Alternative lengthening of telomeres: models, mechanisms and implications

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Abstract | Unlimited cellular proliferation depends on counteracting the telomere attrition that accompanies DNA replication. In human cancers this usually occurs through upregulation of telomerase activity, but in 10–15% of cancers — including some with particularly poor outcome — it is achieved through a mechanism known as alternative lengthening of telomeres (ALT). ALT, which is dependent on homologous recombination, is therefore an important target for cancer therapy. Although dissection of the mechanism or mechanisms of ALT has been challenging, recent advances have led to the identification of several genes that are required for ALT and the elucidation of the biological significance of some phenotypic markers of ALT. This has enabled development of a rapid assay of ALT activity levels and the construction of molecular models of ALT.

End-replication problem
The inability of semi-conservative DNA replication to completely copy the ends of linear DNA molecules. Removal of the RNA primer from the terminal Okazaki fragment on the lagging strand results in incomplete copying of the terminus of that strand, which then provides a shorter template for copying in the next round of DNA synthesis.

DNA-damage response
The coordinated cellular response to DNA damage, including localization of DNA-damage sensing and repair molecules to the site of damage.

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Unlike the circular genomes of many bacteria and archaea, the eukaryotic nuclear genome is packaged into discrete linear chromosomes. Linear chromosomes pose a problem of fundamental biological importance: their ends (telomeres) must be distinguished from chromosome breaks to avoid 'repair' processes that would result in chromosome end-to-end fusions. Such events result in the formation of chromosomes with two or more centromeres that may be pulled to opposite poles during mitosis, causing chromosome breakage and the need for further repair. Repeated cycles of these events would result in rampant genomic instability and, often, in cell death.

Linear chromosomes pose a second fundamentally important problem: their ends cannot be completely replicated by the DNA-replication machinery. Because of this 'end-replication problem', telomeres shorten with each round of DNA replication[1,2]. In the absence of any counterbalancing lengthening processes, the gradual telomere attrition that accompanies cellular proliferation eventually results in excessive telomere shortening and a DNA-damage response (DDR) at the chromosome ends that elicits permanent growth arrest, referred to as senescence[3,4].

The problems associated with segmentation of the eukaryotic genome into linear chromosomes are solved in most eukaryotes, including mammals, by specialized telomeric nucleic acid–protein (nucleoprotein) complexes. Telomeric DNA has a repetitive G-rich sequence (for example, in all vertebrates the sequence is 5′-TTAGGG-3′) that can be synthesized *de novo* by a reverse transcriptase enzyme, telomerase[5]. The DNA is mostly double-stranded, but has a single-stranded terminus that on average is 130–210 bases long in human cells[6] (FIG. 1a). The telomere can fold back on itself, and the single-stranded terminus can invade duplex telomeric DNA, resulting in the formation of a telomere loop (t-loop)[7] (FIG. 1b). The telomeric DNA is bound by the shelterin proteins, some of which specifically recognize double- or single-stranded telomeric DNA (FIG. 1c–e). This nucleoprotein complex prevents the chromosome end from being detected as a DNA double-strand break (DSB)[8].

Most somatic human tissues do not have sufficient telomerase activity to prevent telomere attrition, so their continued proliferation eventually results in senescence. Oncogenesis usually depends on extensive cell proliferation and therefore on avoidance of telomere shortening and senescence[9]. About 85% of all human cancers achieve this through increased activity of telomerase[10], which is therefore a prime target for developing anticancer therapies. Of the remaining 15%, most are able to maintain their telomere lengths in the absence of telomerase by one or more mechanisms referred to as alternative lengthening of telomeres (ALT). Although ALT occurs in common cancers, such as breast carcinomas, it tends to be most prevalent in tumours of mesenchymal origin[11–17]; the reasons for this association are unclear, but human mesenchymal stem cells might have a particular tendency to activate ALT[18]. The tumour types in which ALT is prevalent include glioblastoma multiforme (the most common type of primary malignant brain tumour in adults), osteosarcomas and some types of soft tissue

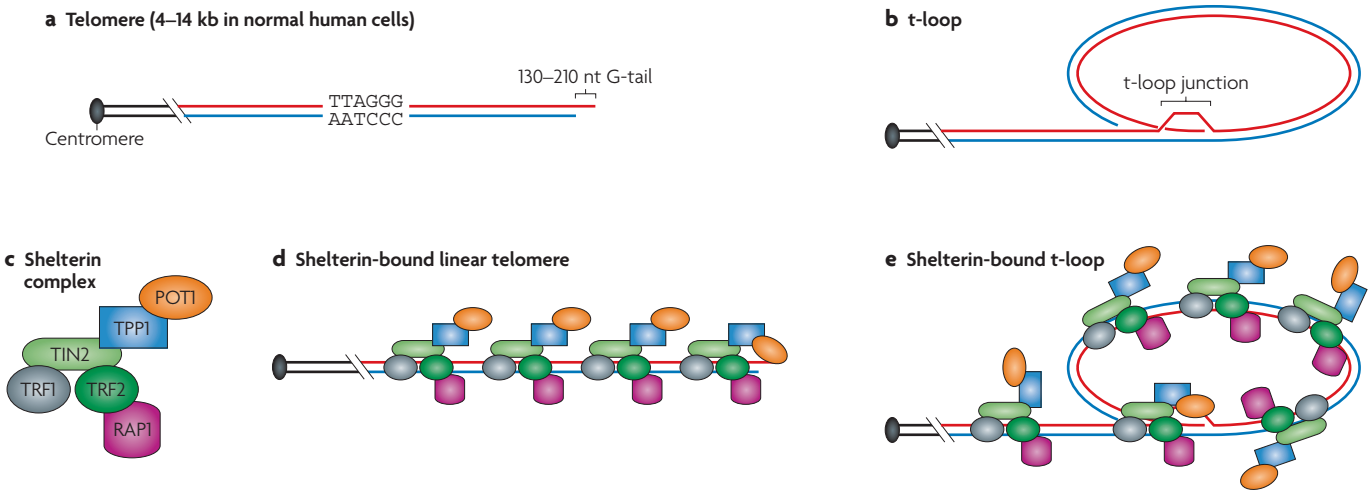

Figure 1 | **Structure of telomeres. a** | Vertebrate telomeres contain repetitive DNA with the sequence (5′-TTAGGG-3′)n. Most of this DNA is double-stranded apart from the terminus, which consists only of the TTAGGG (or 'G-rich') strand. **b** | The telomere can fold back on itself, and the single-stranded terminus can invade duplex telomeric DNA. This results in the formation of a telomere loop (t-loop). **c** | Telomeric DNA is bound by the six-subunit shelterin complex. **d**,**e** | Two shelterin proteins (telomeric repeat-binding factor 1 (TRF1, also known as TERF1) and TRF2 (also known as TERF2)) bind directly to double-stranded telomeric DNA, and one (protection of telomeres 1 (POT1)) binds single-stranded telomeric DNA directly. POT1, TPP1 (also known as ACD), TRF1-interacting nuclear protein 2 (TIN2, also known as TINF2) and RAP1 (also known as TERF2IP) also interact indirectly with double-stranded telomeric DNA through their interactions with other shelterin proteins.

