GENOMIC INSTABILITY IN CANCER

Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins

Paula Martínez and María A. Blasco

Abstract | Mammalian telomeres are formed by tandem repeats of the TTAGGG sequence, which are progressively lost with each round of cell division. Telomere protection requires a minimal length of TTAGGG repeats to allow the binding of shelterin, which prevents the activation of a DNA damage response (DDR) at chromosome ends. Telomere elongation is carried out by telomerase. Telomerase can also act as a transcriptional modulator of the Wnt–β-catenin signalling pathway and has RNA-dependent RNA polymerase activity. Dysfunctional telomeres can lead to either cancer or ageing pathologies depending on the integrity of the DDR. This Review discusses the role of telomeric proteins in cancer and ageing through modulating telomere length and protection, as well as regulating gene expression by binding to non-telomeric sites.

Telomere uncapping
Loss of proper telomere structure owing to either loss of telomeric repeat sequences or alteration in telomere proteins that leads to the activation of the DNA damage response.

The ends of linear chromosomes are formed by a special heterochromatic structure, known as the telomere, which protects them from degradation and repair activities, and which is therefore essential for ensuring chromosome stability[1–9]. Mammalian telomeres are formed by tandem repeats of the TTAGGG sequence that are bound by a specialized six-protein complex, known as shelterin, which has fundamental roles in the protection of chromosomes and in the regulation of telomerase activity at chromosome ends (FIG. 1). The telomerase complex consists of a telomerase RNA component (*TERC*) and the reverse transcriptase catalytic subunit (TERT)[10]. In the past, the study of mice genetically modified for telomerase components was instrumental for demonstrating the role of telomere length in cancer and ageing. Excessive telomere shortening and severe telomere uncapping owing to telomerase deficiency trigger a DNA damage response (DDR) at chromosome ends, which are then recognized as double-strand breaks (DSBs)[1–6,11,12]. In the setting of a competent p53 pathway, mice that lack telomerase activity are resistant to cancer[11,13–20]. By contrast, constitutive telomerase expression in several independent *TERT*-transgenic mouse models results in a modest increase in the incidence of spontaneous and carcinogen-induced cancer[21–23]. The effect of telomerase overexpression on ageing was not addressed until recently owing to the cancer-promoting activity of telomerase. This has been addressed by telomerase overexpression in mice that are genetically engineered to be cancer resistant by means of enhanced expression of the p53, p16 and ARF (also known as CDKN2A) tumour suppressors. In this mouse model, the proportion of mice reaching old age was considerably increased, demonstrating an anti-ageing activity of TERT in a mammalian organism[24]. The fact that TERT-transgenic expression in a *Terc*-deficient background did not affect survival suggests that telomere maintenance is the main mechanism underlying the anti-ageing phenotype of TERT-transgenic mice[24]. Nevertheless, there is emerging evidence for a role for telomerase in stem cell biology that does not involve its telomere maintenance function. In support of a telomere-independent function of TERT, it has recently been shown that telomerase acts as a transcriptional modulator of the Wnt–β-catenin signalling pathway and has RNA-dependent RNA polymerase activity when in a complex with the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*)[25–28].

Shelterin is proposed to have a fundamental role in protecting chromosome ends; however, the role of shelterin components in telomere biology and disease in mammalian organisms has remained unexplored until very recently. The shelterin complex is composed of six core proteins[8,9,29]. Complete abrogation of most of these components results in early embryonic lethality in mice[2,30–35]. The recent availability of several shelterin-transgenic mouse models, as well as the generation of

Telomeres and Telomerase Group, Molecular Oncology Program, Spanish National Cancer Centre (CNIO), Melchor Fernández Almagro 3, Madrid E-28029, Spain. Correspondence to M.A.B. e-mail: mblasco@cnio.es
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At a glance

- Mammalian telomeres are formed by tandem repeats of the TTAGGG sequence bound by a specialized six-protein complex known as shelterin, which has fundamental roles in the protection of chromosomes and the regulation of telomerase activity at chromosome ends. Excessive telomere shortening and severe telomere uncapping trigger a DNA damage response at chromosome ends, which are then recognized as double-strand breaks. Dysfunctional telomeres can lead to either cancer or ageing pathologies depending on the integrity of the DNA damage response. Studies with mouse models that support a role for these proteins in cancer susceptibility and ageing-related pathologies are discussed in this Review.
- Telomere dysfunction causes ageing and also constitutes a driving force for cellular transformation by causing genome instability. Molecular mechanisms underlying telomere-induced genomic instability are described.
- Anti-ageing activity of telomerase has been demonstrated in mice overexpressing TERT genetically engineered to be cancer-resistant by means of enhanced expression of the p53, p16 and ARF tumour suppressors. Telomere-maintenance is the main mechanism underlying the anti-ageing phenotype of TERT-transgenic mice.
- Telomere-independent functions of TERT have recently been described. Overexpression of TERT is a transcriptional modulator of the Wnt–β-catenin signalling pathway and has RNA-dependent RNA polymerase activity when in a complex with the RNA component of mitochondrial RNA processing endoribonuclease (RMRP).
- Roles for the shelterin component RAP1 beyond its roles in telomeres have been uncovered. Mammalian RAP1 is involved in subtelomeric gene silencing and transcriptional regulation, and it also acts as a essential modulator of the nuclear factor-κB (NF-κB)-mediated pathway.
- Telomerase and factors that influence its activity are very attractive targets for the treatment of degenerative diseases and cancer. TPP1 is involved in telomerase recruitment to telomeres. Drugs targeting TPP1 could certainly be a novel strategy for blocking the ultimate goal of telomerase, the lengthening of telomeres.

tissue-specific conditional mouse models, has revealed a role for these proteins in cancer susceptibility and ageing-related pathologies even in the presence of normal telomerase activity and normal telomere length[36–39]. In line with this, the expression of several of the shelterin complex proteins is altered in some human tumours[40–42]. In this Review, we discuss the implications of shelterin components in cancer and ageing. A special emphasis is placed on the recently demonstrated roles for two of the shelterin components, the protection of telomeres protein 1 (POT1)–TRF1-interacting protein 2 (TIN2; also known as TINF2) organizing protein (TPP1; also known as ACD) and repressor-activator protein 1 (RAP1; also known as TERF2IP1) in telomerase recruitment and transcriptional regulation, respectively[39,43–46].

