEJ when the function of TRF2 is partially compromised41.

Paradoxically, components of the c-NHEJ pathway, most notably the Ku70–Ku80 complex, are constitutively present at telomeres49. Ku has a crucial role in protecting telomeres in human cells, and its depletion leads to rapid deletion of telomeric repeats50. By contrast, deleting Ku in mice does not trigger major telomeric defects51,52, hinting at alternative solutions to achieve telomere protection in rodents. Nevertheless, the strong impact of Ku depletion on telomere stability in human cells raises the obvious question of how TRF2 is able to disengage Ku without compromising telomere stability (FIG. 2a). A possible mechanism invokes a recently described interaction between TRF2 and the α5 region of Ku70, which prevents Ku70–Ku80 heterotetramerization53. The TRF2–Ku interaction is thought to block the ability of Ku70–Ku80 to tether opposite DNA ends, which could explain why Ku70–Ku80 association with functional telomeres does not unleash c-NHEJ.

Intriguingly, recent reports suggest that during mitosis, telomeres are highly susceptible to c-NHEJ-mediated fusion54–56. Mitotic cells attenuate c-NHEJ globally by preventing the phosphorylation of RNF8 and 53BP1 by mitotic kinases such as Polo-like kinase 1 (PLK1) and cyclin-dependent kinase 1 (CDK1)55. When this regulation is bypassed, telomeres are fused in an Aurora kinase B-dependent manner. These data corroborate a previous report showing that prolonged mitotic arrest leads to telomere uncapping following the eviction of TRF2 from telomeres, in a process that is dependent on Aurora kinase B54. Future work is likely to shed light on telomere protection in mitosis and reveal why mammalian cells opt for a global shutdown of c-NHEJ, as opposed to simply configuring an extra layer of protection at telomeres.

**Blocking ATR signalling.** The activity of ATR at telomeres is primarily repressed by POT1. Deleting POT1, or blocking its recruitment to telomeres by inhibiting TPP1 or TIN2, induces the formation of ATR-dependent TIFs47,57–60. POT1 binds to telomeric single-stranded DNA (ssDNA), thus preventing the recruitment of RPA (replication protein A), which is a crucial factor for the activation of ATR61 (FIG. 2b). Although POT1 affinity for ssDNA does not exceed the binding affinity of RPA, it has been proposed that the increased local concentration of POT1 at telomeres excludes RPA binding59,60. An alternative model for POT1-mediated inhibition of ATR invokes a cell cycle-regulated RPA-to-POT1 switch mediated by hnRNP A (heterogeneous nuclear ribonucleoprotein A) and TERRA62. According to this model, accumulation of hnRNP A at replicated telomeres promotes the displacement of RPA and the subsequent recruitment of POT1. ATR activation in S phase is also inhibited, by TRF1, which is dedicated to counteracting replication defects at telomeres49 (FIG. 2c). Notably, tethering of TPP1–POT1 to telomeres lacking TRF1 is sufficient to inhibit the TIF response, suggesting that ATR inhibition by TRF1 is dependent on the recruitment of TPP1 and POT1 to telomere DNA64.