Senescence
The permanent removal of a cell from the cell cycle without the loss of viability. Telomere-dependent senescence results from the natural erosion of human telomeres.

Telomerase
The reverse transcriptase that catalytically adds *de novo* telomeric repeats to the chromosome ends.

Shelterin
The six-subunit protein complex that binds specifically to telomeric DNA and regulates telomere function.

Extrachromosomal telomeric DNA
DNA molecules consisting of telomeric repeats that are not associated with the chromosomes. These fragments can be linear, circular, single-stranded, duplex or more complex structures.

Telomeric circle
A double-stranded circular extrachromosomal DNA molecule containing telomere repeat sequences.

Promyelocytic leukaemia nuclear body
A spherical nuclear structure that is associated with several functions, including DNA repair, senescence, apoptosis, viral defence, proteolysis and stress response, and which is named after one of its constitutive components, promyelocytic leukaemia (PML) protein.

sarcomas, and tend to have a particularly poor prognosis. Gaining a more detailed understanding of ALT to enable the development of ALT-targeted treatments or early detection of ALT-positive cancers may therefore be of particular therapeutic value.

Here, we review the current understanding of ALT. Although it seems highly likely that ALT activity in cancer cells is a dysregulated version of a normal process, ALT has been clearly documented only in anomalous situations, such as in cancer and genetically modified organisms. We therefore describe the known phenotypic characteristics of ALT-positive cancer cells and how some of these characteristics are generated, then discuss evidence that ALT involves recombination. We consider two recombination-based models of ALT activity and examine what is known about ALT at the molecular level. Most, if not all of the molecules known to be involved in ALT seem to be present in normal cells, so a question of particular interest is what prevents telomere length being maintained by ALT in normal cells. We also discuss recent findings about altered telomere function in ALT cells, and possible locations of ALT activity in the nucleus.

Phenotypic characteristics of ALT cells

ALT-positive tumours or immortalized cell lines are able to maintain their telomere length throughout many cell doublings in the absence of telomerase activity. The telomeres of ALT cells retain many canonical attributes, including the presence of duplex TTAGGG repeats with single-stranded terminal overhangs of the G-rich strand (G-tails), the presence of the shelterin complex and other telomere-associated proteins, and the ability to form t-loops (FIG. 1).

In addition to these features, ALT cells show a number of unusual characteristics; one of the most striking is an abundance, separate from the chromosomes, of DNA with telomeric sequences. This extrachromosomal telomeric DNA takes many forms, including predominantly double-stranded telomeric circles (t-circles)[19,20], partially single-stranded circles (referred to as C-circles or G-circles depending on whether it is the C-rich or G-rich strand, respectively, that is essentially continuous)[21,22], linear double-stranded DNA[23,24] and very high molecular weight 't-complex' DNA that is likely to contain abnormal, highly branched structures[21].

Telomeric DNA (either chromosomal or extrachromosomal) and associated binding proteins may be found in a subset of promyelocytic leukaemia nuclear bodies (PML nuclear bodies) in ALT cells. PML bodies containing telomeric chromatin are highly characteristic of ALT cells and are therefore referred to as ALT-associated PML bodies (APBs)[25] (BOX 1). Other characteristics of ALT cells include highly heterogeneous chromosomal telomere lengths (ranging from undetectable to extremely long)[26], rapid changes in telomere length[27,28] and a greatly elevated level of recombination at telomeres[29]. Although these phenotypic characteristics of ALT may be useful markers for the presence of ALT activity, they are not all equally specific for ALT, as shown by the contrast between t-circles and C-circles, which is discussed below.

t-circles. It has been proposed that double-stranded t-circles are involved in both ALT and normal telomere biology[30]. It seems likely that t-circles result from the resolution of telomere-loop junctions (t-loop junctions) (FIG. 1b) by recombination enzymes, resulting in free t-circles and

Box 1 | **Are PML bodies the platforms for ALT activity?**

Despite intensive study, the function of promyelocytic leukaemia (PML) nuclear bodies is not entirely clear, but it seems that they are macromolecular platforms that are involved in a number of nuclear processes, including senescence and DNA-damage response (DDR)[102].

PML bodies that contain telomeric DNA and associated telomere-binding proteins are highly characteristic of cells that use the alternative lengthening of telomeres (ALT) mechanism. They are rarely observed in other contexts and so are referred to as ALT-associated PML bodies (APBs)[25]. The telomeric DNA present in APBs can be attached to chromosome ends or extrachromosomal, and there is some evidence that APBs transiently associate with and dissociate from chromosome ends in a dynamic manner[103]. APBs have also been shown to contain recombination proteins that are required for telomere maintenance in ALT, so it is tempting to speculate that ALT activity may occur in these nuclear domains[25].

However, a number of observations seem to be inconsistent with the hypothesis that APBs are the site of telomeric lengthening in ALT cells. The formation of telomeric circles (t-circles) in telomerase-positive human cancer cells by the trimming of overlengthened telomeres was accompanied by the formation of APBs, but no evidence was found for ALT-mediated telomere maintenance[33]. This suggests that the functions of APBs include sequestration of extrachromosomal DNA, which is consistent with previous observations[104]. A cell line in which telomeres are maintained long-term in the absence of telomerase, and therefore in which an ALT mechanism must be used, has no APBs[105], showing that APBs are not essential for ALT activity. Moreover, it has been shown that large APBs form when ALT cells undergo cell cycle arrest or senescence, and that their formation is dependent on proteins — such as heterochromatin protein 1 (HP1) — that are involved in the compaction of chromatin, which would not be expected to facilitate recombination and DNA synthesis[106]. This is consistent with evidence that PML bodies are involved in cell cycle arrest and senescence[107]. Presumably because of the large numbers of telomeres that elicit a DDR in ALT cells[87], when wild-type p53 function is restored they rapidly become senescent and accumulate large APBs in a p21-dependent manner[106]. Methionine starvation of ALT cells also causes growth arrest accompanied by induction of large APBs[108].

However, there is also circumstantial evidence that APBs might be involved in ALT activity. First, in situations in which ALT activity is inhibited, the number of APB-positive cells often decreases[28,50]. Second, the homologous recombination-associated MRN complex (which is made up of meiotic recombination 11 (MRE11, also known as MRE11A), RAD50 and Nijmegen breakage syndrome 1 (NBS1, also known as NBN)) is required for APB formation[108,109]. Third, APBs are suggested to be active sites of ataxia telangiectasia mutated (ATM) and/or ataxia telangiectasia and Rad3-related (ATR) dependent DNA replication[110]. And finally, it has been reported that APBs increase in number and are present in a greater percentage of the cells in G2 phase[28,111] — the stage during which recombination is most active. These data need to be interpreted cautiously, however, given that APBs increase under situations of growth arrest and the cell cycle studies were mostly performed using drug-induced cell cycle arrest, and that under conditions of methionine starvation APB formation is associated with arrest in G0/G1 phase[108].