Factors that influence telomere function

Telomerase: an end to the 'end-replication problem'. During each cell division cycle, telomeres shorten as a result of the incomplete replication of linear DNA molecules by conventional DNA polymerases, which is known as the 'end-replication problem' (REFS 47,48). Telomerase compensates for telomere attrition through the *de novo* addition of TTAGGG repeats by TERT onto the chromosome ends by using an associated RNA component as a template (*TERC*)[49] (FIG. 1). Although telomerase is expressed in embryonic stem (ES) cells, and in most adult stem cell compartments, this is not sufficient to maintain the telomere length that is associated with cell division, and therefore telomere shortening occurs with age in most tissues[7,50–53]. This progressive telomere shortening is proposed to be one of the molecular mechanisms underlying ageing[50,54,55]. Indeed, some diseases that are characterized by the premature loss of tissue renewal and premature death, such as dyskeratosis congenita, as well as some cases of aplastic anaemia and idiopathic pulmonary fibrosis, are linked to germline mutations in *TERC* and *TERT*, which result in accelerated rates of telomere shortening[56–60] (BOX 1).

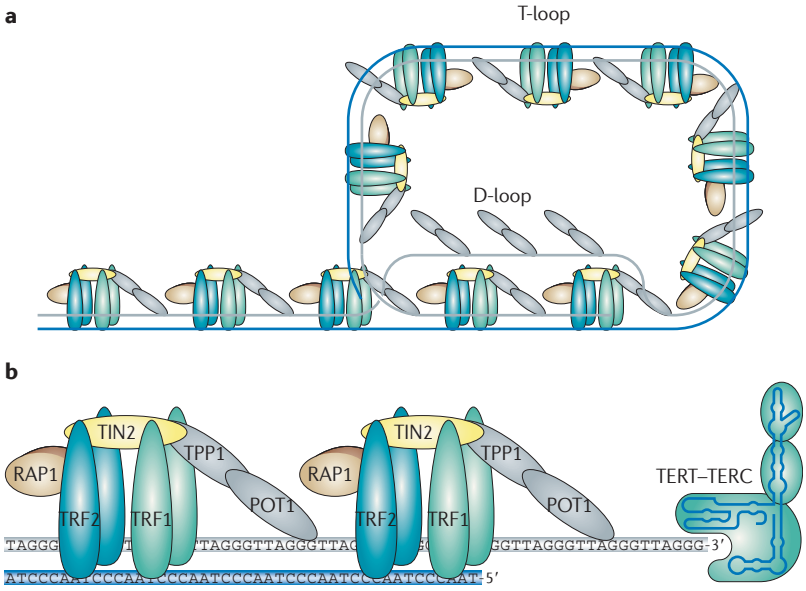
The telomeric chromatin. Telomere elongation mechanisms have been proposed to be regulated by the epigenetic status of the telomeric chromatin[61]. In particular, both telomeric and subtelomeric regions are enriched in histone marks that are characteristic of repressed heterochromatin domains, such as trimethylation of histone H3 at lysine K9 (H3K9m3) and H4K20 (H4K20m3) and binding of heterochromatin protein 1α (HP1α; also known as CBX5), HP1γ (also known as CBX3) and HP1β (also known as CBX1)[62–64]. Also, subtelomeric DNA is heavily methylated[64]. Loss of these heterochromatic marks is concomitant with excessive telomere elongation. In particular, abnormally long telomeres are observed after the loss of H3K9m3 and H4K20m3 heterochromatic marks in cells that are deficient for the histone lysine *N*-methyltransferases SUV39H1 and SUV420H1 (REFS 62,63), as well as on the loss of subtelomeric DNA methylation in cells deficient for DICER1 or the DNA (cytosine 5) methyltransferase 1 (DNMT1), DNMT3A and DNMT3B[65]. In addition, telomere repeat-containing RNAs (TERRAs) or telomeric RNAs (TelRNAs), which are RNAs that originate from telomeric DNA transcription, can associate with the telomeric chromatin, where they are proposed to function as negative regulators of telomere length based on their ability to act as potent inhibitors of telomerase *in vitro*[61,66,67]. In line with this, downregulation of TERRAs occurs during cancer progression, a scenario that requires the efficient elongation of short telomeres by telomerase[67].

Telomeric chromatin is dynamic. Differentiated somatic cells can be reverted to a more pluripotent state to become induced pluripotent stem (iPS) cells through a mechanism known as nuclear reprogramming[68]. The generation of iPS cells involves changes in the epigenetic status of telomeres towards a more open chromatin conformation with a lower density of heterochromatic histone marks, which coincides with increased levels of TERRA, increased telomere recombination and continuous telomere elongation until reaching ES cell telomere length[53]. Although the regulation of telomere lengthening in a chromatin status-dependent manner has not been demonstrated, the observations described above strongly support this possibility.

