NATURE|Vol 447|21 June 2007|doi:10.1038/nature05976

Replication and protection of telomeres

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During the evolution of linear genomes, it became essential to protect the natural chromosome ends to prevent triggering of the DNA-damage repair machinery and enzymatic attack. Telomeres — tightly regulated complexes consisting of repetitive G-rich DNA and specialized proteins — accomplish this task. Telomeres not only conceal linear chromosome ends from detection and inappropriate repair but also provide a buffer to counteract replication-associated shortening. Lessons from many model organisms have taught us about the complications of maintaining these specialized structures. Here, we discuss how telomeres interact and cooperate with the DNA replication and DNA-damage repair machineries.

The protection of genetic information is essential for cells and organisms, because the accumulation of chromosomal aberrations leads to genomic instability. The evolution of linear chromosomes presents a complex puzzle, because chromosome ends need to be protected from enzymatic attack to avoid the loss of genetic information. In addition, all cells have developed mechanisms to detect DNA lesions, and natural chromosome ends need to be hidden from this machinery. The solution in most eukaryotes lies in nucleoprotein complexes known as telomeres, which consist of G-rich DNA repeats covered by specialized binding proteins. The actual terminus of a telomere is not blunt-ended but consists of a single-stranded 3′ protrusion of the G-rich strand (or G strand), known as a G tail or G overhang. These overhangs have been observed in humans, mice, ciliates, yeast, trypanosomes and plants, demonstrating that they are evolutionarily conserved and an essential feature of telomeres.

Loss of telomere function has various consequences in many model organisms, such as loss of the telomere G overhang, resection of the C-rich strand (or C strand), increased levels of recombination at chromosome ends, altered gene-expression patterns, fusion of chromosomes, instability of the genome, growth arrest and cell death. Most of our knowledge about telomere structure and function is derived from studies in a diverse range of organisms, such as the ciliates *Euplotes crassus* (also known as *Moneuplotes crassus*), *Tetrahymena thermophila* and *Oxytricha nova* (also known as *Sterkiella nova*), and the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Kluyveromyces lactis*. In human and mouse cells, the only direct consequences of telomere dysfunction that have been identified so far are degradation of the G strand and/or chromosome fusion. Whether similar phenotypes to those observed in the ciliates and/or yeasts also occur in mammalian cells with impaired telomere function remains to be seen.

Studies in human and mouse cells suggest that the G-rich single-stranded telomere overhang can invade homologous double-stranded telomeric tracts, resulting in a large lasso-like structure, known as a telomeric loop (t-loop)[1] (Fig. 1). This provides an elegant and appealing mechanism by which chromosome ends could be protected. However, at present, it is not clear whether t-loops are present at all chromosome ends, whether they are required for chromosome protection, or whether, instead, they have a role in regulating other features of telomeres (for example, access for the telomere-specific reverse transcriptase, known as telomerase, and therefore telomere length). Telomeres in cells from humans, mice, ciliates, trypanosomes and plants, as well as yeast engineered to have long telomeres, have been shown to have t-loops[2]. Terminal loops are an attractive model for a specialized configuration of chromosome ends; however, it is clear from the ciliate *O. nova* that other equally efficient structures have evolved. *O. nova* chromosome ends are tightly bound by a complex of two proteins of 56 and 41 kDa, efficiently protecting both the single-stranded telomere overhang and the double-stranded telomeric DNA from modifying enzymes[3–6]. This suggests that t-loops have evolved as only one of several means of chromosome end protection.

Mammalian telomeres are associated with the shelterin complex, a complex of interdependent telomeric core proteins consisting of telomeric-repeat-binding factor 1 (TRF1), TRF2, TRF1-interacting protein 2 (TIN2), the transcriptional repressor/activator protein RAP1, protection of telomeres 1 (POT1) and the POT1- and TIN2-organizing protein TPP1 (ref. 7; Fig. 1). TRF1 was originally reported to be involved mainly in the control of telomere length, and TRF2 was mainly implicated in chromosome end protection, by preventing end-to-end fusions[8,9]. Now, however, these distinctions are less clear-cut, because TRF2 has been shown to have a role in telomere length regulation[10], and the targeted deletion of *Trf1* in mice leads to early embryonic lethality. This lethality is probably due to telomere deprotection, because concomitant deletion of the DNA-damage sensor p53 extended the life of the embryos[11]. Such overlapping phenotypes can be explained by the nature of the shelterin complex, which is destabilized by the removal of individual members.

In addition to shelterin, mammalian telomeres interact with a number of other factors that can influence chromosome end integrity and dynamics, such as tankyrase 1 and tankyrase 2, poly(ADP-ribose) polymerase (PARP), meiotic recombination 11 homologue (MRE11), the RecQ-like helicases WRN (Werner's syndrome protein) and BLM (Bloom's syndrome protein), Ku70, Ku86, DNA-dependent protein kinase (DNA-PK; also known as PRKDC), ataxia-telangiectasia mutated (ATM), ATM and Rad3-related (ATR), excision repair cross-complementing 1 (ERCC1), RNA-polymerase σ^{70} factor (XPF) and the DNA-repair protein RAD51D[7] (Fig. 1). This plethora of factors, many of which are involved in DNA recombination and repair, not only demonstrates the flexibility and dynamic nature of the complex but also presents a paradox, because telomeres have long been defined as structures that are protected against becoming substrates for DNA repair or recombination. However, it is becoming increasingly clear that the repair and recombination machineries are an important component of telomere replication, protection and stability. One challenge for the telomere field is to address how these machineries contribute to these different classes of DNA end.