Recently, an intriguing observation was made: expressing a mutated herpesvirus ICP0 protein in ALT cells resulted in greatly enlarged PML bodies, and chromosome ends adhered to the exterior of these bodies[112]. It is not clear to what extent these enlarged PML bodies resemble APBs, which typically contain telomeric DNA at their centre, but in the presence of the mutant ICP0 protein, filamentous bridges were observed to connect telomeres in metaphase spreads, which is consistent with unresolved post-replicative intertelomeric recombination. This suggested that intertelomeric recombination was initiated but not resolved at the enlarged PML bodies.

In another recent study, large foci of telomeric DNA — which were similar to APBs in size and quantity of DNA — were observed in ALT cells in metaphase[87]. These foci were both chromosomal and extrachromosomal and were commonly observed interacting with multiple chromosome ends simultaneously in the presence of recombination proteins. The large telomere foci preferentially localized to telomeres that were eliciting a DDR, suggesting that interaction was occurring at dysfunctional telomeres and possibly between dysfunctional telomeres and extrachromosomal telomeric DNA. Unexpectedly, the large foci only colocalized with PML in 25–55% of occurrences, perhaps consistent with the cyclical degradation of PML bodies that occurs during metaphase. This also suggests that the core of APBs is the telomeric DNA and associated telomere-binding, DNA-repair and recombination proteins, and that this core interacts with and dissociates from PML bodies dynamically.

Given the evidence for and against the involvement of APBs in ALT-mediated telomere lengthening, it seems possible that there may be more than one class of APBs: large APBs that contain compacted chromatin and accumulate under conditions of cell cycle arrest, including senescence, and others that are the sites of ALT activity. This hypothesis awaits testing.

Telomere-loop junction
The DNA structure produced when the single-stranded telomere end is inserted into the duplex telomeric DNA in the formation of a telomere loop.

Telomeric repeat-binding factor 2
A shelterin protein that binds telomeric DNA directly as a homodimer. Its functions include telomere-loop formation, preventing telomere-specific DNA-damage responses and end-to-end chromosome fusions, and inhibiting some forms of telomeric homologous recombination.

Telomere trimming
Telomeres that are overlengthened by telomerase (or presumably by ALT) may undergo rapid shortening events, most likely by telomere-loop junction resolution.

Telomere-loop junction resolution
The processing of a telomere-loop junction by recombination enzymes. This may result in telomere truncation and the production of extrachromosomal telomeric DNA.

truncated telomeres[20]. This reaction is suppressed by the basic domain of a shelterin protein, telomeric repeat-binding factor 2 (TRF2, also known as TERF2), and is dependent on the recombination proteins Nijmegen breakage syndrome 1 (NBS1, also known as NBN) and X-ray repair cross-complementing 3 (XRCC3) in human cells and Rad52 in budding yeast[20,31,32]. The increased abundance of t-circles in ALT cells compared with non-ALT cells[19,20] initially suggested that t-circles result from an aberrant form of recombination that is upregulated in ALT cell lines. However, we recently showed that t-circles also occurred in telomerase-positive human cell lines when their telomeres were artificially elongated by increased expression of telomerase components[33]. The telomeres also became heterogeneous in length in the absence of any other markers of ALT activity[33]. The mean telomere length eventually reached a plateau, and we interpreted the data overall as indicating that human cells have a 'telomere trimming' mechanism that shortens overextended telomeres through telomere-loop junction resolution

(t-loop junction resolution), analogous to the yeast telomere rapid deletion mechanism[34]. The abundant t-circles in ALT cells may therefore result from telomere trimming counteracting otherwise excessive ALT-mediated telomere lengthening events, rather than being directly involved in the ALT mechanism.

C-circles. C-circles, however, seem to be much more specific than t-circles to ALT cells. We recently found that there is a quantitative relationship between the amount of ALT activity and the number of partially double-stranded telomeric circles consisting of an essentially complete C-rich strand and an incomplete G-rich strand[22]. For one ALT cell line it was estimated that there are approximately 1,000 of these C-circles per cell. The origin of C-circles is not clear. We speculate that they are generated by nucleolytic degradation of the G-rich strand of t-circles, but this requires experimental verification. ALT cells also contain G-circles, but these are 100-fold less abundant[22].

A quantitative assay showed that, on average, there are 750-fold more C-circles in ALT cells than in telomerase-positive cell lines or non-immortalized cell strains. Unusual cell lines that maintain telomere lengths in the absence of telomerase (and therefore by definition must use an ALT mechanism) but lack some or most of the usual phenotypic characteristics of ALT cell lines nevertheless contain abundant C-circles, suggesting that C-circles may be the most useful marker of ALT yet identified. In cultured cells that became immortalized spontaneously, there was a temporal correlation between the onset of ALT activity and the appearance of C-circles. Furthermore, when ALT was inhibited, most C-circles disappeared within 24 hours[22]. Assaying C-circle levels may therefore be a useful screen for chemicals that inhibit ALT activity.

Moreover, C-circles were detected in blood samples from patients with ALT-positive osteosarcomas[22]. This promising result suggests that assaying C-circle levels might have utility as a blood test for diagnosing ALT-positive tumours, or for monitoring effectiveness of their treatment. Validation of the clinical utility of the C-circle assay is ongoing.

ALT involves DNA recombination

The existence of a telomerase-independent telomere-length maintenance mechanism (TMM) was first demonstrated in telomerase-null mutant yeast, and the mechanism was found to be dependent on *RAD52*, a gene encoding a homologous recombination (HR) protein[35]. Evidence for the existence of one or more ALT mechanisms in some human cell lines was first provided by the observation that telomere lengths were maintained for many population doublings in the absence of telomerase[26,36], and the first indication of recombination at human telomeres was the finding that telomere lengths sometimes increased or decreased rapidly in telomerase-negative cells[27].

Several studies reported physical evidence of recombination at the telomeres of ALT-positive human cells. For example, a DNA tag in a single telomere was copied to other chromosome ends in ALT-positive cells but not in telomerase-positive cells[37], and some telomeres in ALT-positive cells have complex reorganizations of non-canonical repeats in proximal telomeric regions that are most easily explained by recombination between non-sister telomeres or extrachromosomal sequences[38]. Telomere sister chromatid exchanges (T-SCEs) were found to occur several orders of magnitude more frequently in ALT cells than in telomerase-positive cell lines or normal cells, without an increase in SCE frequency elsewhere in the genome[29,39]. It is not clear, however, that recombination activity is only increased at the telomeres of ALT cells, because although there was no evidence of increased activity in a recombination reporter assay[40], some minisatellite instability was found[17,41,42]. Evidence supporting recombination-dependent telomere maintenance in ALT cells is discussed below.

Recombination-dependent telomere elongation

Although it is generally agreed that telomere elongation in ALT cells requires a DNA recombination step, the mechanism of the lengthening step is uncertain. Two suggested mechanisms for telomere elongation, which are not mutually exclusive, are described here.

