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Telomere and telomerase stability in human diseases and cancer

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1. ABSTRACT

Telomeres are the nucleoprotein structures at the end of linear eukaryotic chromosomes required for genome stability. Telomerase is the specialized enzyme deputed to their elongation. Maintenance of a proper telomere structure, an accurate regulation of telomerase biogenesis and activity, as well as a correct telomere-telomerase interaction and a faithful telomeric DNA replication are all processes that a cell has to precisely control to safeguard its functionality. Here, we review key factors that play a role in the development of these processes and their relationship with human health.

2. INTRODUCTION

Maintaining chromosome end stability is essential for a proper cellular functionality. Several elements synergize in this task: telomeric DNA, telomerase, telomeric proteins and the new recently discovered player TERRA, the set of telomeric transcripts. A fine cross-talk occurs among these elements, making it difficult to consider these components as separate entities. In this review, we will focus primarily on some key elements in telomere and telomerase biology, which are known to play a role in human health. Before entering in the body of the review, we will briefly summarize the main features of telomeres and telomerase and their functional role in human cells. Then, we will get into more details concerning some aspects of telomerase biogenesis, telomere-telomerase interaction and telomere replication, in order to introduce the biological role of genes that are mutated in human diseases. Finally, we will discuss human pathologies due to a failure in telomere maintenance, together with their genetic basis, and germline mutations in telomere genes involved in cancer development.

2.1. Telomeres and telomerase: an overview

Telomeric DNA consists of short repeats; in humans and vertebrates, the repeat unit is the TTAGGG hexanucleotide, which spans from a few kb in adult somatic tissues to about 15 kb, in germinal and fetal cells. The double stranded telomeric sequences end with a 3' single-stranded region of about 100-200 bp (1). The single-stranded DNA can arrange in closed structures such as the t-loop, in which the 3' overhang invades the double-stranded region (Figure 1A) (2), and four strand structures named G-quadruplexes (G4). T-loop formation appears to be important to protect chromosome ends from inappropriate recombination (3). G4 consist of groups of four guanine molecules that form a square planar structure (G-quartets) and then arrange together to form four stranded structures. G4 were first detected *in vitro*, and then demonstrated to occur *in vivo*, both in the telomeric and subtelomeric TTAGGG sequences (4).

Telomeres are elongated by a specialized enzyme, telomerase, which adds telomeric repeats at the 3' overhang; the complementary strand is then

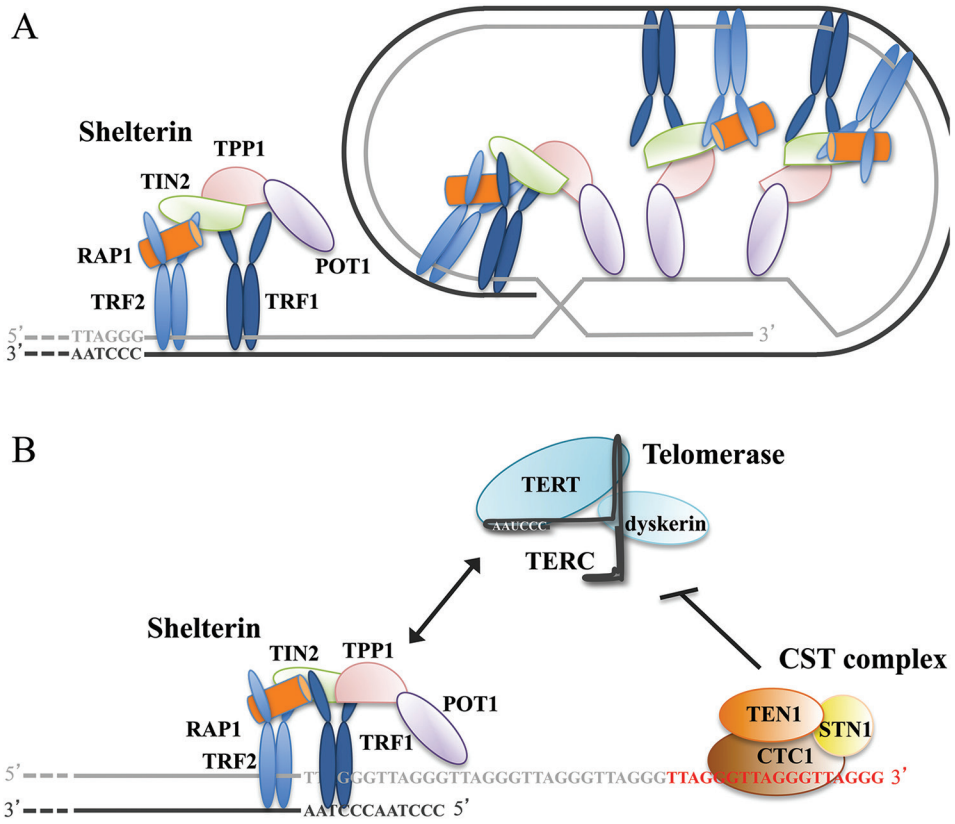

Figure 1. Telomere structure and elongation. A. In humans telomeric DNA consists of many kilobases - up to 15 kb in human cells - of the TTAGGG hexanucleotide. The double-stranded telomeric DNA is represented by 5'-3' G-rich strand (light grey) and its complementary 3'-5 C-rich strand (dark grey). Telomeres end with a G-strand 3' overhang that, invading the double-stranded telomeric sequence, forms a closed structure called telomeric-loop (t-loop). The telomere body is covered by shelterin complexes, made of TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 subunits. TRF1 and TRF2 directly bind to the double-stranded telomeric DNA, while POT1 binds to the single-stranded telomeric portion. B. At the telomere end, the shelterin complex, in particular the TPP1 subunit, works as a bridging factor between telomeres and telomerase. At every cell cycle, telomerase adds several telomeric repeats at the 3' overhang (-TTAGGG- in red). The CTC1–STN1–TEN1 (CST) complex binds to newly synthesized repeats and blocks telomerase activity.