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The end-replication problem and ageing

In 1972, James Watson wrote, "While 5′ to 3′ oriented growth should proceed smoothly to the end of its template, I see no simple way for 3′ to 5′ growth to reach the 3′ end of its template"[12]. Thus, he correctly predicted that the lagging strand of linear chromosomes copied by the semi-conservative replication machinery would not be fully replicated[12]. In 1973, A. M. Olovnikov proposed the 'marginotomy theory of ageing', suggesting that 'telogenes' located at opposite ends of DNA molecules carry no genetic information and fulfil a buffer function. He stated that these telogenes are stochastically shortened during each mitotic cycle, providing a mechanism for ageing[13].

Observations made by L. Hayflick in 1961 suggested that human cells derived from embryonic tissues can only divide about 50 times, and this became known as the Hayflick limit[14]. Since then, the assumption that the Hayflick limit is determined by the initial length of the telomeres and the rate of telomere shortening, as laid out in the mathematical approach of A. M. Olovnikov[13], has been proved experimentally[15,16]. It is well established that critically short telomeres cease to function as protective units and cause the cell to die or to arrest permanently.

Telomeres are now known to have many more roles than simply buffering against DNA loss; however, the initial concept of replication-associated telomere shortening was correct. At present, we refer to the inability of conventional DNA polymerases to replicate linear molecules fully as the 'end-replication problem'. This is caused by the deletion of the RNA primer of the most distal Okazaki fragment and results in the loss of about five bases of terminal genetic material per population doubling[15,17–19]. However, the sequence loss that is predicted to occur as a result of the end-replication problem is considerably less than that which has been observed in primary human cells, which lose about 100–200 bases of TTAGGG repeats per cell division[20–22]. Consequently, replication-associated terminal sequence loss is caused by a combination of the end-replication problem and the processing that must occur to create the G overhang on the telomeres generated by leading- and lagging-strand synthesis.

When telomeres become critically short, they are detected by the cellular DNA-damage repair machinery[23]. As demonstrated in *S. cerevisiae*, chromosomes that lose a telomere are often eliminated, despite checkpoint and DNA-damage repair machineries[24]. In human cells, p53- and RB1 (retinoblastoma 1)-dependent pathways are responsible for monitoring telomere function, whereas p53 seems to be the main sensor in mouse cells[25]. The minimal functional telomere length, and whether this length varies among cell types, has not been clearly defined. But even in senescent human cells, telomeric double-stranded repeats are readily detectable, suggesting that several kilobases of TTAGGG repeats are required at all times. Similarly, when the telomere-protection factor TRF2 is overexpressed in telomerase-negative primary human fibroblasts, telomere-shortening rates almost double, and cells enter senescence with considerably shorter telomeres than control populations, indicating that telomere structure, not telomere length, is the main determinant of functional telomeres[26].

Analysis of signalling from experimentally induced dysfunctional mammalian telomeres and from chromosome ends in senescent cells suggests that the same machinery, the intracellular DNA-damage-monitoring system, recognizes both. Telomeres that are stripped of the protective shelterin complex by expression of a dominant form of TRF2 become associated with factors involved in DNA-damage responses such as the p53-binding protein 53BP1, the histone protein γ-H2AX (phosphorylated H2AX) (see page 951), RAD17, ATM and MRE11, and are visualized as TIF, or telomere-dysfunction-induced foci[27]. Inhibition of the phosphatidylinositol-3-OH-kinase-like kinases ATM and ATR reduces TIF formation, confirming that dysfunctional telomeres are detected by the ATM–p53 pathway[27]. Cells carrying senescent telomeres trigger a cellular response remarkably similar to that elicited by double-strand DNA breaks[28]; in these cells, several DNA-damage-response factors congregate at the eroded telomeres similarly to TIF. These findings underscore the importance of the DNA-damage repair machinery for telomere function, emphasizing the fact that this machinery carries out several essential tasks.

Replicative senescence can be viewed as a mechanism to limit the potential number of population doublings a cell can undergo, hypothetically rendering it a powerful tumour-suppressor mechanism[29]. Each time a cell divides, telomeres shorten as a result of the end-replication problem and end processing. After telomeres have become critically short, they are detected by the DNA-damage repair machinery, and the cell dies or enters senescence. At present, senescence in human cells is regarded as an irreversibly arrested state, effectively inhibiting the generation of immortal cells and therefore cancer formation. As a result, major tumour-suppressive mechanisms need to be deactivated before a cell can overcome this block to immortality. Cells that continue to divide past their normal replicative limit lose all remaining protective telomeric DNA and enter a stage termed crisis. Crisis is marked by massive genomic instability and cell death. Eventually, transformed clones emerge, and although the activation of telomerase is not essential for the acquisition of a transformed phenotype[30], most cells that successfully exit from crisis have upregulated its activity. This observation emphasizes the dual role of telomerase in the immortalization process. On the one hand, reactivation of the enzyme in cells with critically short telomeres allows genomically unstable and immortal clones to be established, which is a major step towards cancer. On the other hand, switching on telomerase in cells that have not reached crisis prevents telomere-mediated genomic instability, which is a hallmark of cancer cells[31]. Consequently, it could be argued that telomerase fulfils a tumour-suppressive role before it contributes to the establishment of immortality (Fig. 2).