Unequal T-SCE model. One model is based on the observation that T-SCEs occur much more frequently in ALT cells than in telomerase-positive cell lines or normal cells[29,39]. The molecular explanation of T-SCEs remains unknown, although SCEs elsewhere in the genome may result from recombinational repair of broken replication forks[43]. There is some evidence that telomeric DNA in ALT cells contains nicks and gaps that may present a structural impediment to DNA replication and therefore result in T-SCEs[21]. Regardless of the mechanism of T-SCEs, their increased frequency in ALT cells has led to the hypothesis that unequal T-SCEs can result in one daughter cell that has a lengthened telomere and therefore a prolonged proliferative capacity, and another daughter cell with a shortened telomere and decreased proliferative capacity[44] (FIG. 2a). This could result in unlimited proliferation of the cell population, provided there is a mechanism for segregating every lengthened telomere into one daughter and every shortened telomere into the other[45,46]. To date, the existence of such a mechanism for the segregation of telomeres has not been established and this model remains hypothetical, although there is some recent evidence for significant non-random sister chromatid segregation in a subset of murine cells[47].

Homologous recombination-dependent DNA replication model. According to another hypothesis, ALT results from the recombination-mediated synthesis of new telomeric DNA using an existing telomeric sequence from an adjacent chromosomal telomere as a copy template[37,48] (FIG. 2b). This is consistent with the observation described above that a DNA tag placed into the telomeres of ALT cells was copied from one telomere to another, resulting in an increase in the number of tagged telomeres[37]. According to this hypothesis,

Telomere-length maintenance mechanism
Any process that extends telomere length to fully compensate for telomere erosion.

Homologous recombination
The genetic exchange between two DNA molecules of identical or very similar sequences. A strand of DNA from one molecule pairs with the complementary strand of the other molecule and vice versa.

Telomere sister chromatid exchanges
Exchanges of DNA between sister chromatids that are limited to the telomere.

Broken replication forks
When a DNA replication fork encounters a structural barrier it may break, resulting in the termination of coordinated DNA polymerization.

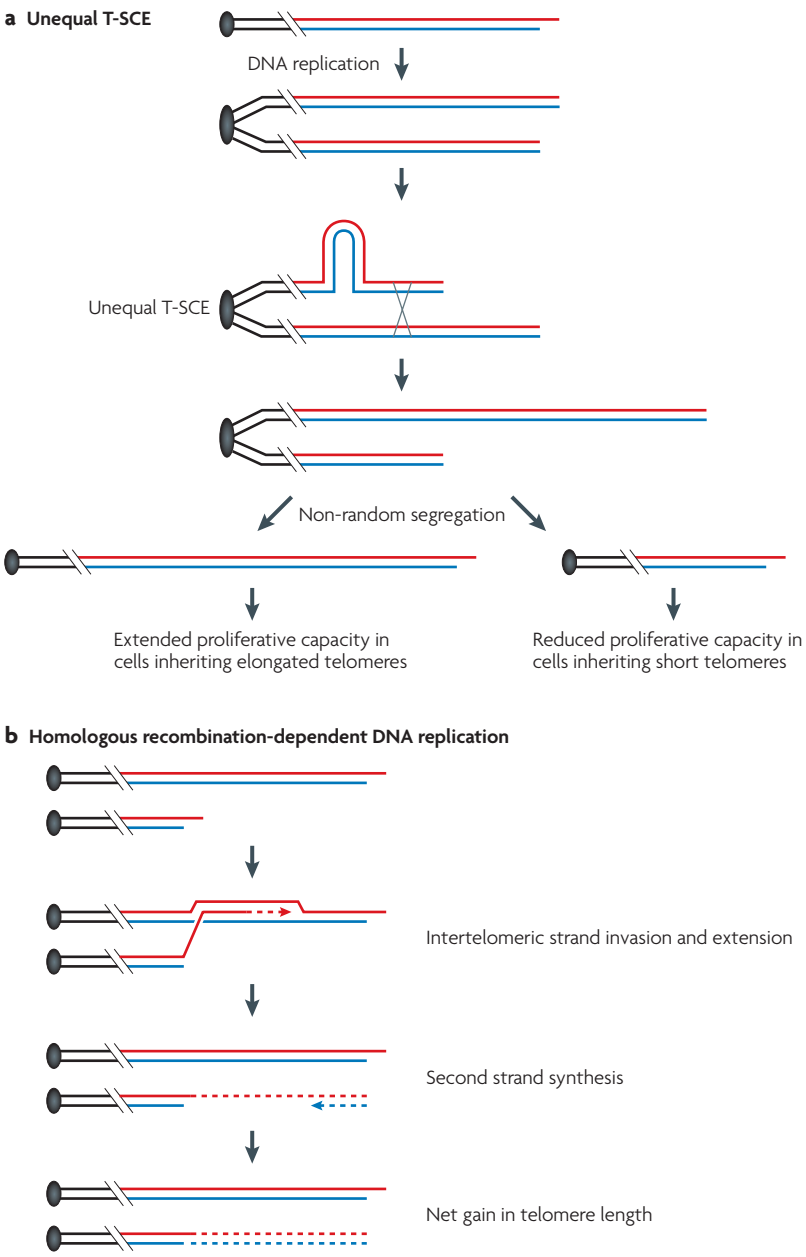

Figure 2 | **Two models of the alternative lengthening of telomeres mechanism.** **a** | It has been proposed that unequal telomere sister chromatid exchanges (T-SCEs) can result in one daughter cell that has a lengthened telomere and therefore a prolonged proliferative capacity, and another daughter cell with a shortened telomere and decreased proliferative capacity. This could result in the unlimited proliferation of the cell population, provided there is a mechanism for segregating the lengthened telomeres into one daughter and the shortened telomeres into the other. It is currently unknown whether such a mechanism for segregation of telomeres exists. **b** | It has also been proposed that alternative lengthening of telomeres results from recombination-mediated synthesis of new telomeric DNA using an existing telomeric sequence from an adjacent chromosomal telomere as a copy template.

telomere-templated DNA synthesis results in a net increase in telomeric DNA. We favour this model because the observed increase in the number of tagged telomeres[37] and the observed DNA sequence changes at telomeres of ALT cells[38] are not predicted by the unequal exchange and asymmetric segregation model.

In this HR-dependent model, the template DNA required for ALT activity is not necessarily the telomere of another chromosome (FIG. 2b). We recently showed that the synthesis of new DNA in a tagged telomere can occur without involving the telomere of another chromosome[49]. This indicates that the telomere can copy itself via t-loop formation (FIG. 3a) or that there is template-directed DNA-copying of one sister chromatid by another (FIG. 3b). It seems reasonable to propose that linear extrachromosomal telomeric DNA may also act as a copy template (FIG. 3c) analogous to the telomere of another chromosome, or that t-circles may be the template for telomeric extension by a rolling circle mechanism[48] (FIG. 3d). The possibility of such a mechanism is consistent with evidence that rolling circle-mediated telomere lengthening occurs in yeast, in which this process can be combined with interchromosomal recombination in a 'roll-and-spread' mechanism[30]. In addition, it has been shown that C-circles serve as an excellent substrate for rolling circle amplification *in vitro*[22], and it seems reasonable to speculate that they may also serve as a substrate for rolling circle-mediated elongation of telomeres in ALT cells *in vivo*. Annealing of a telomeric G-rich overhang to a single-stranded region of a C-circle followed by DNA polymerization from the chromosome end would promote rapid synthesis of G-rich telomeric DNA by rolling circle replication at the chromosome end.