**Box 1 Classical non-homologous end joining (c-NHEJ) versus alternative NHEJ (alt-NHEJ)**

Cells use two mechanistically distinct end-joining pathways to repair DNA double-strand breaks (DSBs)44,45. C-NHEJ leads to minimal sequence alterations at the repair junctions, whereas alt-NHEJ (also known as microhomology-mediated end joining (MMEJ)) causes extensive deletions (as well as insertions) that scar the break sites following repair (see the figure). C-NHEJ is active throughout the cell cycle and is initiated when the Ku70–Ku80 heterodimer binds to DNA ends with high affinity. Ku then recruits the Ser/Thr kinase DNA-PKcs (DNA-dependent protein kinase catalytic subunit) to phosphorylate a number of downstream targets, including the terminal end-processing enzyme Artemis that cleaves single-stranded overhangs, and DNA ligase 4 (LIG4) and the scaffold protein XRCC4, which catalyse the ligation of DNA ends. Alt-NHEJ, which is most active in the S and G2 phases of the cell cycle, is dependent on signalling by poly(ADP-ribose) polymerase 1 (PARP1) and relies on 5′–3′ resection of DNA by MRN (MRE11–RAD50–NBS1) and CtBP-interacting protein (CtIP). Base pairing at the resected ends drives their annealing to promote synapsis of opposite ends of a DSB. Annealed ends are subject to fill-in synthesis by the low-fidelity DNA polymerase θ (Pol θ), which stabilizes the annealed intermediates and promotes end joining, primarily by LIG3. Alt-NHEJ introduces deletions and insertions that scar the break sites following repair. The deletions are caused by extended nucleolytic processing, whereas the insertions result from the activity of Pol θ.
Figure 2 | How shelterin protects telomeres. a | TRF2 (telomere repeat-binding factor 2) represses ATM (ataxia telangiectasia mutated) signalling and classical non-homologous end joining (c-NHEJ). TRF2 promotes the formation of the protective telomere loop (t-loop) structure, which hides chromosome ends from ATM and c-NHEJ. In addition, TRF2 inhibits 53BP1 (p53-binding protein 1) accumulation by blocking RNF168-mediated ubiquitylation by activating the deubiquitylase BRCC3 (BRCA1/BRCA2-containing complex, subunit 3). Last, TRF2 blocks the dimerization of the Ku complex, thereby preventing the activation of c-NHEJ. b | POT1 (protection of telomere 1) represses ATR (ataxia telangiectasia and Rad3-related) signalling by competing with RPA (replication protein A) for single-stranded DNA (ssDNA) binding at telomeres. c | TRF1 inhibits ATR activity during telomere replication with the help of TPP1–POT1. TRF1 also counteracts replication fork stalling at telomeric secondary DNA structures (such as quadruplex DNA (G4)) with the help of RTEL1 (regulator of telomere elongation helicase 1) and BLM (Bloom syndrome, RecQ helicase-like), thereby protecting against telomere fragility. RTEL1 is recruited to replicating telomeres by interacting with PCNA (proliferating cell nuclear antigen). d | Alt-NHEJ (alternative-NHEJ), which is dependent on DNA ligase 3 (LIG3), PARP1 (poly(ADP-ribose) polymerase 1) and DNA polymerase θ (Pol θ), is repressed in a redundant manner by shelterin and the Ku70–Ku80 complex. e | The generation of telomere 3’ overhang involves TRF2-dependent recruitment of the nuclease Apollo to resect double-stranded ends. Leading and lagging ends are then resected by EXO1 (exonuclease 1) to generate long single-stranded overhangs, which are subsequently filled in by Pol α–primase and the CST (CTC1–STN1–TEN1) complex. f | Aberrant resection of uncapped telomeres is carried out by the enzymatic machinery that processes double-strand breaks (DSBs) — the nucleases CšTıP (CšTıP-interacting protein) and EXO1 and the helicase BLM — and is repressed redundantly by shelterin and 53BP1. iDDR, inhibitor of the DNA damage response.
A joint effort to suppress alt-NHEJ at chromosome ends.

Early evidence for the activation of the alt-NHEJ pathway at mammalian telomeres emerged from the analysis of telomerase-deficient mice. Specifically, chromosome end-to-end fusions following telomere attrition were evident even when core components of the c-NHEJ pathway (DNA ligase 4 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs)) were deleted. These experiments hinted that alt-NHEJ could be responsible for processing dysfunctional telomeres in the early stages of tumorigenesis. Analysis of telomere fusion junctions in human tumours revealed hallmarks of alt-NHEJ repair — frequent microhomologies and extensive deletions — further implicating this error-prone repair pathway as operating at dysfunctional telomeres. Genetic manipulation of shelterin in mouse cells indicated that alt-NHEJ is repressed in a redundant manner. Specifically, ligase 3-mediated telomere fusions were maximally observed when the shelterin complex was completely depleted in Ku70–Ku80-deficient mouse cells. The mechanism by which redundant suppression of alt-NHEJ is achieved has not been fully established. It is possible that the activity of alt-NHEJ is dependent on signalling by both ATM and ATR, which manifests when the entire shelterin complex is lost. In agreement with this idea, co-depletion of TRF2 and TPP1 — which activate the two kinases, respectively — is sufficient to trigger efficient alt-NHEJ activity. Similarly, the mechanism by which Ku inhibits alt-NHEJ remains unknown. It has been proposed that Ku has a higher binding affinity to DSBs than PARP1 (poly(ADP-ribose) polymerase 1) and could therefore block alt-NHEJ by repressing PARP1-mediated signalling. Alternatively, Ku might exert its effect by inhibiting 5'end resection, which is a prerequisite for alt-NHEJ-mediated repair.

Polishing the end: the art of making overhangs.