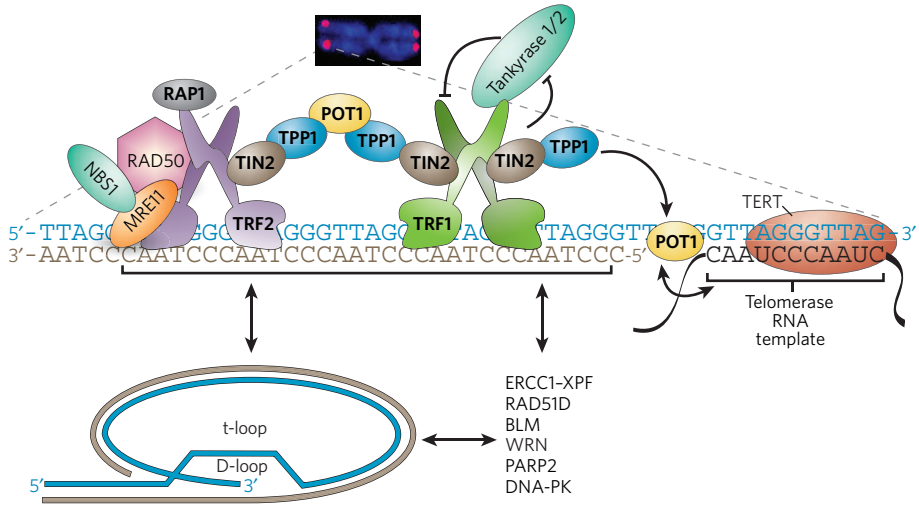


Figure 1 | The mammalian telomeric complex. The fluorescence image shows the location of a telomere within a chromosome. Mammalian telomeres consist of TTAGGG repeats with a single-stranded 3′ overhang of the G-rich strand. Specific protein complexes bind to the double- and single-stranded telomeric DNA. The components of the shelterin complex are shown in bold text. The single-stranded overhang can invade the double-stranded portion of the telomere, forming protective loops — such as t-loops with displacement loops (D-loops) — at the invasion site. The telomerase complex (which contains the telomerase RNA template and the reverse transcriptase TERT) interacts with the overhang and is regulated by shelterin and other telomeric proteins[7]. Other factors that can interact with telomeres are listed. Bidirectional arrows indicate interactions.

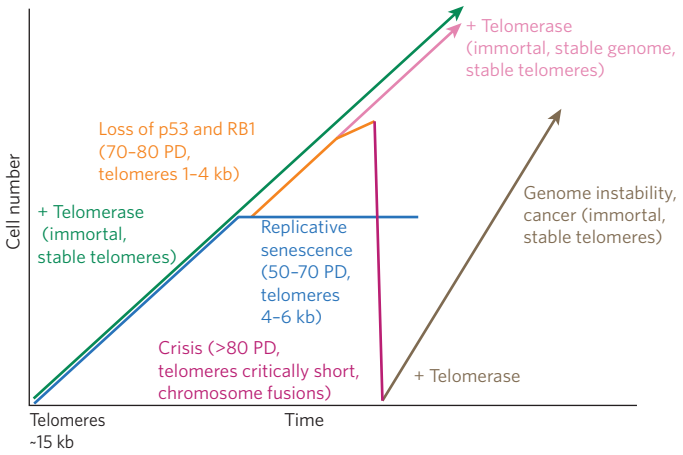


Figure 2 | Telomere shortening, senescence and cancer. Primary cells divide exponentially, and telomeres shorten from ~15 kilobases (kb) until they reach a critical length, 4–6 kb. Irreversible cell-cycle arrest then occurs (blue). Activation of telomerase before senescence allows cells to divide indefinitely and maintain a stable genome (green). If, instead, the p53 and RB1 pathways are suppressed, cells continue dividing (orange) until end protection is completely lost, resulting in telomeric crisis, cell death and massive genomic instability (dark pink). If telomerase is activated before erosion is complete, this rescues the genome from instability by re-establishing telomere maintenance (light pink). Activation of telomerase after the accumulation of mutations results in an unstable genome, allowing clones that carry multiple mutations to escape cell death (that is, to become immortal). Such cells are predisposed to oncogenic transformation (brown). PD, population doublings.

Approximately 10% of human tumours rely on a telomerase-independent method to maintain their telomeres. Known as ALT (alternative lengthening of telomeres), it is based on recombination between telomeres[32] (Box 1). Tumours resulting from *Wrn*$^{-/-}$ mouse cells without telomerase activity readily engage the ALT pathway, and although the exact molecular mechanism is not understood, preliminary findings point to aberrant homologous recombination as the underlying cause[33,34].

Despite the finding that expression of the catalytic subunit of telomerase readily immortalizes primary cells without seeming to cause genomic instability[16,35], the *in vivo* evidence for telomere involvement in human ageing is limited to correlations. For example, the mean length of telomeric restriction fragments from DNA isolated from sperm cells is considerably longer than comparable fragments isolated from replicating cells *in vivo*[36], and it has recently been demonstrated that senescent cells account for up to 15% of the cell population in the skin of aged baboons[37]. Many such correlations have been documented, and although they show that telomeres shorten with age, it is unclear whether telomere shortening causes ageing *in vivo*.

Little is known about the role of telomeres during the ageing process of differentiated cells and in organisms that do not contain mitotic cells. Although it has been suggested that telomere elongation extends the lifespan of the nematode *Caenorhabditis elegans*[38] (in which cells do not undergo mitosis after development is complete), this is probably a secondary effect of *hrp-1* overexpression in these animals, because clonal wild-type nematode strains with varying telomere length did not show any differences in organismal ageing and lifespan[39]. Consequently, and in line with Watson's and Olovnikov's concepts of the end-replication problem, it is unlikely that telomeres have a major role in the ageing of non-dividing cells.

