Telomere dysfunction and genome instability

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1. ABSTRACT

The nucleoprotein complexes that cap the very ends of the eukaryotic chromosomes, named telomeres, are indispensable for cell viability. Telomeric DNA shortens in each cell division until it cannot exert end-protective functions in human somatic cells. Additionally, several proteins have been described to play a key role in telomere homeostasis preventing chromosome extremities to be recognized as double-stranded breaks. When telomeres become dysfunctional, either through excessive shortening or due to defects in the proteins that form its structure, they trigger p53/pRb pathways what limits proliferative lifespan. Impairment of telomere function together with a compromised senescence/apoptosis response leads to chromosome instability. Fusions between dysfunctional telomeres or even between dysfunctional telomeres and double-stranded breaks can initiate breakage-fusion-bridge cycles. Initially, telomere fusions were proposed to cause only structural abnormalities. Nevertheless, changes in chromosome number have also emerged as a possible consequence of alterations in end capping. Here we review the main aspects of telomeres and telomere-based chromosome instability, highlighting why they have been proposed as a driving force for tumourigenesis.

2. TELOMERES AND TELOMERASE

Telomeres are specialised nucleoprotein structures located at the ends of eukaryotic chromosomes. Telomeres protect chromosome extremities from being recognized as double-stranded breaks (DSBs), and thus from triggering a DNA damage response (DDR). Furthermore, telomeres avoid improper nucleolytic degradation at chromosome ends what would lead to genetic losses. These roles make telomeres key players in chromosome structure and function as well as in cell viability (1). Telomeric DNA consists of an array of six G-rich nucleotides of different sequence and length depending on the species. Nonetheless, telomeric repeats are highly conserved among different organisms, from protozoa to vertebrates, which means conserved functions (2). Telomeres end in an essential 3' single-stranded overhang ranging from 100 to 200 nucleotides. Electron microscopy studies suggested this overhang loops back and integrates into the duplex repeat tract, forming a "t-loop" (3,4). Different proteins have been described as playing important roles in the regulation of telomere length maintenance and in the formation of the protective end-cap that prevents chromosome fusions. The mammalian telomeric core complex has been termed shelterin and includes proteins that bind directly to the telomeric DNA (TRF1, TRF2 and
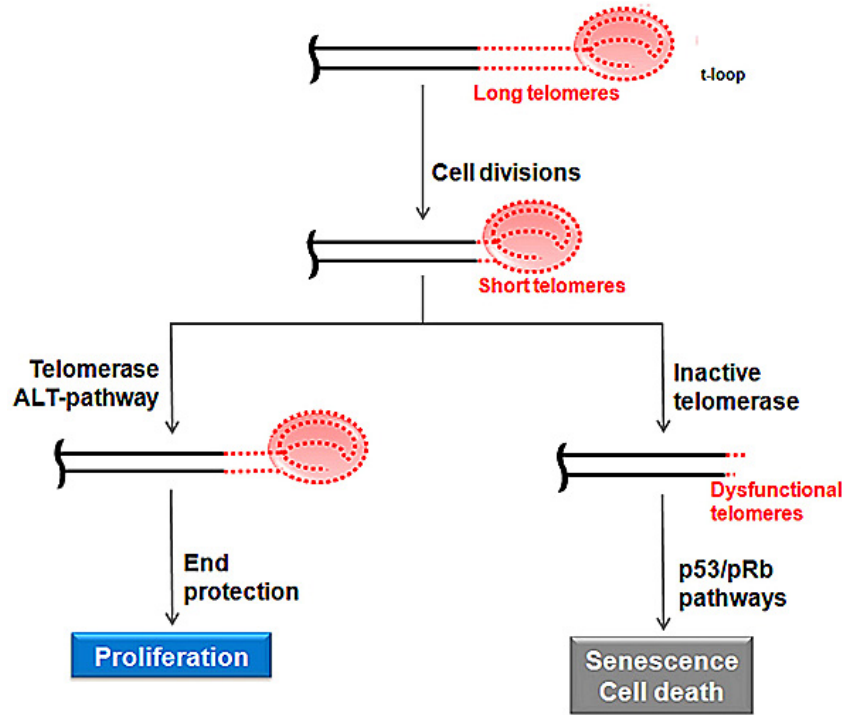
Figure 1. Telomeres cap the end of the chromosomes. When telomeres are long enough they can fold back and form a t-loop structure that protects chromosome extremities. As cells divide, telomere sequences shorten because of the end-replication problem. Exceptionally, some cells maintain telomere length, which enables them to protect the chromosome ends and continue proliferating. However, in the absence of a mechanism that allows for telomere elongation, telomere shortening ultimately leads to dysfunctional telomeres that cannot exert end protective functions. In this context, p53/pRb pathways are triggered and cells cease to divide.

POT1) and telomere-associated proteins that are recruited to telomeres by the former (TIN2, TPP1 and Rap1) (5,6). Additionally, proteins that are more commonly involved in DNA repair are also found at telomeric ends. Examples include the DNA-PK and MRN complexes, PARP1, PARP2, Tankyrase 1, Tankyrase 2, ATM, ERCC1/XPF, RAD51D, WRN and BLM (7-9).