Telomere ends are subject to two forms of nucleolytic processing, each of which is carried out by independent machineries and regulated differently. Following telomere replication, physiological processing of telomere ends generates a 3’ overhang, a crucial structure for telomere protection. This is mediated by a number of factors, including the shelterin subunits TRF2 and POT1B (in mice). TRF2 recruits the Apollo nuclease to resect blunt leading-strand ends and create short overhangs, whereas lagging-strand overhangs result from the removal of the RNA primer from the terminal Okazaki fragment. Subsequently, a long-range resection step is carried out by EXO1 (exonuclease 1), which acts on both leading and lagging strands and transiently elongates the overhang. Finally, overhang length is fine-tuned to an optimal length (∼50–300 nucleotides) with the help of the CST complex, which, in the case of mouse telomeres, is recruited by POT1B to assist during fill-in synthesis. It is important to note that the genetic analysis of 3’ overhang generation was primarily carried out in mouse cells, and whether human POT1 functions similarly to mouse POT1B remains to be determined.

In addition to the aforementioned physiological processing of telomeres, dysfunctional telomeres are subject to aberrant degradation. Hyper-resection of uncapped telomeres is inhibited by shelterin and by 53BP1, which is a general repressor of DNA end resection at DSBs. Deletion of TRF2 in 53BP1-null cells leads to an extended telomere overhang, mediated by ATM and dependent on the endonuclease ChiP (CtBP-interacting protein). A more substantial resection takes place following the deletion of both TRF1 and TRF2 and the creation of shelterin-free telomeres in 53BP1-deficient cells (Fig. 2f). The unmitigated resection of shelterin-free telomeres is executed by CtIP and EXO1 and is aided by the helicase BLM.

How the replication machinery navigates TTAGGG repeats.

TTAGGG repeats are prone to forming stable secondary structures (including quadruplex (G4) DNA) that challenge the replication machinery as it progresses through telomeric DNA. Among the various shelterin subunits, TRF1 has a major role in assisting the semi-conservative replication of telomeres. The function of RTEL1 during telomere replication is mediated by an interaction with the replication clamp PCNA (proliferating cell nuclear antigen). Inhibiting the RTEL1–PCNA interaction increases the incidence of replication fork stalling and telomere fragility. BLM is recruited to telomeres by direct binding to TRF1 and suppresses telomere fragility. Studies have also implicated WRN, another RecQ helicase, in facilitating lagging-strand telomere synthesis, although WRN does not function in the same pathway as TRF1. A parallel pathway that assists in the replication of telomeres is executed by CST. Inhibition of individual CST components compromises replication fork restart and leads to telomere fragility. The activity of CST is independent of TRF1, and the complex functions by assisting Pol α–primase activity at telomeres. Last, TRF2 is also thought to facilitate telomere replication by relieving topological constraints that would otherwise hinder replication–fork progression.

We currently know many of the players that assist telomere replication and counteract telomere fragility, but the dynamic interplay between the different factors assisting the replisome to progress through telomere DNA is unknown. In addition, the nature of the fragile aberrancy itself, and whether it is the result of altered packaging of the chromatin or actual DNA breaks and chromatin gaps, remains a mystery that in the future may be solved by super-resolution microscopy.

Homologous recombination at telomeres: keeping up with the neighbours.

The activity of the homologous recombination pathway at telomeres may seem to be less harmful than that of NHEJ, but it can affect cellular
survival when it alters telomere length. Homologous recombination at telomeres manifests in three major forms: exchange of sequence between sister (chromatid) telomeres (telomere sister chromatid exchange (T-SCE)), aberrant excision of t-loops (t-loop homologous recombination), and recombination that leads to alternative lengthening of telomeres (ALT).

T-SCE has detrimental consequences when an unequal exchange happens, in which case a daughter cell inherits a short telomere and suffers the deleterious impact of telomere uncapping. Loss-of-function analysis in mouse cells revealed that shelterin contributes to the repression of T-SCE together with the Ku complex, which is a general repressor of recombination in mammalian cells. Depletion of TRF2, RAP1 or POT1 in the context of Ku70–Ku80 complex deficiency stimulates exchange of sequences between telomeres on sister chromatids. The mechanism by which these factors inhibit T-SCE is unknown. With regards to POT1, it is conceivable that its binding to telomere ssDNA counteracts the loading of homologous recombination factors — RPA and, subsequently, RAD51 — thereby inhibiting recombination. RAP1 and TRF2 possibly stabilize the telomeric double-stranded DNA (dsDNA)–ssDNA junction and block strand invasion during homologous recombination. Consistent with this idea, in vitro studies indicate that TRF2 has a greater preference for binding to junction sites when bound to RAP1.

