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Review

# Telomeres and chromosomal instability

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**Abstract.** Telomeres are distinctive structures, composed of a repetitive DNA sequence and associated proteins, which enable cells to distinguish chromosome ends from DNA double-strand breaks. Telomere alterations, caused by replication-mediated shortening, direct damage or defective telomere-associated proteins, usually generate chromosomal instability, which is observed in senescence and during the immortalization process. In cancer cells, this chromosome instability could be extended by their ability to 'repair' chromosomes and terminate in break-fusion-bridge cycles. Dysfunctional telomeres can be healed by activation of telomerase or by the 'alternative mechanism' of telomere lengthening. Activation of such telomere maintenance mechanisms may help to preserve the integrity of chromosomes even if they play a role in chromosomal instability. This review focuses on molecular processes involved in telomere maintenance and chromosomal instability associated with dysfunctional telomeres in mammalian cells.

**Key words.** Telomere; chromosomal instability; telomere-associated proteins; alternative lengthening of telomeres; tumorigenesis.

## Introduction

Telomeres are specialized DNA-protein structures located at the ends of linear chromosomes. In mammalian cells, the telomeric sequence is a repetition of a hexanucleotide motif, TTAGGG, which serves multiple functions, including prevention of fusion or degradation of chromosomes and facilitating chromosome segregation. In normal somatic human cells, the telomere length progressively shortens, and the cell population eventually undergoes senescence. At this stage, chromosomal instability is maximal and characterized by great number of telomeric fusions. Although most cells die, the rare cells that have bypassed crisis are generally aneuploid, senescence express telomerase activity, have a stable telomere length [1] and can potentially divide indefinitely. The protection and maintenance of human chromosome ends require the function of two related factors, TRF1 and TRF2, and several interacting proteins. Components of the nonhomologous end-joining (NHEJ) DNA repair system may also be implicated in telomere protection. The loss of chromosomal integrity is attributed either to a functionally 'uncapped' state of the telomeres or when telomeres reach a threshold length in the absence of telomerase activity [2] and lead to the formation of chromosomal abnormalities (CAs), a general characteristic of cancer [3]. Active telomerase, which adds TTAGGG repeats, plays a role in allowing cell proliferation in most human cancer cells, but an alternative mechanism (ALT) of telomere lengthening, probably via homologous recombination and copy switching, also seems to be involved [4].

## Proteins and telomere maintenance

Telomeric DNA and telomere-specific binding proteins both play an essential role in stabilizing chromosome ends by forming a cap structure which protects chromosomes

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from degradation and terminal fusions [5]. In mammals, telomeric DNA located at the extremity of chromosomes exhibits a 3′ overhang. This single-strand DNA has been shown to form a T-loop structure by invading the double-stranded region of the telomeric DNA [6]. The T-loop model provides the sequestration of the G-strand overhang, which could otherwise activate DNA damage checkpoints and DNA repair enzymes. Formation and stabilization of this structure are dependent on telomeric proteins such as TRF1 and TRF2 and, as more recently described, on proteins implicated in the general repair machinery (table 1). Disruption of telomere function results in formation of dicentric chromosomes and other abnormalities created through end-to-end fusions [7]. In senescent and in tumor cells, dicentric chromosomes, rings and sister chromatid fusions are correlated with critically short telomeres [8]. Much evidence suggests that the status of the telomeric complex, as well as the length of telomeric sequence, is crucial for chromosomal stability [2].

**Telomerase**

In most eukaryotes replenishment of telomeres is carried out by a nuclear ribonucleoprotein called telomerase which adds simple telomeric sequences onto preexisting 3′ overhangs [9, 10]. In human cells, this reverse transcriptase is constituted by a catalytic component (hTERT) associated with an RNA subunit h(TR). The hTR subunit is ubiquitously expressed, whereas hTERT is only expressed in embryonic stem (ES) cells and germ cells. The observation that 90% of tumors exhibit telomerase activity emphasizes that the lengthening of telomeres by telomerase is the process preferentially used to maintain the proliferative potential of cells. Some evidence, however, points to a role of telomerase in chromosome end protection which is independent of its ability to effect telomere elongation. Human fibroblasts and endothelial cells ectopically expressing telomerase reverse transcriptase can bypass replicative senescence despite a lack of telomere elongation [11, 12]. Recent experiments, using a new telomerase antibody, report for the first time that hTERT is active in normal human fibroblasts during their transit through S phase [13]. This hTERT activity suggests that telomerase and telomere structure are dynamically regu-

Table 1. Telomere-associated proteins identified in mammalian cells.

| Name | Localization/interaction | Functions at telomeres | Gene localization | References |
|---|---|---|---|---|
| Specific telomeric proteins | | | | |
| Telomerase | direct interaction with TTAGGG overhang | telomere elongation | 5p15.33 | 69 |
| Pot1 | binds specifically to the G-rich strand of telomeric DNA | telomere-length maintenance<br>telomere protection | 7 | 70 |
| TRF1 | present in T loops and in APBs<br>double-stranded TTAGGG repeats binding | negative regulator of telomere length (dependent on telomerase) | 8q13 | 71, 72 |
| TRF2 | present in T loops and in APBs<br>double-stranded TTAGGG repeats binding | negative regulator of telomere length (independent on telomerase)<br>chromosomal integrity maintance | 16q22.1 | 6, 44, 73 |
| TANK1/2 | putative localization at telomeres through its interaction with TRF1 | TRF1 ribosylation<br>positive regulator of telomere length | 8p23.1/10q23.3 | 28, 29 |
| TIN2 | TRF1-binding protein | positive regulator of telomere length | 14q11.2 | 24, 74 |
| RAP1 | TRF2-binding protein<br>associated with APBs | telomere length regulator | 16 | 75 |
| PINX1 | TRF1/Pin2-interacting protein | potent telomerase inhibitor | 8p23 | 22 |
| Nonspecific telomeric proteins | | | | |
| Ku70/Ku86 | found associated to telomeric repeats | negative regulator of telomere length<br>role in telomere capping | 2q35/22q11-q-q13 | 76–78 |
| DNA-PKcs | direct or indirect telomeric DNA association | capping and putative postreplicative telomeres processing role | 8q11 | 57, 79 |
| Rad50/ NSB/MRE11 | DNA-repair complex that binds TRF2<br>associated with APBs | putative role in T-loop stabilization | 5q31/8q21.3/11q21 | 49, 80–83 |
| Rad51 | APB localization in ALT cells | putative role in telomere maintance by HR in ALT cells | 15q15.1 | 84, 85 |
| WRN/BLM | binds 3′ overhang<br>physically interacts with TRF2 | telomere structure maintance | 8p12-p11.2/15q26.1 | 86, 87 |
| P53 | single-strand overhang binding T-loop junction in vitro | putative role in telomere structure maintenance apoptosis signaling pathway | 17p13.1 | 88, 89 |
| ATM | TRF1-interacting protein | signaling of telomere-mediated function<br>telomere chromatin structure | 11q22-3 | 90, 91 |