Normal human somatic cells progressively shorten their telomeres with each round of cell division due to incomplete replication, the so-called end replication problem. DNA polymerase synthesizes *de novo* DNA strands in 5'-3' direction and a 5'-end RNA primer is needed in order to start elongating. While the new DNA strands are generated, the RNA primers are removed and the DNA polymerase fills the gaps by adding free nucleotides. However, removal of the distal RNA primer from the lagging strand gives rise to a gap that the enzyme cannot complete (10,11). This end replication problem, together with telomere end processing (12) to form the t-loop structure, promotes telomere shortening in subsequent cell cycles. Other factors such as oxidative stress (13,14) and the increased sensitivity of subtelomeric regions to DSBs (15) contribute to the loss of telomere repeats. Telomeres progressively shorten in proliferating cells until they are unable to protect the end of the chromosomes. When excessive telomere attrition occurs, cells cease dividing by triggering p53/pRb-dependent apoptosis and/or

senescence "Figure 1" (16). Inactivation of these checkpoints leads cells to crisis, a stage characterised by extensive telomere shortening chromosome fusions and eventually massive cell death. This telomere length-dependent growth inhibition has been proposed as the primary mechanism for tumour suppression *in vivo* (17,18) because it represents a barrier for unlimited cellular proliferation and genome instability (19,20).

Telomerase is the enzyme responsible for *de novo* addition of telomere repeats to the very ends of chromosomes and thus it compensates for the loss of telomere sequences (21). Greider and Blackburn discovered telomerase in 1985 in the model organism *Tetrahymena thermophila* (22). In humans, the core enzyme consists of two subunits: an RNA component (hTR), which serves as the template for telomere synthesis, and a catalytic protein, the telomerase reverse transcriptase (hTERT). Telomerase is not ubiquitously detected but is expressed in certain cell types such as the germline, activated lymphocytes, adult stem cells and somatic cells during early embryogenesis. Adult somatic cells show undetectable or low levels of telomerase activity, which contributes to telomere shortening in each cell division (21,23). Given that a minimum length of telomeric sequences is a requisite for cells to become immortal, over-expression of telomerase has been found in more than 90% of human cancers (24,25). A telomerase independent mechanism for telomere
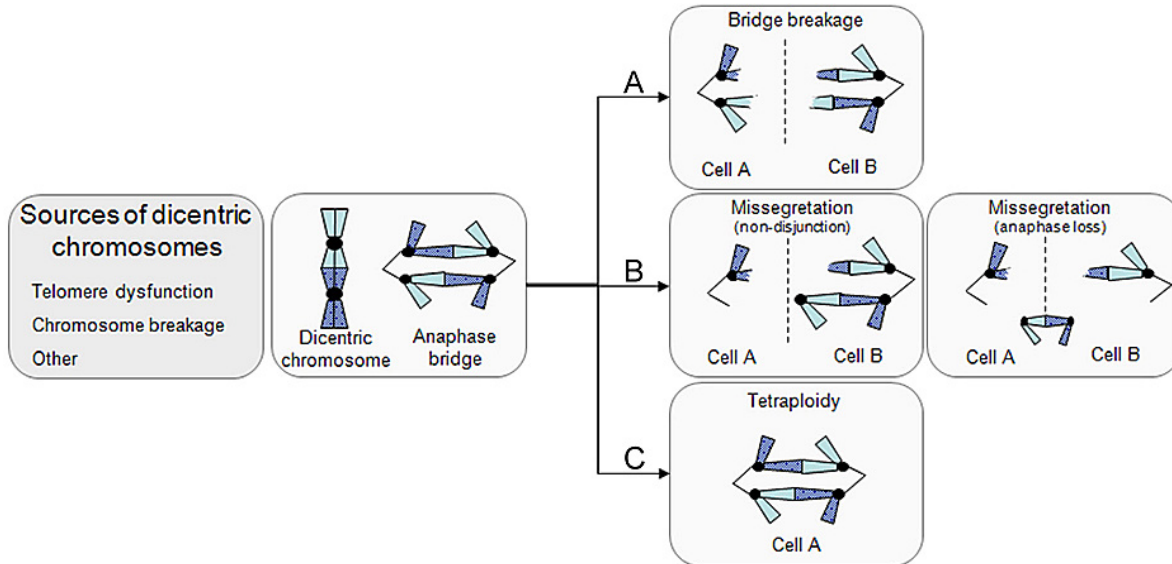
Figure 2. Telomere dysfunction and chromosome breakage, among others, generate reorganized chromosomes such as dicentric chromosomes by illegitimate end-joining. These rearranged chromosomes with two centromeres constitute a source of chromosomal instability when a chromatin bridge is formed at anaphase. (A) Opposite pulling forces exerted on the bridged chromatin can ultimately break it, originating new broken ends that are susceptible to further reorganization. (B) Another possible fate of chromatin bridges is the generation of aneuploidy, either by non-disjunction or anaphase loss events. (C) Tetraploidization has also been proposed as an alternative outcome, resulting from the presence of anaphase bridges.

maintenance, termed ALT (alternative lengthening of telomeres), has also been described in human cancer cells in which telomeres are maintained through recombination events "Figure 1" (26-28).

3. WHY ARE SHORT TELOMERES CONSIDERED A SOURCE OF CHROMOSOME INSTABILITY?

As referred to above, short telomeres can arise from progressive telomere attrition in dividing cells, which leads to the accumulation of uncapped chromosome arms. If p53/pRb pathways are impaired, cells may proliferate despite the acute telomere attrition and they may eventually undergo breakage-fusion-bridge (BFB) cycles. BFB cycles might appear when two uncapped chromosomes fuse their extremities. The result of these illegitimate fusions is a dicentric chromosome i.e. a chromosome with two centromeres. During mitotic cell division, centromeres of sister chromatids are pulled to opposite poles in order for each daughter cell to maintain the ploidy of the species. When the two sister chromatids of a dicentric chromosome twist, the two centromeres of each chromatid are pulled to opposite poles at anaphase creating a chromatin bridge. Anaphase-bridges may then be resolved in different ways "Figure 2" (29). If the bridge breaks due to the tension generated when the microtubules pull chromatin to opposite spindle poles, daughter cells will carry a broken chromosome susceptible of new fusions and bridges in the following cell cycles "Figure 2A". Thus, BFB cycles are a driving force for chromosome instability (CIN). CIN arises when the capacity for maintaining the integrity of the chromosomes is lost. The result of CIN is structural and numerical abnormalities that accumulate in cells as they divide, giving rise to reorganized karyotypes. Eventually,