Telomere replication

The end-replication problem correctly predicts that linear DNA molecules shorten during every replicative cycle. Consequently, in the absence of a mechanism to maintain the absolute ends, chromosomes eventually lose the protective cap provided by their ends, resulting in the loss of genomic integrity. To counteract replication-associated telomere shortening, telomerase evolved. Telomerase is a specialized reverse transcriptase complex and can add G-rich telomeric repeats to the absolute ends of chromosomes using its own internal RNA template, effectively stabilizing telomere length. In *S. cerevisiae*, telomeres switch between extendable and non-extendable states[40]. In human cells, the limited amount of telomerase contributes to telomere length homeostasis, because increased amounts of telomerase change telomere length settings to a different equilibrium[41]. In *S. cerevisiae*, deletion of the gene encoding the telomerase RNA template (*TLC1*) or the catalytic subunit (*EST2*) leads to gradual telomere shortening and growth arrest[17–19]. Similarly, mice lacking the gene encoding either the telomerase RNA (*Terc*) or the reverse transcriptase domain (*Tert*) gradually lose their telomeres over several generations, resulting in degeneration of highly proliferative cell populations and sterility[42–44]. Expression of human TERT in fibroblasts causes telomere elongation and renders the cells immortal, effectively avoiding telomere-shortening-dependent replicative senescence[16].

No origin of replication has been detected in telomeres, rendering the closest origin, resident in the subtelomeric region of the chromosome, the starting point for the replication of chromosome ends. Passage of the replication fork through the telomere is thought to generate a blunt-ended leading-strand product and a lagging-strand product with a short 3′ G overhang (Fig. 3). In the presence of telomerase, and during new telomere synthesis, the actions of the conventional replication machinery and telomerase are closely coordinated. Inhibition of C-strand synthesis in the ciliate *E. crassus* by using aphidicolin, a specific inhibitor of DNA polymerase-α and DNA polymerase-δ, leads to a general lengthening of the G strand, thereby showing that C- and G-strand synthesis is coordinated[45]. Similarly, addition of new telomeres by telomerase in *S. cerevisiae* requires not only extension of the 3′ G-rich end by telomerase but also fill-in synthesis of the C strand by DNA primase, DNA polymerase-α and DNA polymerase-δ. G-strand polymerization by telomerase is inhibited in *S. cerevisiae* if DNA polymerase-α and DNA polymerase-δ are inactive, suggesting that telomerase needs to interact with the lagging-strand synthesis machinery to be active[46]. An excellent candidate for regulating the coordination between telomerase and the conventional DNA polymerases is the Cdc13 complex, which attracts telomerase to chromosome ends in *S. cerevisiae*. Cdc13 binds to single-stranded G-rich telomeric DNA and then recruits telomerase[47,48]. Several studies have suggested that, subsequently, lagging-strand synthesis fills in the C strand, and then inhibits telomerase in a Cdc13-dependent manner[49,50].

Little is known about coordinated C- and G-strand synthesis in mammalian cells, but activation of a temperature-sensitive allele encoding DNA polymerase-α causes elongation of the G tail and of the telomere overall, suggesting that coordination of telomerase with the replication machinery is a common feature in all organisms[51].

The G-rich and repetitive nature of telomeric DNA complicates replication as well, because it potentially allows the formation of secondary structures, such as G quartets[52]. Consequently, telomeric proteins support the progressing replication fork, allowing efficient telomere synthesis. In *S. pombe*, Taz1, the homologue of TRF1 and TRF2, is required for telomere replication. Without Taz1, replication forks stall at telomeric sequences, regardless of whether the repeats are located at the ends or in the interior of chromosomes[53]. This suggests that the Taz1-dependent telomere-replication phenotype is due to characteristics of the telomeric sequence itself and not to its position on the chromosome. In human cells, the RecQ-like helicase WRN contributes to efficient telomere replication. Overexpression of a helicase-defective *WRN* allele causes the occasional loss of telomeres generated by the lagging-strand machinery, and telomerase expression compensates for this loss of telomeric sequence, implicating telomere maintenance in the pathology of Werner's syndrome[54]. Accordingly, targeted deletion of *Wrn* in mice leads to phenotypes that resemble the human Werner's syndrome only when telomerase is also deleted[55]. In summary, it is becoming increasingly clear that telomere replication and telomerase-dependent telomere elongation are highly coordinated processes and that telomeric proteins have essential roles in the regulation of these processes.

The generation of telomere overhangs

After telomere replication is completed, the newly generated lagging-strand telomere carries a short 3′ overhang, resulting from the removal of the most distal RNA primer used for Okazaki fragment synthesis. It is not clear whether this distal primer is placed at the absolute terminus of the chromosome or a few bases from the end, therefore allowing an overhang that could be longer than the length of the RNA fragment. By contrast, leading-strand synthesis is expected to continue until it reaches the end of the template, resulting in blunt-ended products (Fig. 3). In ciliate, yeast and human cells, overhangs can be detected at both ends of the chromosomes, suggesting that there are regulated mechanisms for G-tail generation[56–60]. Currently, no candidates for overhang-generating nucleases have been identified in any organism, but the field is looking to ciliates for clues, because much of the work on telomere replication was pioneered in these organisms.

T. thermophila maintains G overhangs with defined sequence and length on both chromosome ends[56]. Because both the G strand and the C strand are processed accurately in the absence of telomerase, it has been suggested that C-strand resection works in collaboration with G-strand cleavage to generate a functional telomere end. These steps potentially include more than one nuclease, and when the telomeric sequence is artificially changed, the terminal nucleotides were not altered, suggesting that the nuclease activities do not show sequence preference and that additional factors, such as proteins that bind to the single-stranded overhang, regulate specificity[61].

