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Review

Telomere dysfunction in genome instability syndromes

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Abstract

Telomeres are nucleoprotein complexes located at the end of eukaryotic chromosomes. They have essential roles in preventing terminal fusions, protecting chromosome ends from degradation, and in chromosome positioning in the nucleus. These terminal structures consist of a tandemly repeated DNA sequence (TTAGGG in vertebrates) that varies in length from 5 to 15 kb in humans. Several proteins are attached to this telomeric DNA, some of which are also involved in different DNA damage response pathways, including Ku80, Mre11, NBS and BLM, among others. Mutations in the genes encoding these proteins cause a number of rare genetic syndromes characterized by chromosome and/or genetic instability and cancer predisposition. Deletions or mutations in any of these genes may also cause a telomere defect resulting in accelerated telomere shortening, lack of end-capping function, and/or end-to-end chromosome fusions. This telomere phenotype is also known to promote chromosomal instability and carcinogenesis. Therefore, it is essential to understand the interplay between telomere biology and genome stability. This review is focused in the dual role of chromosome fragility proteins in telomere maintenance.

Keywords: Telomere dysfunction; Genome instability syndromes; DNA sequence

Contents

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1. Introduction

Telomeres, the nucleoprotein complexes at the end of eukaryotic chromosomes, are specialized structures that protect chromosome ends and participate in a number of processes of a great cellular relevance [1]. Their significance in human pathology is becoming of crucial importance because of their role in cellular senescence and carcinogenesis [2]. In addition, an increasing number of cancer predisposition syndromes have dysfunctional telomeres as a common trait.

A few base pairs (50–200 bp) of telomeric DNA sequence tend to be lost with each cell division. This progressive shortening can be compensated for by telomerase, a specialized enzyme that remains absent in most normal somatic cells but that becomes activated in the majority of tumour, germ and immortal cells hence allowing further proliferation with the acquired risk of mutation accumulation [3]. The machinery responsible for telomere maintenance is composed of a subset of proteins that directly bind to telomeres or have an either positive or negative indirect regulatory role. Some of these proteins are very important for their dual function as they also participate in different DNA damage response pathways that altogether cross-talk in a complex network of tumour suppressor pathways [4]. A deficiency in any of these proteins leads to serious disorders of genetic instability ultimately leading to cancer predisposition.

Syndromes with both telomere and DNA damage response defects are Ataxia Telangiectasia (AT; caused by mutations in *ATM*), Nijmegen breakage syndrome (NBS, originated by defects in *NBS1*), Bloom syndrome (BS) and Werner (WS) syndrome (caused by defective helicases BLM and WRN, respectively), and Fanconi anaemia (FA; a genetic disorder with at least 12 disease-related genes involved). In addition, other DNA damage response proteins, such us DNA–PKcs or Ku, are also known to play a role in telomere stability. This review is focused in the dual role of these chromosome fragility proteins in telomere maintenance.

2. Telomere structure and shortening

Telomeres, from the Greek for telo (end) and mere (part), are specialized nucleoprotein complexes localized at the end of linear chromosomes and are required for cell viability, chromosome stability, protection from end-to-end fusions and nucleolytic degradation of chromosome ends, chromosome localization in the nucleus, chromosome segregation in anaphase, recombination of homologous chromosomes in meiotic cells and repair of DNA double strand breaks [1–3,5,6]. Telomeres also play an important role as they distinguish natural DNA ends from DNA ends resulting from breakage events [7,8]. Telomeres should avoid being detected as broken chromosomes since the latter trigger DNA damage checkpoints and subsequent repair pathways [9].

Telomeric DNA is characterized by being a G-rich double stranded DNA composed by short fragments tandemly repeated with different sequences depending on the specie. Human telomeric DNA consists of repetitive hexanucleotide sequences $(TTAGGG)_n$ that span 5–15 kb [10,11] ending in a G-rich 3′-single-strand overhang evolutionary conserved among eukaryotes [12,13] that folds back to form a ‘‘T-loop’’ stabilized by several telomeric proteins [13,14].

Telomeres have been reported to shorten as a function of age and in vitro and in vivo cell division [15,16]. As somatic cells age, telomeres progressively shorten with each round of replication because of the ‘‘end-replication problem’’ at the 5′-end of the DNA

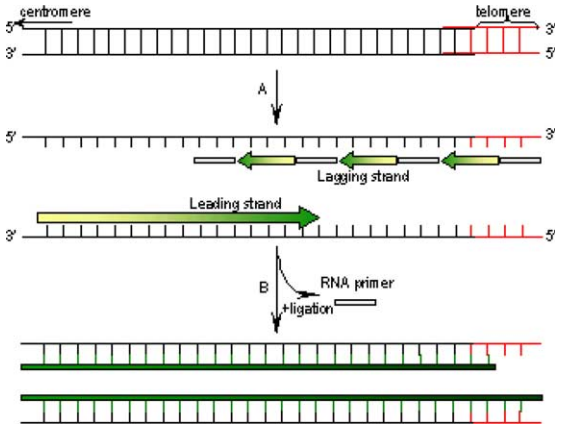

Fig. 1. Schematic model of the "end-replication problem": (A) template strands open up as to allow DNA polymerase to access and synthesize both leading and lagging (via Okazaki fragments) strand in $5' \rightarrow 3'$ direction. (B) RNA primers are eliminated, gaps are filled and DNA fragments ligated except the most terminal part that cannot be replenished, and thus, this chain shortens leaving unreplicated part of the telomeric sequence and a 3′-G-rich single-stranded overhang.

lagging strand. This shortening has been proposed to be the mitotic clock that sets the limit of a cell life span and so the mechanism that regulates the number of times a cell can divide before entering senescence [17,18]. This is due to the fact that conventional DNA polymerase can only synthesize in $5' \rightarrow 3'$ direction and so the 5′-end of the lagging strand will be shortened when compared to its template strand once the most terminal Okazaki's fragment-RNA primer is eliminated [16] (Fig. 1). The action of a $5' \rightarrow 3'$ exonuclease on the C-A rich strand also contributes to this phenomenon [19,20].