synthesized through conventional DNA replication mechanisms. Telomerase works as an RNA dependent DNA polymerase, it contains both the RNA molecule with the telomeric template sequence (TERC) and the protein catalytic subunit (TERT) (5). Recently, it has been discovered that telomeric DNA is also transcribed. The telomeric RNA, TERRA, is synthesized starting from subtelomeric loci towards chromosome ends, it contains UUAGGG repeats extending from about 100 bp to 9 kb and is bound to telomeres (6). Besides the replication and transcription machineries, telomeric DNA interacts with specific proteins, the shelterin complex and the more recently discovered CTC1–STN1–TEN1 (CST) complex (Figure 1B) (7,8). The shelterin proteins TRF1 and TRF2 directly bind the telomeric duplex DNA, while POT1 binds the single strand overhang. TPP1 interacts with POT1 and enhances its binding to telomeres. TPP1 also plays a prominent role in recruiting telomerase to telomeres (see Section 3.1.). TIN2 localizes at telomeres through its binding to TRF1 and TRF2. It links TRF1 with TRF2 and also bridges these proteins to TPP1-POT1, thus connecting the double-stranded and the single-stranded portions of telomeres. Finally, RAP1 associates with telomeres binding to TRF2. Telomeric proteins are essential for genome stability and loss of any shelterin components can have dramatic consequences on cellular viability (9). The CST complex caps telomere ends independently of shelterin proteins, it binds to newly synthesized G-rich overhangs and has probably a role in terminating telomeric DNA synthesis, leading to the switch to C-strand synthesis (see Section 4.1.) (10).

Telomeres are essential protective chromosome structures. They compensate for the inability of replicative DNA polymerases to completely replicate the 3' ends of linear DNA molecules, avoiding the loss of unique genetic material at each cycle of DNA replication (11). They protects DNA ends from the attack of nucleolytic enzymes and DNA repair enzymes, allowing the discernment of natural DNA ends from DNA double strand breaks. Ligation between two chromosome ends has deleterious consequences, causing the formation of

dicentric chromosomes, which are unstable at anaphase and lead to genomic rearrangements (12). Telomeric DNA must retain a certain length to be functional. Telomere length homeostasis depends on cellular divisions and telomerase activity. In humans, telomerase is active in embryonic cells, in adults, is present in germ line and stem cells, while is virtually absent in somatic cells (13). Telomerase activity in germ cells, embryonic and stem cells assures that telomeres are maintained long enough to allow tissue renewal during the entire life of a multicellular organism. In fact, telomere shortening impairs stem cell proliferation, and thus their ability to regenerate tissues (14). In contrast, in somatic cells, telomeres shorten at each cell division (15). Somatic cells grown *in vitro* have been paradigmatic to study the consequences of telomere shortening on cellular functions. In the middle of last century, Hayflick and Moorhead (16) discovered that somatic cells in culture had a limited lifespan, ceasing to divide after a defined number of replications. Un-dividing cells were metabolically active and changed their shape becoming large and stellate. When telomeres and telomerase were molecularly identified (17-19), it became clear that telomere shortening below a threshold length was the cause of the replicative block (20). Telomeres falling below a threshold length are not distinguished from DNA double strand breaks anymore and trigger the DNA damage response, which blocks cellular replication (21). This cellular state was named cellular or replicative senescence (22). Cellular senescence is implicated in organismal aging, while its bypass in cancer development. In fact, telomerase activation, in the vast majority of tumors or, more rarely, the activation of recombination-based alternative telomere lengthening (ALT) mechanisms, allows telomere maintenance, which is the prerequisite for the indefinite replicative capacity of malignant cells (23,24).

TERT expression is the major determinant for telomerase activity in organismal tissues. In fact, while TERC is ubiquitously expressed, TERT is repressed in most somatic tissues and its expression correlates with telomerase activity (25,26). Exogenous expression of TERT in several somatic cell types is sufficient to restore telomerase activity and allows the bypass of replicative senescence, confirming the prominent role of TERT regulation in telomere maintenance (27) Importantly, a large body of evidence indicates that TERT and TERC play additional role in cellular physiology besides telomere maintenance (28,29).

3. TELOMERASE BIOGENESIS AND RECRUITMENT AT TELOMERES

Telomerase adds telomeric repeats to the 3' telomeric DNA overhang. TERT and TERC are the core subunit of telomerase, they have been highly conserved during evolution and are sufficient to synthesize telomeric repeats *in vitro*. *In vivo*, they associate with accessory proteins, which differ among different species, and play a central role in orchestrating telomerase biogenesis and activity (30).

TERC contains an "H/ACA box", a motif that marks RNAs interacting with the RNA binding protein dyskerin (31), and a CAB (Cajal body) box (32), required for Cajal body localization of the telomerase complex (33,34). Dyskerin is essential for proper assembly and stability of telomerase and is a subunit of the telomerase holoenzyme (35). It binds to nascent TERC together with NOP10, NHP2 and NAF1; substitution of NAF1 with GAR1 promotes nucleolar localization of the TERC-H/ACA-RNP (36). NOP10, NHP2 and GAR1 are found associated with the mature telomerase holoenzyme and probably play additional functions, still to be clarified, in regulating its stability and trafficking (37). Purification of dyskerin complexes from cancer cells revealed that it is also associated with TCAB1. TCAB1 is a component of the active telomerase holoenzyme; in fact, it is associated with all the three known telomerase components: TERC, TERT and dyskerin, and immunodepletion of this protein leads to the loss of TERC and telomerase activity from cellular extracts. TCAB1 localizes to Cajal bodies and could be responsible for TERC localization in these sub-nuclear structures, as well as for TERC/telomere co-localization (38).

TERT is translated in the cytoplasm and imported into the nucleus through the nuclear pores, in association with the chaperon proteins HSP90 and p23 (39). The telomerase holoenzyme is assembled during S phase by association of TERT with the TER–H/ACA RNP complex. Telomerase assembly is facilitated by the ATPases pontin and reptin (40), which bind to dyskerin and TERT independently and then dissociate from the active complex. Recent evidence suggests that the complex is assembled in the nucleolus and retained in this organelle through the interaction between hTERT and the nucleolar protein nucleolin; subsequently, thanks to the interaction with TCAB1, it is directed to Cajal bodies and telomeres (41).