In human cells, in the absence of telomerase, the leading-strand daughter telomeres carry longer overhangs than the telomeres synthesized by the lagging-strand machinery[62]. However, when the catalytic subunit of telomerase is introduced, similar lengths are found at both leading and lagging daughter telomeres. Moreover, both daughter telomeres have conserved terminal nucleotides at their 5′ ends. Small-interfering-RNA-dependent knockdown of expression of the shelterin component POT1 in human tumour cells randomizes the last nucleotides of the 5′ telomere end, suggesting that this single-stranded TTAGGG-binding protein is involved in regulation of terminal specificity[63]. This process differs for the 3′ end, where the terminal residues seem to be much more variable in the absence of telomerase. Overexpression or suppression of individual components of the telomere-binding protein complex or of the multisubunit telomerase complex might already disturb the equilibrium at telomeres, limiting the conclusions that can be drawn about the *in vivo* situation from such experiments.

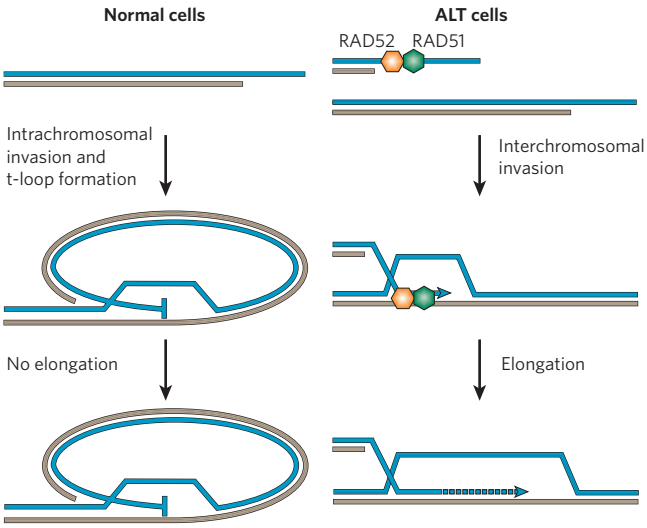
Eventually, detailed knowledge about overhang length and base specificity of leading- and lagging-strand telomeres in the presence and absence of telomerase will provide insight into the coordination of telomerase activity at telomeres. There are several possible ways in which this coordination might occur. Both daughter telomeres are nucleolytically recessed in the 5′ to 3′ direction after replication, but the efficiency varies at each strand, potentially rendering the leading-strand telomere with a longer overhang[62]. The presence of telomerase complicates processing, because telomerase could take advantage of the short overhang at the lagging strand before nuclease action, specifically elongating telomeres replicated by the lagging-strand machinery. However, if telomerase acts after overhangs are generated on both strands, more than one fill-in step by the lagging-strand machinery and more than one resection step might be required to generate functional chromosome ends. The field of telomere research is in agreement that well-regulated overhang generation is an essential step for telomere function and thus for chromosome protection; however, at this stage, only the surface of this complex problem has been scratched.

Telomeres and the DNA-damage repair machinery

The protective features of telomeres are lost when chromosome ends become uncapped, through the mechanisms described here. These subsequently dysfunctional telomeres are then subject to DNA repair by non-homologous end joining (NHEJ) or homologous recombination.

In accordance with a requirement for the NHEJ pathway (which depends on Ku, DNA-PKcs (the catalytic subunit of DNA-PK), and DNA ligase IV and its cofactor XRCC4) for the processing of dysfunctional telomeres, DNA ligase IV is required for end-to-end fusion of critically short or dysfunctional telomeres. DNA ligase IV fuses telomeres in *S. pombe* lacking *taz1* (a homologue of the mammalian *TRF* genes)[64], in *S. cerevisiae* with mutated *TEL1* (which encodes a protein kinase) or *MEC1* (which encodes a signal transducer)[65], and in mouse cells that lack TRF2 and therefore contain uncapped chromosome ends[66]. Ku contributes to telomere protection in *S. cerevisiae*, *S. pombe* and mammalian

Box 1 | ALT, homologous recombination and BIR at telomeres



A subset of immortalized cells do not show telomerase upregulation and use a recombination-based pathway known as alternative lengthening of telomeres (ALT) to maintain chromosome ends. One of the main characteristics of 'ALT cells' is the presence of promyelocytic leukaemia (PML) bodies, which are subnuclear structures that contain telomeric DNA, telomeric proteins and factors involved in DNA recombination and repair. ALT cells have heterogeneous telomere lengths, ranging from critically short telomeres to telomeres of up to 100 kb, as well as telomeric DNA circles of 1–60 kb[94]. It has been suggested that the homologous-recombination machinery is responsible for the amplification of the telomeric sequences in ALT cells, because a selection marker introduced into telomeres of ALT and non-ALT cells spreads throughout telomeres only in ALT cells[33]. ALT cells show an increased rate of sister chromatid exchange[95,96], suggesting that the homologous-recombination pathway is involved. Overexpression of a mutant *TRF2* allele in non-ALT cells generates t-loop-sized DNA circles that depend on the ERCC1–XRCC3 complex[81]. This phenotype resembles the ALT-associated DNA circles and again suggests a possible role for homologous recombination in the ALT pathway.

The molecular mechanism of the ALT pathway is far from understood but resembles break-induced replication (BIR). BIR is a gene-conversion mechanism that is induced only when one DNA end invades a homologous sequence and initiates DNA replication with the homologous sequence as template. Proteins such as RAD50, RAD52 and MRE11 are involved in BIR[97]. It is feasible that invasion of the single-stranded telomere overhang into double-stranded TTAGGG repeats is a BIR-like situation. Consequently, it is possible that the telomere maintenance mechanism used by ALT cells is similar to that proposed for BIR. The figure compares telomere structure and regulation in non-ALT cells and ALT cells.