Figure 3 | The three facets of telomere homologous recombination: T-SCE (telomere sister chromatid exchange), t-loop (telomere loop) homologous recombination and ALT (alternative lengthening of telomeres).

a | Exchange of sequence between sister chromatid telomeres (marked in red and green) is inhibited by RAP1 (repressor activator protein 1), POT1 (protection of telomere 1) and Ku70–Ku80. b | T-loop homologous recombination is blocked by TRF2 (telomere repeat-binding factor 2). TRF2 recruits RTEL1 (regulator of telomere elongation helicase 1) during S phase to unwind the t-loop and therefore protect it from being cleaved by structure-specific endonuclease subunit SLX4. In addition, TRF2 inhibits t-loop excision by inhibiting the activity of NBS1–XRCC3 (Nijmegen breakage syndrome 1–X-ray repair complementing defective repair in Chinese hamster cells 3). c | Telomere repeats have the propensity to form stable quadruplex (G4) DNA structures, which would impede replication fork progression. It has been proposed that ATRX (α-thalassaemia/mental retardation syndrome X-linked) unwind G4 DNA, enabling the deposition of histone H3.3 and ultimately assisting replication fork progression. The activity of ATRX at telomeres inhibits various ALT (alternative lengthening of telomeres) phenotypes including T-SCEs, formation of telomere circles, intrachromosomal telomere recombination and formation of APBs (ALT-associated promyelocytic leukaemia nuclear bodies). DAXX, death domain-associated protein; HR, homologous recombination; PML, promyelocytic leukaemia; RPA, replication protein A.
Aberrant telomere homologous recombination leads to t-loop excision owing to the activity of DNA repair protein XRC3, NBS1 and SLX4 (FIG. 3b). The t-loop configuration poses a challenge to the replication machinery, as it needs to be unfolded for the replication fork to progress through telomeres, otherwise it could be excised. In addition to promoting t-loop formation, TRF2 protects t-loops from illegitimate homologous recombination. TRF2 recruits RTEL1 to unwind t-loops in S phase93. Deleting RTEL1 or inhibiting its interaction with TRF2 allows SLX4-mediated t-loop excision, resulting in the formation of double-stranded telomere circles (t-circles) and rapid telomere loss94,95. TRF2 also protects the t-loop from XRC3- and NBS1-mediated cleavage96-99. Notably, telomere trimming by XRC3 occurs in normal cells that possess long telomeres, including in the male germ line100,101. This mechanism has been proposed to provide an additional layer of telomere length regulation, although how it is kept in check to avoid rampant telomere shortening remains elusive.

ALT is activated in a subset of human tumours that lack telomerase activity102 to maintain the length of the telomere repeats103. Telomeres maintained by ALT typically cluster in ALT-associated PML bodies (APBs)104, have increased expression of TERRA105 and display elevated levels of T-SCE106. A hallmark of ALT is recombination between telomeres on separate chromosomes, which was demonstrated experimentally by interchromosomal copying of a telomere-embedded neomycin tag107. ALT telomeres are highly heterogeneous in length108 and are littered with non-canonical repeats, probably owing to recombination with subtelomeric regions109. Survival of ALT cells is compromised when homologous recombination factors (RAD51, MRN, RAD9, RAD17, RPA and others) are inhibited, confirming their dependency on ALT for telomere maintenance (for a review, see REF. 109).

What triggers ALT and why it is activated in a subset of tumours remains unknown. A strong candidate is the histone chaperone ATRX, which is mutated in a large majority of cells and tumours that exploit the ALT pathway110,111. Reintroducing ATRX into ALT cells suppresses T-SCE, APB and c-circle formation, and inter-chromosomal telomeric recombination112,113 (FIG. 3c), ATRX is part of the SWI/SNF family of ATP-dependent helicases114 and associates with chromatin by binding to sites of histone H3 Lys9 trimethylation (H3K9me3), which are enriched at telomeric DNA115. ATRX also interacts with the histone chaperone DAXX (death domain-associated protein), allowing the deposition of the histone variant H3.3 at repetitive sequences of telomeres and pericentromeres116,117. The mechanism by which ATRX protects TTAGGG repeats from aberrant recombination remains unclear. However, several lines of evidence suggest that it may relate to telomere replication. First, in vitro studies indicate that ATRX binds to and unwinds G4 DNA118. Second, ATRX-deficient ALT cells accumulate increased levels of RPA at telomeres117,119. Last, re-expression of ATRX in ALT cell lines reduces the frequency of replication fork stalling120. Taken together, these studies suggest that both the helicase-unwinding activity of ATRX and the histone chaperone properties of the ATRX–DAXX complex are likely to counteract telomere recombination by resolving stable secondary structures that would otherwise impede fork progression. It is important to emphasize that ATRX depletion by itself is not sufficient to induce ALT112,113, indicating that additional genetic alteration(s) are necessary. Interestingly, deletion of the gene encoding the histone chaperone ASF1 is sufficient to trigger ALT-like phenotypes in telomerase-positive cells121, and binding of the nucleosome-remodelling deacetylase (NuRD) complex to variant repeats found at telomeres in ALT cells creates a permissive environment for recombination122. Such observations reinforce the notion that alteration in chromatin status renders telomeres conducive to homologous recombination. In addition to chromatin environment, the presence of TERRA–telomeric DNA hybrids was proposed to affect ALT — reduced levels of these RNA–DNA hybrids leads to a significant reduction in homologous recombination-mediated telomere elongation123.