The shelterin complex. The shelterin complex has been proposed to modulate telomerase activity at chromosome ends[8,9,29]. The shelterin complex is composed of six core proteins, telomeric repeat binding factor 1 (TRF1; also known as TERF1) and TRF2 (also known as TERF2), TIN2, POT1, TPP1 (also known as TINT1, PTOP and PIP1) and RAP1 (REF. 8) (FIG. 1). TRF1, TRF2

Mismatch repair pathway
DNA repair mechanism that corrects mispaired nucleotides that originate during DNA replication and recombination.

and POT1 bind directly to telomeric DNA repeats, with TRF1 and TRF2 binding to telomeric double-stranded DNA, and POT1 to the 3′ singled-stranded G overhang. TRF1 and TRF2 do not interact, and they bind telomeric DNA independently. In particular, both proteins bind telomeric duplex DNA with a high specificity for the 5′-YTAGGGTTR-3′ sequence, both as homodimers and as oligomers[8,9,69–71]. POT1 possesses high specificity for the single-stranded telomeric DNA sequence 5′-TAGGGTTAG-3′, thereby binding to the G-strand overhang, as well as to the displaced G-strand at the D-loop[9,72–74]. POT1 interacts with the TRF1 complex via protein–protein interactions, and this interaction is thought to affect POT1 loading on the single-stranded telomeric DNA[74]. Although human cells contain only one *POT1* gene, mouse cells have *Pot1a* and *Pot1b*[31,35,75]. The two mouse POT1 proteins are highly homologous, and can associate with telomeric DNA, but they have distinct functions at telomeres[31]. TIN2 can bind TRF1 and TRF2 through independent domains and recruits the TPP1–POT1 complex, thus constituting the bridge between the different shelterin components[76–78]. TPP1 binds TIN2 and POT1 through its carboxy-terminal domain and its central domain, respectively[77,79]. It has been established that TPP1 recruits POT1 to telomeres[33,80]. In addition, the amino terminus of TPP1 contains a telomerase-interacting domain, suggesting a role for TPP1 in the recruitment of telomerase to chromosome ends[81]. Finally, RAP1 forms a complex with TRF2, and this association is essential for RAP1 binding to telomeres[2,82,83]. In spite of its telomeric location, three independent groups recently showed that RAP1 is dispensable for telomere capping but prevents telomere recombination and fragility[44,84]. Thus, RAP1 is not a telomere protective protein, in marked contrast to TRF2, TRF1, POT1 and TPP1 (REFS 2,37,39,44,85). *Rap1* abrogation did not affect the telomeric heterochromatic structure, telomeric transcription or the telomeric localization of the other shelterin components but did increase homologous recombination at telomeres[84]. Although *Rap1* deletion did not have a dramatic effect on telomere length in immortalized mouse embryonic fibroblasts (MEFs), mouse epidermis lacking RAP1 did show a 26% reduction in mean telomere length and showed an increase in γH2AX DNA damage foci[44], suggesting a possible role of RAP1 in telomerase regulation.



Telomeric roles
- Protection and recombination (TRF2, TRF1, TIN2, TPP1 and RAP1)[8,12,36]
- G-strand protection (POT1)[31,35,75]
- Length regulation (TRF2, TRF1, TIN2, TPP1 and RAP1)[8,12,36]
- Inhibition DDR (TRF2, TRF1, POT1 and RAP1)[8,36,44,84]
- Telomere replication (TRF1)[37,85]
- Telomerase recruitment (TPP1)[43,39]
- Telomere lengthening (TERT–TERC)[10]

Extra-telomeric roles
- Subtelomeric silencing (RAP1)[44]
- Transcriptional regulation (RAP1)[44,45]
- NF-κB signalling (RAP1)[45]
- Wnt signalling (TERT)[25,27]
- RNA polymerase (TERT–RMRP)[26]
- Stem cell mobilization (TERT)[22,28]

Figure 1 | **Telomere structure and functional roles of the telomeric proteins.** **a** | Schematic model of the shelterin complex bound to a telomere in a T-loop configuration. Telomeres contain a double-stranded region of TTAGGG repeats and a 150–200 nucleotide-long single-strand of the G-rich strand. The G-strand overhang (grey strand) invades the doubled-stranded DNA region of the telomere to form a protective telomere T-loop, with a displacement D-loop at the invasion site[8]. **b** | Schematic representation of telomere-bound proteins, the shelterin complex and telomerase. The shelterin complex binds to the telomere in a T-loop configuration. This complex is composed of telomeric repeat binding factor 1 (TRF1; also known as TERF1), TRF2 (also known as TERF2), repressor-activator protein 1 (RAP1; also known as TERF2IP1), the protection of telomeres protein 1 (POT1), TIN2 (also known as TIFN2) organizing protein (TPP1; also known as ACD), TIN2 and POT1 (REF. 8). TRF1, TRF2 and POT1 bind directly to telomeric DNA repeats, with TRF1 and TRF2 binding to telomeric double-stranded DNA and POT1 to the 3′ singled-stranded G-overhang[69–74]. TIN2 binds TRF1 and TRF2 through independent domains and recruits the TPP1–POT1 complex, constituting the bridge among the different shelterin components[76,78,200]. Telomerase is a two-partner enzyme, the catalytic subunit (TERT) and the RNA template (*TERC*), which recognizes the hydroxyl group (OH) at the 3′ end of the G-strand overhang and elongates the telomere[10]. The specific functions, telomeric and extra-telomeric, that are associated with each shelterin component and the telomerase are highlighted in yellow boxes. DDR, DNA damage response; NF-κB, nuclear factor-κB.

Telomere dysfunction and genomic instability

Genomic instability is a prominent characteristic of most, if not all, cancer types that has an essential role in tumorigenesis by accelerating the accumulation of genetic changes that are responsible for cancer cell evolution[86,87]. In a smaller subset of cancer types, particularly in hereditary non-polypopsis colon cancer, genomic instability is observed at the nucleotide level, with nucleotide insertions, deletions and substitutions due to mutations in the mismatch repair pathway[87]. However, genomic instability is most frequently manifested as gross chromosomal aberrations and changes in ploidy. Chromosomal aberrations observed in tumours could be either a late by-product of extensive cellular deregulation in already transformed cells or, alternatively, could constitute early events that cause malignant transformation. Several laboratories have provided evidence supporting the involvement of chromosomal aberrations in the early events of malignant transformation, as genomic instability facilitates the acquisition of malignant traits through the alteration of key genes and thus fuels cancer progression[88–90]. In line with this, critical telomere shortening that is associated with excessive proliferation of preneoplasic cells might be an important source of genomic instability[91–96]. In particular, the study of cells and mice that are deficient for telomerase or some of the shelterin proteins supports

Oral leukoplakia
The most common premalignant or potentially malignant disorder of the oral mucosa characterized by presenting several degrees of epithelia dysplasia. Defined as a white patch or plaque of the oral mucosa that cannot be characterized clinically or pathologically as any other disease.