lated. Therefore, telomerase binding at telomeres, rather than bulk telomere elongation per se, might protect very short telomeres from recognition as damaged DNA.

## TRF1 and associated proteins

In mammalian telomeres, two TTAGGG repeat binding factors, TRF1 and TRF2, have been discovered [14, 15]. In vitro, both TRF proteins bind to double-stranded telomeric DNA through a conserved myb domain located in their carboxy-terminal domains, but they differ in their N termini rich in either acidic residues (TRF1) or basic residues (TRF2). TRF1 and TRF2 negatively regulate telomere length. Overexpression of TRF1 or TRF2 in vivo induces a gradual decline in telomere length [16, 17]. The formation of the T loop was proposed to involve both TRF1 and TRF2. TRF1 has the ability to induce binding and pairing of duplex telomeric DNA [18], whereas TRF2 induces the invasion of the 3′ overhang into duplex telomeric DNA [6]. Thus, the T-loop-based mechanism for telomere protection would predict that both TRF1 and TRF2 are crucial for length homoeostasis of human telomeres.

TRF1 prevents access of telomerase to the telomeres. Expression of a dominant-negative allele of TRF1 leads to telomere elongation [16]. In this system, TRF1 does not affect telomerase activity, and according to the current model, a large amount of telomeric protein would be recruited on long telomeres, blocking telomerase-mediated elongation and thus leading to a decrease in telomere length. A similar model has been proposed for telomere length homeostasis in yeast [19]. Recent chromatin immunoprecipitation (ChIP) experiments showed that TRF1 interacts with a single-strand telomeric DNA-binding protein: POT1 (protection of telomeres 1). A mutant form of POT1 abrogates TRF1-mediated control of telomere length and induces rapid and extensive telomere elongation. TRF1 binding at telomeres increases the POT1 binding on single-stranded DNA, which could sequester telomere terminus and thus block telomerase from elongating the telomeric DNA. Alternatively, POT1 can interact with the base of the T loop and could stabilize telomeres in a closed conformation [20]. However, another group showed that overexpression of different variants of POT1 leads to telomere elongation by telomerase [21]. They propose that POT1 could displace the T loop and might allow telomerase access to the 3′ ends of human telomeres. A TRF1/Pin2 interacting factor is PinX1, which has been shown to interact with hTERT and modulate its activity directly [22]. Pin2 is the 20-amino acid-deleted isoform of TRF1 and is more abundant than TRF1 in the cell [23]. PinX1 represents a novel class of proteins that can regulate telomerase activity. Indeed, overexpression of PinX1 inhibits telomerase activity, shortens telomeres and induces crisis. Moreover, depletion of PinX1 increases tumorigenicity in nude mice, suggesting that PinX1 is a putative tumor suppressor [22]. TRF1 function is regulated by TIN2 and two homologous proteins, TANK1 and TANK2. Binding of TRF1 to telomeric repeat sequences is enhanced by TIN2. A mutation in TIN2 protein leads to telomere elongation, suggesting that TIN2 is a negative regulator of telomere length [24]. TANK1 and TANK2 have a poly(ADP-ribose) polymerase (PARP) catalytic domain. These modifications inhibit TRF1 binding to telomeric sequences [25], and overexpression of TANK1 in tumor cell lines causes telomere elongation [26]. Thus, TANK1 might inhibit TRF1 binding and might be a positive regulator of telomere elongation. Recent data show that TANK1 overexpression leads to TRF-1 degradation by the proteasome. This degradation is required to keep TRF1 off the telomere and is dependent on TANK1-PARP activity. This is the first telomeric protein shown to be targeted by the ubiquitin-proteasome pathway [27]. Like TANK1, overexpression of TANK2 in the nucleus releases TRF1 from telomeres, suggesting that in vivo TANK2 could play a role at telomeres [28]. Nevertheless, overexpression of TANK2, but not TANK1, causes rapid cell death by necrosis [29]. Different data show that TANK2 associates and colocalizes with TANK1; thus, both tankyrases might function as a complex and probably mediate overlapping functions [30]. Controversial studies concerning the role of PARP in controlling telomere length have been carried out [31, 32]. PARP1 deficient mice developed by Wang et al. were found to have dramatically shorter telomeres and an increased chromosome fusion frequency and aneuploidy [33]. However, PARP1 knockout mice from De Murcia's laboratory displayed normal telomere length and a slight increase in end-to end fusions compared with wild-type mice [34]. Such discrepancy could be attributed to the difference in genetics background of these PARP1-deficient mice. PARP1 deficiency in $P53^{-/-}$ genetic background results in heterogeneity of telomere length [35]. Together with recent finding that PARP1 interacts with telomerase [36], these observations suggest the involvement of PARP1 in the telomere length regulation.