Every time a somatic human cell divides, about 50–200 bp of terminal DNA are lost [17,21] until eventually telomeres are so short that no further cell divisions can happen. This phenomenon, suggested to be a protection mechanism against cancer and known as replicative senescence, usually takes place after ∼50 population doublings and then the cells become senescent, are unable to divide further, their genetic expression is altered and most of them will finally die (crisis). A sufficiently short telomere (or an unprotected telomere) may be the signal for replicative senescence in normal cells and then the cell will stop proliferating [15,17,22,23].

Nonetheless, this elevated telomere shortening rate cannot be fully explained by the replication problem alone. Some authors have revisited this model and proposed another one that predicts an S-phase-specific exonuclease acting after replication and hence exacerbating the 3′-overhang length compared with the 5′-ends in a cell cycle-dependent fashion [5,19]. It has also been proposed that deficient DNA repair of certain lesions promotes telomere erosion [24]. For example, telomeres accumulate single-strand breaks produced by oxidative stress as this kind of damage is less efficiently repaired at telomeres [24,25]. In addition, epigenetic factors have also been reported to modulate telomere length [26,27]. None of these hypotheses are mutually exclusive and all of them may take place simultaneously.

When telomeres reach a critical shortening and cannot fulfil their normal functions, the resulting genomic instability allows acquisition of further mutations that in most of the affected cells promotes cellular death by apoptosis [28]. Derived from these observations, it would be predicted that activation of mechanisms that maintain telomere length would promote immortalisation [29]. Consistent with this, the majority of tumour and immortal cells up-regulate the enzyme telomerase that extends and stabilizes telomeric ends.

3. Telomere maintenance

Telomerase was first described by Carol Greider and Elizabeth Blackburn in 1985 in *Tetrahymena* [30], but it was not until 1989 when it was identified in humans [31]. Telomerase is a specialized DNA polymerase that adds telomeric arrays onto chromosome ends to compensate natural telomeric loss [3,30,32]. This enzyme is composed of two subunits, a catalitic protein subunit with retrotranscriptase activity (hTERT) and a RNA subunit (hTR) with a sequence complementary to the telomere sequence. This RNA component is used as a template to synthesize de novo telomeric sequences by reverse transcription at the very end of chromosomes [33].

The observations that (i) in spermatocytes and some human tumour cells, telomere erosion and elongation seems to be balanced with no apparent loss of telomeric DNA; (ii) telomeres are shorter in many

tumours than in its adjacent tissues; (iii) telomeres shorten during cell division and (iv) in vivo telomeres are shorter in older people than in young people, provide evidence that telomerase is active in both tumour and germ-line cells, but not in normal somatic cells [16,25,34,35]. Telomerase activity has also been detected in some haematopoietic, epidermic and non-carcinogenic hepatic cells, supporting the notion that telomerase per se is not oncogenic [36–40]. However, in these tissues, telomerase activity is insufficient to maintain telomere length through life indefinitely and telomeres become progressively shorter with aging.

Apart from its role in stabilizing the telomeric repeats, it has been proposed that telomerase activity is a requirement for immortalisation of human cells [18,41]. During senescence, and thereafter (crisis), once telomeres become so short that cannot carry out their roles, most cells die because of their acquired genomic instability, but a few overcome crisis through activation of a mechanism that maintains telomere length although critically short [34,37,38,42]. Thus, telomerase activity has become a requirement for cells to become immortal, maintain their telomeres, acquire other mutations causing oncogenesis and develop tumours. That is the reason why telomerase is a prime candidate as a target for novel therapies against cancer.

Although most human tumours and immortal cell lines have active telomerase, there are a few immortal cell lines and tumours that are telomerase negative. Their main feature is a high degree of heterogeneity in telomere size, ranging from almost undetectable to extremely long telomeres, with more than 50 kb [43–45]. This telomere length maintenance mechanism is known as alternative lengthening of telomeres (ALT) [46]. Despite the fact that ALT and telomerase can coexist in the same cells, there exist repressive factors that act in somatic hybrids from ALT and telomerase positive cells that abolish the ALT phenotype, but these factors remain to be elucidated [47].

Derived from studies with yeasts lacking telomerase, a recombination-mediated mechanism for telomere maintenance has been reported [48]. This pathway involves Rad52p and to some extent it could be extrapolated to eukaryotes [49]. Supporting the hypothesis of recombination as the mechanism for telomere lengthening in ALT cells, it has been shown that these cells contain specific nuclear structures termed ALT-associated promyelocitic leukemia (PML) bodies. These bodies consist of telomeric DNA and some telomeric proteins including Rad51 and Rad52 among others [50]. Although it has recently been demonstrated that in ALT cells telomeric DNA is copied to other telomeres, probably by recombination, and some participant proteins have been elucidated, all the components and mechanisms involved in this process are yet to be identified [51].