Chromatid dicentrics
Aberrant dicentric chromosome that results when a chromosome lacking a telomere or with a dysfunctional telomere replicates and its sister chromatids fuse at their ends. At anaphase, the fused sister chromatids break owing to the presence of two centromeres.

a model in which telomere dysfunction, either owing to the loss of telomeric repeats or owing to the loss of the telomere protective structure, causes genome instability and thereby affects tumorigenesis[3,11,37–39,41,97,98]. Several of the molecular mechanisms that trigger genomic instability and that are related to telomere defects are discussed below (FIG. 2).

Breakage–fusion–bridge cycles. When telomeres become critically short or unprotected owing to shelterin defects, they can trigger a DDR that is dependent on an ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR) at chromosome ends, which are then recognized as DSBs (FIG. 2). Similar to DSBs, uncapped telomeres are 'repaired' by the activation of the homologous recombination and non-homologous end joining (NHEJ) pathways leading to telomere length changes and terminal deletions or to end-to-end fusions, respectively[12,99]. Interestingly, end-to-end fusions can arise from the activation of either the classic (C-NHEJ) or the alternative (A-NHEJ) NHEJ pathways depending on how telomeres are rendered dysfunctional[100]. Thus, fusions arising on TRF2-depletion are mediated by the C-NHEJ pathway, and fusions induced by TPP1–POT1 depletion are mediated by the A-NHEJ pathway[100]. However, fusions triggered by shortened telomeres in telomerase-null mice have been reported by some authors to be C-NHEJ dependent[101] but others have claimed that these are A-NHEJ dependent[100,102].

End-to-end fusions between sister chromatids (chromatid dicentrics) or fusions that involve different chromosomes (such as dicentric chromosomes or multicentric chromosomes) result in prolonged periods of chromosome instability involving breakage–fusion–bridge (B–F–B) cycles. A sister chromatid B–F–B cycle is initiated when a chromosome with critically short or uncapped telomeres replicates, and the resulting sister chromatids fuse at the end, forming a bridge during anaphase, which eventually breaks during cell division as the two centromeres are pulled in opposite directions (FIG. 2b,c). Successive B–F–B cycles will lead to further terminal deletions and amplifications, generating arrays of inverted repeats that are typical of the amplified regions that are found in human cancer[103]. When the fusion occurs between chromosomes, this leads to many of the chromosome rearrangements that are observed in cancer cells, namely dicentrics, rings, translocations, large duplications, double-minute chromosomes, amplifications and terminal deletions. These cycles continue until the chromosome acquires a new telomere, a process known as telomere healing (FIG. 2).

Defects in telomeric DNA replication. Telomere damage can also occur from problems in telomeric DNA replication. Telomeres present challenges for the replication machinery, including topological interference by the T-loop and the formation of higher-order DNA secondary structures, such as G-quadruplexes[104,105]. Moreover, telomeric DNA is a poor substrate for nucleosome assembly *in vitro*, and telomeric chromatin structure is highly heterochromatic[7,106]. Thus, replication forks stall naturally at mammalian telomeres and require an ATR-dependent restart for replication to complete[107]. It has recently been proposed that telomeres resemble fragile sites, and shelterin, in particular TRF1, is essential for preventing telomere breakage that is associated with replication fork stalling at telomeres[37,85]. *Terf1* deletion leads to end-to-end fusions and to a high incidence of telomeres with multiple telomeric signals (MTS)[41,98] (FIG. 2). Supporting the idea that telomeres are bona fide fragile sites, hypomorphic mutations in ATR lead to an increase in MTS and telomere recombination[108]. The DDR kinase ATR primarily functions to prevent replicative stress and genomic instability, and ATR depletion leads to increased chromosome breakage, both in humans and in mice[109,110]. As common fragile sites are prone to breakage, it is therefore expected that on oncogenic signalling telomeres are probable sites of genome instability initiation.

Terf1 deletion in mice produces embryonic lethality at the blastocyst stage; however, no defects in telomere length or telomere capping are detected at this stage[32] (BOX 2). More recently, the use of conditional *Terf1* alleles demonstrated that *Terf1*-deleted cells show rapid induction of senescence. Senescence occurred concomitantly with abundant phosphorylated H2A histone family, member X (γH2AX) foci located on telomeres,