Ataxia teleangectasia (AT) is an autosomal recessive disorder, and cells derived from AT patients show an accelerated loss of telomeres and elevated spontaneous frequencies of chromosomal aberrations, including end associations [37]. The gene responsible for AT, ATM [38], a master controller of cellular pathways orchestrating the response to DNA damage, has been reported to interact and phosphorylate TRF1 [39]. A mutation in the ATM gene increases the fraction of telomeres bound to the nuclear matrix. This may be the reason why AT cells have a higher frequency of end-to-end associations [40]. Inhibition of Pin2/TRF1 in AT cells by stable expression of mutants increases telomere length as shown in other cells. Surprisingly, Pin2/TRF1 mutants reduce radiosensitivity

and complement G(2)/M checkpoint defects [41]. These results demonstrate a critical role for Pin2/TRF1 in the ATM-dependent regulation of telomere and DNA damage response. Protein level and subcellular localization of Pin2/TRF1 are tightly regulated during the cell cycle. In addition, overexpression of Pin2/TRF1 induced abortive mitosis and apoptosis in cells containing short telomeres [39]. These results, together with the demonstration of Pin2/TRF1 interaction with microtubules, indicate that Pin2/TRF1 also played an important role in mitotic progression [42]. The telomeric role of ATM was further analyzed using ATM and telomerase double knockouts [43]. These mutants showed increased telomere erosion and genomic instability. However, the incidence of T cell lymphomas associated with ATM deficiency was reduced. A general proliferation defect was evident in all cell types and tissues examined, and this defect extended to tissue stem/progenitor cell compartments provides a basis for accelerated aging and premature death of the compound mice. Thus, ATM deficiency and telomere dysfunction act together to impair cellular and whole-organism viability, supporting the view that aspects of AT pathophysiology are linked to the functional state of telomeres and the impairment of stem/progenitor cell reserves [43].

**TRF2 and associated proteins**

The major known function of TRF2 at telomeres is to stabilize G-strand overhang and thus to protect chromosomes from end-to-end fusions [44]. Although TRF2 protein is able to induce by itself the formation of the T loop in vitro [6], it is likely that its role at telomeres in vivo requires other factors. When TRF2 is inhibited, 15% of telomeres are fused, and telomeric DNA persists at the site of fusion, demonstrating that the TTAGGG repeat is not sufficient for telomere integrity [44]. Molecular analyses suggest that the telomeres are ligated together by a DNA ligase IV-dependent NHEJ mechanism [45]. Indeed, it was demonstrated that G-overhang loss precedes ligation [44]. Telomere fusions can occur before and after DNA replication, resulting in dicentric chromosomes and chromatid dicentrics, respectively [44]. Bailey et al. using chromatid orientation fluorescent in situ hybridization (CO-FISH) determined that it is usually the telomere created by leading strand DNA synthesis which is implicated in dicentrics [46]. One possible explanation for preferential fusion would be that the DNA synthesis of the leading strand, unlike for the lagging strand, generates an appropriate blunt end substrate for NHEJ ligation. Thus, in the absence of TRF2, the cell fails to regenerate 3′ overhang, and chromosome extremities remain unprotected due to their inability to be processed into the T loop. In a subset of human cell types, TRF2 inhibition results in P53- and ATM-dependent apoptosis rather than senescence. These results suggest that telomeres lacking TRF2 molecules are recognized as damaged DNA [47].

Recent experiments reported that overexpression of TRF2 protein induces telomere shortening without accelerating senescence. Thus, TRF2 protects critically short telomeres from end-to-end fusion and delays senescence entry. It seems that the protected status of telomeres, in addition to the telomere loss, is important for entry into senescence [48].

The complex Rad50/MRE11/NBS1 is a binding partner of TRF2 [49]. The presence of this complex with a nuclease activity at telomeres is particularly interesting with regard to the formation of the 3′ overhang [50]. However, mammalian cells lacking the MRE11 complex are not viable [51]; conditional deletion would be required to determine the contribution of this complex at telomeres.

In human cells, WRN [the protein which is deleted in Werner syndrome (WS)] physically interacts with TRF2 [52]. In vitro, TRF2 also demonstrates a high affinity for BLM (the protein deleted in Bloom syndrome) protein [53]. TRF2 interaction with either WRN or BLM results in stimulation of their helicase activities. The premature senescent phenotype observed in WS cells does not seem to be induced by abnormally short telomeres but may still be related to telomere dysfunction [54]. The molecular role of these proteins at the telomeres needs to be elucidated, but they are likely involved in the cellular response to dysfunctional telomeres.

**DNA repair proteins**

The fact that DNA repair proteins implicated in NHEJ or in the homologous recombination (HR) process are located at telomeres would suggest an involvement of these proteins in structure and telomere length maintenance. The role of proteins specific to HR will be detailed below.

The DNA-PK complex consists of a heterotrimeric subunit which carries the catalytic activity (DNA-PKcs) and DNA-binding proteins called Ku (Ku70 and Ku86). The Ku proteins bind to double-strand breaks (DSBs) and recruit DNA-PKcs to the break, whereas XRCC4 and ligase IV are involved in the final ligation step. Studies performed on mouse embryo fibroblasts (MEFs) from DNA-PK$^{-/-}$ mice showed telomeric DNA fusions with normal telomere length, anaphase bridge and normal G-strand overhang [55–58]. Recently, by generating mice doubly deficient in DNA-PKcs and in telomerase, Blasco's group showed accelerated telomere shortening. In addition, DNA-PKcs is required for apoptosis induction by critically short telomeres [58]. These results suggest that DNA-PKcs interacts with telomerase to maintain telomere length, as well as to signal short telomeres as DNA damage, triggering apoptosis and facilitating end-to-end fusions of short telomeres. Experiments performed in the hamster cellular system showed that the lack of

DNA-PKcs also increases the gene amplification phenomenon [59].
In yeast, Ku has an essential role at telomeres. Ku deficiency leads to telomere shortening, loss of telomere clustering, loss of telomere silencing and deregulation of telomeric G overhang [60, 61]. Furthermore, yeast Ku moves from the telomere to the DSB upon induction of damage, suggesting a link between DNA repair and the telomeres [62, 63]. In mammals, Ku associates with telomeric repeats [57] and can interact with TRF1 and TRF2 [64, 65]. Recent studies, carried out on late-generation telomerase-deficient and Ku86-deficient mice, point to a role of Ku86 in mediating chromosomal fusions and apoptosis induction in cells with critically short telomeres, although it does not induce telomere shortening or deregulation of G overhang as shown by the analysis of Ku86-deficient mice [66, 67]. All these results suggest that Ku86 could contribute to render telomeres less accessible to telomerase or DNA repair activities.
Studies in mice demonstrate that the loss of single copy of *lig4* promotes development of soft tissue sarcomas that possess clonal amplifications, deletions and translocations. These results demonstrate that reduced activity of the NHEJ system gives rise to chromosomal aberrations that drive non-lymphoid tumorigenesis [68].