Repression of both telomerase and ALT mechanisms results in telomere shortening and finite proliferative capacity. In addition, cellular immortalisation and escape from crisis strictly depend on any of these two telomere maintenance pathways. These observations highlight the importance that telomere maintenance mechanisms harbour for anti-cancer therapy and drug development.

4. Telomere proteins

Despite the fact that telomeric ends and DNA double strand breaks (DSB) somehow resemble each other, the cellular machinery responds very differently to both structures. Thus, it seems obvious to hypothesize that telomeric structures are organized in a given way so as to be ignored by the DNA repair machinery. A series of studies on different species have reported an elevated number of telomere binding proteins. The most striking paradox is that many of these proteins are also DSB repair proteins [52–54].

Preceded by work on eukaryotes finding large telomeric DNA–protein complexes [55–57], and expecting that essential telomeric proteins might be evolutionary conserved, Zhong et al. identified in 1992 a specific telomeric DNA-binding protein called TTAGGG repeat factor 1 (TRF1) [58]. TRF1 forms a homodimer through its Myb-related domains and has been proposed to be an inhibitor of telomerase to control telomere elongation [59], although it does not affect telomerase activity or expression. TRF1 could act indirectly by emitting a negative signal to telomerase through some other proteins or by binding to telomere tracts thus impeding telomerase access to its natural substrate [59]. One of these mediator proteins could be Tankyrase, which binds to TRF1 via its ankyrin domain and when overexpressed releases TRF1 from the telomeres promoting thus its elongation [60].

In 1997, another telomere binding protein with Myb-related domains was described and termed TRF2 [61,62]. TRF2 maintains telomere integrity, as inhibition of TRF2 results in loss of G-strand overhangs and induces covalent fusion of chromosome ends [59,63,64]. Another function of mammalian telomeres that has been reported to be mediated by TRF2 is the activation of the apoptotic response and cell cycle arrest involving the ATM/p53 pathway, in a manner similar to the response to DSB [65].

Electron microscopic analysis revealed that a high proportion of chromosomes form a T-loop structure at their very end, as previously mentioned. These structures are formed through strand-invasion of the single-strand G-rich overhang folded back into the double strand telomeric sequence. This formation is mediated by TRF1 and TRF2. Although the exact biochemical activities that may take place are not known, it has been reported that TRF2 binds at the tail–loop junction in a very specific manner while TRF1 binds along the T-loop. This particular conformation creates a difference between chromosome ends and DSBs thus preventing telomere attrition and the activation of the DNA repair machinery avoiding the ulterior phenotypes associated with telomere impairment [14].

In mammals, the preferred way to repair DSBs is through an error-prone pathway termed non-homologous end joining (NHEJ) using DNA–PKcs/Ku80 as the key proteins involved in it [66–69]. Ku is an abundant heterodimer formed by Ku70/Ku80 subunits that play a key role not only in NHEJ but also in V(D)J recombination, DNA replication, transcriptional regulation, telomere maintenance, replicative senescence, cell cycle regulation and tumour suppression [70–77]. Despite being one of the main NHEJ repair proteins, Ku80/70 tether along the telomeric sequence through interaction with TRF1 providing essential telomeric capping, independent of the other member of the complex, DNA–PKcs. This observation is corroborated by the fact that a high frequency of telomeric fusions appears in metaphase spreads from mouse embryonic fibroblasts (MEFs) deficient for Ku80 [50,59,78–80]. Ku is also a negative regulator of telomerase [81].

DNA–PKcs is a serine/threonine protein kinase that contains a phosphoinositol 3-kinase (PI3-K) domain, resembling ATM, another protein with a negative effect in telomere maintenance and chromosome instability [82–85]. DNA–PKcs participates in telomere capping, not in telomere maintenance, as is apparent from work using mice defective for DNA–PKcs whose cells have abnormally long telomeres and an increased percentage of terminal fusions compared with mice with different backgrounds [86–88]. This indicates that DNA–PK complex proteins are involved both in telomere length regulation and end capping. Resembling Ku80, DNA–PKcs interacts with telomerase [89] and poly(ADP-ribose) polymerase-1 (PARP-1) [90], and it was suggested that it is involved in the processing of telomeres produced by leading strand synthesis [87].

Other repair proteins that also bind to human telomeres are the triplex forming proteins Mre11/Rad50/NBS1 (MRN complex) together with their associated protein, BRCA1 [91]. This complex co-immunoprecipitates together with TRF2 although it is thought that only a small fraction of the MRN complex associates with telomeres. An intriguing characteristic is that albeit Mre11 and Rad50-binding to TRF2 is cell cycle-independent, NBS1 recruitment to telomeres is S-phase specific [88]. The role of this complex on mammalian telomeres is not known although it could have a role in the release of a telomerase substrate. This same complex has been demonstrated to co-localize with PML bodies in ALT cells, especially in late S-G_2 phase of the cell cycle [92] and together with some other proteins involved in homologous recombination (HR). This observation could point for a function of the MRN complex in telomere lengthening in ALT cells (Fig. 2).