Box 1 | **Human diseases linked to telomere dysfunction**

- Dyskeratosis congenita is a rare disorder that is characterized by a triad of clinical symptoms: dystrophic nails, skin hyperpigmentation and oral leukoplakia[116]. Mucocutaneous signs are present in infancy. Bone marrow failure follows in the first or second decade of life, and aplastic anaemia is usually fatal. Other premature ageing symptoms such as pulmonary diseases, dental abnormalities, oesophagostenosis and alopecia are often associated with the disease[116]. The disease follows the three different modalities of inheritance: X-linked recessive, autosomal dominant and autosomal recessive. The most severe form of dyskeratosis congenita manifestation is found in Hoyeraal–Hreidarsson syndrome (X-linked), which presents as a multisystemic disorder that is characterized by mental retardation, microcephaly, intrauterine growth retardation and aplastic anaemia[116]. Patients with dyskeratosis congenita show a higher incidence of spontaneous cancer, abnormally short telomeres and increased chromosomal instability[116]. Mutations in the three main components of telomerase holoenzyme have been linked to dyskeratosis congenita; namely in dyskerin (*DKC1*) telomerase reverse transcriptase catalytic subunit (*TERT*) and in the telomerase RNA component (*TERC*) (for reviews see REFS 40,116). In addition, mutations in the telomerase-associated proteins, nucleolar family A member 3 (NOP10; also known as NOLA3) and NHP2 (also known as NOLA2)[189,190], as well as in TRF1-interacting protein 2 (TIN2; also known as TINF2)[114] have also been detected.
- Acquired aplastic anaemia is a serious bone marrow disorder that is characterized by hypocellular bone marrow and low cell counts. Patients with acquired aplastic anaemia present with leukocytes with considerably shorter telomeres than age-matched controls. Mutations in both telomerase components, *TERT* and *TERC*, as well as in telomeric repeat binding factor 1 (*TRF1*; also known as *TERF1*), *TRF2* and *TIN2* have been linked to the disease[116,191].
- Idiopathic pulmonary fibrosis (IPF) is a progressive disorder that is characterized by a cough, dyspnea, impaired gas exchange and reduced lung volume. Pathologically, IPF lungs present with patchy fibrosis, interstitial inflammation and collagen depositions. A considerable proportion of patients with dyskeratosis congenita develop IPF. Mutations in telomerase components have been found in 15% of patients with familiar IPF[56,116].
- Cartilage hair hypoplasia (CHH) is a pleiotropic disorder of bone growth that is characterized by a short stature with other skeletal abnormalities, hypoplastic hair, ligamentous laxity, immune deficiency and neuronal dysplasia of the intestine[173]. Patients with CHH show a predisposition to lymphomas and other cancers. Mutations in *RMRP*, encoding a structural RNA molecule, have been linked to CHH[173].

Dicentric chromosome
Chromosome with two centromeres that results from the fusion of two monocentric chromosome pieces.

Multicentric chromosome
Chromosome with multiple centromeres that results from the fusion of several chromosome pieces.

Ring
Aberrant monocentric chromosome the arms of which have fused together to form a ring.

Double-minute chromosome
Acentric chromatin circle of variable size that consists of multiple copies of a short rearranged DNA segment that has undergone amplification.

G-quadruplex
Four-stranded structure formed by nucleic acids rich in guanine that consists of a square arrangement of guanines stabilized by Hoogsteen hydrogen bonds. Can form within or between G-rich strands of telomeric DNA.

Fragile site
Genomic region prone to replication fork stalling in which gaps and breaks frequently occur and is thus a hotspot for recombination events.

Multiple telomeric signal (MTS)
An aberrant structure at telomeres that is enhanced by conditions known to cause replication fork collapse, such as aphidicolin treatment. By immunofluorescence technique, an MTS is seen as several fluorescent dots instead of just one.

Hypomorphic mutation
A type of mutation that results in an altered gene product with reduced enzymatic activity or a lower expression level than the wild-type allele.

Chromosome concatenation
Aberrant chromosomal structure in which several chromosomes are fused.