## Chromosomal Instability in Replicative Senescence

In most human primary cells, telomeres shorten after each replication round. When the telomeres reach a critical length, cells stop dividing and enter senescence. Hayflick first suggested that diploid fibroblasts undergo a limited number of divisions in culture, after which they stop proliferating [92]. Primary cells undergo a finite number of divisions, arrest the cell cycle and reach mortality stage 1 (M1) [93]. The replicative potential of primary cells can be extended by viral oncogene (e.g. $AgT_{SV40}$) transformation, despite the dangerous shortening of telomeres. Cells presenting critically short telomeres would cease to divide and enter crisis (mortality stage 2, M2). Senescent cells can persist in culture for months to several years, remaining metabolically active, but unable to synthesize DNA. They also undergo morphological changes such as cell enlargement, flattening and the expression of $\beta$-galactosidase activity at pH 6 [94].
Replicative senescence seems to be a genetically dominant phenotype as shown by a large number of somatic cell fusion studies in which proliferating normal cells were fused with immortal tumor-derived cells. To establish how many distinct genes play a part in cellular senescence, several immortal cell lines were fused with each other [95]. The ability of some, but not all, fusion pairs to produce hybrid cells that are not immortal has led to the identification of four complementation groups of replicative immortality, indicating that at least four genes are required for the induction of senescence. These findings are confirmed by chromosome transfer studies identifying different genes that induce a senescent-like phenotype in immortal cell lines [96–99].
In cultured human fibroblasts, the frequency of chromosomal abnormalities involving telomeric regions, especially dicentric chromosomes, rises dramatically as cells approach replicative senescence [7, 100–102]. Telomere length has been found to be a good indicator of the potential replicative capacity of cultured human fibroblasts [103] and a correlation between the frequency of dicentric chromosomes and telomere length in senescent human cells has been demonstrated [7]. The frequency of dicentrics increases, and cells enter M1. Both events appear to be triggered when telomere length reaches its minimum size of approximately 1.5 kb. Thus, it might be suggested that dicentric formation occurs through a direct end-to-end fusion when telomeres erode sufficiently.
Senescent fibroblast karyotypes present abnormalities. Telomere fusions occur as shown by the genesis of dicentric chromosomes. In senescent cells, dicentric chromosome number rises far more than the number of other chromosome aberrations such as fragments, gaps and breaks [101]. Moreover, abnormal mitosis occurs during colony expansion and is characterized by dicentric and unbalanced translocations. Both the frequency of mitosis carrying telomeric associations and the number of telomeric associations per mitosis have been shown to increase with culture passages [104]. Finally, cytogenetic analysis of cultured fibroblasts obtained from centenarians (that is from individuals very close to the maximum expected life span) does not reveal any peculiar structural chromosome anomaly, but some telomeric associations arose in cells approaching senescence [105]. In some studied cell lines, particular chromosomes seem to be preferentially involved, indicating that dicentric formation is a result of changes specific to those chromosomes, rather than involving general factors in the cell [101]. Also, in human dermal fibroblasts, certain chromosomes are more implicated than others in rearrangements involving specific telomeric regions at late passages after irradiation with heavy ions [106].
The causal factor that drives a primary cell to enter replicative senescence is connected with telomere attrition and telomerase absence. In this context, telomeres shorten and lose their end stabilization function. In $mTERC^{-/-}$ p53 mutant mice, loss of telomeric function has been shown to cause breakage-fusion-bridge cycles [107, 108], which result in the loss of chromosomal material and chromosomal instability.
Primary MEFs from the $mTERC^{-/-}$ mouse strain were used to investigate whether dysfunctional telomeres, either due to telomere shortening to a critical length and/or loss of their end-capping function, lead to a senescence-

like arrest in mouse primary cells [109]. This mouse strain lacks telomerase due to a targeted deletion of the gene encoding the telomerase template RNA [110]. Actually, after four to six generations in the absence of telomerase, the telomeres of these mice are as short as those in human cells before senescence. mTERC$^{-/-}$ MEFs with short telomeres have a reduced proliferative capacity as well as a reduced ability to immortalize spontaneously in culture. These results indicate that telomere shortening limits the proliferative potential of cultured mouse fibroblasts.

Not only the telomerase, but also other telomeric proteins, such as TRF2, seem to be implicated in the induction, or in the prevention, of replicative senescence. Overexpression of TRF2 in human fibroblasts results in accelerated telomere shortening, without promoting premature senescence [48]. TRF2 accelerates telomere shortening in several fibroblast strains regardless of the status of p53 or the p16-RB pathways. TRF2 also accelerates the entry into crisis in SV40 large T and HPV16-E6 and E7 cultures, probably by an earlier induction of the excessive genome damage responsible for crisis. But its overexpression in human fibroblasts lacking p53 and RB function (that grow beyond normal senescence setpoint and continue to lose telomeric DNA, resulting in frequent chromosome end fusion and other chromosomal damage) creates a measurable reduction in chromosome end fusion and associated chromosomal damage. All these data suggest that overexpression of TRF2 can protect very short telomeres, and this would be the mechanism by which TRF2 can extend the lifespan and alter the senescence setpoint of human primary cells. Collectively, these data argue that replicative senescence is caused by a change in the status of the telomeric complex rather than by a complete loss of telomeric sequences. One possibility is that critically short telomeres in senescent human cells no longer bind sufficient TRF2 to achieve a protective state. Alternatively, binding of TRF2 may facilitate the recruitment of other proteins required for telomere protection and suppression of senescence.