In the absence of telomerase, a subset of yeast cells elongate their telomeres through amplification of telomeric and subtelomeric repeat sequences. This recombination maintenance pathway depends on RAD52 (ref. 98), suggesting that homologous recombination can elongate telomeres in a telomerase-independent manner.

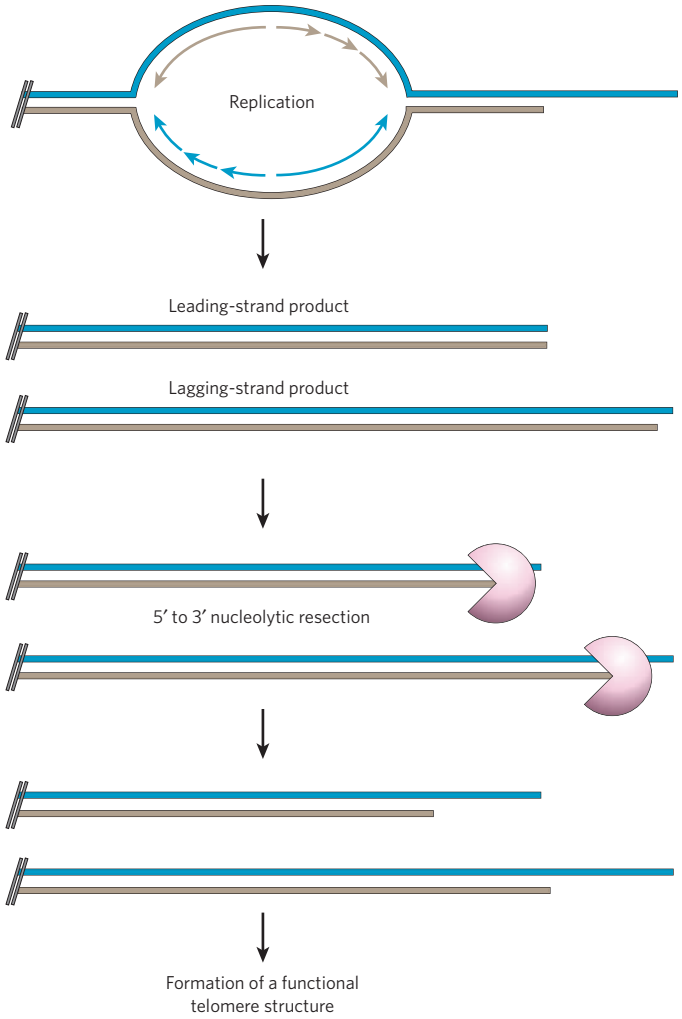


Figure 3 | End replication and processing. There are 3′ G overhangs at both ends of the chromosome, and these are thought to be generated by 5′ to 3′ nucleolytic activity. Semi-conservative replication of telomeres generates a blunt-ended leading-strand product and a lagging-strand product with a short overhang. Nucleolytic digestion (pink) in the 5′ to 3′ direction then generates G overhangs, which allow the formation of a functional telomere structure (not shown). The short overhang generated by lagging-strand synthesis could be sufficient for a functional telomere, so it has been proposed that only the leading-strand product undergoes nucleolytic digestion.

cells. In all of these organisms, Ku associates with telomeric DNA, regulates telomerase and inhibits inappropriate recombination and repair events at telomeres[67,68].

In *S. cerevisiae*, the single-stranded telomeric DNA-binding factor Cdc13, which also has a prominent role in telomerase recruitment, is central to chromosome end protection[69]. A protective complex of Cdc13, Stn1 and Ten1 protects the telomere from excessive degradation of the C strand[47,70,71], resulting in long G tails, which trigger Rad9-dependent cell-cycle arrest[72,73].