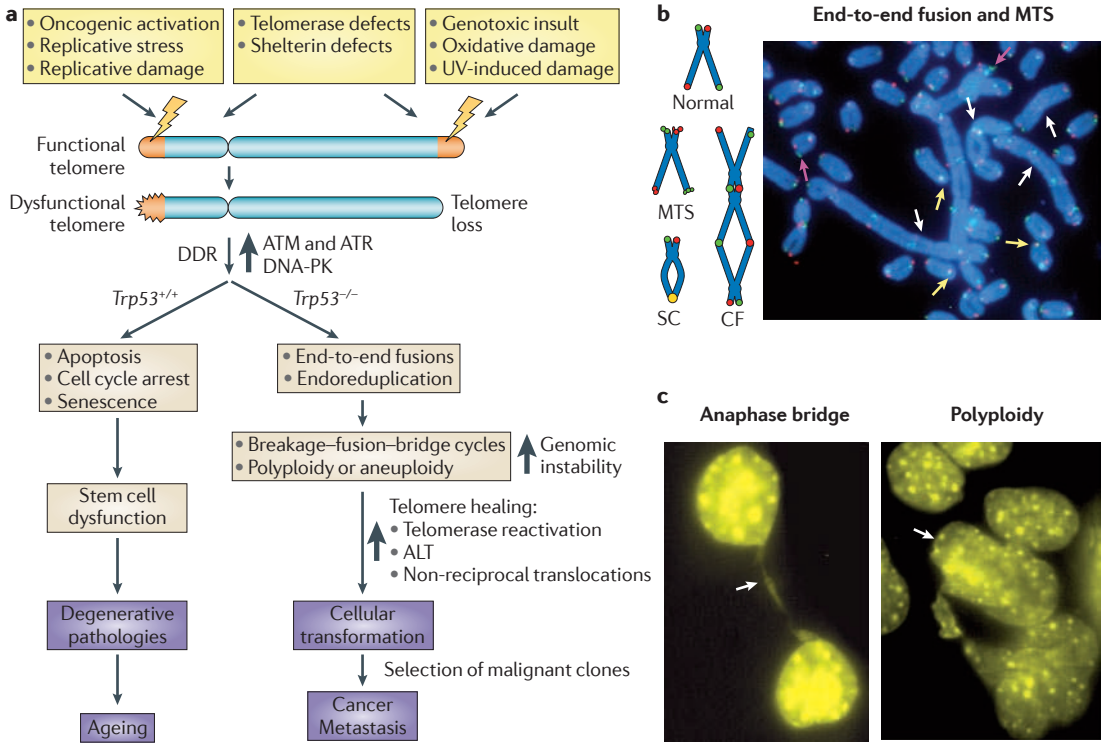


Figure 2 | **Telomere dysfunction as a driver of genomic instability. a** | Telomeric DNA damage originates from various mechanisms. Oncogene-induced replicative stress may cause telomere loss owing to the intrinsic telomere shortening that is associated with replication, as well as an elevated incidence of stalled replications forks at telomeres that resemble fragile sites[37,85,107,108]. Mutations in telomerase and in shelterin components may result in either telomere loss or severe telomere uncapping[12,36]. Telomeric DNA is highly susceptible to genotoxic damage[119,122]. Dysfunctional telomeres, either owing to critically short telomeres or to uncapping, elicit a DNA damage response (DDR) by activation of upstream kinases, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR)[8,12]. DDR activation may drive cells towards two opposing outcomes depending on the p53 and p21 status. Consequent activation of the tumour suppressor p53 induces cell cycle arrest, apoptosis or senescence, negatively affecting stem cell functionality and causing tissue degeneration and ultimately organ failure. In *Trp53*-deficient cells, the damage proceeds unchecked and no cell cycle arrest and senescence or apoptotic response will take place. The activation of the ATM–ATR kinase pathways leads to mitotic block. The cells are then able to bypass mitosis and re-enter S phase of the cell cycle, becoming tetraploid[132]. Tetraploidization can readily initiate genomic instability owing to the presence of multiple centrosomes that will give rise to the random distribution of chromosome-originating aneuploid daughter cells in mitosis. Activation of either the classic or the alternative non-homologous end joining (NHEJ)pathways results in end-to-end fusions that initiate successive cycles of breakage–fusion–bridges. On telomere healing, either by telomerase reactivation or by homologous recombination-based mechanisms, such as the alternative lengthening of the telomere (ALT), stable malignant clones will be generated, giving rise to metastasic tumours. **b** | Representative example of chromosomal aberrations induced by telomere uncapping: chromosome fusions and concatenation (CF; white arrow), sister chromatid fusions (SC; yellow arrow) and multitelomeric signals (MTS; purple arrow) are indicated. **c** | Representative images of an anaphase bridge and polyploid cell induced by telomere uncapping. The cells in parts **b** and **c** are immortalized mouse embryonic fibroblasts (MEFs) deleted for telomeric repeat binding factor 1 (TRF1; also known as TERF1). UV, ultraviolet.

phosphorylation of ATM, as well as phosphorylation of the ATM and ATR downstream checkpoint kinases CHK1 and CHK2 (REFS 37,85). Abrogation of the p53 and RB pathways in these cells bypasses senescence and leads to chromosome instability, including sister chromatid fusions, chromosome concatenation and the occurrence of MTS[37]. In spite of elevated telomere fusions and increased telomere fragility, *Terf1*-deleted MEFs show normal telomere length, suggesting that TRF1 is not essential for telomere length maintenance but has an important role in telomere protection[37]. In an analogous manner, ES cells conditionally deleted for *Terf1* showed normal telomere length but increased telomere fusions[111].

Mice conditionally deleted for *Terf1* in stratified epithelia, *Terf1Δ/Δ K5-cre* mice (BOX 2), show perinatal lethality and severe skin morphogenesis defects that are concomitant with increased telomere damage signalling, activation of the p53–p21 and p16 pathways, and cell cycle arrest *in vivo*[37]. Interestingly, stratified epithelia from *Terf1*-deficient mice also showed preneoplastic lesions (dysplasia and hyperkeratosis) as early as 1–6 days after birth, suggesting that TRF1-driven telomere damage could promote the early stages of tumorigenesis. Importantly, p53 deficiency in *Trp53*$^{-/-}$/*Terf1Δ/ΔK5-Cre* mice rescued hair follicle stem cell defects and skin hyperpigmentation, and also increased

Box 2 | **Mouse models underscoring telomere dysfunction as a driving force in cancer development**

The importance of telomere dysfunction as a mechanism for chromosome instability in cancer was first demonstrated by studies in telomerase-deficient mice. In particular, telomere shortening in the absence of telomerase activity is a potent tumour suppressor mechanism, even in cancer-prone backgrounds such as the *Ink4a/Arf*-deficient mice[14–17,40,54,192]. An exception to the tumour suppressor activity of short telomeres are mice lacking both telomerase and p53, which show a higher incidence of human-like carcinomas[3,97]. Recent work highlights that telomere uncapping, even in the presence of normal-length telomeres, is also a potent driver of carcinogenesis. In contrast to telomerase-deficient mice (knockout mouse models for the reverse transcriptase catalytic subunit, *Tert*, and the RNA component *Terc*) that survive to adulthood, complete abrogation of telomeric repeat binding factor 1 (*Terf1*; which encodes TRF1), *Terf2*, protection of telomeres protein 1 (*Pot1a*), adrenocortical dysplasia homologue (*ACD*; which encodes TPP1) and *Terf1*-interacting nuclear factor 2 (*Tinf2*; which encodes TIN2) results in early embryonic lethality[2,30–35]. The recent availability of several shelterin-transgenic mouse models, as well as the generation of tissue-specific conditional mouse models, has revealed a role for these proteins in cancer susceptibility and ageing-related pathologies even in the presence of normal telomerase activity and normal telomere length[37]. Similarly, expression of TRF1, TRF2, TIN2 and POT1 is altered in some human tumours[40]. In particular, a deregulated expression of TRF1, repressor-activator protein 1 (RAP1) and TPP1 has recently been described for patients with chronic lymphocytic leukaemia[42].