Inhibition of TRF2 was used to examine how mammalian cells enter senescence in response to telomere dysfunction. A dominant-negative TRF2 allele expression induces a premature senescence indistinguishable from replicative senescence in primary fibroblasts [111]. This finding, together with the demonstration that overexpression of TRF2 can delay senescence, suggests that the initiating signal for the induction of senescence emanates from chromosome ends lacking sufficient TRF2 protection.

As mentioned above, cells from AT patients exhibit elevated frequencies of spontaneous chromosomal aberrations, including end associations [37]. Altered telomere interactions could influence the senescence signaling mechanism, since AT cells undergo premature senescence. In addition, AT cells show telomere loss. Telomere length reduction has been linked with chromosome end associations. It could be a consequence of a breakage not repaired near or within the telomere while chromosomes migrate in opposite directions. The expression of hTERT in AT fibroblasts extends their life span without changing the frequency of telomeric end associations as well as the occasional appearance of SA-β-Gal+ cells [112]. The presence of end-to-end associations in hTERT-transformed AT cells suggests that these cells could still have altered telomere nuclear matrix interaction, and that may influence the premature entry in senescence.

Since abnormalities in the replication and/or chromatid separation of telomeric regions may affect the integrity of chromosomal ends, the behavior of several telomeres in cultured human fibroblasts during early and late cell passages was studied using fluoroscence in situ hybridization (FISH) experiments [113]. The results show that delay in replication and/or separation of several human telomeric regions occur in primary fibroblast cells as they approach replicative senescence. Incomplete DNA replication, failure to separate sister chromatids, or improper condensation at telomeric regions would all be expected to compromise chromosomal integrity, if these cells progress to anaphase. Since a high percentage of dicentric chromosome and other chromosome abnormalities have been observed in senescent cells, these findings provide a potential mechanistic model to explain these observations. Moreover, the inactive X chromosome, which replicates late, shows an accelerated shortening of telomeric repeats [114]. Since the rate of aneuploidy in cultured lymphocytes increases with advancing age, with an extreme overinvolvement of the X chromosome in women [115], this observation is also consistent with a potential causative role of telomere shortening in the X chromosome aneuploidy in aging women.

## Chromosome aberrations

The first specific chromosomal aberration observed in a tumor was the Philadelphia chromosome in chronic myelogeneous leukemia (CML), in 1960. Previously, abnormal chromosomes had already been observed in tumor cells, but the relationship with the tumoral phenotype was only admitted in the late 1970s. The presence of chromosomal abnormalities is well known as a fundamental feature of tumor progression [3]. However, cytogenetic analysis has revealed that the pattern of CA is largely different from one type of tumor to another, with aberrations which are specific or common to several tumors [116–118]. In general, these chromosome changes are translocations, gene amplifications, losses and gains of whole chromosomes or large portions of chromosomes, leading to fusion genes, modification of gene copy number or deregulation of the expression of various oncogenes.

**Chromosomal abnormalities in tumors**

The number and type of chromosome changes might depend on their temporal occurrence and on the probability of promoting neoplastic transformation. Mutations promoting growth stimulation induce tumors most of time when secondary events provide a selective advantage [119]. This introduced the primary and secondary changes concept, which is well developed in hematological malignancies [120]. In general, primary changes are balanced, recurrent and most of time the sole abnormality, but they represent an essential step in tumorigenesis. Secondary changes are numerous, unspecific and give advantages during tumor progression. However, this 'two-steps' view of tumorigenesis depends on the affected cell [121, 122]. On the one hand, hematological neoplasms and some bone and soft tissue tumors typically arise through balanced translocations which are directly involved in the tumoral phenotype. Characteristic illustrations are the t(9;22) translocation in the CML or the t(11;22) in the Ewing sarcoma [123, 124]. On the other hand, the vast majority of cancers occurring in human adults are carcinomas of epithelial origin. These tumors present highly rearranged karyotypes with perpetuating modifications during tumor progression, so that high-grade solid tumors exhibit a higher number of CA and no chromosomal change can be regarded as a common primary event [125]. However, the repeated occurrence of several changes favors the hypothesis of two karyotypic evolutionary processes. For example, in most colorectal tumors, monosomy 17p and 18 were found, and the karyotypic evolution involved mainly several additional monosomies due to unbalanced rearrangements or losses, as well as gains of chromosomes and amplifications. In this group of tumors, the mean number of chromosomes remains close to 46 and is called the monosomic type. In the other tumors, either a monosomy 17 or a monosomy 18 was found. The karyotypic evolution involved essentially trisomies resulting from gains and leading to the trisomic type with the mean chromosome number close to 51 [126, 127]. Thus, chromosomal variability in malignant cells may be due to an inherent genetic instability caused by the dysfunction of genes controlling cell division or cell death, including DNA repair [128–133], and also by the founder cell effect. Recent studies have strengthened the proposal that mutational alterations conferring instability occur early during tumor formation, then influencing the karyotypic evolution [134, 135].

**Telomere-driven chromosomal instability in cancer cells**

The shortening of telomeres or mutation of telomeric sequences can lead to the formation of telomeric fusions between chromosome arms [136–141]. Experiments carried out on *Tetrahymena* showed a role of telomeres in chromosome separation during mitosis. Expression of a telomerase RNA with an altered template sequence causes a severe delay or block in completing mitotic anaphase. Although the sister chromatids begin to separate and are pulled apart as anaphase progresses, they are unable to segregate to daughter poles that lead to cell death. Mutant telomeres could be inaccessible to factors that normally act to separate sister chromatids [141].

In some cancer cells, loss of telomeric repeats may occur at a high rate, leading to high frequencies of telomere associations. These fusions lead to anaphase bridges at the next cell division and then promote the breakage-fusion-bridge (BFB) cycle mechanism generating numerous chromosome aberrations [142]. During mitosis, the two centromeres of a dicentric chromosome are frequently pulled towards opposite spindle poles, producing a break. Broken ends, corresponding to chromosomes lacking one telomere, remain unstable until they are capped [143]: either by fusion resulting in a novel chromosomal rearrangement or by capture of a telomere associated with a nonreciprocal translocation of another chromosome fragment as described in tumor epithelial cells and mouse fibroblasts [143–145]. These new fusions could generate new unstable chromosome structures, perpetuating the BFB cycle and chromosomal instability. This BFB cycle triggered by telomere dysfunction has been shown to play an important role in epithelial carcinogenesis in mice [107, 146] and in human tumors [108, 147], where a negative correlation has been established between the length of telomeres and their involvement in chromosome aberrations. Anaphase bridges can also cause whole chromosome losses or the collapse of the cytokinetic process, leading to numerical chromosome aberrations. Many of the characteristic genetic abnormalities in tumor cells, including gene amplification, aneuploidy and loss of heterozygosity can be explained by the chromatid BFB cycle model [148]. This telomere-driven chromosomal instability seems to occur early in the tumorigenesis since CA involving telomeric regions are widely seen in tumors of low grade or in young people [108, 136, 149, 150].