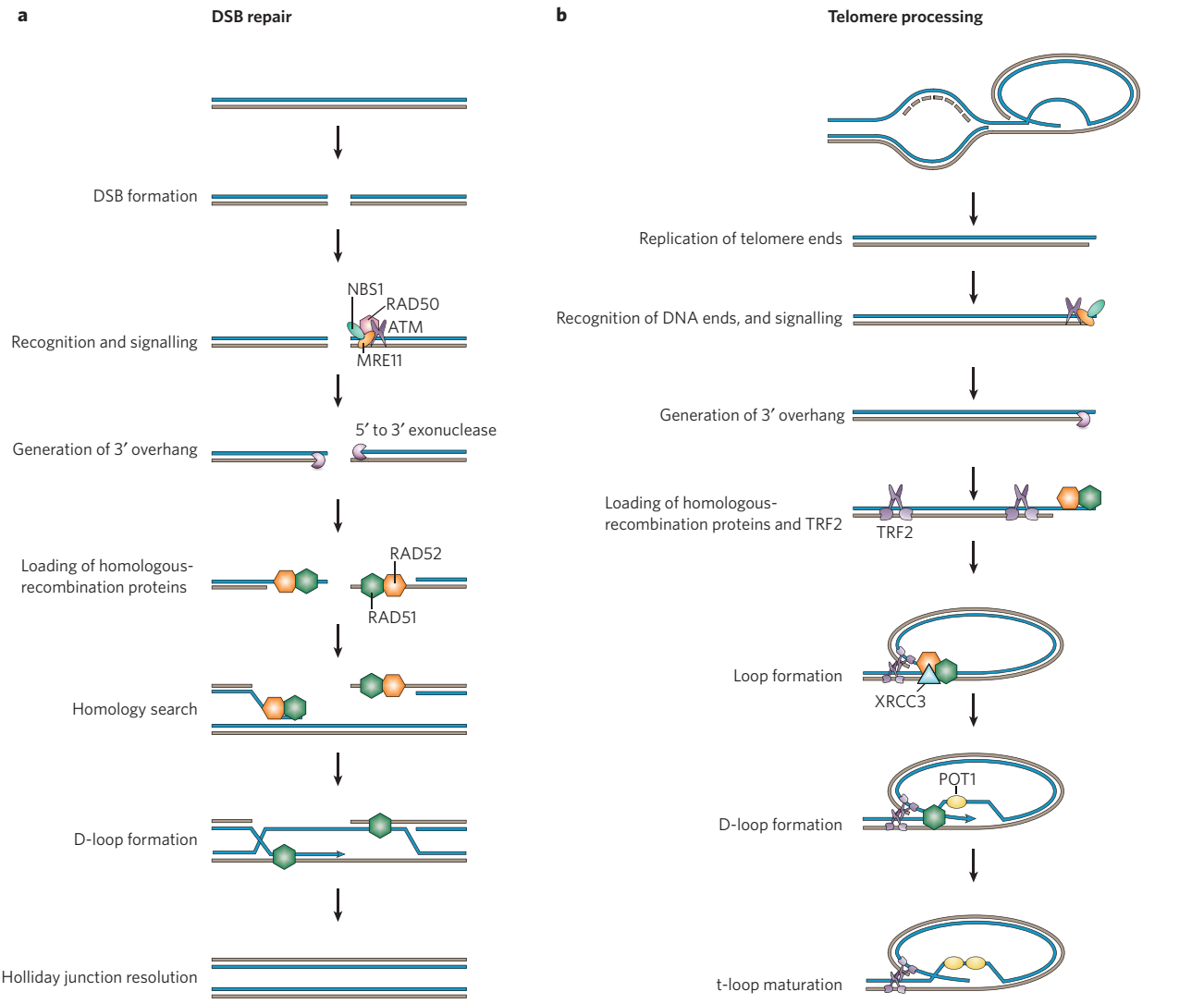
Loss of the *S. pombe* double-stranded telomere-binding protein Taz1 results in uncontrolled elongation of single- and double-stranded telomeric tracts[74], as well as loss of viability and telomere fusion in yeast arrested in the G1 phase of the cell cycle[64]. Chromosome end fusions are also a consequence of loss of Pot1, a single-stranded telomere-binding protein, which is a homologue of the α-subunit of the *O. nova* end-binding factor[75]. *S. pombe* cells lacking Pot1 rapidly lose telomeric and subtelomeric DNA and become inviable, although a subset of cells survive and have circularized chromosomes. Human POT1 regulates telomere length in a telomerase-dependent manner, relaying information from shelterin to the telomerase complex[75–77]. Mouse cells have two POT1 proteins, POT1A and POT1B, suggesting recent expansion of the telomeric complex in rodents[78,79]. POT1A is sufficient to repress DNA-damage signalling at telomeres in the absence of POT1B. However, lack of POT1B leads to a substantial increase in the length of the G tail in a telomerase-independent manner, a phenotype that could not be rescued by overexpression of POT1A, establishing that the two proteins have distinct roles in telomere protection. In addition to POT1, the mammalian factor TRF2 has a substantial role in protecting telomeres from NHEJ and homologous recombination. Telomeres in mouse and human cells that lack TRF2 lose the G tail, are detected as damage sites, and are substrates for DNA-ligase-IV-dependent fusion[9,66,80]. However, telomeres in cells that lack both DNA ligase IV and TRF2 do not show overhang loss, although they are still recognized as DNA damage, indicating that the loss of the G tail is a consequence of NHEJ and is not required for the DNA-damage pathways at telomeres[80]. In addition, overexpression of a human *TRF2* allele encoding a protein that lacks the amino terminus but retains the DNA-binding region causes the rapid shortening of telomeres and the generation of extrachromosomal telomeric DNA circles[81]. These circles can readily be detected in cells in which the ALT pathway is engaged (Box 1) and depend on the homologous-recombination protein XRCC3, which is involved in the resolution of Holliday junctions (junctions between four DNA strands), suggesting that TRF2 protects telomeres not only from NHEJ but also from homologous-recombination-based deletion of large stretches of DNA.

It is now well established that the natural chromosome ends need to be protected from inappropriate repair, so it seems paradoxical that several proteins involved in the detection or repair of DNA lesions localize to telomeres. Such localizing proteins include the MRX/N complex (Mre11–Rad50–Xrn2 in yeast and MRE11–RAD50–NBS1 in mammals) and the protein kinases Tel1 and Mec1 in *S. cerevisiae* or their mammalian homologues, ATM and ATR, all of which have crucial roles in DNA-damage signalling and telomeric integrity. Deletion of *MRE11* or *RAD50* in *S. cerevisiae* leads to gradual telomere shortening, which does not increase when *EST2* is also deleted, placing the MRX complex in the same pathway as telomerase[82], probably recruiting the enzyme to telomeres[83]. The finding that MRX localizes to telomeres in late S phase suggests that this complex is required to prepare the telomere for telomerase-dependent replication[84]. Co-deletion of MRN components and *rad3* (the homologue of *MEC1* and *ATR*) in *S. pombe* results in telomere loss and chromosome circularization[85], again placing the complex in a pathway that provides protection against telomere loss. Similarly, Tel1 and Mec1 can be found at *S. cerevisiae* telomeres[86], and deletion of the genes encoding both molecules causes telomere loss, a phenotype that can be overcome by attracting active telomerase to the chromosome ends[87]. At the same time, Tel1 protects telomeres against NHEJ-dependent fusion, because the frequency of telomeres fused to an inducible double-strand break increases sharply in a strain that lacks both Tel1 and Tlc1 (ref. 88). In humans, cells derived from patients with cancer-prone syndromes such as the Nijmegen breakage syndrome or ataxia-telangiectasia (which carry mutations in *NBS1* and *ATM*, respectively) show accelerated telomere shortening and chromosome fusions[89,90]. Similarly to yeast, the MRN complex and ATM have been detected at telomeres[91,92], suggesting dual roles in DNA-damage signalling and telomere maintenance for these factors.