HR proteins such as Rad54 and Rad51D are also involved in telomere length maintenance [93,94]. Rad54 plays a role in telomere capping since when absent, a high frequency of end-to-end fusions and accelerated shortening is observed. An intriguing observation is the fact that double mutants $Rad54^{-/-}$/DNA–$PK^{-/-}$, compared to single mutants, show an even higher proportion of fusions. This means that the fusion-forming mechanism is not likely to involve HR. Regarding Rad51D, it also plays a role in protecting chromosome ends from telomere erosion both in telomerase positive and negative cells [94]. Thus, all the evidence leads to the conclusion that the two major mechanisms of DSB repair are involved in telomere stability

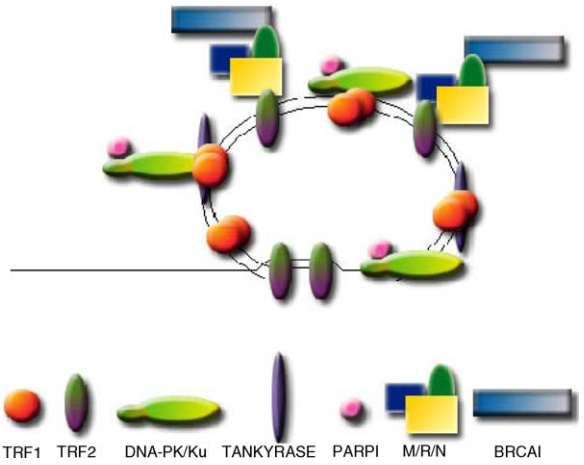

Fig. 2. T-loop ending of telomeres with some of its associated telomeric proteins.

5. Telomeres and cancer

As previously discussed, in most human somatic cells, progressive telomere shortening occurs with each cell division up to a point termed ‘‘replicative senescence’’ [95]. This observation has led to the suggestion that telomeres act as a mitotic clock that limits further cell division [41]. If a cell bypasses this first checkpoint via inactivation of p53 or pRb [96] it can divide further with consequent extensive telomere erosion. These telomeres will lose their protective functions causing chromosomal fusions, continuous ‘‘breakage-fusion-bridge’’ cycles, derived chromosome imbalances, gene amplifications, and ultimately leading to the generation of complex non-reciprocal translocations, a hallmark feature of adult solid tumours and genomic instability in general [97]. Damage-induced cellular responses are also activated during this process referred as ‘‘crisis’’ [34]. The final fate of crisis is cell death but a subset of cells may overcome this block. Clones emerging from crisis invariably either reactivate telomerase or the ALT mechanism allowing telomere length maintenance, extended life-span, and thus, immortality [34,42, 98]. Consistent with this, two supportive facts have been observed: (i) telomerase activity appears in most tumours and immortal cell lines [38,99–102], although in some other cases, down-regulation of additional proteins such as p16 or inactivation of the Rb/p16 pathway is needed for this immortalisation [103] and (ii) all the above mentioned events are typical of tumour cells and tumour progression [104,105]. It is remarkable that apart from its role in tumourigenesis, telomeres seem to be involved in premature aging [106] and those related diseases, some of which will be further discussed [17,84, 85,107,108].

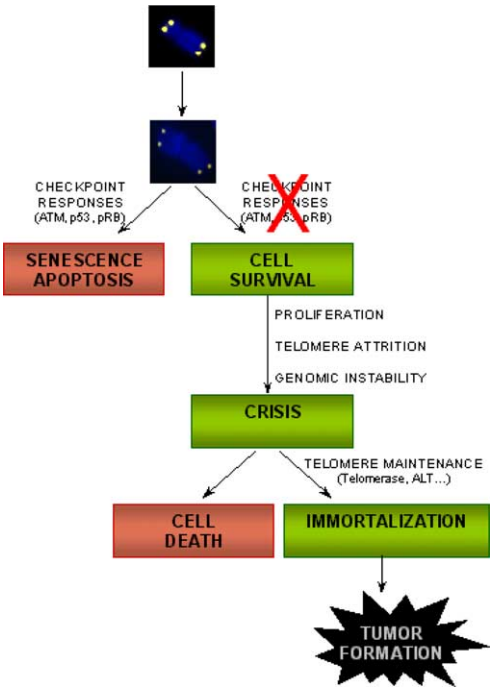

Fig. 3. Cellular responses to telomere shortening. Once telomeres shorten beyond a critical length, if cellular checkpoints are intact, cells activate apoptosis. Conversely, if checkpoints are bypassed, cells survive, continue proliferating and telomeric attrition goes on up to ‘‘crisis’’. Then, if a telomere maintenance mechanism is activated, cells might acquire the immortal but genetically unstable status and continue dividing giving rise to a tumor.

All this evidence leads the conclusion that telomere dysfunction would appear to have two outcomes in somatic cells depending on the integrity of checkpoint mechanisms: respond to checkpoints with senescence/apoptosis or proliferate resulting in genomic instability. The rare cells that continue to proliferate and emerge from crisis, having accumulated additional mutations, genetic lesions and inactivated tumour suppressor checkpoints, are those that will ultimately develop cancer in a Darwinist positive selection (Fig. 3).

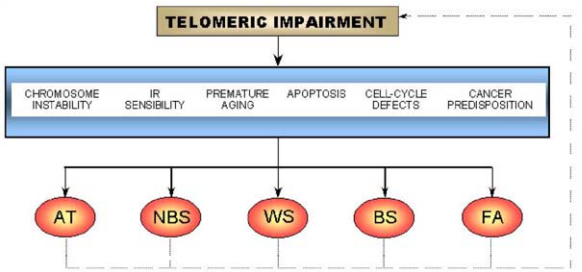

Fig. 4. An integrated view of the interplay between chromosome instability syndromes and telomere biology. Consequences of telomeric anomaly are common factors within a series of malignancies that deal with genomic instability. The proteins that when defective cause these syndromes are in turn directly or indirectly involved in telomere maintenance.