Cajal bodies accumulate telomerase thanks to TCAB1-TERC binding, and in S-phase, a fraction of Cajal bodies colocalizes with telomeres. Controversial data are available relatively to the requirement of intact Cajal bodies for telomerase recruitment at telomeres. In fact, while in HEK 293T cells exposed to siRNAs against the Cajal bodies' protein coilin telomerase localization at telomeres was abolished and hTERC foci were not observable (42), recent studies on a coilin-knockout HeLa cell line, created using targeted zinc-finger nuclease-mediated insertional mutagenesis, gave an opposite result (43). In these cells, telomerase activity was normal and no telomere shortening was detected, suggesting that Cajal bodies are dispensable for telomerase activity.

Thus, further work is needed to clarify the role of intact Cajal bodies in telomerase biology, also taking into account that mouse TERC does not accumulate in Cajal bodies, despite containing a CAB box (44). The paper by Chen *et al.* (43) opens up the possibility that some components of Cajal bodies, rather then Cajal bodies *per se*, are required for telomerase functionality. Indeed, evidence has been reported that TCAB1 itself is directly involved in driving telomerase to telomeres (42).

3.1. Telomeric proteins and telomere-telomerase interaction

Telomerase trafficking between sub-nuclear structures is certainly fundamental for telomerase localization and activity at telomeres, but it is not the only player in this process. As mentioned above, telomeric DNA is bound to the shelterin complex, whose subunits control telomere length. A large body of evidence indicates that overexpression of shelterin components leads to telomere shortening, while their depletion causes telomere lengthening (7). A crucial role for telomerase activity at telomeres is played by TPP1 and POT1, which have a dual function in the regulation of telomere elongation. In fact, if TPP1 or POT1 downregulation, or the loss of their interaction, are associated with telomere elongation (45,46), *in vitro* studies have shown that TPP1 and POT1 increase telomerase activity and processivity, aiding the telomerase translocation step (47-49).

The double action of TPP1 and POT1 at telomeres can be explained by the capacity of TPP1 to interact not only with telomeres, but also with telomerase (50), working as a bridging factor between the two participants in chromosome end maintenance. Investigations on telomerase recruitment at telomeres, both with immunofluorescence approaches and chromatin immunoprecipitation experiments monitoring TERC and TERT association with telomeres, showed that depletion of TPP1 led to a reduction in telomerase foci colocalizing with telomeres (51). Zhong *et al.* (52) found that in cells depleted for TPP1, TERT was present in a small number of bright foci positive for coilin, suggesting that in the absence of the shelterin protein, telomerase was arrested in Cajal bodies e did not reach telomeres. To confirm that TPP1 is actually a recruitment factor for telomerase, the authors tethered a TPP1-lacI fusion protein to a lacO array stably integrated into a single intrachromosomal genomic locus. They showed that the TPP1 fusion protein localized at the internal chromosome locus and still retained the ability to bind telomeres. At the internal locus, it could recruit telomeres and TERT, competing for telomerase binding at telomeres, confirming an active role of TPP1 in TERT recruitment.

TPP1 interacts with TERT through its oligonucleotide/oligosaccharide-binding (OB) fold domain (50,51), and in particular a region of the domain, called TEL patch (TPP1 glutamate (E) and leucine (L)-rich patch), was found to be required for telomerase binding and enzyme processivity (53,54). Overexpression of a TPP1 protein mutated in the OB fold domain not only impaired telomerase localization at telomeres, but also led to telomere shorting indicating a functional meaning for TPP1-TERT binding (52). TPP1 action at telomeres cannot do without TIN2; in fact, TIN2 knockdown diminishes TPP1-POT1 amount at chromosome ends and eliminates TPP1 telomerase recruitment at telomeres (51,55,52). In contrast, POT1 seems to be dispensable for telomerase hiring at telomeres. It is worth mentioning that cell-cycle dependent TPP1 posttranslational modifications, such as phosphorylation of specific residues and protein ubiquitylation have been implicated in telomerase recruitment and TPP1 localization at telomeres, respectively (56,57).

A recent study performed on human embryonic stem cells using genome engineering techniques allowed the analysis of the consequences of specific mutations in the TPP1 TEL patch domain, without the disruption of the shelterin complex at telomeres (58). This study provided evidence that the TPP1 TEL patch domain is essential not only for telomerase recruitment, but also for telomerase activity. Furthermore, it highlighted an additional TPP1 function not yet disclosed by studies performed in tumor cells. The authors found that the expression of a TPP1 protein lacking the POT1 binding domain could complement a TTP1 protein deleted for the TEL patch domain, suggesting that POT1 is replaceable for telomere elongation. However, telomeres were kept at a very short length compared to the physiological one. This implicates that, on the one hand, the TPP1-POT1 complex is important for telomerase activity, may be increasing enzyme processivity, and on the other hand, in the absence of POT1, TPP1 set the feedback regulation of telomerase activity at telomeres at a new shorter telomere length.

4. TELOMERE REPLICATION

4.1. Telomeric DNA elongation and C-strand synthesis

To be accessible to telomerase, the t-loop in which the 3' telomeric overhang is organized has to be dismantled. The RTEL1 helicase has been shown to play a major role in this process (59). In fact, in the absence of this helicase, t-loops are incorrectly resolved and telomeric circles are generated by the SXL4 nuclease complex (see also next Section for RTEL1 functions in telomere biology). The 3' telomeric overhang is then extended by telomerase. This occurs after passage of the replication fork through telomeres (60). In equilibrium conditions, when telomere length is maintained within a specific range, every telomere is elongated at each cell cycle by a single telomerase molecule, which adds about 60 nucleotides (61). Fill-in of the telomeric C-strand is uncoupled by telomerase action and is accomplished in

late S (60). Thus, after telomeric 3' overhang elongation, two problems arise: what does make telomerase stop adding TTAGGG repeats to chromosome ends? What recruits DNA polymerase alfa (Pol-a) at the overhang, given that the semi-conservative DNA replication complex has likely left the telomeric DNA?