Telomere-maintenance proteins in ALT cells

A number of recombination proteins have been shown by genetic analyses to be necessary for telomere maintenance in ALT cells. We propose here that these proteins can be classified as those required for ALT-mediated telomere elongation and those required for preventing telomere loss.

Telomere elongation: MRN complex. The components of the MRN complex (meiotic recombination 11 (MRE11, also known as MRE11A), RAD50 and NBS1) were the first proteins to be identified as necessary for ALT-mediated telomere maintenance[50,51]. MRN is a DNA-damage sensor that recruits the ataxia telangiectasia mutated (ATM) protein, one of the master controllers of cell cycle checkpoint signalling pathways, to a DSB and facilitates 5′ to 3′ resection of the DNA ends to create 3′ overhangs for the strand invasion necessary for HR[52]. MRN normally localizes to telomeres during the S and G2 phases by direct interaction with TRF2 (REF. 53), and this may contribute to G-tail formation[54–56]. According to the HR-dependent DNA replication model of ALT, telomere elongation requires DNA polymerization from a 3′-overhanging strand of telomeric DNA that has invaded an adjacent chromosomal telomere, a sister telomere, a t- or C-circle, or a t-loop[37,49] (FIGS 2b,3a–d). It is therefore likely that MRN promotes ALT activity by recruiting ATM to telomeres, initiating the recombination process and processing the chromosome end to form an extended telomeric 3′ overhang that can invade adjacent telomeric DNA, which can then be used as a copy template for extension of the telomere.

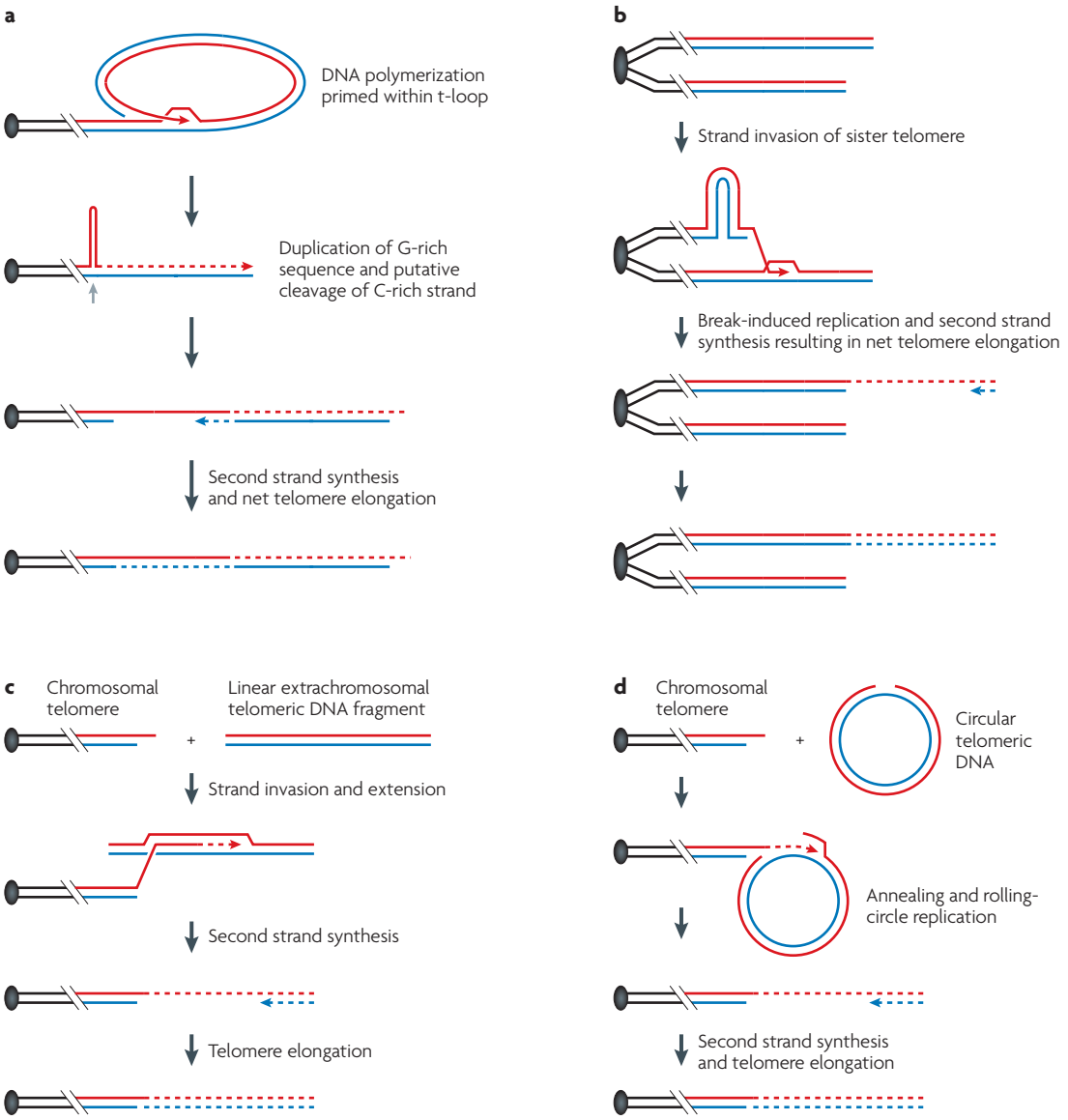

Figure 3 | **Alternative copy templates for recombination-mediated synthesis of telomeric DNA.** As an alternative to an adjacent chromosomal telomere (as shown in FIG. 2b), it is proposed that the copy template used for alternative lengthening of telomeres-mediated telomere lengthening may also be: the same telomere through telomere-loop (t-loop) formation (**a**); the telomere of the sister chromatid (**b**); linear extrachromosomal telomeric DNA (**c**); or circular extrachromosomal telomeric DNA (**d**). The light grey arrow indicates the site of putative cleavage of the C-rich strand.

Break-induced replication
Homologous recombination-mediated DNA repair that involves a 3′ overhang from a one-ended DNA break invading a homologous sequence on the end of another chromosome. This primes DNA replication to copy the sequence of the invaded chromosome onto the distal end of the invading chromosome.

Inhibiting MRN function in ALT cells by overexpression of a protein (SP100) that sequesters NBS1 (REF. 50), by constitutive knockdown of individual MRN subunits through short hairpin RNA (shRNA) expression[51] or by repeated transient transfection of small interfering RNAs (siRNAs) against RAD50 (REF. 57) resulted in telomere shortening. The rate of telomere erosion in MRN-inhibited ALT cells was similar to that in cells devoid of any TMM[50,51,57]. In long-term MRN-inhibition experiments, telomere shortening eventually ceased and short but stable telomere lengths were maintained with no reduction in cell viability, whereas in siRNA experiments, telomere shortening eventually resulted in an increase in cells that were senescence-associated β-galactosidase positive, and presumably senescent. The discrepancy may reflect the presence of residual ALT activity due to incomplete depletion of MRN in long-term expression experiments or the presence of redundant pathways that fulfil the function of MRN in ALT cells. Consistent with the possibility of redundant pathways, Mrx (the budding yeast MRN orthologue) promotes but is not essential for break-induced replication (BIR) in *Saccharomyces cerevisiae*[58]. The mechanism of BIR is proposed to be similar to the HR-mediated DNA replication model of ALT, but in BIR the copy template is a sister chromatid or another chromosome, and subtelomeric regions may also be copied to repair the broken chromosome.