All of these observations indicate that functional telomeres require interaction with DNA-damage repair proteins, suggesting that the DNA-damage repair machinery is involved in replication of telomeres, protection of functional chromosome ends, and detection of, and signalling from, dysfunctional ones.

Similarities between the proteins responsible for the detection and repair of DNA lesions and those found at functional and dysfunctional telomeres suggest that, for a cell, the difference between a DNA break and a telomere is less pronounced than previously assumed. The finding that functional telomeres are detected by the DNA-damage repair machinery in every cell cycle, and the presence of the homologous-recombination machinery at telomeres in G2 phase, suggests that this pathway is involved in telomere end processing[92,93] (Box 2).

Box 2 | Telomere maintenance and double-strand-break repair



The repair of a double-strand break (DSB) by the homologous-recombination repair pathway involves well-characterized molecular steps and proteins (see figure, part **a**). After a DSB is detected, the MRN complex (MRE11–NBS1–RAD50) is one of the first repair factors to be recruited, and this is followed by MRN-dependent activation of the protein kinases ATM and ATR[99]. Activation of these protein kinases results in signals that lead to the recruitment of processing factors, which generate 3′ single-stranded overhangs. The exposed overhangs are coated by replication protein A, which protects the DNA against degradation and inhibits the formation of secondary structures (not shown). Next, the single-stranded DNA invades homologous duplex DNA sequences, forming a displacement loop (D-loop) by homologous pairing and strand exchange, a process catalysed by RAD51 and stimulated by RAD52, RAD54 and RAD55–RAD57. Using the homologous sequence as a template, the invading strand primes DNA synthesis, generating a Holliday junction. The genetic information is restored after the Holliday junction is cleaved by RAD51C–XRCC3-dependent resolvase activity[100].

Detection of ends, generation of 3′ overhangs and invasion of homologous sequences are essential steps for DSB repair, and these steps seem strikingly similar to the predicted order of post-replicative telomere processing and of t-loop formation (see figure, part **b**). Recently, we suggested that functional human telomeres in primary fibroblasts interact with proteins of the DNA-damage repair machinery[92]. MRE11, NBS1 and activated ATM localize to telomeres from late S phase until G2 phase, suggesting that telomeres are detected as DNA damage. The damage signal is localized to chromosome ends, because neither stabilization of p53 nor phosphorylation of CHK2 (checkpoint 2 homologue; also known as CHEK2) can be observed in the nucleus, suggesting that the signal is well controlled, and does not lead to cell-cycle arrest. All of these results promote the hypothesis that telomeres are not always hidden from the DNA-damage repair machinery and suggest that telomeres require a close relationship with the DNA-damage-response pathways for function, fuelling the idea that the processing of telomeres is similar to the processing of DNA breaks.

In a manner analogous to that of DSB processing, telomeres recruit RAD51, RAD52 and XRCC3 before mitosis, potentially resulting in a search for homologous DNA sequences, followed by strand invasion. However, invasion of another chromosome by a telomere overhang can lead to a deleterious phenotype. TRF2 is a good candidate for involvement in avoidance of inter-telomere invasion, because this protein can form t-loops *in vitro* by keeping the telomere end and the duplex DNA of the same telomere in proximity. This model is supported by the finding that in an *in vitro* assay with telomeric substrates, not only is the homologous-recombination machinery required for efficient invasion but also the telomeric protein TRF2 (ref. 93). Consequently, this TRF2 activity, together with the homologous-recombination machinery, potentially facilitates the formation of the D-loop that forms the core of the t-loop structure and provides a substrate for POT1.

Conclusions

The field of telomere biology has progressed considerably from the simplistic view that telomeres function only as non-coding buffer zones at the ends of linear chromosomes. We now view telomeres as highly specialized and regulated complexes in which length and structure determine integrity and function. Despite the open questions about the requirements of t-loops for end protection or telomere length regulation, it has been accepted that the G-rich 3′ single-stranded overhang is required for telomere function.

However, the cell not only needs to control the formation of the 3′ overhang (and therefore the adoption of a functional telomere structure) but also needs to monitor telomere length, as demonstrated by the finding that critically short telomeres lose their protective function.

A similar bipolar relationship is observed between telomeres and DNA-damage-response pathways. On the one hand, the intracellular DNA-damage repair machinery is required to detect dysfunctional telomeres, which are consequently processed like any other double-strand break. On the other hand, the DNA-damage repair machinery is required for telomere replication and telomere protection, and it also seems to be essential for the formation of a functional telomere structure.

Taken together, all of these observations demonstrate that Watson and Olovnikov were correct when they suggested a problem in the replication of terminal DNA. The telomere, ageing and cancer fields have since managed to advance our understanding of the problem considerably, although it has not yet been solved. ■

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Acknowledgements We are indebted to V. Lundblad for constructive comments on the manuscript, the National Institutes of Health for funding (J.K.) and the Leukemia and Lymphoma Society for a long-term postdoctoral fellowship (R.E.V.).

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