In order for ALT telomeres to engage in interchromosomal recombination, they must first disengage from their cohered sister chromatid and move across the nucleus to meet a telomere on another chromosome. ATRX was proposed to regulate this choice between inter- and intratelomeric recombination. In the absence of ATRX, cohesion between sister telomeres persists, prompting an increase in T-SCE124. In addition, HOP2 (homologous-pairing protein 2 homologue), a protein that is normally required for synopsis of meiotic chromosomes, was recently shown to promote rapid and directional movement of telomeres over micrometre distances before their synopsis with recipient telomeres during ALT125.

**Using telomeres to discover DDR genes**

The realization that inhibition of shelterin activity marks telomeres as sites of DSBs provides an opportunity to interrogate various aspects of the DDR using telomeres as an experimental system. As discussed above, removal of particular shelterin components activates specific DNA damage signalling pathways and repair mechanisms. Accordingly, shelterin manipulation provides a tractable system that has been used to gain insight into the mechanistic basis of DSB repair in mammalian cells.

**Ingredients to make sticky ends**

Different approaches have been used over the years to isolate factors that bind to functional as well as dysfunctional telomeres. A quantitative telomeric chromatin isolation protocol (QTIP) was applied to identify differences in telomeric chromatin composition between cells with functional telomeres and cells with telomeres depleted of TRF2 and POT1 (REF. 28). In a similar approach, proteomics of isolated chromatin segments (PICH)29 was used to compare functional telomeres to those rendered dysfunctional following the removal of TRF2 (REF. 29). These approaches confirmed that dysfunctional telomeres are recognized as site of DNA damage and recruit the same repair factors that are associated with DSBs at other sites in the genome. Moreover, these methods and others helped
Telomere biology disorder (TBD). One of a set of pathologies that are defined by the presence of short telomeres.

A promiscuous polymerase for a sloppy repair pathway. The robust fusions observed in shelterin-free and Ku70–Ku80-deficient cells enabled investigation of the basis of the increased mutagenicity of alt-NHEJ. Using deep sequencing, non-TTAGGG nucleotide insertions were identified at the junction between two fused telomeres\textsuperscript{135} (FIG. 5). Such random insertions had previously been identified in other cells in which alt-NHEJ is active\textsuperscript{136} and provide a molecular signature for this repair pathway. To identify the enzymatic activity responsible for these insertions, a number of low-fidelity DNA polymerases were inhibited in shelterin-free, Ku80-null cells. This led to the identification of the translesion DNA polymerase Pol θ\textsuperscript{137} as a key alt-NHEJ factor that catalyses the addition of random nucleotides at fusion junctions\textsuperscript{135} and stimulates the joining of opposing ends of a broken DNA\textsuperscript{138,139} (FIG. 5). Depletion of mammalian Pol θ hinders alt-NHEJ at uncapped telomeres, blocks non-reciprocal chromosomal translocations in mouse embryonic stem cells\textsuperscript{138} and inhibits repair of endonuclease-induced DNA breaks\textsuperscript{138–140}. The function of this translesion polymerase is conserved in Drosophila melanogaster\textsuperscript{141} and Caenorhabditis elegans\textsuperscript{142}. Notably, Pol θ inhibition in mammalian cells was marked by an increase in homologous recombination\textsuperscript{138,139}, indicating that the erroneous polymerase potentially influences the choice of DSB repair pathway in S phase, when both homologous recombination and alt-NHEJ are most active\textsuperscript{139}.

Pol θ is overexpressed in several human cancers\textsuperscript{143–144}, especially those with homologous recombination deficiency\textsuperscript{139}. Interestingly, depletion of Pol θ in BRCA-mutant tumour cells resulted in significant accumulation of chromosomal aberrations and unrepaired breaks, and compromised cellular survival\textsuperscript{138,139}. Given the increased mutagenicity of alt-NHEJ, it is tempting to speculate that this compensatory mechanism shapes the genome of homologous recombination-defective cancers and therefore influences tumour progression and resistance to therapy.