6. Telomeres in genome instability syndromes

Chromosome aberrations, genomic instability and cancer predisposition are the hallmarks of a number of syndromes in which the defective genes play important role in recognizing, signalling and/or repairing DNA damage, DNA processing, cell cycle regulation, apoptosis and/or telomeric maintenance. Defects in any of these genes can lead to cancer predisposition and to a number of clinical defects. These syndromes share various features at the clinical and cellular level that can be explained in most cases by interactions and molecular links among them, creating a network of pathways involved in DNA repair and DNA damage response [4].

In this review, we will focus on the genomic instability syndromes that display some common features due in part to the fact that the corresponding gene products are directly or indirectly involved in telomere biology. Although in most cases the exact function that corresponding gene products play on telomeres is yet undefined, what it seems clear is that the compromised telomeric stability in these syndromes is a major drawback for the maintenance of chromosomal integrity. Defining the way in which these proteins take part on telomeric maintenance will help us to understand the molecular biology of these diseases, and therefore, to develop knowledge-based therapies (Fig. 4).

7. Ataxia telangiectasia

AT is a rare, pleiotropic, autosomal recessive inherited disease with a complex clinical phenotype. Its main features usually appear in the second year of life and are progressive neuronal degeneration (cerebellar ataxia), ocular telangiectasia, immunodeficiency, hypogonadism, genomic instability, premature ageing, short stature, mild diabetes mellitus, and cancer predisposition (particularly lymphomas and leukaemia) [109–111]. The frequency of *ATM* gene carriers is about 1/100 and the estimated frequency of affects is about 1/40,000. Female carriers have been reported to be predisposed to breast cancer [112,113]. At the cellular level, the AT associated features are chromosomal instability, lack of radio-resistant DNA synthesis, hypersensitivity to ionising radiation (IR), cell cycle-checkpoints impairment, shortened telomeres, defective response to growth stimuli, and a high level of reactive oxygen species [110,114–116].

The gene causing this disease, *ATM* is a tumour suppressor gene that was first identified in 1995 by positional cloning on chromosome 11q22-23 by Savitsky et al. [117]. The predicted ATM protein belongs to the family of the PI 3-kinases that are involved in mitogenic signal transduction, meiotic recombination, and cell cycle control [118,109]. Other members of this family are DNA–PKcs, and ATR (Ataxia Telangiectasia and Rad3 related), a defective protein in Seckel syndrome involved in UV-damage and stalled replication fork signalling [119].

ATM responds rapidly to DSBs by phosphorylating a subset of proteins involved in different signaling pathways [109]: ATM phosphorylates p53, a key player at the G1/S cell cycle checkpoint; ChK2 kinase, with a role at G2/M checkpoint; Mdm2 protein; Brca1, which is involved in the S-phase and G2/M cell cycle checkpoints; inactivates CtIP, a negative regulator of BRCA1 and also phosphorylates the DNA repair proteins NBS1 and FANCD2, among other substrates [109,120–124].

A possible role of ATM in telomere maintenance has been extensively studied during the last decade as there exist a large subset of direct and indirect results that support this hypothesis: (i) ATM shares some degree of homology with the yeast Tel1 gene that participates in telomere metabolism [83,125,126]; (ii) cells derived from AT patients show an elevated frequency of chromosomal aberrations, with end-to-end associations the most frequent [85,127,128]; (iii) primary fibroblasts both from human patients and $Atm^{-/-}$ mice seem to undergo premature senescence

in culture [129,130]; and AT cells also show an abnormal response to agents inducing reactive oxigen species (ROS) and a high level of oxidative stress [115,131], a phenomenon linked to telomere shortening.

Metcalfe et al. observed an accelerated telomere shortening in AT accompanied by a high number of terminal fusions [108]. This observation could be a plausible explanation for the genomic instability previously observed in the cells. This telomeric attrition and accelerated senescence also involves p53, one of the ATM substrates [132] and as it was shown with the double knock-out mice $Atm^{-/-}/Terc^{-/-}$, both ATM and telomeres have a role at organismal phenotype level, with ATM being proposed as a protective factor of telomeres [133,134]. A striking finding regarding telomeres and AT cells was the presence of an excess of extrachromosomic telomeric signals. On metaphases, these can be seen as extrachromosomic signals that represent broken telomeres or defective replication intermediates (i.e. single-strand G-rich fragments) that correlate with shorter telomeres and elevated frequency of terminal fusions [84]. A possible explanation of this phenomenon could be provided by the excess of oxidative stress observed in these cells [135]. An increased production of ROS accelerates telomere shortening, as lesions, produced at telomeric level (specially, 8-OxoG) are abnormally repaired [24,25] and telomeres are prone to be attacked by oxidative radicals because of their G-rich sequence [136].

8. Nijmegen breakage syndrome

NBS is a rare autosomal recessive disease. The *NBS1* gene is located in chromosome 8q12 [137] and positional cloning and biochemical approaches identified the *NBS1* gene encoding the p95 protein, also named nibrin [138,139]. Although NBS1 does not share homologies with any other known protein, it contains two domains: a breast cancer carboxy terminal (BCRT) domain and a fork-head associated (FHA) domain that appears in some proteins with roles in cell cycle checkpoints and DNA repair [140]. This syndrome is clinically characterized by microcephaly, a distinct facial appearance, growth retardation, immunodeficiency, progressive mental retardation, and a strong predisposition to lymphoid malignancy and respiratory tract infections [141,142].