In 2009, Miyake *et al.* (62) and Surovtseva *et al.* (63) independently reported that a complex homologous to *S. cerevisiae* CST (cdc13, Stn1and Ten1) is also found at telomeres of higher eukaryotes. *S. cerevisiae* lacks a shelterin-like complex and CST fulfils the end-protection role, binding the 3' telomeric overhang in a sequence-specific manner (64,65). Moreover, yeast CST coordinates telomeric DNA replication, interacting first with telomerase and then with DNA Pol-a, for the synthesis of the C-rich strand (66-68). In humans, proteins homologous to yeast Stn1 and Ten1 have been identified, while the third subunit of the complex, named CTC1 (conserved telomere maintenance component 1) has been isolated on the basis of its association with Stn1 and shows little sequence similarity with cdc13 (62). Both CTC1 and Stn1 were first described in humans as accessory factors for Pol-a-primase (69-71), indicating a role for these proteins in DNA replication.

CST complexes are structurally similar to RPA heterotrimers, at the DNA sequence levels, for biochemical features and crystal structures (8). Both CST and RPA use OB fold domains to bind to DNA and interacting proteins. Human CST binds to single-stranded DNA without sequence specificity and all the subunits are required for high affinity binding. CST is found at the telomeric 3' overhang and protects the single-stranded telomeric region independently of POT1 (62). Evidence has been reported that depletion of either human CST subunits in cancer cells leads to telomere lengthening, indicating that CST is a negative telomerase regulator. Chen *et al.* (10) showed that CST binding to the telomeric G strand peaks after DNA replication and the complex inhibits telomerase activity both impeding primer accessibility to the enzyme and restraining TPP1/POT1 positive activity on telomerase, clearly indicating a role for CST in terminating telomerase action at telomeres.

Besides this, human CST has also been found involved in solving the second problem, that is the filling in of the telomeric C-strand after G-strand elongation. In fact, depletion of Stn1 leads to a delay in G-strand shortening in late S, because of a defect in C-strand filling (72,73). The precise mechanism by which CST participates in this process is still to be uncovered but, given its association with the DNA Pol-a-primase complex, it is plausible that CST facilitates the association of this complex to telomeres for C-strand fill-in. Although to be proved, TPP1-CST interaction might position CST at telomeres after G-strand elongation and facilitates Pol-a recruitment or regulation (10,73).

4.2. Replication of the telomere body and telomere fragility

The repetitive and heterochromatic nature of the telomeric DNA duplex poses several constrains to the replication machinery. In the presence of telomeric DNA secondary structures, such as G4 and t-loops, replication fork can stall and a robust system to re-initiate fork progression is needed (74). In accordance with this observation, several proteins have been identified that are requested for a successful telomeric DNA replication in addition to the standard replication factors, among which shelterin proteins and specific DNA helicases (Figure 2). Replication problems at telomeres give rise to chromosomal alterations that resemble those occurring at fragile sites (75,76). Fragile sites are chromosomal regions that challenge DNA replication, in particular under conditions of replication stress (reviewed in (77)). The common fragile sites, for example, are prone to break when cells are subjected to low doses of aphidicolin, a DNA polymerase inhibitor (78). Fork stalling and collapse can lead to chromosomal breaks. Fragility can determine genomic instability causing chromosome rearrangements, as DNA amplifications or deletions, indicating an important link between fragile sites and cancer (79).

On telomeres, the fragile phenotype is revealed by numerous, spatially separated, telomeric fluorescence *in situ* hybridization (FISH) signals at a single chromatid end that give a broken or incompletely condensed appearance to telomeres. Sfeir *et al.* (76) identified TRF1 as the crucial factor to evade telomere fragility. In fact, in immortalized TRF1 knockout mouse embryonic fibroblasts (MEFs), up to 20% of the mitotic cells exhibited abnormal telomeric FISH patterns, *versus* less than 5% of control cells. As described for intrachromosomal sequences, also telomeres show an aphidicolin-induced fragility, exacerbated in TRF1 deficient cells, confirming that telomere fragility is due to replication problems (76). The repression of telomere fragility by TRF1 depends on its DNA binding capacity that, in turn, is essential to recruit specific helicases during the S-phase (76,80). Analysis of metaphase spreads from helicase-deficient cells allowed the identification of BML and RTEL1 as the specific TRF1 partners in preventing telomere fragility. In fact, both mouse BML deficient cells and RTEL1 deficient embryonic stem cells showed severe and mild telomere fragility, respectively; furthermore, BLM or RTEL1 shRNAs induced the telomere fragile phenotype in MEFs (81,76). Interestingly, RTEL1 and BLM double-knockout cells showed an additive effect on telomere fragility compared to single ones, suggesting the use of distinct mechanisms in resolving secondary structure (59). On the other hand, the effect of BML or RTEL1 deletion was epistatic with TRF1 knockout, indicating that TRF1 acts in the same pathway upstream to helicases (76,80). As regards RETL1, its interaction with PCNA seems to be important to prevent fork stalling or collapse (82).

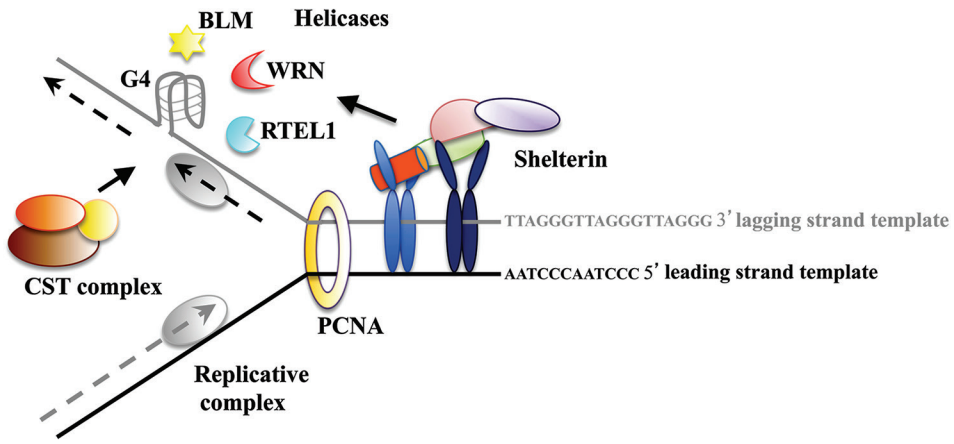

Figure 2. Telomere replication. Semi-conservative replication of the double-stranded telomeric DNA can be hampered by secondary DNA structures, such as G4s. Specific helicases, recruited at telomeres by shelterin proteins, and the CST complex are required for an efficient telomere replication.