As mentioned above, the BFB cycle could generate amplification (fig. 1). Gene amplification is a common consequence of genome instability in tumor cells and can be the basis of oncogene activation and drug resistance [151–154]. Several groups have provided evidence that DNA breakages play an important role in the initiation of gene amplification [155, 156]. In fact, chromosome breaks might result in a global change in DNA compaction [157]. Moreover, amplification rates can be increased by the proximity of chromosome fragile sites [158] and by a delay in mitotic chromosome condensation [159] that is sometimes induced by the phosphorylation of histone H3 [160, 161]. Gene amplification mostly concerns the genes located close to the chromosome breakpoint. The high level of amplification indicates the repet-

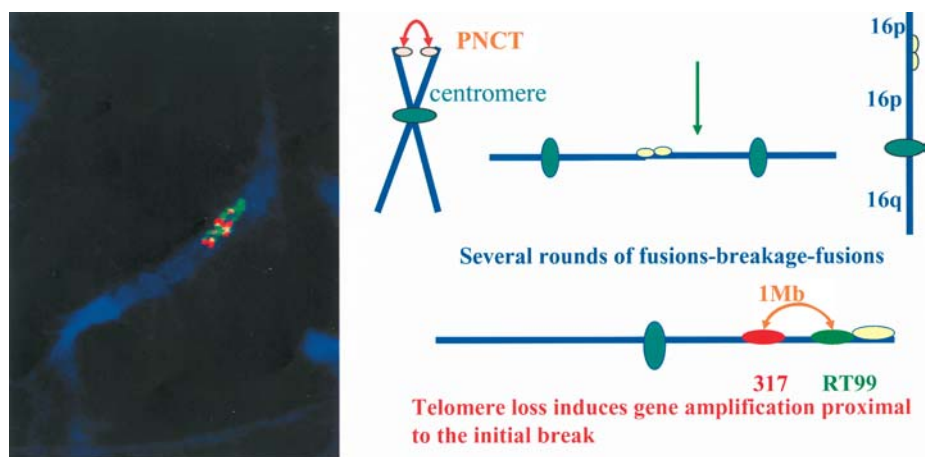


Figure 1. Gene amplification: consequence of telomere loss (from [162]. The human cell line was transfected with plasmids containing telomeric repeat sequences that 'seed' new telomeres when integrated on the end of a 'marker' chromosome (chromosome 16). Cells presenting one lost telomere were subcloned. Fusions of sister chromatids and BFB cycles occur where the telomere is lost. To investigate the localized amplification, metaphase spreads were hybridized simultaneously with the fluoroscein-labeled cosmid RT99 (green) adjacent to the integration site, as well as an additional rhodamine-labeled cosmid 317H7 (red) that is located 1 Mb from the integration site. The chromosomes were stretched to detect the hybridization signals generated by the two probes. Most hybridization signals involved the proximal sequences, with only occasional signals involving sequences located at more than 1 Mb from the initial telomere loss.

itive occurrence of BFB cycles in the same location, predicting that these regions may be hot spots for chromosome breakage [162]. Even if the mechanisms promoting translocations remain unknown, several studies have pointed out the role of the recombination of specific sites like Alu sequences [163], immunoglobulin genes [164, 165] or enzyme recognition sites [166]. All these data strongly suggest that the chromatin structure, the sequence and the chromosome condensation of particular chromosome regions are closely related to their involvement in formation of chromosome aberrations.

It was demonstrated that cells with mitotically unstable chromosomes are eliminated rapidly [167]. In addition, critically short telomeres can induce cellular senescence [168]. These responses to cellular damage are largely dependent on the p53 protein implicated in the maintenance of cell cycle arrest and in the activation of the different proteins of the machinery of DNA repair. Recent analysis carried out on telomerase knockout mice submitted to radiation emphasizes the role of telomere length on radiation-induced chromosomal instability. Short telomeres fused with radiation-induced breaks causing the increased chromosomal instability observed in this mouse [169]. When the mutations are too great in number or in consequences, cell death or a program of apoptosis occurs. The elimination of unstable cells plays a key role in tumor suppression, avoiding the growth of cells with a proliferative advantage [170]. On the one hand, telomere shortening induces cell death and can lead to tumor suppression, while on the other hand, it could initiate chromosome instability and promote tumorigenesis. Thus, telomere shortening can have two outcomes with opposite predicted effects on tumorigenesis.

## Telomerase and tumorigenesis

In 85–90% of tumors, telomerase is involved in the selective process of the tumorigenesis [7, 171, 172]. On the one hand, TERT expression is associated with nontelomeric functions. Indeed, ectopic expression of telomerase could enhance cell survival in the face of proapoptotic cellular stress [173] and regulate the transcription of a set of genes implicated in cell growth and in the DNA repair system [174–176]. Recently telomerase was shown to increase tumorigenic potential in mice, without any influence on telomere length [177, 178], and in human fibroblasts high expression of telomerase elicits senescence growth arrest [179]. These findings support the notion that telomerase might have novel and telomere-independent functions. On the other hand, the most recognized function of telomerase concerns maintenance of the telomeres. The acquisition of telomerase activity and the rescue of telomere integrity also have two contradictory effects: stabilizing chromosome ends reduces genomic instability, [180]; conversely, bypassing the M2 limit confers immortal growth properties on tumor cells [181, 182].