CIN would facilitate gains and/or losses of genes involved in growth control, senescence, apoptosis and checkpoints, which would ultimately create an environment that may promote tumourigenesis (30). The link between telomere loss and the accumulation of dicentric chromosomes was established by Counter *et al.* in 1992 (31). Evaluation of human embryonic kidney cells at early population doublings (PDs) revealed less than 1% of dicentric chromosomes in metaphase spreads. However, after inactivation of p53/pRb pathways by oncogenic transformation, these chromosome abnormalities increased dramatically as cells divided and reached crisis. In contrast, the number of dicentric chromosomes decreased in a population that emerged from crisis and showed telomerase activity, which stabilizes telomere length. These experiments suggested that telomere shortening and the resulting accumulation of uncapped chromosomes underlay this type of chromosome rearrangement (31).

Strikingly, the number of G-rich repeats in human telomeres is chromosome- (32,33), cell type- (34) and donor-specific (35,36). Studies in human somatic cells demonstrated that as telomeres do not simultaneously reach a critical length, fusions occur preferentially between those uncapped chromosome extremities (36-44). Human mammary epithelial cells (HMECs) are an attractive useful model for evaluating *in vitro* telomere-dependent CIN. These cells derive from healthy breast tissue samples and lack telomerase activity, which implies a finite lifespan. Nonetheless, HMECs spontaneously inactivate *CDKN2A*, which codifies for the CDK-inhibitor p16 when they are cultured (45). Analysis of HMECs at different PDs revealed that end-to-end fusions increased as cells divided, these fusions not being random, but affecting those

chromosomes with the shortest telomeres (36,44). Furthermore, HMECs showed an increase in anaphase-bridges and structural CIN as the culture progressed, which correlated with gradual telomere erosion (36,45-47). This phenotype was reverted when HMECs were immortalised and telomerase activity was detected (46, our unpublished data, 2011). Importantly, BFB cycles may also take place when just one chromosome shows an unprotected end. In this case, fusions will occur between sister chromatids after DNA replication, thus originating a isodicentric chromatid (36,44,48,49). This type of reorganization was evaluated using a plasmid containing a selectable marker gene that was immediately adjacent to a telomere (48). The authors determined that, when this telomeric sequence was lost, sister chromatids fused at their ends, formed a bridge during anaphase, and broke when the two centromeres were pulled in opposite directions (48). After DNA replication in the following cell cycle, the sister chromatids fused once again and thus the cycle continued until a new telomere was added to the free end, which provoked the accumulation of extensive DNA amplifications (8,50). Therefore, it is noteworthy that the loss of just a single telomere is capable of inducing CIN in multiple ways, affecting large regions of the genome (51).

Anaphase-bridges originated by telomere-telomere fusions have also been suggested to lead to the loss of whole chromosomes, creating large scale genetic imbalances. Alterations in chromosome segregation cannot emerge after anaphase-bridge breakage. Instead, they arise when dicentric chromatids detach one or both of their centromeres from the spindle pole due to the mechanical tension generated by bridged chromatids (42,47,52). While detachment from one pole gives rise to a non-dysjunction event -a hyperploid and a hypoploid daughter cells are formed-, loss of both anchorages –anaphase loss- gives rise to two hypoploid daughter cells "Figure 2B". Non-disjunction of dicentric chromatids is expected to produce the same rate of daughter cells with gains and losses of chromosomes. Nonetheless, different studies have reported that chromosome losses are more frequently detected (47,52-54). One possible explanation relies on the fact that the hyperploid cells generated through a non-disjunction event contain the additional chromosome in the form of an unstable dicentric. When these cells re-enter mitosis, a new anaphase-bridge may be generated and depending on its resolution, the number of chromosomes in the two new daughter cells might vary. Thus, the initial hyperploid cell population would be underscored, whereas the hypoploid population would be continually enriched, which correlates with the observed data (47,55). Furthermore, the hypoploidy is maintained in the successive cell divisions. Irrespective of the mechanism –non-disjunction event or anaphase loss-, it is being revealed that anaphase-bridges generated by end-to-end fusions might lead to aneuploidy.

Finally, tetraploidization has been envisaged as an additional consequence of dysfunctional telomeres by endoreduplication in a p53-defective background. Human (56) and mouse (57) cells were able to by-pass mitosis and re-enter the S phase, thus re-duplicating their genome without a previous nuclear and citoplasmatic division. This telomere-based tetraploidization is not related to anaphase-bridge resolution. It is the result of the persistent DNA damage response generated (57) and/or the lack of p53 function (56) which prevents cells entering mitosis and retain them at G2 phase. Eventually, cells undergo another round of DNA replication due to expression and degradation of components involved in cell cycle control (57). Nonetheless, anaphase-bridges formed by end-to-end fusions have also been proposed as driving to tetraploidy by abrogation of cytoplasmatic cell division "Figure 2C" (45, our unpublished data, 2011).

The evidence exposed above highlights the importance of telomere homeostasis in maintaining genome integrity. Progressive telomere shortening in a permissive cell environment by impairment of p53/pRb checkpoints may be a source of both structural and numerical chromosome abnormalities (58). More importantly, as telomerase in itself has been reported not to induce cell transformation (59), it might be speculated that its contribution to telomere capping by adding *de novo* telomeric sequences would have a positive effect in stabilizing the karyotype, thus decreasing the telomere-based chromosome instability. Considering all the above, together with the observation that telomeres are shorter in tumour cells than in non-tumour cells, it is tempting to speculate that telomerase activation is a late event in the onset of cancer (60). Initially, a certain degree of chromosome instability is needed in order to establish gains and/or losses of different genes that regulate cell survival and proliferation. Later, telomerase prevents telomeres from continuing to erode and fuse and the highly reorganized karyotype stabilizes. Nonetheless, evidence suggests that chromosome instability due to telomere loss can continue in cancer cells despite the expression of telomerase (15,61).