Chromosome orientation FISH (CO-FISH) allowed the evaluation of telomere fragility distinguishing leading and lagging strands: in TRF1 knockout cells the frequency of multiple telomeric signals was equivalent at both sister chromatid ends (76); on the contrary, BLM loss gave a strong strand bias toward lagging strand fragility, suggesting that BLM is required to uncoil G4 DNA occurring in the lagging strand during DNA replication (80). Treatment of cells with the G4 DNA-stabilizing drug TMPyP4 (83) established a direct correlation between G4 formation during DNA replication and the fragile-site phenotype; in fact, exposure of RTEL1 deficient cells to TMPyP4 worsened the fragile telomere phenotype, pointing out that G4 DNA secondary structures are a major source of telomere fragility in the absence of RTEL1 (59).

Besides BLM, other RecQ family helicases, such as RECQL4 and RECQL1, have been linked to telomere fragility. RECQL4 is associated, as Werner (WRN) and BLM, to autosomal recessive disorders characterized by genomic instability and cancer predisposition (84,85). Cells with mutations in the *RecQL4* gene from Rothmund-Thomson Syndrome patients show a high frequency of fragile telomeres as well as cells depleted for RECQL4; moreover, induced replication stresses enhance fragility in the absence of RECQL4 (86). It has been demonstrated that RECQL4 neither acts on G4, nor is sufficient *per se* to dismantle telomeric t-loop; however, it is likely that RECQL4-WRN complexes are present at telomeres, where they can work synergistically to resolve telomeric t-loops very efficiently. RECQL1 is also involved in telomere body replication and RECQL1-depleted cells show a high frequency of fragile telomeres (87). Mutations in RECQL1 are not yet described in association with human diseases (87).

Finally, evidence has been reported that also the CST complex is involved in the replication of the duplex telomeric DNA (88). In fact, depletion of CTC1 or STN1 leads to the appearance of fragile telomeres and STN1 knockdown causes a delay of telomeric DNA replication, indicating that these proteins participate in the restart of replication after fork stalling. Interestingly, Stewart *et al.* (88) have also shown a more general role for CST in the replication of the human genome, aiding replication restart in replication stress conditions.

Telomere fragility and genes involved in the suppression of this phenotype have a large implication in human health (see also next Section). Martinez *et al*. (75) provided evidence suggesting that telomere fragility, in particular due TRF1 depletion, can also have an impact on organismal health. These authors showed that *TRF1* deletion in mouse stratified epithelia causes a very high frequency of telomeric aberrations, among which telomere fragility, that determine skin hyperpigmentation, epithelial dysplasia and perinatally death, even in the absence of telomere shortening. In mice in which *TP53* was also knockout, an increase in cancer development was found. Moreover, Schoeftner's group demonstrated that TRF1 targeting by miR-155 in breast cancer cells increases telomere fragility and genome instability (89). In estrogen receptor positive breast cancers, a correlation was found between miR-155 overexpression, TRF1 downregulation and poor prognosis, suggesting a possible link between telomere fragility and tumor progression.

5. TELOMERE DISORDERS

5.1. Clinical features

Given the essential role of telomeres in cellular physiology, it is not surprising that a failure in telomere length maintenance can lead to complex pathologies. Moreover, given the complexity of telomere length maintenance and the elevated number of genes involved in this process, it is not unexpected that mutations in

Table 1. Hereditary disorders due to defects in telomere biology genes. References can be found in the text

Telomere disorder	Onset	Gene (protein/RNA)	Mode of inheritance
Dyskeratosis congenita	Childhood	*TERT* (TERT)	Autosomal dominant
		TERC (TERC)	Autosomal dominant
		DKC1 (DKC1)	De novo X linked recessive
		TINF2 (TIN2)	De novo Autosomal dominant
		NOP10 (NOP10)	Autosomal recessive
		NHP2 (NHP2)	Autosomal recessive
		WRAP53 (TCAB1)	Autosomal recessive
		CTC1 (CTC1)	Autosomal recessive
		RTEL1 (RTEL1)	Autosomal recessive
Hoyeraal-Hreidarsson syndrome	Childhood	*DKC1* (DKC1)	X linked recessive
		TINF2 (TIN2)	Autosomal recessive
		ACD (TPP1)	Autosomal recessive
		RTEL1 (RTEL1)	Autosomal recessive Autosomal dominant (rare)
Revesz syndrome	Childhood	*TINF2* (TIN2)	Autosomal recessive
Coat Syndrome	Childhood	*CTC1* (CTC1)	Autosomal recessive
Idiopathic pulmonary fibrosis	Adulthood	*TERT* (TERT)	Autosomal dominant
		TERC (TERC)	
Aplastic anaemia	Adulthood	*TERT* (TERT)	Autosomal dominant
		TERC (TERC)	
Liver cirrhosis	Adulthood	*TERT* (TERT)	Autosomal dominant
		TERC (TERC)	

several different genes can be responsible for monogenic pathologies linked to alterations in telomere biology. These disorders, listed in Table 1, are characterized by short telomeres and proliferation failure in a variety of tissues (90-93). Since short telomeres are present in germ cells, and thus in embryos, multiple organs can be affected, even if the presentation of each pathology shows peculiar features, which range from the involvement of several organs and childhood onset, as for example in diskeratosis congenita (DC), to the interest of one main organ and adult onset, as in idiopathic pulmonary fibrosis (PF). Before entering into the details of the genetic basis of these diseases, we will give a brief description of their main clinical features.

DC has been the first human disease whose pathogenesis has been found directly associated with an alteration in telomere maintenance and short telomeres, whose length generally falls below the first percentile for age (94). DC is a multisystemic disease with an early onset, whose main clinical manifestations are the diagnostic triad of abnormal skin pigmentation, nail dystrophy and oral leukoplakia, together with bone marrow failure, the principle cause of mortality, and a high risk of cancer development (95,96). DC mode of inheritance varies, X-linked, autosomal dominant or autosomal recessive forms have been described, as well as *de novo* cases. More severe forms of DC have been identidied, namely Hoyeraal-Hreidarsson (HH) and Revesz syndromes (RS) (97,98). These syndromes share with DC short telomeres and several clinical features, moreover both HH and RS can have cerebral abnormalities and intrauterine growth retardation, and RS also presents with bilateral exudative retinopathy and intracranial calcifications. The onset is earlier than for DC and death occurs in the first decades of life. Coats plus syndrome, also known as cerebroretinal microangiopathy with calcification and cysts (CRMCC), is another syndrome characterized by short telomeres; patients show symptoms common with DC and its related disorders, such as intracranial calcifications, bilateral exudative retinopathy and intrauterine growth retardation, as well as bilateral retinal

telangiectasias, bone abnormalities and gastrointestinal vascular ectasias (99).