Telomeres gone bad

Alterations in the activity of telomere-associated proteins are important factors in the onset of human diseases. Dyskeratosis congenita, the prototypical telomere biology disorder (TBD), is caused by mutations in genes involved in telomere length regulation. To date, known mutations causing dyskeratosis congenita have been found in telomerase genes (telomerase RNA template component (TERC) and telomerase reverse transcriptase (TERT)), the TERC-regulating gene H/ACA ribonucleoprotein complex subunit 4 (also known as dyskerin), and the genes encoding the shelterin component TIN2 and the helicase RTEL1, as well as in TCAF1 (also known as WRAP53), NOP10 and NHP2 (REFS 145–154). Dyskeratosis congenita patients have critically short telomeres and display a plethora of symptoms that range from impaired tissue regeneration capacity to cognitive defects. Severe variants of dyskeratosis congenita include Hoyeraal–Hreidarsson syndrome and Revesz syndrome. Less clinically severe variants, such as subsets of apparently isolated aplastic anaemia or pulmonary fibrosis,
have also been recognized as TBDs. The genetic basis of TBDs, as well their clinical manifestations and implications, have recently been discussed in an excellent review. Here, we focus exclusively on how alterations in telomere length and in telomere-associated proteins affect genomic stability and have an impact on cancer development.

**The good the bad and the ugly: telomeres and cancer.** In certain types of cancer, telomere dysfunction is considered to be a key trigger for chromosomal instability and a promoter of tumorigenesis. Rapid proliferation of pre-neoplastic cells leads to gradual telomere shortening, which ultimately triggers a DDR, inducing cellular senescence and/or apoptosis. This illustrates the tumour suppressor function of telomere shortening that limits the proliferative potential of cancer cells.

It is estimated that the accrual of five dysfunctional telomeres in a cell is sufficient to elicit a DDR and induce senescence. Despite losing their end protection, telomeres in senescent cells remain non-fusogenic, and it has been reasoned that this is due to the retention of few molecules of TRF2 that block end joining. Inactivation of p53 and/or RB pathways allows cells to bypass senescence, leading to telomere attrition and formation of dicentric chromosomes. This is known as telomere crisis, and it is estimated that ~50% of chromosome end-to-end fusions are completely devoid of TTAGGG.

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**Figure 4 | Telomeres as a tool to investigate DNA end resection and classical non-homologous end joining (c-NHEJ).**

(a) An overview of the assay to monitor c-NHEJ and DNA end resection at telomeres. Southern blot analysis allows the visualisation of telomere fusion events. Genomic DNA is cleaved with frequently cutting restriction enzymes, resolved on a denaturing gel and hybridized with a radiolabelled telomere probe. As TTAGGG repeats are not cut by restriction enzymes, they are resolved in the context of dysfunctional telomeres in TRF2-deficient cells. Double-strand break (DSB) sensing by the MRE11–RAD50–NBS1 (MRN) complex triggers a signalling cascade by recruiting autophosphorylated ataxia telangiectasia mutated (ATM) and phosphorylates the histone variant H2AX at Ser139 (REF. 197), which recruits MDC1 (mediator of DNA damage checkpoint protein 1) to sites of breaks. ATM then phosphorylates the histone variant H2AX at Ser139, which then recruits the effector proteins RIF1 (RAP1-interacting factor 1) and PTIP (Pax transactivation domain-interacting protein), both of which bind to phosphorylated 53BP1. RIF1 functions in part by recruiting REV7 (also known as MAD2L2) to sites of breaks, where it inhibits end resection.

(b) A schematic representing key players that promote c-NHEJ and block DNA end resection, focusing on factors that were studied in the context of dysfunctional telomeres in TRF2-deficient cells. Double-strand break (DSB) sensing by the MRN complex triggers a signalling cascade by recruiting autophosphorylated ataxia telangiectasia mutated (ATM) and phosphorylates the histone variant H2AX at Ser139 (REF. 197), which recruits MDC1 (mediator of DNA damage checkpoint protein 1) to sites of breaks (ATM). ATM then phosphorylates the histone variant H2AX at Ser139, which then recruits the effector proteins RIF1 (RAP1-interacting factor 1) and PTIP (Pax transactivation domain-interacting protein), both of which bind to phosphorylated 53BP1. RIF1 functions in part by recruiting REV7 (also known as MAD2L2) to sites of breaks, where it inhibits end resection.

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repeats. Although most cells succumb to telomere crisis, rare survivors reactivate telomerase (or engage ALT) to replenish telomere repeats and proliferate indefinitely.