4. CHROMOSOME INSTABILITY IS EXACERBATED WHEN CRITICALLY SHORT TELOMERES COEXIST WITH DOUBLE-STRANDED BREAKS

DNA is continuously exposed to exogenous and endogenous stress. Probably, the most dangerous agents are those that inflict DSBs in the DNA. The main DNA repair pathways involved in resolving DSBs in eukaryotic cells are non-homologous end joining (NHEJ) and homologous recombination (HR). Two protein kinases, namely ATM and ATR, are major players as transducers of damaged DNA. Once they are activated, a signalling cascade is initiated, leading to DNA restoration (62). ATM elicits NHEJ response, which is the preferred mechanism for repairing DSBs in mammalian cells (63). Similarly, an ATM/ATR-dependent DDR is triggered by uncapped chromosomes, thus dysfunctional telomeres are recognised as conventional DSBs (64). DDR at dysfunctional telomeres usually results in the activation of canonical (65) and non-canonical NHEJ (66,67). Irrespective of the repair pathway operating, an unprotected chromosome may join its sister chromatid or may fuse to another uncapped chromosome in a defective p53/pRb background. But what happens when chromosomes with dysfunctional telomeres
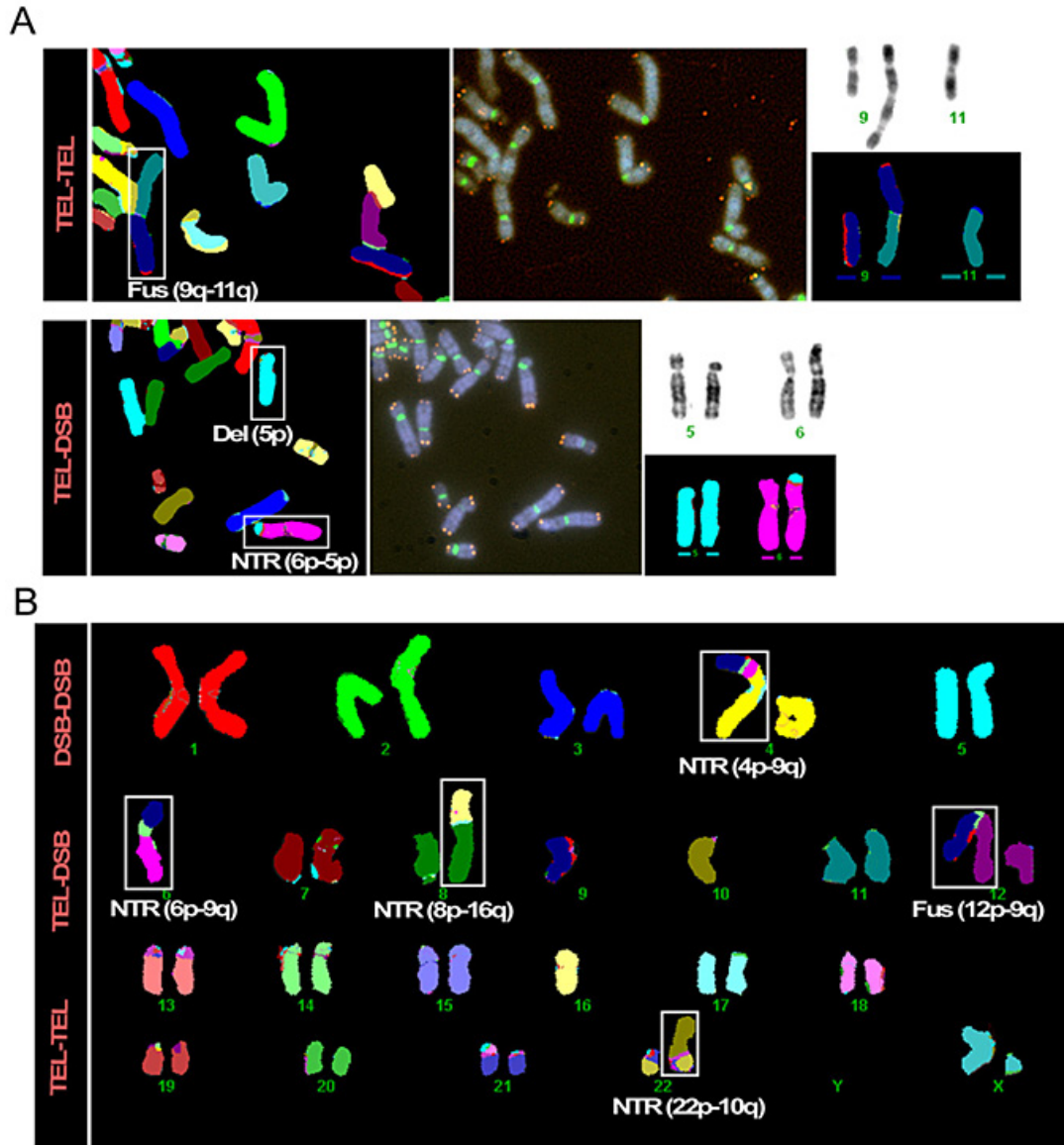
Figure 3. Dysfunctional telomeres elicit a damage response (DDR) and because they are recognized as conventional double-stranded breaks (DSBs) they can join other dysfunctional telomeres (Tel-Tel) or broken extremities (Tel-DSB) if they coexist in a cell. (A) Portions of metaphase spreads hybridized with whole-chromosome paintings (left images) as well as pan-telomere and pan-centromere probes (middle images), and the corresponding partial karyotypes (right images) are shown. The metaphase spread in the upper panel harbors a Tel-Tel fusion between q arms of chromosomes 9 and 11. The metaphase spread in the lower panel harbors a Tel-DSB fusion, where the short arm of chromosome 6 has joined the distal broken portion of 5p. The deleted short arm of chromosome 5, lacking telomeric signals, is observed. (B) Example of a whole-chromosome painting karyotype that includes Tel-Tel [Fus(12p-9q)], Tel-DSB [NRT(4p-9q), NRT(8p-16q), NRT(22P-10q)] and DSB-DSB [NRT (6p-9q)] chromosome reorganizations, among others. All the samples derive from late population doublings of human mammary epithelial cells.