Activated telomerase produces a dynamic balance between elongation and shortening and tends to result in homogeneous telomere length. The resultant stabilization of telomere length promotes chromosome stability. In hTERT-immortalized cells, telomeres are usually shorter than those in the normal cells from which they were derived [183–185], and in some cases they continue to shorten further than the telomeres of transformed but nonimmortal cells that entered crisis. This suggests that telomerase may protect chromosomes from end-to-end fusions, not only by lengthening the telomere but also by a capping function [11, 173, 186, 187].

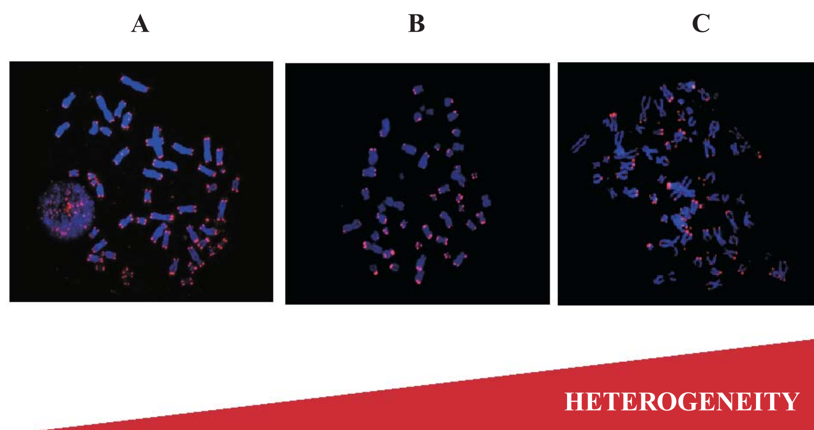


Figure 2. Telomeres heterogeneity in primary fibroblast (*A*), senescent fibroblast (*B*) and ALT tumoral fibroblast (*C*).

Genetic instability, leading to the malignancy of cells by the disruption of genes controlling the cell cycle, can occur in telomerase-positive cells, such as immature or hematological cells. In this case, the stabilization of broken chromosomes by telomerase is an early event, occurring before the critical shortening of the telomeres causing the chromosome instability. The resulting karyotype is often quite normal, with few anomalies and without overinvolvement of telomeric regions. Conversely, in rather differentiated cells, numerous mutations have been accumulated, and telomeres are short enough to induce end-to-end fusions, leading to the BFB cycle mechanisms and maintaining chromosome instability. In this case, telomerase activation occurs late in tumorigenesis, after the initiation of the genetic instability [102, 143, 181, 182, 188, 189], and tumors may display complex karyotypes with numerous and nonrecurrent chromosome abnormalities [107, 122]. This category is the most represented in tumors of epithelial origin, the vast majority of human cancers.

## Chromosomal instability and ALT phenotype

Human cells possess at least one additional mechanism for telomere maintenance, alternative lengthening of telomeres (ALT), which is independent of telomerase and relies on homologous recombination and copy switching [190–192]. The large majority of human tumor cells acquire immortality through expression of the catalytic subunit of telomerase (hTERT) [193], whereas fewer cells activate this alternative lengthening mechanism of telomere maintenance [194].

The ALT pathway in human cells is associated with marked variability in telomere length, rapid telomere lengthening or deletion of several kilobases, and the presence of specific nucleoprotein structures called ALT-associated PML bodies or APBs (fig. 2) [191]. All human ALT+ cancers and cell lines analyzed to date have a very wide telomere length distribution. Some chromosome ends have telomeres that are undetectable by FISH, while others within the same cell have very long telomeric sequences [173, 190, 195, 196].

Scheel et al. demonstrated that ALT cells present an increased number of complex nonreciprocal chromosomal rearrangements compared with telomerase-positive cell lines, suggesting that in ALT cells the occurrence of elongated and shortened telomeres and signal-free chromosome ends could give rise to chromosomal end-to-end associations and BFB cycles [4, 197, 198]. ALT+ cells present a high frequency of drastic telomere length changes. Some chromosomes with telomere sequences less than 200 bp showed a rapid and heterogeneous increase of up to 50 kb, correlating with the occurrence of increased chromosomal instability, and especially chromosome fusion. In a telomerase-negative human cell line with a tagged telomere [190], a correlation was found between the rapid changes in telomere lengths and the frequency of chromosome fusions. Intertelomeric recombination events in human ALT cells were evidenced by targeting a DNA tag into the telomeric region [192]. Up to five tagged telomeres in one cell were observed by FISH analysis after 63 population doublings. This phenomenon was not seen when the tag was located in the subtelomeric region. Taken together, these results focus on the recombination-based pathway for telomere maintenance in ALT cells. Moreover, *Saccharomyces cerevisiae* knockout for the telomerase RNA gene displays telomere shortening, and the cells eventually die. Survivors were found to maintain their telomeres by a mechanism that was dependent on RAD52, which encodes a protein that is involved in homologous recombination. Further analyses identified two classes of telomerase-null survivors. Type I survivors undergo amplification of a subtelomeric sequence (called Y′) and type II survivors have telomeres with lengths that are very heterogeneous, but are increased overall [199–201]. Both survivor types required RAD52; however, RAD51,

RAD54 and RAD57 are required in type I survivor, whereas RAD50, RAD59 and SGS1 are needed for type II [202–204]. The existence of a type I analogue ALT mechanism in human cancers remains to be elucidated, whereas type II analogue human cells have been characterized [205]. Telomeres of the yeast type II cells therefore resemble those of human ALT cells. After immortalization of ALT cells, the subtelomeric regions of specific chromosome ends were sequenced by polymerase chain reaction. The results underscore the involvement of a sequence from a donor, which is copied to a recipient telomere. A class of complex telomere mutations, described only in ALT+ cells, are defined by replacement of the progenitor telomere at a discrete point, called fusion point, with a different repeat array [205]. No such complex mutations have been retrieved in normal, precrisis or telomerase-positive cell lines, confirming that these mutations are specific to ALT+ cells. A high frequency of simple intraallelic telomere mutations in ALT+ cells has been observed, indicating that such mutations contribute to telomere instability, putatively through a reduction in mismatch repair. Studies on yeast have suggested that a defect in mismatch repair may remove a barrier to homologous recombination between telomeres and therefore contribute to the ALT pathway for telomere maintenance [206].