It was first proposed by McClintock that dicentric chromosomes undergo repeated cycles of breakage–fusion–bridge leading to chromosomal rearrangements. Almost eight decades later, the fate of human dicentric chromosomes derived from telomere fusions was traced using live-cell imaging. Surprisingly, dicentric chromosomes form anaphase bridges that persist through mitosis and are processed by the cytoplasmic nuclease TREX1 (three prime repair exonuclease 1). Clones that survive this crisis stage display chromothripsis and kataegis, which are localized hypermutation events often found in cancer genomes. Interestingly, previous work suggested a crucial role for DNA ligase 3 in the survival of cells undergoing telomere dysfunction. It is therefore possible that DNA ligase 3 is required for processing of TREX1-generated DNA breaks, leading to hypermutagenesis.

This paradigm of telomere dysfunction and cancer has been tested in vivo using the telomerase-knockout mouse. When combined with p53 mutations, later generations of telomerase-null mice display accelerated tumour formation and a shift in the tumour spectrum towards mostly carcinomas, characterized by non-reciprocal translocations and chromosome fusions. One caveat with the telomerase-knockout mouse model is that constitutive telomerase deficiency constrains tumour progression. In order to firmly establish the function of telomere dysfunction in malignancy and metastasis, an inducible telomerase allele was studied in the context of a PTEN mouse model of prostate cancer. Reactivation of telomerase in tumour cells that have already experienced telomere dysfunction was sufficient to suppress DNA damage signalling, and, importantly, resulted in the formation of highly metastatic tumours that invade the bone.

In addition to these animal studies, evidence in support of a role for telomere dysfunction during tumorigenesis came from the analysis of telomeres in cells derived from cancer patients at different stages of the disease. Specifically, telomere fusions, which were detected molecularly using a PCR-based method, were evident in chronic lymphocytic leukaemia (CLL) and breast cancers, and were found to be predictive of poor prognosis. The reactivation of human telomerase is crucial for malignant progression. Telomerase activity has been detected in ~90% of human cancers, and its inhibition limits the survival of human cancer cells. Mutations in the promoter region of TERT are among the most prevalent mutations in cancers. The first mutations identified in a genome-wide association study of melanoma patients are in close proximity to the TERT transcription start site and create a binding motif for the ternary complex factor (TCF) and E-twenty-six (ETS)-domain transcription factors. Subsequent sequence analysis identified similar point mutations in the TERT promoter in a wide range of cancers; in glioblastomas, the mutations facilitate the recruitment of the multimeric GA-binding protein (GABP) transcription factor to the TERT promoter region. In many cases, the mutations correlate with increased TERT transcription and enhanced telomerase activity. The TERT promoter mutations were recently engineered into human embryonic stem cells (hES cells) using the CRISPR–Cas9 editing system. In pluripotent cells, which already express TERT, the mutations did not increase telomerase activity, but they prevented telomerase silencing upon the development of cancer.

Figure 5 | The mechanism by which DNA polymerase θ (Pol θ) promotes alternative non-homologous end joining (alt-NHEJ). Sequence analysis of shelterin-free telomeres in Ku-deficient cells identified random nucleotide insertions at telomere fusion junctions. Subsequent genetic studies identified Pol θ as a key alt-NHEJ factor that promotes the joining of dysfunctional telomeres. Following double-strand break (DSB) formation or telomere uncapping, DNA ends are resected to create short 3’ overhangs. On the basis of in vitro experiments, genetic studies and sequence analysis of fusion junctions, Pol θ seems to be capable of extending the 3’ single-stranded DNA (ssDNA) using a combination of template-dependent as well as template-independent activities, the latter potentially mediated through a snap-back intermediate. The incorporation of random nucleotides at sites of breaks is predicted to increase the level of microhomology, thereby promoting the synopsis of opposite ends of a DSB. Annealed intermediates are then subject to fill-in synthesis by Pol θ, a step that would stabilize the duplexed DNA. Ultimately, the DNA is joined by DNA ligase 3.

Chromothripsis
A mutational phenomenon that involves catastrophic shattering and rebuilding of chromosomes, leading to multiple clustered chromosomal rearrangements.