coexist with conventional DSBs? Dysfunctional telomeres are expected to fuse not only with each other but also to join conventional DSBs "Figure 3A". Accordingly, fusions between uncapped chromosomes and DSBs have been detected both in mouse (68) and human (36,69) cells. In a telomere-dysfunction environment, at initial stages, the main chromosome alterations should be telomere-to-telomere fusions. However, as the culture progresses, end-

to-end fusions may enter BFB-cycles leading to DSBs formation. As telomeres continue eroding due to cell divisions, the probabilities of uncapped chromosomes joining DSBs increase through the culture. Afterwards, repeated BFB cycles of telomere-telomere and telomere-DSBs rearrangements results in a high proportion of broken chromosomes and increased DSBs-DSBs chromosome aberrations "Figure 3B" (36). As we will discuss below,

dysfunctional telomeres are the result not only of proliferation-dependent attrition, but also of impairment of its nucleoprotein structure. Therefore, the illegitimate recombination events between telomeres and DSBs might occur in any situation that implies defects in protecting chromosome extremities (70,71). Consequently, the progressive accumulation of dysfunctional telomeres plays a major role in chromosome aberration formation, which results in higher rates of CIN in proliferating cells.

Importantly, the fact that dysfunctional telomeres interfere with the proper repair of conventional DSBs also has implications for the sensitivity of cells to damaging agents. Cells with critically eroded telomeres showed more sensitivity to ionizing radiation and chemical agents than cells with a normal telomere length (69,72-75). Considering that telomeres shorten in each cell division, it might be predicted that the level of DNA damage sensitivity on cells will depend on the age of the individuals (69,75). Various epidemiological studies reinforced this hypothesis by detecting a higher incidence in radio-induced tumours with increasing ages at exposition (76-78).

5. TELOMERE SHORTENING IS NOT THE ONLY SOURCE OF TELOMERE-DEPENDENT CHROMOSOME INSTABILITY

In addition to telomere shortening, there are other factors that contribute to telomere-based chromosome instability. It has been reported that it is not the length of telomeres *per se* that is responsible for telomere dysfunction, but the inefficiency of capping the end of the chromosomes (79). In this regard, short telomeres are unable to protect the chromosome extremities when they do not contain sufficient telomeric repeats to support telomeric-associated proteins. Moreover, impairment of any of these proteins that form the telomere structure, regardless of telomere length, is also associated with alterations in t-loop formation and/or maintenance. Conventional fluorescence *in situ* hybridization (FISH) analysis of metaphase spreads using telomeric and centromeric probes has shown that fusions caused by telomere attrition lack telomeric DNA in the fusion points. On the contrary, it is present at the junction point when end-to-end fusions are the consequence of telomere-associated protein defects (49).

### 5.1. The shelterin complex and DNA repair proteins

The shelterin complex is constituted by TRF1, TRF2, POT1, TIN2, TPP1 and Rap1 proteins. They are abundant at chromosome ends and are not involved in other functions other than protecting chromosome extremities (5). Different studies have shed light on their roles in preserving telomere length and telomere homeostasis. TRF1 (Telomeric Repeat binding Factor 1) (80) and Rap1 (the human homolog of the yeast telomeric protein Rap1) (81) are major players in telomere length regulation. TIN2 (TRF1-interacting protein 2) and TPP1 (also known as TINT1/PTOP/PIP1) are bridge molecules that connect the different shelterin components to each other (5). TRF2 (Telomeric Repeat binding Factor 2) and POT1 (Protection Of Telomeres 1) are the most extensively evaluated

proteins in relation to telomere capping. TRF2 binds to the double-stranded telomeric DNA. It has been suggested that TRF2 inhibits improper recombination events at chromosome ends (82) by leading to t-loop formation (83), thus preventing end-to-end fusions. Transduction with a TRF2 dominant-negative allele promoted end-to-end fusions between chromosomes, chromatids and nonreciprocal translocations (65,82,84-88). These telomere fusions are generated through NHEJ pathway, as $Lig4^{-/-}$ $/p53^{-/-}$ double knock out mouse embryonic fibroblasts (MEFs) transduced with a TRF2 dominant-negative allele displayed neither telomere fusions nor dicentric chromosomes despite the lack of TRF2 (87). In addition to TRF2, POT1 was predicted to be involved in preserving the t-loop structure (89), mainly because it preferentially binds to the 3' overhang (90). In this regard, deficiencies in POT1 led to anaphase-bridges, chromosome fusions and breaks in human (91-93) and mouse (94,95) cell lines, although to a lesser extent than TRF2 deficiency. Importantly, telomere fusions in cells depleted in POT1 seem to be mediated by non-canonical NHEJ (67). Furthermore, TRF2 (96) and POT1 (97-99) may be involved in telomere length regulation as well.