Pathologies less severe and with a later onset than those described above, but which can still be associated with a defect in telomere maintenance, are aplastic anemia (AA), PF and some non-alcoholic/non-infection liver disease (90,100,91-93). Interestingly, organs involved in these pathologies are also compromised in the more severe telomere syndromes. About 10% of AA cases and 5-10% of PF cases are due to dominant germ-line mutations in *TERT* or *TERC*. The majority of cases are isolated, however, the analysis of family history often reveals the presence of features observed in DC patients. *TERT* or *TERC* mutations were described for the first time in patients with non-alcoholic/non-infection liver disease by Calado *et al.* (101), studying 5 families with liver, haematological and autoimmune disorders. Subsequently, *TERT* or *TERC* constitutional variants were found in about 4% of patients with cirrhosis, suggesting that short telomeres can predispose to liver alterations (102).

Despite AA, PF and liver diseases can be considered separate clinical entities, it is not uncommon that patients harbouring a mutation in a telomere metabolism gene actually show signs of the different diseases. For example, PF patients mutated in *TERT* or *TERC* show a higher risk to develop bone marrow failure and liver disease compared to patients with PF due to other aetiology. Similarly, PF can frequently occur in telomerase deficient aplastic anemia patients, when are treated with toxic drugs for bone marrow transplantation (90). Thus the common feature at the basis of the diseases, short telomeres, can lead to the overlapping of affected organs in patients mainly diagnosed for a specific pathology.

Another possible feature of telomere syndromes is genetic anticipation, due to progressive telomere shortening in successive generations, given that telomere length is heritable (103). Families with an autosomal dominant mutation in a telomerase gene have been described, in which the age of disease development decreased from adulthood to childhood in subsequent generations, while the severity of the diseases dramatically increased (104,105).

5.2. Genetic basis

Table 1 summarizes the genes involved in the different telomere disorders described in the previous paragraph. Nine telomere biology genes have been found so far associated with telomeric disorders. Mutations in *TERT* or *TERC* are common to all the pathologies, while the involvement of each of the nine genes has been described only in DC.

Germ-line mutations in the X-linked *DKC1* gene, which encodes for the dyskerin subunit of telomerase, have been the first identified genetic cause of DC and have also been described in HH patients (106-108). DC mutations in dyskerin are mainly missense mutations, which preserve its pseudouridylation function that is required for viability, while compromise its ability to support telomerase biogenesis. At the functional level, mutations in *DKC1* determine a reduction in the steady state levels of TERC, and thus in telomerase holoenzyme biogenesis and telomere maintenance, but they do not impair telomerase specific activity (109).

A defective allele of *TERT* or *TERC* can be sufficient to cause a telomere syndrome, because haploinsufficiency is sufficient to perturb telomere length (94,105). Although TERT disease-associated mutations cause telomere shortening *in vivo*, many of them do not significantly alter telomerase activity *in vitro* (110), suggesting that either a subtle enzymatic defect can be sufficient to produce telomere shortening over years, or that mutations affect telomerase functions that are essential *in vivo* but not *in vitro*, like recruitment at telomeres.

NOP10 and NHP2 are part of the dyskerin complex and it is therefore not unexpected that mutations in their encoding genes can cause DC (111,112). As for mutations in dyskerin, mutations in *NOP10* and *NHP2* cause a marked reduction in TERC levels. TCAB1 is another protein involved in telomerase biogenesis whose encoding gene, *Wrap53*, has been found mutated in DC (113). Four mutations were found in two unrelated compound heterozygotes, healthy parents and siblings carried a single mutated *Wrap53* allele, indicating an autosomal recessive mode of inheritance. Mutations in *Wrap53* cause a marked reduction in TCAB1 levels and prevent the correct telomerase localization in Cajal bodies, leading to TERC accumulation in nucleoli and hampering telomere elongation by telomerase. Recently, Freund *et al.* (114) demonstrated that the DC-associated mutations found in *Wrap53* prevents the correct folding of TCAB1 by the chaperon protein TRiC (115,116). mutant TCAB1 can associate with TRiC but, in contrast to the wild type protein, remain linked to TRiC and do not progress towards the acquisition of the mature form able to bind TERC (114). Misfolded proteins are probably eventually released by TRiC and destroyed after failing to pass a quality control process. The discovery of a proteostasis pathway for the correct folding of TCAB1 adds a new layer of complexity to telomerase biogenesis and to the biological processes required for telomere maintenance.

TIN2 and TPP1 are the only shelterin proteins so far known to be involved in telomere disorders. Mutations in the TIN2 encoding gene, *TINF2*, cause about 10-20% of the DC cases, they have also been found in HH and RS patients, as well as in children with severe aplastic anemia (117-120). TIN2 mutations fall outside the TPP1 binding domain and do not impair

telomere capping, suggesting that they affect telomere lengthening by telomerase independently of TPP1 (121). TIN2 mutations cause a very severe telomere shortening, greater than telomerase subunit mutations, and cluster within a conserved region of the protein. Canudas *et al.* (122) showed that this region contains the binding site for the heterochromatin protein 1 (HP1) and TIN2-HP1 interaction is disrupted in DC patient cells. In the absence of TIN2-HP1 binding, sister chromatid cohesion at telomeres is prejudiced and telomere shortening occurs, although with a mechanism that has still to be understood.

Mutations in *TERF2IP*, encoding for TPP1, were found in a patient affected by HH (123). The proband was a compound heterozygote showing very severe symptoms and very short telomeres. One allele harboured a missense mutation in the TPP1-TIN2 binding motif, whose functional meaning is not completely clear. In fact, it seemed to affect TPP1-TIN2 interaction only modestly and did not appear to significantly compromise TPP1 binding at telomeres or impact on telomerase recruitment and processivity. In contrast, the second mutation, a single-aminoacid deletion falling in the TEL-patch domain, clearly compromised both telomerase recruitment and processivity leading to telomere shortening (123).