Telomere elongation: SMC5–SMC6 complex. The eight-subunit structural maintenance of chromosomes 5 (SMC5)–SMC6 complex is also involved in HR, and three of the subunits (SMC5, SMC6 and methyl methanesulfonate-sensitivity 21 (MMS21, also known as NSMCE2)) are required for ALT[57]. Repeated transient transfection of ALT cells with siRNAs against SMC5 and MMS21 resulted in gradual telomere shortening consistent with inhibition of telomere elongation[57]. MMS21 is an E3 SUMO ligase that is required for response to DNA damage[59], and the action of the SMC5–SMC6 complex in ALT seems to involve MMS21-mediated sumoylation of shelterin components[57]. MMS21 can sumoylate TRF1 (also known as TERF1), TRF2, TRF1-interacting nuclear protein 2 (TIN2, also known as TINF2) and RAP1 (also known as TERF2IP). Also, the catalytic activity of MMS21 is necessary for APB formation in ALT cells, and expression of mutant TRF1 in which the lysine sumoylation target residues were replaced by arginine reduced the number of TRF1-positive APBs. The mechanism of action for SMC5–SMC6 in ALT is unknown, but if APBs are structural centres for telomere extension in ALT cells (BOX 1), SMC5–SMC6 may facilitate telomere recruitment to APBs through sumoylation of shelterin proteins. This suggests that SMC5–SMC6 acts upstream of MRN by first recruiting telomeres to APBs in which MRN may initiate recombination. Alternatively, SMC5–SMC6 may work downstream of MRN by promoting telomere extension in APBs following MRN-dependent strand invasion.

Proteins that prevent telomere loss in ALT cells. Other proteins, including flap endonuclease 1 (FEN1)[60], MUS81 (REF. 61), Fanconi anaemia group D2 (FANCD2) and Fanconi anaemia group A (FANCA)[62], have been identified that seem to be required for maintenance of telomeres in ALT cells but differ from the MRN and SMC5–SMC6 complexes in that overall telomere shortening does not occur when they are depleted. We suggest that these proteins have a telomere maintenance function rather than, or in addition to, being involved in ALT-mediated telomere elongation. Interference with this maintenance function may result in acute cellular effects that make it difficult to test whether these proteins are also involved in telomere elongation.

In addition to an elongation mechanism, telomere length maintenance encompasses processes to prevent rapid loss of telomeres. One mechanism by which telomeres can be truncated is through difficulties encountered during DNA replication. Because telomeric DNA is replicated from subtelomeric origins, replication is unidirectional towards chromosome ends, with the G-rich strand serving as the lagging-strand template (lagging telomere) and the C-rich strand serving as the leading-strand template (leading telomere). In the event of a stalled or broken telomere replication fork, distal telomeric sequences may be lost unless the fork can be repaired.

Telomere loss due to failure of normal DNA replication has been best described in primary human cells deficient for the Werner syndrome RecQ helicase (WRN) or the nuclease FEN1 (REFS 63,64). In WRN- or FEN1-deficient cells, leading telomeres are synthesized to completion while sister telomere loss (STL) of some lagging telomeres occurs. The mechanism of the WRN STL phenotype is thought to be that, in the absence of WRN activity, G quadruplexes accumulate in the G-rich template strand and cause failure of lagging-telomere replication. FEN1 interacts directly with WRN and is a structure-specific endo- and exonuclease functioning in lagging-strand replication, HR and the restart of stalled replication forks[65]. FEN1 is also likely to function in the same telomere maintenance pathways as WRN[64]. The FEN1 and WRN STL phenotype can be rescued by exogenous telomerase activity[63,64], presumably because telomerase is able to lengthen the shortened telomeres.

In ALT cells, WRN is not essential for telomere maintenance[66,67], but FEN1, MUS81, FANCD2 and FANCA are essential — all of which are proteins that have a normal role in recombinational repair of stalled or broken replication forks[65,68,69]. Several lines of evidence support the conclusion that these proteins repair broken telomeric replication forks in ALT cells. Both FEN1 and MUS81 were found to localize to telomeres in ALT cells during the G2 phase, when recombinational repair of telomeric DNA is proposed to occur[70], and depletion of MUS81 resulted in decreased cell division in ALT cells[61]. Depletion of any of MUS81, FANCA and FANCD2 decreased endogenous T-SCEs in ALT cells, suggesting that the repair of broken forks was reduced[61,62]. Depletion of MUS81, FANCA and FANCD2 also increased the numbers of telomere signal-free chromosome ends in ALT cells but did not reduce overall telomere length, in contrast to the effect of decreased MRN or SMC5–SMC6 function[61,62]. Depletion of FEN1 in ALT cells increased the number of telomeres showing a DDR and undergoing fusion[60], which are expected outcomes of rapid telomere loss.

Topoisomerase 3α (TOP3A) may also have similar functions in repairing stalled replication forks, as TOP3A depletion results in several phenotypes resembling those of FEN1 and MUS81 depletion[71]. However, TOP3A depletion also causes concomitant depletion of TRF2 in ALT cells[71]. It is unclear why this occurs, but TRF2 reduction in ALT cells would also account for some phenotypes observed following telomere loss, such as end-to-end fusion of chromosomes and reduced cell growth[72]. Therefore, it is unclear whether the telomeric DNA phenotypes result directly from decreased TOP3A activity or decreased TRF2 protein levels.

It is interesting to speculate whether ALT cells may be particularly susceptible to inhibition of repair of stalled replication forks. First, ALT telomeres may have nicks or gaps that are structural impediments to DNA replication[21]. Moreover, it is possible that ALT cells are particularly susceptible to complete telomere loss, because most ALT cells already contain substantial numbers of very short telomeres[48] (although this might be partially counteracted by increased mobility of short telomeres in ALT cells, which may increase their probability of interacting with a suitable substrate for ALT-mediated elongation[73]). Disrupting fork repair

Sister telomere loss
When one sister chromatid telomere of a metaphase chromosome end is not replicated owing to errors in DNA polymerization. This results in a telomere signal-free chromosome end.

may therefore result in telomeres that are so short that they elicit chromosome end-to-end fusions, genomic instability and cell death. In addition, when a telomere is completely lost, rectification of this situation requires the addition of telomeric sequences to a non-telomeric chromosome end, a process called 'chromosome healing'. Telomerase can perform chromosome healing, and although there is some evidence that chromosome healing occurred in the ALT-positive KB319 fibroblast line[74], it is not known whether ALT heals chromosome ends as efficiently as telomerase.