In addition to the shelterin complex, there are other proteins that transiently associate with telomeres and are crucial for telomere homeostasis. Although they are less abundant at chromosome ends, defects in some of them are linked to telomere fusions and/or telomere shortening. Strikingly, these proteins are mainly implicated in the different pathways that mediate DNA repair (7-9,100). NHEJ and HR proteins are key factors in processing telomeres. The importance of NHEJ proteins in telomere maintenance is represented by the components of DNA-PK complex (Ku70, Ku86 and DNA-PKcs proteins) which are associated with telomeres in mammalian cells (101-105). Inactivation of Ku86 caused accelerated telomere attrition and a high degree of end-to-end fusions with the presence of telomeric sequences in the fusion points in human (106,107) and mouse (104,108-110) cells. Moreover, *Ku86* has been defined as an essential gene for cell viability due to its role in protecting telomeres from nucleolytic attack (111) and from recombination events (82). The role of Ku70 in telomeres seems to be related to recombination as well: it inhibits HR between sister chromatids during t-loop formation (5). Regarding DNA-PKcs, the presence of telomeric DNA in telomere-telomere fusions scored in $DNA\text{-}PKcs^{-/-}$ mouse fibroblasts (86,108,112-114), also suggests that it collaborates in the protection of t-loop structure. Similarly, telomere attrition and an increase in end-to-end fusions, anaphase-bridges and complex chromosome aberrations have been scored in cells that are deficient in HR repair proteins such as Rad51D (115) and Rad54 (116). Proteins involved in DNA repair pathways other than NHEJ and HR also play a role in telomere maintenance and chromosome stability. Some examples include Rad9, ERCC1, XPF, Fanconi anemia proteins and members of the PARP family (8, 20,100,117).

Nonetheless, the relationship between DDR proteins and telomeres is not only limited to DNA end processing or telomere length control (118). Both WRN
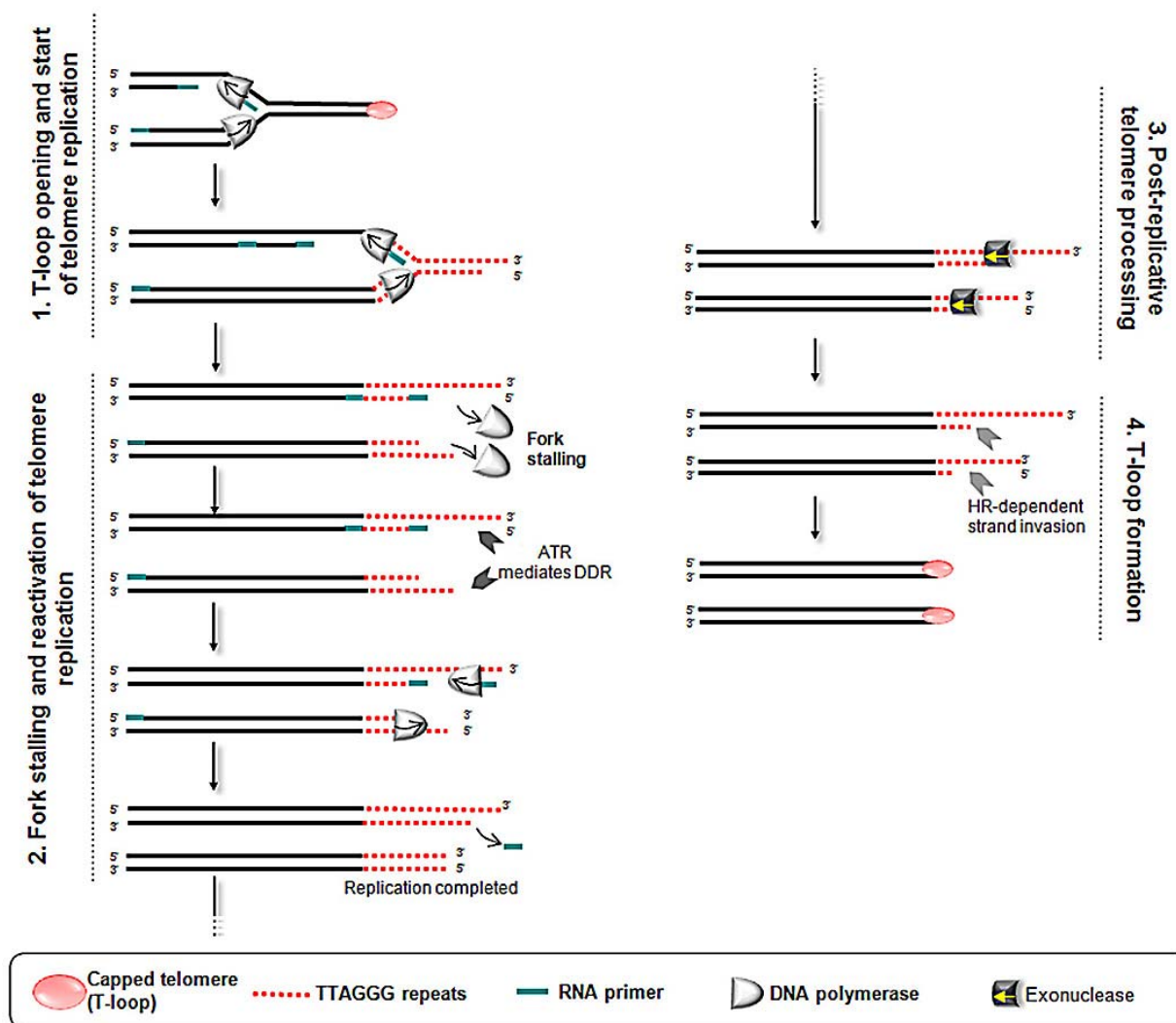
Figure 4. Telomere synthesis is initiated at S-phase and extended until replication fork stalling-like structures interfere with the replication machinery. As a consequence, a single-stranded DNA is generated, which elicits an ATR-dependent DNA damage response (DDR) that recruits DNA factors capable of restarting the replication at telomeres. When accomplished, the lagging strand gives rise to a short 3' tail whereas the leading strand finishes in a blunt end. At G2, both telomeres are processed by exonucleases in order to form a 3' overhang. This situation triggers a local ATM-dependent response and homologous recombination (HR) related proteins are accumulated at the end of the chromosomes. Eventually, the overhang loops back and integrates into the double-stranded DNA, forming the t-loop.