Another telomere capping protein involved in telomere syndromes is the CST subunit CTC1. Bi-allelic mutations in *CTC1* have been found in the majority of patients with Coat plus and in some DC affected individuals (124-127). So far, no mutations in the other CST components, namely Stn1 and Ten1, have been found associated with human pathologies. The *CTC1* mutational spectrum includes missense mutations, in frame deletions and putative intronic splicing donor site mutations, together with nonsense and reading frame shift mutations, which result in premature stop codons and non-functional proteins. None of the patients so far described carried two presumed complete loss-of-function alleles, suggesting that severe homozygous *CTC1* mutations would be lethal.

A variable telomeric phenotype characterizes CTC1 defective patients. While in some of them very short telomeres were found (124,125), others showed a normal telomere length (126,127), suggesting that telomere dysfunction may be related to telomeric structural defects other than telomere length loss. To better understand the molecular defects linked to *CTC1* mutations, Chen *et al.* (128) functionally characterized 11 CTC1 proteins carrying point mutations or small deletions found in patients. The authors showed that all the mutations, despite differently affecting CTC1 functions, caused the accumulation of internal single-stranded gaps of telomeric G-rich DNA, suggesting that the CTC1 role in telomeric DNA replication (129) could be impaired in the patients. Thus an incomplete lagging strand synthesis during telomeric DNA replication could be the molecular basis of the Coat Plus/DC cases mutated in *CTC1*.

Defects in telomeric DNA replication can also be at the basis of telomere syndromes mutated in *RTEL1*. Mutations in *RTEL1* have been found both in DC and HH patients (130-134). RTEL1 dismantles t-loops and counteracts telomeric G-quartets promoting telomere replication. *RTEL1* mutations are mainly autosomal recessive, but can also show an autosomal dominant pattern of inheritance. In one family, two siblings characterized by very short telomeres inherited a truncating mutation from their mother, who had telomeres below the normal length, but was still healthy at the time of the analysis (131). Genetic anticipation is likely the explanation for the manifestation of the disease in the probands.

Mutations in RTEL1 can affect its PCNA interaction (PIP) domain; in mouse cells, abrogation of RTEL1-PCNA interaction leads to accelerated senescence, defects in DNA replication, as well as in replication of telomeres, which could also account for the telomere defects in the patients (82). Lack of the PIP domain does not affect t-loop disassembly by RTEL1 (82). This function is likely impaired by mutations falling in the RTEL1 harmonin N-like/PAH helical fold domain, which could work as a center of interaction with other proteins (135), since cells from patients harboring these mutations show a high frequency of t-circle formation (134). Finally, a peculiar cellular phenotype has been described in patients carrying mutations in a cysteine-rich C terminal domain named C4C4-type RING-finger motif (130,133,134). Patients' cells showed telomeres very heterogeneous in length, a high frequency of telomere sister chromatid exchanges and telomere loss, resembling the phenotype of cells maintaining telomeres through the ALT mechanisms.

The genes described in this paragraph explain the genetic basis of telomere syndromes in about 60-70% of the affected families (92).

5.3. Mechanisms of telomere syndromes and cancer predisposition

Given the role of short telomeres in limiting cellular proliferative capacity, it is not unexpected that telomere shortening in highly proliferative tissues, as the hematopoietic compartment, can lead to stem cell exhaustion and organ failure. Bone marrow failure is actually one of the major clinical feature of telomere syndromes, however, this class of diseases can also involve organs with a low cellular turnover, like lungs (136). Evidence from mice models with reduced telomere length suggests that short telomeres can make lung cells more susceptible to a second hit, which would be required for the development of the pathology, in the absence of a high proliferation rate (136). This two hit

model could also explain why PF is a adulthood disease and the development of PF in aplastic anemia patients exposed to toxic drugs. Second environmental hits can also explain the variability in the age of symptom onset in different patients.

Telomere exhaustion in highly proliferating cells is at the basis of bone marrow failure in DC, thus it can be surprising that DC patients also show an increased frequency of cancer development, in particular for haematological malignancies, which requires cellular proliferation. This paradox can be explained by the double role of short telomeres, which, on the one hand, can arrest cellular proliferation and, on the other hand, increase genome instability, possibly leading to alterations in the expression of cancer related genes and tumor development (137). Although the issue is still debated and can variably apply to different types of cancers (see section 9), evidence has been reported that short telomeres are positively associated with cancer in the general population (138,139). Given the low telomerase activity in DC cells, it is possible that compensatory mechanisms occur in cancer cells to increase enzyme activity, or that alternative telomere lengthening mechanisms are activated for telomere maintenance in DC patients tumors. As far as the first point is concerned, Jongmans *et al.* (140) showed that mitotic recombination between *TERC* alleles in six DC patients led to the reversion of the genetic defect and somatic mosaicism in blood cells. Revertant cells could be the possible cells involved in cancer development. However, to our knowledge, no clear data on telomere maintenance in tumors from patient with telomere syndromes are available.

As previously mentioned telomere shortening in DC and related syndromes can lead to genetic anticipation of the disease manifestation. Evidence has been reported that telomere shortening can also be found associated with genetic anticipation in hereditary cancer syndromes, as Li-Fraumeni (LF) and hereditary breast cancer syndromes (141,142) and Von Hippel–Lindau (VHL) disease (143). The relationship between telomere shortening and these diseases is not completely clear, however a few explanations can be envisioned. LF syndrome is due to germiline mutations in the *TP53* gene and Tabori *et al* (141) speculates that the absence of p53 in somatic and germline cells could allow the escape from cellular senescence of cells with short telomeres, thus leading to telomere shortening in successive generations. Hereditary breast cancer syndrome is mainly caused by mutations in *BRCA1* and *BRCA2*. These genes are involved in DNA double strand break repair through homologous recombination (144), but also in telomere metabolism (145,146). *BRCA2*, in particular, has been found to play a role in telomere replication. In fact, in mouse cells conditionally deleted for *BRCA2*, Badie *et al.* (145) found telomere shortening and an increase in multiple telomere signalling at chromosome ends, a hallmark of telomere fragility due replication defects. Finally, very little is know about the possible involvement of the *VHL* gene, whose germline mutations cause the VHL disease, in telomere shortening, except for the recent observations showing that it plays a role in DNA damage response (147,148).