Further investigation has revealed that cells derived from NBS patients display characteristic abnormalities similar to those observed in AT, including spontaneous and induced chromosomal instability, sensitivity to IR, fail to induce p53 at the G1/S boundary, defective cell cycle checkpoints and lack of radioresistant DNA synthesis [143–146]. This is the reason NBS has long been considered as a variant of AT. However, their clinical manifestation differs in some points and they are also genetically distinct [137]. The overlapping of NBS at the clinical and cellular level with other syndromes with impaired response to DSBs, together with the association of NBS1 with phosporylaled histone H2AX at the site of damage shortly after IR exposure, suggests that NBS might be caused by a defective response to DSBs [139,147]. It is known that NBS1 is phosphorylated in response to UV, IR, methylmethane sulphonate or hydroxyurea. NBS1 phosphorylation is ATM-dependent only after treatment with IR for the subsequent activation of the S-phase checkpoint. In the other mutagens, ATR could be the kinase in charge [148–151].

As previously mentioned, the NBS1 product has been found to interact with two proteins to form a complex: Mre11 and Rad50 [138]. The presence of NBS1 is essential for the formation of this complex as NBS1 deficiency abrogates the IR-induced complex assembly [139,152]. These three proteins are all involved in the processing of IR-induced DSB and in other processes such as meiotic recombination (VDJ), rearrangement and telomere maintenance [153]. Because these processes are defective in the NBS patients' cells, chromosomal aberrations accumulate and immunodeficiency occurs.

From studies in yeast, it is known that the Mre11 complex participates in the maintenance of telomeric length, producing ssDNA, thus making telomeres more accessible to telomerase. In the case of telomerase deficient yeasts, the MRN complex is also needed for the recombination pathway of telomere enlargement [154–156]. In mammals, the MRN complex binds to TRF2 specifically during S-phase in the case of NBS, and cell cycle independently in the case of Mre11 and Rad50, indicating a role in telomeric replication or T-loop formation [91]. In cells from

NBS patients, both telomere shortening and premature senescence are observed, but reintroduction of hTERT alone is not sufficient to restore the telomeric defect. The fact that both NBS and hTERT need to be introduced to recover a wild type cellular phenotype, might suggest that NBS1 plays a role in telomere extension more than in any other step of telomere biology [157].

As in yeasts, a role of MRN complex has been reported for telomeric ends in human ALT cells. The interaction depends on the direct association of NBS1 with TRF1 at PML nuclear bodies, as was demonstrated both by co-immunoprecipitation and yeast two-hybrid analysis. This association takes place at the G_2 phase of the cell cycle and it is at that point when DNA synthesis is detected at the PML bodies [158]. NBS1 is necessary not only for the whole complex to congregate at PML bodies, but for the association of other proteins involved in the assembly of these nuclear bodies such as BRCA1 and the subsequent DNA synthesis at these sites [92]. A recent report describes a novel role of NBS1, which is related to telomere-derived cellular responses. Eller et al. reported that after introduction into the cell of DNA oligonucleotides homologous to telomere overhangs so as to mimic unprotected telomeres, the cell triggers an S-phase checkpoint mediated by NBS1, activates p53 via ATM and undergoes apoptosis [159].

9. Bloom syndrome

BS is a rare recessive disorder associated with growth retardation, immunodeficiency and increased risk of malignancy at an early age [160]. This disorder is represented by a single gene, *BLM*, mapped to the long arm of chromosome 15 (15q26.1). This gene encodes a 1417 amino acids protein. The same as WRN, BLM protein shows similarity to sequences of RecQ subfamily of ATP-dependent DNA helicases, including RecQ, Sgs1, Rqh1, and the human RECQL [161,162]. Analysis of *recQ* mutants has indicated that RecQ proteins participate in DNA repair, replication, and HR, but act as suppressors of illegitimate recombination [163,164]. BLM protein is a member of a large complex termed BRCA associated surveillance complex (BASC), that also contains other members of the replication, recombination and repair machinery, as for example PCNA, RAD51, BRCA1, ATM, MRN complex, MSH2 and others [165].

The clinical features of this syndrome are pre- and postnatal growth deficiency (small body size), sun-sensitivity, facial skin lesions (telangiectasic, hypo- and hyperpigmented skin), immunodeficiency with increased susceptibility to infections and respiratory illness in particular, and an increased susceptibility to cancer, particularly leukemia [160,161]. BS cells show marked genomic instability; in particular, hyper-recombination between sister chromatids and homologous chromosomes [166] although they also exhibit insertions, deletions, loss of heterozygosity, telomere associations and chromosome figures such as quadriradials [167]. BS cells are hypersensitive to UV, hydroxyurea, alkylating agents and up to some extent, to IR. BLM is phosphorylated by ATM and also by ATR although the exact function in preserving genome integrity still remains to be elucidated [168,169]. This observation together with the fact that BLM suppresses DSB during replication probably avoiding inappropriate recombination, supports the involvement of BLM in the DNA repair machinery [170].