(119) and NBS1 (120) helicases seem to be involved in telomere replication by resolving structures that would otherwise block DNA synthesis. Mutations in *WRN* and *NBS1* genes give rise to Werner or Nijmegen Breakage syndromes, respectively (8), and chromosome instability based on telomeric dysfunction is associated with them (121-124).

It is important to consider that several proteins have been described as interacting and collaborating with each other in maintaining the nucleoprotein structure of telomeres. Although the results summarized here reflect a

direct alteration in the end of chromosomes when one of them is missing, it is important to highlight that they are inter-dependent. Thus, impairment of any of them may affect the function of other factors, which would engender higher rates of alteration in telomere homeostasis (125).

5.2. The cross-talk between telomeres and DNA damage response

Telomeric DNA replicates during S phase and in late S/G2 phase both chromosome extremities need to be processed for t-loop formation in order to safeguard the chromosomes from eliciting a DNA damage response.

Paradoxically, when DNA polymerization finishes, the end of the chromosomes are recognized as DSBs and an ATM/ATR-dependent DDR is initiated. Although activation of the signalling cascade is incompatible with cell proliferation and leads to cell cycle arrest (126), this response has been described to be essential for t-loop formation. Verdun *et al.* (127,128) proposed a model to explain the interplay between the DDR and telomere homeostasis "Figure 4". At late S phase, ATR is recruited at chromosome ends due to the presence of single-stranded DNA originated by replication fork stalling-like structures. The activation of ATR at telomeres promotes the accumulation of DNA replication factors that restart telomeric DNA synthesis (128). Nonetheless, as mentioned above, the DNA polymerase cannot completely synthesize the lagging strand telomere and the leading strand telomere gives rise to a blunt end. Consequently, post-replicative telomeres, which remain in an opened state, need to be processed during G2 for proper 3' overhang and t-loop formation. This is mediated by ATM, which initiates a DDR response at uncapped chromosome extremities (127). But how does this model reconcile with the known ATM-dependent end-to-end fusions generated in response to uncapped chromosomes? This model argues that although ATM is activated, it does so locally. Accordingly, substrates of ATM, such as p53 and CHK2, were found to be non-phosphorylated after ATM activation (127). The main player in preventing ATM-dependent NHEJ activation at telomeres is the protein TRF2 (127). In absence of TRF2, chromosomes are maintained uncapped and activation of the DDR elicits telomere-telomere fusions regardless of telomere length (65,84-88).

In order to process the chromosome ends to form the 3'overhang, different nucleases are recruited to the telomeres.Recent studies are attempting to shed light on this process. Nevertheless, the mechanisms that underlie the 3'overhang generation and length regulation are still unclear (6,129,130). It is believed that 3' single-stranded tails are generated by C-strand resection and it has been proposed that Pot1b may act as a negative regulator in mouse cells (6). SNM1B/Apollo, a 5'-3' exonuclease, has been suggested to have a role in leading strand telomere processing. Immortalized *SNM1B/Apollo* knock out MEFs showed a reduction of 3' overhang signals when compared to controls and an increased in chromatid-type fusions involving leading strand telomeres (131). Of note, these fusions were independent of ATM function despite the lack of TRF2 (131). Mre11, a 3'-5' exo- and endonuclease, which is a component of the MRN complex, is also involved in telomere processing (67).

After telomere processing, the t-loop structure must be restored. This close configuration resembles the Holliday junction intermediates generated by HR-dependent DNA repair. Since the HR machinery has the ability to generate displacement loops *in vitro* and also localizes at telomeres *in vivo*, it was proposed that HR is required for the formation of the t-loop structure (128). After telomeres have been processed, a 3' single-stranded tail is generated. This event provokes the recruitment of several ATM-dependent factors that promote the invasion

of its double DNA strand (127). At this level, it has been suggested that TRF1 and TRF2 could promote DNA invasion at the same telomeres (128), avoiding inter-chromosome recombination events. Moreover, the shelterin protein POT1, which binds to single-stranded telomeric DNA, could repress HR at telomeres, as it is transiently released just prior to t-loop formation (94,95). Once the telomere nucleoprotein structure is re-established, POT1 binds to the single-stranded DNA competing against RPA, a protein that stabilizes single-stranded DNA intermediates, and avoiding an ATR-dependent DNA damage response (132).

In summary, both TRF2 and POT1 play major roles in chromosome end protection by inhibiting two independent pathways mediated by ATM (133) and ATR (132), respectively. Lack of either TRF2 or POT1 promotes the accumulation of DDR proteins at telomeres, giving rise to telomere-induced foci (TIFs) (64,92,134,135). The activation of DDR leads to end-to-end fusions as the pathways that drive to recombination events are not inhibited (65,82,84-88,91-95). The final fate of cells with defective TRF2 or POT1 is senescence and/or apoptosis (84,92,94,136). This phenotype supports that not only telomere shortening but also impairment of the proteins that formed its structure leads cells to cell-cycle arrest and/or apoptosis (64). Nonetheless, the role of the shelterin factors in telomere protection does not seem to be limited to TRF2 and POT1 and needs further studies. It is important to consider that other shelterin members may collaborate in telomere end protection. They all form a structure that regulates telomere maintenance by connecting all the proteins to each other (137). Therefore, it is not surprising that the efficiency of POT1 in preventing a DDR relies on TPP1. It favours its binding to the telomeric DNA (132,138,139) as well as modulating TRF2-DNA interaction (137). Moreover, Tpp1-Pot1a/b complex acts as a regulator of the SNM1B/Apollo nuclease activity (129). It has also been discussed that lack of TIN2 may also elicit a POT1-mediated ATR-dependent DNA damage response (5). Similarly, targeted deletion of *Trf1* leads to embryonic lethality. It has been suggested that lack of TRF1 causes telomere deprotection, this fact being the reason for the premature death of mice (140).