If recombinational repair of replication forks is necessary for telomere maintenance in ALT cells, why then is WRN not essential for ALT[66]? This may be because other RecQ helicases, such as BLM, provide redundant function in ALT cells. In budding yeast, Sgs1 is the only RecQ helicase and it is essential for the yeast equivalent of ALT[75,76]. Intriguingly, when cells from mice that have been telomerase-null for sufficient generations to have undergone substantial telomere shortening are also made WRN-null, they activate ALT more readily after loss of p53 function than comparable cells with wild-type WRN[77]. This suggests that moderate levels of broken telomeric replication forks may promote intertelomeric recombination and lead to telomere elongation provided that the cause of the broken forks does not also inhibit recombination-dependent telomere elongation.

Why does ALT not maintain telomeres in all cells?

If, as the evidence suggests, telomere elongation in ALT cells is a recombination-mediated template switching mechanism[37], why are not all cells able to prevent telomere shortening through an ALT mechanism? Normal somatic cells have substantially lower levels of extrachromosomal telomeric DNA than ALT cells, but why do they not use other chromosome ends or t-loops as copy templates? Telomeric DNA would be expected to be highly recombinogenic, given that it consists of long tracts of repetitive sequence and terminates in a single-stranded 3′ overhang. It seems that normal cells contain factors that prevent ALT from maintaining the lengths of their telomeres: when normal cells are fused with ALT cells, the ALT mechanism is repressed in the resulting hybrids[78]. ALT therefore results from loss of a normal function.

The identity of the factors that normally repress ALT is essentially unknown, but there are some hints that the shelterin proteins TRF2 and protection of telomeres 1 (POT1) may contribute. Shelterin proteins exert their function by regulating the activity of other proteins that localize to chromosome ends[8]. In telomerase-positive mouse cells, POT1 and TRF2 have anti-recombinogenic properties[79–82]. *In vitro*, TRF2 promotes the formation of t-loops and four-strand DNA junctions and protects these structures against enzymatic cleavage, suggesting that TRF2 has an essential role in regulating telomeric recombination by promoting t-loop formation but preventing resolution of telomeric recombination intermediates[83–85]. Recent evidence suggests POT1 has an important role in regulating the replication of the G-rich strand, so inhibiting POT1 function may increase the number of broken replication forks and T-SCEs or other telomeric recombination events[86].

In ALT cells, shelterin proteins bind not only to telomeric DNA at chromosome ends but also to extrachromosomal telomeric DNA. The total quantity of telomeric DNA in ALT cells is significantly increased, whereas the total levels of TRF2 seem to be slightly lower or unchanged[87]. It is not known whether this results in decreased saturation of shelterin proteins on telomeric DNA, but it seems possible that the change in ratio of telomeric DNA to total cellular content of binding proteins could result in a relative deficiency of the latter, which could contribute to decreased repression of telomeric recombination. MRN, WRN, MUS81, FEN1 and TOP3A all bind TRF2, which suggests that reducing relative TRF2 saturation may limit control over these proteins at chromosomal telomeres. Therefore, reduced shelterin-protein saturation of ALT telomeres could be the cause and/or consequence of ALT. Perhaps initially rare stochastic processes that result in recombination-mediated lengthening of a telomere can result in small changes in the ratio of telomeric DNA to telomere-binding proteins, leading to increasing dysregulation of telomeric recombination, further accumulation of telomeric DNA and, finally, fully established ALT activity.

It has also been suggested that the epigenetic state of the subtelomeric region can control telomeric recombination[88]. Although it is uncertain whether subtelomeric methylation affects ALT activity in human cells[89–91], this remains an important area of research.

Telomere capping function in ALT cells

If the saturation of shelterin components is decreased at ALT telomeres, it might be expected that chromosome end protection would also be impaired. When telomeres are dysfunctional, a telomere-specific DDR analogous to a DSB response[3,92] and/or chromosome fusions resulting from covalent ligation of chromosome ends[72,93,94] may occur. Strikingly, telomeres that spontaneously elicit a DDR but repress fusions are common in ALT cells[87]. We interpret these data as indicating that telomeres can adopt three distinct states with various levels of chromosome end protection (FIG. 4). 'Closed-state' telomeres repress both DDRs and fusions; 'intermediate-state' telomeres are susceptible to a DDR but repress fusions; and 'uncapped' telomeres are fusogenic and presumably elicit a DDR before ligation. We speculate that the distinctions among these states are as follows. Closed-state telomeres form a DDR-preventing protective structure that intermediate-state telomeres fail to adopt. DSB repair proteins, including MRE11, mediator of DNA damage checkpoint 1 (MDC1) and p53-binding protein 1 (TP53BP1), therefore localize to intermediate-state telomeres, but fusions are actively prevented by the retention of shelterin proteins, probably TRF2 and its binding partner RAP1. TRF2 and RAP1 inhibit covalent fusion of telomeric DNA *in vitro* and in cell lines, which suggests that these proteins directly inhibit covalent ligation of telomeric DNA[95,96]. Uncapped telomeres lack sufficient levels of shelterin to inhibit fusions.

Protection of telomeres 1
A shelterin subunit that binds single-strand G-rich telomeric DNA. It heterodimerizes with the shelterin subunit TPP1 (also known as ACD) and regulates telomerase access to chromosome ends, protecting the telomere against a telomere-specific DNA-damage response and inhibiting some forms of telomere recombination.