The cellular functions of BLM reported to date are abundant and diverse: BLM can catalyse branch migration of Holliday junctions which prevents the collapse of replication forks [171]. BLM has also been found to localize within PML bodies in ALT cells, and accumulates in the S-phase of the cell cycle [172,173]. It is also needed for nuclear localization of the MRN complex after replication fork arrest [174]. Related to the abovementioned interactions, there exists a interplay between BLM and p53 proteins as both co-localize at stalled replication forks in a BLM-dependent manner together with Rad51. Inactivation of both p53 and BLM leads to a higher HR or sister chromatid exchanges than with BLM inactivation alone, hence suggesting that both proteins participate in complementary pathways during the process of replication fork resolution [175]. It could be said that BLM functions in the early response to DNA damage and promotes the ulterior recruitment of repair proteins at the stalled forks [176].

The *Saccharomyces cerevisae* SGS1 gene encodes a RecQ-like DNA helicase homologous to human WRN and BLM. In telomerase negative (est2) yeast cells also lacking *SGS1*, a high rate of telomeric shortening, accelerated senescence and poor growing

efficiency, due in part to a G_2/M cell cycle arrest, have been observed. The fact that introduction of murine *Wrn* gene restores the G_2/M arrest, suggests the hypothesis that the telomeric role that SGS1 plays might be conserved in mammals [177,178]. As previously mentioned, this hypothesis was confirmed for BLM in ALT cells [173,179–181]. In these cells, BLM interacts with TRF2 (either directly or indirectly) through its RQC motif resulting in an increased telomeric length. A plausible explanation could be that BLM protein unwinds the G-rich 3′-overhangs allowing the initiation and strand-invasion mechanism between telomeric sequences carried out by TRF2. This event would be part of the recombination-mediated telomere elongation process [181].

Contrary to the behaviour observed in ALT cells, in telomerase positive/$BLM^{-/-}$ cells, there is an increase in telomeric length. This would suggest that BLM somehow limits the amount of telomeric DNA, maybe by processing the 3′-end of the telomeric repeats [182].

10. Werner syndrome

WS is an autosomal recessive disease characterized by premature aging and associated symptoms including atherosclerosis, osteoporosis, greying and thinning of the hair, diabetes mellitus, bilateral ocular cataracts, and a high incidence of several types of tumours, particularly sarcomas, although to a more limited extent than in BS patients [183–185]. Hallmark features of cells derived from WS patients include genomic instability (chromosome translocations, deletions and rearrangements) and hypersensitivity to certain DNA damaging agents that cause replication arrest and/or DSB at the replication fork, such as camptothecin or 4-nitroquinoline 1-oxide [186–189]. The gene defective in WS encodes a protein, WRN, with $3' \rightarrow 5'$ helicase function that shares some homology with other RecQ helicases and with $3' \rightarrow 5'$ exonuclease activity, what makes it different from other proteins of the RecQ family [190,191]. This protein participates in replication, DNA repair, recombination and transcription [192,193]. WRN localizes predominantly in the nucleoli, but at the S-phase of the cell cycle relocates to replication foci and sites of DNA damage [193–195].

In detail, WS cells exhibit premature replicative senescence, a defect in S-phase progression and hyper-recombination [196]. Consistent with this, its yeast homologue, SGS1 suppresses illegitimate recombination, suggesting an evolutionary conserved function [163]. Using its helicase activity, WRN protein binds with replication protein A (RPA) to unwind DNA duplexes during replication [196]. The coordinated action with RPA is also of importance for the general understanding of telomere biology [197]. WRN helicase activity also appears to have a role in resolving Holliday junction structures [198,199]. Regarding its function on DNA repair, WRN interacts with Ku and DNA–PKcs which, in turn, modulates WRN exonuclease activity, avoiding excessive processing of non-compatible ends and, thus, undesired gene deletions [200–204]. WS cells also exhibit large deletions, chromosome rearrangements and shortened lifespan, although in general, this chromosomal defect is not as dramatic as the observed in other genome instability syndromes [187,205].

Similar to the above mentioned syndromes, WS cells display elevated rates of telomere shortening and deficient telomere repair, accelerated telomere shortening and a wide range of telomere lengths [206]. After introduction of telomerase catalytic subunit hTERT, the defect in premature replicative senescence is corrected [207–210]. However, telomerase does not completely restore the chromosomal instability observed in WS cells. These observations suggest that telomere impairment alone is not the only cause of the elevated rate of the chromosomal rearrangements that happen in these cells. Other evidence that supports its potential role on telomeres and correlates it with DNA repair is the association of WRN with the Ku heterodimer that, as stated before, participates in telomere balance, resulting in the WRN exonuclease activity enhancement [211,212]. Although WS cells display an accelerated telomere shortening, telomere lengths at the time of senescence are longer than in normal cells [207] so the premature senescence observed might not be directly triggered by short telomeres, although it seems clear that there exists a telomeric defect since telomerase is capable of restoring the senescence defect [209,212]. There are several plausible explanations for this: (i) WRN could act as a protective protein that caps the end of telomeres that, when unprotected, activate the senescence pathways; (ii) WRN helicase

is the key function as it confers an accessible substrate to polymerases in telomeres [213]; or (iii) there is a failure to resolve stalled replication forks at telomeres causing an increase in the rate of DSBs production at these sequences [214].

Regardless of the mechanism involved, there is no doubt that telomere dysfunction plays a role in some of the cellular defects observed in this syndrome, although it is almost sure that chromosomal instability displayed in WS cells is not directly caused by the telomere defect.