6. EVIDENCE FOR A CONNECTION BETWEEN TELOMERE-BASED CHROMOSOME INSTABILITY AND CANCER *IN VIVO*

The relationship between telomeres and CIN *in vivo* was first established in mouse models in 1997 (141). Unlike humans, laboratory mice possess long telomeres and constitutive telomerase activity. Mice lacking the RNA component of telomerase (*mTerc*) exhibit progressive telomere shortening and eventually chromosome instability (end-to-end fusions and aneuploidy) as successive generations of telomerase null mice are obtained (71,141,142). Despite presenting gross chromosome aberrations, mice lacking telomerase showed reduced levels of tumour formation and/or progression not only when telomerase was missing but also when its deficiency coexisted with impairment of other factors related to

tumourigenesis (71,143-146). Importantly, these mouse models have an intact p53 pathway, which limits cell proliferation and protects cells from the adverse consequences derived from dysfunctional telomeres (144). Nevertheless, in the absence of appropriate checkpoints, short telomeres contribute to the high chromosomal instability that is characteristic of human tumors. Accordingly, late generation $mTerc^{-/-}/p53^{+/-}$ mice presented a high incidence of carcinomas, which showed high frequencies of chromosomes lacking telomeric signals, end-to-end fusions, anaphase-bridges, lagging chromosomes, nonreciprocal translocations and numerical chromosome aberrations (147,148). All these cytogenetic abnormalities were similar to those identified in human tumours (147,148). However, none of the alterations described above were detected in early generation mice that shared the same genetic background (147). In conclusion, telomere-based CIN fuels epithelial carcinogenesis when the appropriate checkpoints fail. This type of tumour is absent in mice when telomeres are functional and p53 pathway is not impaired (147); nonetheless, it is the most frequently detected in human adults. Thus, cells with dysfunctional telomeres and impaired cell cycle checkpoints seem to be more prone to initiating neoplastic processes. Of note, murine cells only depend on p53 response in order to bypass crisis and continue proliferating despite acute telomere shortening (149,150). This is not the case for human cells: they must inactivate both p53 and pRb pathways (16). In this regard, telomere dysfunction-dependent CIN has been claimed to be a tumour promoter when the senescence/apoptosis checkpoints are abrogated. Alterations in gene dosage due to CIN may engender remarkable changes in gene expression patterns that induce cells to transformation (151). In addition to mTERC- p53-deficient mice, other strains carrying defective genes related to telomere homeostasis have been generated to evaluate the impact of telomere dysfunction in carcinogenesis (6,152,153). These different genetically-engineered mouse models have been proven to be an invaluable tool for the study of telomere function. Some examples have already been reviewed here and include mice with alterations in proteins involved in DNA repair pathways.

Finally, an attempt was made to reconcile the observations in mouse models and human cells grown *in vitro* with the onset of human epithelial cancers. Different studies carried out in several tumour tissue samples agreed with regard to the impact of telomere length and telomere-dependent CIN on tumourigenesis and/or tumour progression. Short telomeres are a feature of tumour cells but they have also been detected in cancer precursor lesions from prostate (154), pancreatic (155) and colon (156) tissues. Similarly, CIN has been observed in colorectal neoplasias prior to malignant transformation (157). When both events were evaluated, dysfunctional telomeres and CIN were found in premalignant lesions (53,58,158) as well as in biopsies from a great variety of tumour tissues (46,58,61,159), telomere shortening being correlated to chromosome instability.

All this evidence highlights the relevance of telomere dysfunction and the resulting CIN in epithelial tumour development. Nonetheless, high levels of chromosome abnormalities compromise cells' viability. Although most cells would die due to alterations in genes involved in essential cell functions, the massive genetic instability associated with this stage might allow unusual cells to rapidly accumulate the genomic alterations needed for malignant transformation and reactivate telomerase in order to divide indefinitely. Telomerase reactivation would occur later in the carcinogenic process, once telomere dysfunction had lead to the constellation of genomic alterations needed for malignant transformation.

7. CONCLUSION

Telomeres are essential structures in eukaryotic chromosomes. They limit cell proliferation and uncontrolled cell divisions defining the deadline of cell viability. In addition, they are also an important source of chromosome instability when p53/pRb pathways are compromised. Although only a few examples have been described here, the complex network that modulates telomere integrity is still somewhat unclear and further work is needed in order to unravel the proteins and mechanisms that underlie telomere function. Nonetheless, evaluation of different proteins that participate in telomere structure reinforces the idea that failures in eukaryotic chromosome end capping as well as extremely short telomeres elicit a DDR that may lead to increasing CIN due to illegitimate rejoining of broken ends. Thus, our current knowledge highlights the importance of telomere homeostasis in cell fitness. Indeed, telomeres have been proposed as possible biomarkers of cancer, hereditable and age-related diseases or ageing itself.

8. ACKNOWLEDGMENTS

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Abbreviations: DSBs: double-stranded breaks, BFB cycles: breakage-fusion-bridge cycles, DDR: DNA damage response, hTR: human telomerase RNA component, hTERT: human telomerase reverse transcriptase, CIN: chromosome instability, PDs: population doublings, HMECs: human mammary epithelial cells, NHEJ: non-homologous end joining, HR: homologous recombination, FISH: conventional fluorescence *in situ* hybridization, MEFs: mouse embryonic fibroblasts, TIFs: telomere-induced foci

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