Kataegis
Clustered point mutations that localize to particular regions of certain cancer genomes.
The proliferation of tumours is supported by the reactivation of telomerase, which stabilizes chromosome ends and prevents rearrangement due to breakage–fusion–bridge cycles. POT1 mutations are associated with mild chromosome fusion phenotype, which is predicted to induce chromosomal elongation. POT1 mutations also manifest in a telomere dysfunction in cancer. POT1 mutations induce telomere fragility and are associated with considerable telomere elongation. Two independent pathways trigger telomere dysfunction in cancer: A novel way in which cancer cells do business. The sequencing of cancer genomes highlighted a potentially novel mechanism capable of inducing telomere dysfunction and promoting genomic instability in tumours. Acquired mutations in POT1 were noted in CLL (chronic lymphocytic leukaemia), and shortly thereafter, missense variants of POT1 were identified in familial melanoma, gliomas, mantle cell lymphomas, and T-cell leukaemia/lymphoma. Interestingly, analysis of the clonal evolution of several mutations in CLL patients suggested that POT1 mutations arise early in CLL development and are likely to contribute to disease progression. Despite their prevalence among many different cancer types, the mechanism by which POT1 mutations induce telomere dysfunction and influence tumour progression is not fully understood. Limited functional analyses indicate that POT1 mutations lead to telomere elongation, increased telomere fragility and mild telomere fusion phenotype. Chromosome end-to-end fusions can instigate breakage–fusion–bridge cycles, leading to increased genomic instability in POT1-mutated tumours. The observed telomere fragility suggests that replication defects triggered by POT1 alterations constitute a novel type of tumour-promoting mechanism. Future experiments are necessary to reveal how the identified cancer-associated POT1 mutations induce telomere fragility.

The identification of POT1 mutations raises the question of whether other shelterin subunits, especially ones that induce telomere fragility, are mutated in cancers. Nonsense mutations in TPPI and RAP1 were recently detected in melanoma. Although no TRF1 mutations have been detected so far, it is noteworthy that mice with reduced TRF1 levels have increased incidence of lymphoid tumours, and deletion of TRF1 in p53-null keratinocytes leads to squamous cell carcinomas. In the same way, mice carrying a mutation in RTEL1, which affects telomere replication, display accelerated tumorigenesis.

But humans are not mice! A recurrent concern in evaluating mouse models of human diseases is that humans, after all, are not mice. Telomere biology is not an exception and there are significant differences between human and mouse telomeres that need to be taken into consideration when evaluating mouse models of telomere dysfunction. Two key differences are the length of telomeres and the regulation of telomerase. Mice have significantly longer telomeres compared with humans, and they express telomerase in most cell types. As a result, telomere shortening is not a limiting factor in the lifespan of a mouse cell or in murine differentiation of hES cells.

**Figure 6** Two independent pathways trigger telomere dysfunction in cancer. A | Telomere attrition induces telomere dysfunction and promotes gross chromosomal rearrangement due to breakage–fusion–bridge cycles (a non-reciprocal translocation is shown). Telomerase reactivation is a key event that stabilizes chromosome ends and supports the proliferation of tumours. B | Recurrent mutations in the TERT promoter are common in many cancers and seem to create a de novo binding site for the transcription factor GABP (GA-binding protein transcription factor). C | Deficiency in the shelterin subunit POT1 (protection of telomere 1) represents a novel mechanism that triggers telomere dysfunction in cancer. POT1 mutations induce telomere fragility and are associated with considerable telomere elongation. POT1 mutations also manifest in a mild chromosome fusion phenotype, which is predicted to induce chromosomal instability and augment tumour progression. D | POT1 mutations cluster primarily in its oligonucleotide-binding (OB) fold domains and are widespread across many tumour types.
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DNA DAMAGE REPAIR

38. In this publication, the authors demonstrate that ATM and ATR signalling are repressed by TRF2 and POT1, respectively, and that efficient end joining of deprotected telomeres is dependent on DNA damage signaling.
42. This publication highlights a mechanism by which TRF2 inhibits ATM signalling and identifies the IDDR domain of TRF2 as important for RNF168 inhibition.
45. This report demonstrates that deprotection of telomeres by inhibiting the function of human TRF2 leads to chromosome fusions. (2016).


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Removal of shelterin reveals telomeres.

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Wang, Y., Ghosh, G. & Hendrickson, E.A. Ku86 extensive deletion and microhomology, and can result in lymphocytic leukaemia and report the incidence of telomeres.


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This report identifies ALT as a telomerase-independent mechanism that maintains telomeres in a subset of tumors.


This publication shows that dicentric chromosomes can persist through mitosis and are resolved 3–20 hours after anaphase by the cytoplasmic nucleosome TREC1. This is thought to promote chromothripsis and kataegis.


Using telomere-deficient cell lines, the authors provide in vivo evidence that telomere erosion in a p53-null background promotes the development of epithelial tumours that display complex chromosomal rearrangements, including non-reciprocal translocations.


In this report, the authors identify a germline mutation in the promoter of TERT, which creates a binding motif for ETS transcription factors.


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Competing interests statement
The authors declare no competing interests.