11. Fanconi anaemia

FA is a rare autosomic recessive genetic disorder characterized by a high number of developmental defects, progressive bone-marrow failure, a 1000 fold risk of developing acute myeloid leukemia, a high predisposition to develop solid tumours, and an increased of both spontaneous and DNA cross-linkers-induced chromosome instability [215]. The estimated frequency of carriers is 1 in 300, although in some ethnic groups, as for example, the population of South Africa, Ashkenazi Jews and some ethnical groups in Spain (unpublished observation), this frequency is much higher due to founder effects and consanguinity [216–218].

This syndrome is very heterogeneous phenotypically and genetically, making its clinical diagnosis rather difficult [219]. Thus, hypersensitivity of FA cells to the clastogenic effect of diepoxybutane or mitomycin C provides a unique marker for a conclusive diagnosis [220]. Among the most common birth defects or features are short stature, skeletal anomalies in thumbs, forearms, hips and ribs, kidney malformations, ''café-au-lait'' spots on skin, microcephaly, microphthalmia, mental retardation, gastro-intestinal difficulties, hypogonadism in males and defects in tissues separating chambers of the heart.

Unlike the previously discussed syndromes, at least 12 genes are involved in FA: *FANCA*, *FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL* and *FANCM* [4,221–223]. The most prevalent complementation group is FA-A, accounting for ~65% of all patients, followed by FA-C and FA-G [224]. The key protein in the FA pathway is FANCD2, whose activation via monoubiquitination depends on the formation of an upstream core complex formed by nine sub-units: FANCA, -B, -C, -E, -F, -G, -I, -L and -M [4,225,226]. FANCD2 monoubiquitination takes place in response to cross-linking agents such as DEB or MMC and the activated form associates with BRCA1 in damage-induced foci [225]. FA cells deficient for *FANCD2* are thus sensitive to mitomycin C but also have a degree of sensitivity and an abnormal S-phase check-point in response to IR due to the fact that FANCD2 is phosphorylated by ATM with the mediation of NBS1 [224,227]. FANCD2 phosphorylation can also be achieved via ATR but in response to interstrand cross-links [228]. These observations highlight the central role of FA in several DNA repair/damage response pathways [4].

It is common knowledge that telomeres are involved in proliferation capacity [229], and telomere integrity is essential for the normal functionality of haematological system as late-generation telomerase knock-out mice show a diminished haematopoietic production [220,230]. Both haematopoietic stem cells and lymphocytes express telomerase in a regulated manner, although this up-regulation is insufficient for preventing telomere erosion [40,231] and may lead to a poor proliferation of haematological cells. This is supported by the correlation between excess telomere shortening and haematopoietic system defects in a number of genetic syndromes including FA [232,233].

The nature of this telomeric shortening is controversial as there might be different sources of telomeric erosion. Our laboratory's work, demonstrated that not only a telomeric shortening is observed in peripheral blood samples from FA patients, but also a high proportion of extra-chromosomic TTAGGG signals and an increased number of telomeric fusions compared with healthy individuals, resembling AT [234]. This could be explained not only because of replicative shortening but also as a result of excessive telomere breakage in FA lymphocytes. FA cells have a defect in oxygen metabolism (hipersensitivity to high levels of oxygen tensions) and increased formation of 8-OxodG due to a high burden of intracellular ROS [235] and it is known that free radicals accelerate telomere shortening [236]. This evidence suggests redox by-products as one of the most probable sources of telomere shortening on FA cells although a more direct role of FA proteins cannot be disregarded. This

role could be mediated via the interaction of FA with MRN and with proteins involved in HR repair [237]. Regarding the end-to-end fusions found on FA lymphocytes, they are not associated with an abnormal TRF2 end-capping activity, as TRF2 binds to telomeres independently of the FA pathway [234]. Extensive efforts are underway to unravel the role that telomeres play on FA or that of FA proteins play on telomere biology.

12. Concluding remarks

Telomere maintenance has emerged as a key biochemical process in the regulation of cancer and aging. The mechanism involved in telomere homeostasis compromises a number of proteins and telomerase, a highly specialized enzyme. But this is not an as specific a process as it could seem on the first sight: most of the ''telomeric'' proteins are indeed DNA repair/damage response proteins with relevant functions in different cellular pathways. Defects in these proteins cause a variety of syndromes (FA, AT, NBS, WS and BS) with shared defects, as most of the affected pathways are interconnected [4].

As detailed in this chapter, telomere impairment is a common trait among these disorders and, derived from this defect, an array of secondary signs might come up to complete the characteristic phenotypic features linked to these diseases. In the case of AT and FA, the responsible proteins are thought to play a direct or indirect protective role for telomeres since, when defective, a high rate of end-to-end fusions and accelerated telomere shortening is observed. Further studies are, however, required to determine if the excess of telomere shortening in FA is related to the replicative history of the bone-marrow cells. The MRN complex is involved in telomere extension both in telomerase positive and negative cells through binding to TRF2. Also, BLM binds to TRF2 in ALT cells resembling WRN, but it has a negative role in telomerase positive cells by limiting the amount of telomeric DNA. It is well established that a telomere defect underlies in WS cells, but the exact function of WRN in telomere biology is yet unknown.

As a conclusion, we could point out that although a long way remains to elucidate the exact mechanisms by which chromosome fragility proteins modulate telomere maintenance, an essential part of the road has already been walked. Further research on this topic will lead us to a better understanding not only of the mechanism of telomere maintenance but also of the molecular biology of the pathways preventing chromosome instability and hence avoiding cancer development in the general population.

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