However, the precise role played by recombination in ALT lengthening of telomeres is still unknown. Both break-induced replication and rolling circle amplification have been proposed as mechanisms through which telomeres are elongated in yeast. In human cells, four mechanisms have been proposed so far: intertelomeric replication, T loop, rolling circle and linear extrachromosomal telomeric repeat (ECTR) homologous recombination [196]. In intertelomeric replication, the single-stranded overhang DNA at the end of one telomere invades double-stranded DNA of another telomere and uses it as a copy template [207]. T loop could contribute to telomere length variation in ALT cells in several ways: a telomere can be lengthened by using itself as a copy template in the T loop. In the case of crossover events, ECTRs can be formed after loop-mediated excision, as circular or linear forms, leading to telomere shortening which could account for the rapid reduction in telomere length seen in ALT cells [190] and in hybrid cells in which ALT is repressed [195]. Moreover, the resulting ECTR is involved as copy template for homologous recombination at the ends of other chromosomes resulting in continuous telomere length fluctuations [196]. These mechanisms might have different efficiencies in maintaining telomere integrity and in chromosome stability. Thus, ALT cells maintain their telomeres via striking length variations, whereas activated telomerase produces a dynamic balance between elongation and shortening and tends to result in telomeres of somewhat homogeneous length.

Further indirect evidence that the ALT mechanism might involve homologous recombination came from the particular composition of APBs. PML bodies are constituted by protein accumulations in the nuclei of most normal cells. These proteins are involved in a wide variety of functions such as tumor suppression, cell cycle regulation, senescence and apoptosis [208]. In all human ALT cell lines examined to date, APBs are distinguished from other PMLs by the presence of telomeric DNA, telomeric binding proteins such as TRF1and TRF2 [85], and a wide range of proteins involved in DNA recombination and replication, such as RAD51, RAD52, RPA, MRE11, RAD50, NBS1, BLM and WRN [71, 209]; reviewed in [196]. There is a temporal correlation between the immortalization event and the occurrence of APBs [85]. Conversely, when ALT is repressed in somatic cell hybrids, APBs eventually disappear [195]. Moreover, a tight correlation between the presence of APBs and ALT phenotype has been pointed out [85]. The telomeric DNA in APBs may be a subset of the total ECTRs that have been detected in various types of cells [210, 211]. In general, ECTRs are not detectable in telomerase-positive cell lines or in normal human cells [210]. Several proteins have been identified as associated with APBs, but whether they are required for the human ALT pathway or not still needs to be elucidated. It seems possible that the function of APBs is to repair or to eliminate telomeric DNA which is recognized as genomic damage by the cell. A recent study identified BLM as involved in telomeric DNA synthesis via a functional helicase domain, exclusively in ALT cells. These results strongly suggest that BLM could facilitate recombination-driven amplification of telomeres in ALT cells [53].

Thus the presence of these recombination-involved proteins within APBs emphasizes recombination-based telomere lengthening in ALT+ cells. The specific genetic requirements that promote the ALT pathway are not so far elucidated due to difficulties in genetic analyses of human cells. An inducible ALT system, where intermediate pathways steps could be analyzed, still needs to be established. Telomerase and ALT telomere maintenance mechanisms are equivalent in terms of immortalization and full tumorigenesis capability. However, their efficiency in tumorigenesis as well as chromosome stability may be quite different. Despite their long mean telomere length, most ALT cells contain some very short telomeres, which are more prone to end-to-end fusion events than telomerase-maintained telomeres and so might be a source of chromosome instability.

Emerging studies emphasize that activation of telomere maintenance strategies is not an obligatory step for tumorigenesis in human cells. Rudolph et al. reported that acquisition of a telomere maintenance mechanism is not strictly required for early stage carcinogenesis in human fibroblasts but may occur as a later event, restoring genomic stability to a permissive level for tumor progression. This hypothesis may be supported by the observation

that one patient with osteosarcoma showed metastasis telomerase$^{-}$/ALT$^{+}$ when primary tumor was telomerase$^{-}$/ALT$^{-}$ [212].

Unexpectedly, the absence of detectable telomere maintenance mechanisms was observed both in a subset of osteosarcoma [212] and in a clone derived from ALT cells that express a mutant form of hTERT incapable of elongating telomeres in vivo [M. A. Cerone et al., personal communication]. This clone shows suppression of the ALT pathway, absence of telomere shortening and no amplification of the subtelomeric regions. This could suggest that telomere maintenance mechanisms in human cells could be more diverse than previously thought.

## Conclusion

As is now well documented the telomeres in normal human cells shorten progressively during successive cellular divisions. This telomere erosion has been attributed to the inability of the general DNA replication machinery to replicate completely the very ends of the chromosomes. Thus, telomeres may be lost completely or shortened to such an extent that they no longer protect the ends of chromosomes. The protecting function of these nucleoprotein elements requires not only a minimum size but also an adequate structure, which involves numerous proteins. In addition to telomeric proteins, a growing number of proteins involved in DNA repair machinery have been demonstrated to interact with telomeres. When telomere function is altered, cells stop dividing and develop a senescent phenotype which has the characteristic of a high frequency of telomere associations. This telomere dysfunction is necessary for the initiation of carcinogenesis, as it induces chromosomal instability. The chromosomal instability remaining during tumor progression is balanced by the activation of telomere maintenance mechanisms. It was first thought that telomere length was maintained by the telomerase activity in cancer cells. A second mechanism defined as ALT was then described and seems to involve homologous recombination. Recent publications point to the possibility of immortalization without the involvement of any detected telomere maintenance mechanism.

Understanding these complex telomere maintenance mechanisms and the resulting chromosomal instability is primordial in designing new drugs and in overcoming the inefficiency of anti-telomerase-based therapeutics. Investigation of the potential correlation between telomere maintenance mechanisms and chromosomal instability may aid definition of additional biomarkers helpful for tumor-type classification and then development of appropriate therapeutic protocols.

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