Genotype	Cancer phenotype	Ageing phenotype	Refs
Terc$^{-/-}$	Reduced incidence of cancer	Premature ageing: decreased proliferative potential of adult stem cell populations, alopecia, intestinal atrophy, hair greying, infertility, heart dysfunction, bone marrow aplasia, kidney dysfunction and defective bone marrow	11,15–20
Terc$^{-/-}$ *Trp53*$^{-/-}$	Cancer prone	Increased mobilization of adult stem cells with dysfunctional telomeres. Rescue of small body size phenotype. Lower organismal survival	3,193–195
Terc$^{-/-}$ *Ink4a*$^{-/-}$/*Arf*$^{-/-}$	Delayed tumour onset; maintain lymphoma and sarcoma range		196
K5-Tert	Cancer prone	Increased proliferative response in stratified epithelia	22,23
K5-Tert super-p53 super-p16-ARF	Resistant to cancer	40% increase in median longevity, reduction in aging-associated pathologies, improved neuromuscular coordination, increased glucose tolerance and a better fitness of epithelial barriers	24
Terf1$^{-/-}$	Embryonic lethal		32
K5-*Terf1*	Slightly increased susceptibility to skin carcinogenesis protocols	Premature skin deterioration, hyperpigmentation and alopecia	123
Terf1$^{\Delta/\Delta}$ K5-Cre	Rapid development of pre-neoplastic lesions at 1–3 days of age	Perinatal death, epithelia degenerative pathologies, hyperpigmentation hyperkeratosis, defective hair follicle and sebaceous gland development	37
Terf1$^{\Delta/\Delta}$ K5-Cre p53$^{-/-}$	Increased incidence of spontaneous squamous cell carcinomas	Rescue of survival, hair development and skin defects	37
Terf2$^{-/-}$	Embryonic lethal		2
Mx1-*Terf2*	Not reported	Not reported	34
K5-*Terf2*	Increased susceptibility to spontaneous and induced skin cancer	Premature skin deterioration, hyperpigmentation and alopecia	41
K5-*Terf2 Terc*$^{-/-}$	Accelerated skin carcinogenesis	Severe premature skin deterioration, hyperpigmentation and alopecia	98
Tpp1$^{-/-}$	Embryonic lethal		33
Acd (hypomorphic)	Not reported	High perinatal death, developmental defects, hyperpigmentation, alopecia, infertility, adrenocortical dysplasia and malformations of the skeletal and genitourinary system	113
Acd Trp53$^{-/-}$	Increased carcinoma incidence	Rescue of survival and of the acd phenotypes except germ cell atrophy	38
TPP1$^{\Delta/\Delta}$ K5-Cre	Epithelia dysplasia	Perinatal death, epithelia degenerative pathologies, hyperpigmentation, hyperkeratosis, defective hair follicle and sebaceous gland development	39
Pot1a$^{-/-}$	Embryonic lethal		35
Pot1b$^{-/-}$	Not reported	Not reported	31,75
Pot1b$^{-/-}$ *Terc*$^{+/-}$	Not reported	Hyperpigmentation and fatal bone marrow failure	137
Tin2$^{-/-}$	Embryonic lethal		30
Rap1-knock in	Embryonic lethal; defective NF-κB signalling in heterozygosis	Not reported	45
Rap1$^{-/-}$	Not reported	No effects on viability or fertility	84
Rap1$^{\Delta/\Delta}$ K5-Cre	Not reported	Mild skin hyperpigmentation	44

Cyclobutane pyrimidine dimer (CPD)
Interstrand DNA lesion formed when two adjacent pyrimidines are joined across their 5–6 double bonds owing to the UV radiation excitation of one of the pyrimidines.

Nucleotide excision repair (NER)
DNA repair mechanism that removes UV radiation-induced helix-distorting lesions such as pyrimidine dimers and 6,4 photoproducts. Two subpathways exist: global genomic NER and transcription-coupled NER.

Xeroderma pigmentosum
Autosomal recessive genetic disorder linked to mutations in components within the NER pathway. Patients are deficient in repairing UV radiation-induced DNA damage and are prone to develop skin cancers and several skin malignancies such as keratoses, hyperpigmentation and blistering.

Spindle assembly checkpoint (SAC)
Monitors proper chromosome attachment to spindle microtubules. SCA prevents anaphase until all chromosomes are properly attached to the spindle. To achieve proper segregation, the two kinetochores on the sister chromatids must be attached to opposite spindle poles. It is composed of mitotic checkpoint proteins MAD and BUB. These reside on kinetochores and show changes in phosphorylation and localization as cells proceed through mitosis. Failure of SAC can result in aneuploidy.

mouse survival, indicating that proliferative defects associated with *Terf1* abrogation are mediated by p53 (BOX 2). Moreover, long-lived *Terf1*$^{-/-}$;*Trp53*$^{-/-}$-double-null mice spontaneously develop invasive and genetically unstable squamous cell carcinomas (SCCs) in the tail and ear skin (BOX 2). The SCCs lacking TRF1 invaded the dermis and were genetically unstable, as indicated by the presence of multinucleated giant cells and anaphase bridges[37]. These results suggest that TRF1 functions as a suppressor of both cancer and ageing by preventing telomere-induced genomic instability in proliferating cells. A role for TRF1 as a potential anticancer target is still unknown. In this regard, future studies should address the effect of deleting *Terf1* in the context of pre-existing tumours to clarify whether *Terf1* deletion may contribute to stopping or promoting cancer growth. Two scenarios could be envisioned: on the one hand, in a tumour suppressor-proficient background, the severe proliferative defects of cells lacking TRF1 would impede the replicative stress caused by activating oncogenic mutations. On the other hand, oncogene-induced replicative stress, together with telomere uncapping, could fuel neoplasic transformation by successive mitotic cycles of B–F–B and thereby augment tumorigenesis. Further work should be carried out in order to address this matter.

In line with defects in shelterin components as drivers of genome instability, mice harbouring a hypomorphic mutation in *Tpp1* show adrenocortical dysplasia (ACD) and skin hyperpigmentation, which are also rescued by p53 deficiency[38,112,113] (BOX 2). Moreover, similar to that observed for *Terf1* deficiency, *Trp53* deficiency also leads to increased carcinomas in mice with ACD[37,38].