6. GERMLINE MUTATIONS IN TELOMERE BIOLOGY GENES AND CANCER DEVELOPMENT

As described in the previous section, germline mutations in telomere biology genes can lead to complex hereditary disorders, which comprise cancer predisposition among their clinical features. Recent studies have shown that mutations in *TERT* and some shelterin component genes can be associated with hereditary melanoma and predispose to different types of cancer.

Analysis of mutations co-segregating with the disease in cancer prone families has often allowed the identification of genes playing a broad role in cancer development, including sporadic cancer forms. Melanoma family studies, for example, led to the recognition of *CDKN2* and *CDK4*, encoding for the cell cycle regulator genes $p16^{INK4a}$ and cyclin-dependent kinase 4, respectively, as high penetrance melanoma susceptibility genes. *CDKN2* is responsible for 20-40% of the melanoma familial cases (149), while *CDK4* have been found in few families worldwide (150). In the attempt to find other melanoma predisposing genes, Horn *et al.* (151) analysed a large family with 14 melanoma affected siblings through linkage analysis and high-throughput sequencing and found a mutation in the *TERT* promoter, which fully segregated with the disease. The mutation led to the creation of a new binding site for Ets transcription factors and for the ternary complex factors (TCFs) Elk1 and Elk2. *In vitro* experiments indicated an up to two fold greater transcriptional activity for the mutated promoter compared to the wild type one, which could determine an increased TERT expression in tissue expressing Ets and TCF. To this regard, it is worth mentioning that some affected melanoma family's members developed additional tumors, including cancer of the ovaries, which are tissues with high TCF Elk1 expression. Somatic mutations in the *TERT* promoter generating an Ets/TCF binding sites, different from the familial one, were also found at high frequency (around 70%) in cell line and sporadic melanomas (151,152). A possible link between *TERT* promoter mutations, Ets/TCF and melanoma is given by the observation that the Elk1 and Elk4 members of the TCF family, are downstream targets of *BRAF*, the oncogene frequently activated in melanoma (153,154). A link that could possibly hold true in papillary thyroid carcinoma (155).

Rare germline mutations in shelterin component genes, namely *POT1*, *ACD* and *TERF2IP*, the last two encoding for TPP1 and RAP1, respectively, have also been found to co-segregate with the disease in melanoma families of different origins (156-158). Most melanoma associated *POT1* variants were inherited in an autosomal dominant manner. They were missense mutations falling within the N-terminal regions of the protein, which contains the OB fold domains that mediate the interaction between POT1 and the telomeric DNA overhang (159,156,157). For some of these variants, *in vitro* experiments showed that indeed *in vitro* translated POT1 carrying the mutated aminoacid completely lacked the capacity to form complexes with the telomeric DNA (156). At the telomeric level, in blood cells of *POT1* mutated melanoma patients, an increased telomere length was found, which is a considered a risk factor for melanoma, together with an increase in the frequency of fragile telomeres, a feature linked to telomeric DNA replication problems and associated with increased cancer development in mice (75,160,156,157). Besides melanomas, different types of tumors were also found in some *POT1* mutation carriers or their relatives, including breast cancer, small cell lung cancer, endometrial cancer and brain tumors, suggesting that *POT1* can be a susceptibility gene for other tumors besides melanoma (156).

Similarly, melanoma families showing germline mutations in *ACD* and *TRF2IP* co-segregating with the disease showed an enrichment in different types of cancers (e.g. breast, cervical, ovary colon, bowel and lung cancer), suggesting that alterations in the shelterin complex can predispose to the development of a broad spectrum of neoplasia (158). Indeed, most *ACD* mutations fell in the TPP1-POT1 binding domain and likely compromised TPP1-POT1 heterodimer formation. One *TRF2IP* mutation resulted in a truncated TPP1 protein lacking the TRF2 binding motif, which is expected to be unable to the bind to shelterin complex.

Taken together, all the studies presented so far strongly suggest that alterations in telomere metabolism are strongly predisposing to susceptibility to melanoma, but also to other forms of cancer. To this regard, it is worthwhile mentioning that somatic mutations, primarily occurring in the *POT1* region coding for the OB fold domain, have been found in sporadic cases of chronic lymphocytic leukemia (CLL) (161). *POT1* is frequently altered in CLL, being mutated in about 5% of the tumors analysed (161). Mutations are in heterozygosis, indicating that mutated POT1 works in a dominant-negative manner. *In vitro* experiments with HeLa cells exogenously expressing mutant POT1 proteins showed that the mutated protein localizes at telomeres, although it is unable to bind single-stranded telomeric DNA. In tumor cells from CLL patients carrying POT1 mutations, Ramsey *et al.* (161) found a higher frequency of telomeric fusions with telomeric signals at the junction site compared to control tumors, but no differences in the frequency of telomeric fusion without TTAGGG signalling. This is in agreement with the fact that POT1 mutations do not cause telomere shortening, but possibly telomere elongation. Moreover, the authors also found a modest but significant increase in telomere fragility, strongly suggesting an elevated level of genomic instability in mutated POT1 bearing tumors.

7. PERSPECTIVE

Advancing knowledge on telomere and telomerase biology has disclosed more and more factors involved in the determination of the structure, biogenesis and function of these key cellular components. Moreover, evidence has emerged that not only a proper maintenance of telomere ends is essential for genome stability, but also a faithful replication of the telomere body is important for the safeguarding of genome integrity and several proteins have been identified that are required for this task. The strict relationship between telomeres, telomerase and cancer appeared clear very soon in the telomere field. More recently, *TERT* and some shelterin component genes have been established as cancer susceptibility genes, making even more complex the relationship between telomeres and cancer. In addition, a spectrum of disorders due to premature telomere shortening has been recognized and the number of genes involved in telomere and telomerase biology causative of these diseases has rapidly increased in recent years. The connection between telomeres, telomerase and human health is thus obvious and a continuous crosstalk between basic research on genes and mechanisms involved in telomere metabolism and the medical management of human pathologies related to telomere maintenance will possibly help in developing new strategies for therapeutic interventions against these diseases.

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