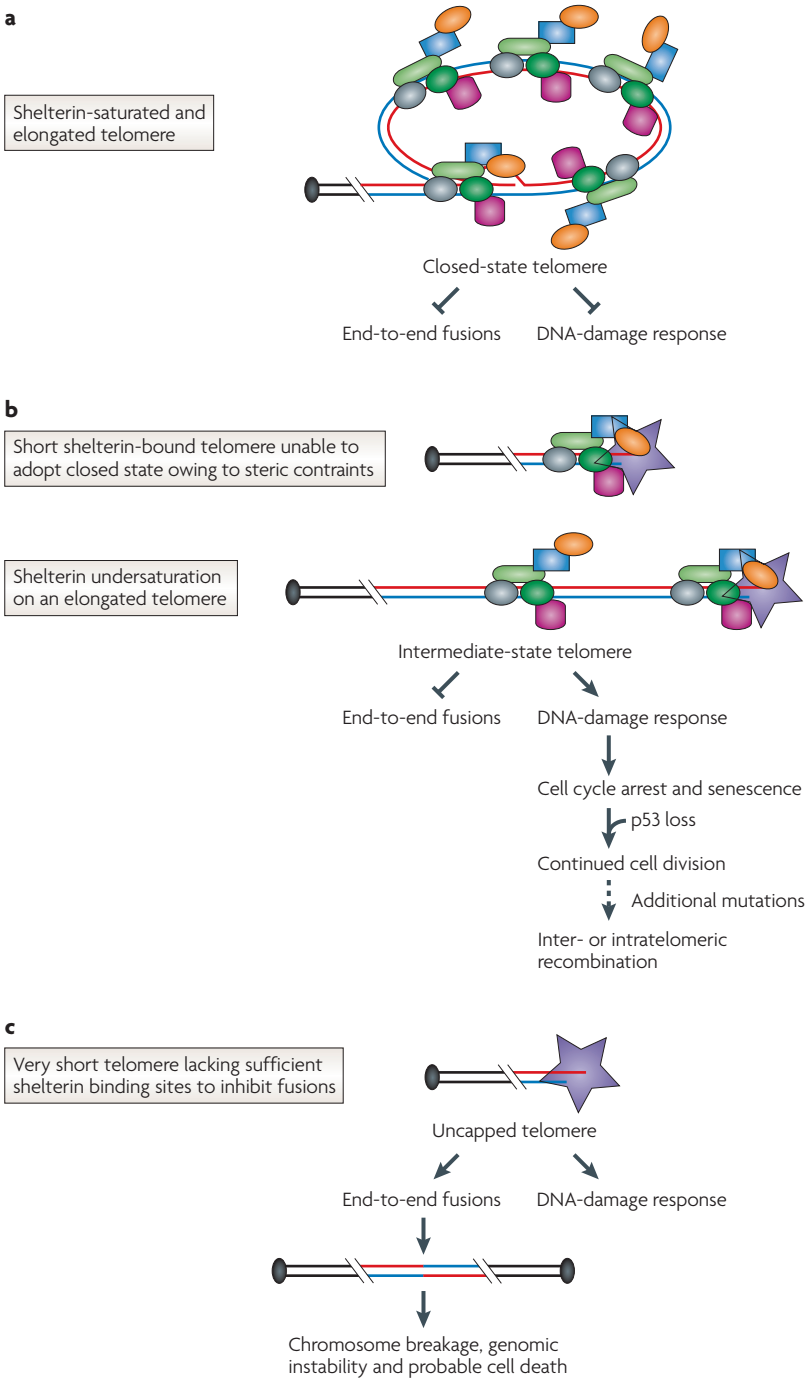

Figure 4 | **Closed-state, intermediate-state and uncapped telomeres.** In this model of telomere function and dysfunction, it is proposed that the fully capped — or closed — telomere state (**a**) prevents a DNA-damage response (DDR) and chromosome end-to-end fusions through the combination of a structural conformation (such as a telomere loop (t-loop)) and the presence of sufficient shelterin proteins. An intermediate-state telomere (**b**), for reasons that are essentially unknown (but might include relative undersaturation of the telomere with shelterin proteins), is able to prevent end-to-end fusions but not a DDR. The presence of a DDR usually results in p53-dependent cell cycle arrest. Genetic events involved in activation of the alternative lengthening of telomeres usually include the loss of p53, which permits cell proliferation in the presence of intermediate-state telomeres. Additional, unknown events (indicated by the dashed arrow) result in the de-repression of recombination-mediated telomere elongation. In the fully uncapped telomere state (**c**), chromosome ends retain insufficient levels of shelterin to inhibit chromosome end-to-end fusions.

In telomerase-positive cells with functional shelterin proteins, the proportion of spontaneously occurring intermediate-state telomeres is inversely proportional to telomere length and telomerase activity, consistent with a central tenet in telomere biology that greater telomere length confers greater end protection. ALT cells have a significantly greater percentage of intermediate-state telomeres than telomerase-positive cells. Some of the intermediate-state telomeres in ALT cells are quite long, indicating that telomere-length-independent dysfunction occurs spontaneously in these cells[87]. Given that p53 acts in the major response pathway to telomere dysfunction and that loss of p53 is required for cells to be refractory to telomere DDR-induced cell cycle arrest[97,98], it is not surprising that the majority of ALT cell lines and tumours lack normal p53 function[99].

There is circumstantial evidence linking the presence of intermediate-state telomeres in ALT cells to ALT activity. Spontaneous telomeric DDR activity and ALT activity are both repressed when ALT and telomerase-positive cells are fused to form hybrids, indicating that both result from a loss of normal function[87]. Introduction of exogenous telomerase activity into ALT cells usually fails to repress either telomeric DDR activity or ALT activity, despite extending the shortest telomeres and eliminating telomere signal-free chromosome ends[28,87]. This is consistent with the observation that telomere dysfunction in ALT cells can be telomere length-independent, and with previous observations that both elongated and short telomeres can undergo rapid elongation in ALT cells[27].

Consistent with an undersaturation of TRF2 at the telomeres of some ALT cell lines, expressing exogenous TRF2 in ALT cells decreases the number of spontaneous intermediate-state telomeres[87]. This effect is dependent on ATM, a protein that is known to interact with TRF2 and that has a key role in TRF2 dysfunction-induced DDRs[100]. However, TRF2 overexpression does not inhibit the overall DDR in ALT cells, suggesting that the exogenous TRF2 effect is telomere specific. We are currently testing whether TRF2 overexpression can also inhibit ALT activity.

TRF2 overexpression only partially suppresses the telomere DDR in ALT cells, and spontaneous telomeric DDRs occur in ALT cells lacking ATM activity[87]. POT1-related telomere dysfunction in mammals is signalled through ataxia telangiectasia and Rad3-related (ATR), which is presumably still intact in these cells[101], and the question therefore arises as to whether ALT cells also have a functional deficiency of POT1. However, reagent limitations have made it difficult to test this to date.

DDR signalling precedes recombination at sites of chromosomal damage, and it is likely that the DDR signal emanating from intermediate state telomeres promotes intertelomeric recombination. However, a telomere DDR alone is not sufficient to initiate telomere recombination. Telomerase-positive cell lines with low telomerase activity and short telomeres also show elevated numbers of intermediate-state telomeres. Changes (which have yet to be identified) in addition to the intermediate telomeric state are therefore necessary to promote ALT activity in human cells.

Concluding remarks

The phenotypic characteristics associated with ALT are useful for determining whether a cell line or tumour is likely to be ALT-positive, but it has been observed that some of these characteristics can be induced artificially in the absence of ALT activity. Of the known characteristics, C-circles seem to be the best available indicator of whether ALT activity is present, and there also seems to be a quantitative relationship between the number of C-circles and the amount of ALT activity.

There is general agreement that the ALT mechanism depends on recombination, but the process through which telomere elongation occurs is uncertain. The available evidence seems to fit best with a model of ALT activity in which single-stranded telomere ends invade double-stranded telomeric DNA or anneal to single-stranded telomeric DNA, use it as a template for synthesis of new telomeric DNA and thereby elongate themselves. The copy template may be the same telomere (through t-loop formation), the telomere of a sister chromatid or another chromosome, or one of the many forms of extrachromosomal telomeric DNA present in ALT cells.

The proteins known to be required for ALT activity are present in normal cells, in which they are required for normal DNA recombination and repair functions. The mechanism through which normal cells prevent their telomeres being maintained by ALT activity is unknown, but somatic cell hybridization studies have shown that activation of ALT involves loss of one or more normal repressor functions. This is presumably one of a series of changes required for the dysregulation of ALT activity (FIG. 4), which, in the great majority of cases, includes loss of wild-type p53 function. Loss of p53 function permits the accumulation in ALT cells of telomeres that have an intermediate state: they permit a DDR (and presumably recombination events) but inhibit end-to-end fusions. However, intermediate-state telomeres are not sufficient to permit ALT activity, and additional changes need to be identified. Understanding ALT and how it is normally controlled will underpin the development of therapies for targeting cancers that depend on this mechanism for their continuing growth.

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Competing interests statement
The authors declare no competing financial interests.

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