Deletion of *Tin2* in mice results in early embryonic lethality[30] (BOX 2), mirroring that of *Terf1*- and *Terf2*-deficient mice[2,32]. Mutations in *TERF1*, *TERF2* and *TIN2* have been identified in patients with bone marrow failure syndromes, but mutations in *POT1*, *RAP1* and *TPP1* have not yet been reported[114–116]. Further analysis of TIN2 function in preserving genome stability will require the development of conditional or tissue-specific knockout mouse models.

The susceptibility of telomeric DNA to genotoxic damage. Telomeric DNA has an increased susceptibility to single-strand DNA damage that is induced by oxidative stress owing to the fact that guanine triplets are highly sensitive to oxidation[117–119] (FIG. 2). Oxidative damage levels at telomeres correlate with rates of telomere loss during subsequent rounds of DNA replication[120,121]. Additionally, owing to their pyrimidine-rich nature, mammalian telomeres are hypersensitive to ultraviolet (UV) radiation-induced DNA damage, as they have sevenfold more cyclobutane pyrimidine dimers (CPDs) than the rest of the genome after UV irradiation. The G-strand is highly prone to form TT-CPDs and the C-strand to form CC-CPDs and CT-CPDs[122]. CPDs are repaired by the nucleotide excision repair (NER) pathway, which removes the damaged DNA fragment. Shelterin components, in particular TRF2 and TRF1, may repress NER at telomeres[41,123]. On the one hand, TRF2 physically interacts with a complex of the NER pathway that includes the DNA repair endonucleases ERCC1 and xeroderma pigmentosum complementation group F (XPF; also known as ERCC4)[124]. In addition, TRF1- and TRF2-overexpressing mice show an accelerated rate of telomere shortening that is fully rescued by simultaneous XPF deficiency, supporting a role of NER at telomeres[41,123]. These observations led to a model in which overexpression of TRF2 and TRF1 causes an aberrant recruitment of XPF at telomeric DNA, thereby causing telomere shortening and a concomitant depletion of XPF from non-telomeric DNA, leading to an enhanced sensitivity to UV radiation damage at extra-telomeric regions[41,123,125]. Mice overexpressing TRF2 in stratified epithelia, *K5-Terf2* mice (BOX 2), show an increased susceptibility to spontaneously developing skin tumours and are prone to UV radiation-induced carcinogenesis, which is analogous to the skin phenotypes of mice deficient in components of the NER pathway[41,126,127]. In this regard, TRF2 expression is increased in human skin carcinomas[41]. Interestingly, telomerase deficiency dramatically accelerates TRF2-induced epithelial carcinogenesis, coinciding with a higher chromosomal instability and higher burden of DNA damage. Telomeric recombination and alternative lengthening of the telomere (ALT)-associated promyelocytic leukaemia (PML) bodies (APBs) were augmented by TRF2 overexpression[98], suggesting a role for TRF2 in controlling telomere recombination.

In addition to TRF1 overexpression (*K5-Terf1* mice) leading to XPF-mediated telomere shortening in the mouse epidermis[123] (BOX 2), overexpressed TRF1 co-localizes with the spindle assembly checkpoint (SAC) proteins BUBR1 (also known as BUB1B) and MAD2 (also known as MAD2L1) and results in aberrant mitosis[123]. Thus, TRF1 and TRF2 act within the same pathway to control telomere length in mammals, resulting in XPF-dependent telomere shortening when overexpressed. The higher susceptibility to UV radiation-induced DNA damage and the predisposition to skin cancer of both the *K5-Terf2* and the *K5-Terf1* mice suggests that upregulation of TRF2 and TRF1 levels may constitute a potent oncogenic insult *in vivo*.

Cell cycle control and endoreduplication. Limiting genome replication to once per cell cycle is essential for maintaining genomic stability. Cancer cells are usually aneuploid, with highly variable chromosome numbers, ranging from hypodiploidy to tetraploidy and hypertetraploidy[128,129]. Telomere shortening and telomere dysfunction have been shown to trigger polyploidization[130,131]. Polyploidization induced by telomere dysfunction is dependent on the DDR, as inhibition of ATM and ATR kinases or their downstream effectors CHK2 and CHK1 hampers the formation of polyploid cells[132,133]. Overexpression of the ATR-activating domain of topoisomerase DNA II binding protein 1 (TOPBP1), which activates ATR in the absence of DNA damage[134], also induces tetraploidization, indicating that polyploidization is a consequence of DNA damage signalling rather than a consequence of the DNA lesions themselves[132]. POT1 is involved in repressing the ATR pathway[135,136].

Deletion of both *POT1* orthologues *Pot1a* and *Pot1b* in MEFs with a defective p53 pathway, leads to the progressive accumulation of polyploid cells containing several centrosomes and a high incidence of metaphasic diplochromosomes and quadruplochromosomes[132]. Double-knockout cells for *Pot1a* and *Pot1b* show increased DNA damage foci at telomeres, endoreduplication and early induction of senescence. At the mechanistic level, these cells enter the S phase of the cell cycle without progression through mitosis. Subsequent activation of the ATM and ATR signalling cascade prevents activation of cyclin-dependent kinase 1 (CDK1)–cyclin B, thereby blocking entry into mitosis and prolonging G2 until the cells eventually switch to a state resembling G1. The tetraploid cells resulting from persistent telomere damage re-established a normal cell cycle profile on restoration of telomere protection[132,133]. Single knockouts revealed that POT1A and POT1B have distinct roles[31,35,75]. Hockemeyer and co-workers[31] found that POT1A was required to inhibit a DDR at the telomere, and POT1B had the ability to regulate the amount of single-stranded DNA at telomeres in a telomerase-independent manner[31]. Deletion of *Pot1a* or *Pot1b* did not result in telomere length changes[31]. However, others have reported that *Pot1a*-deficient cells exhibit overall telomere lengthening and 3′ overhang elongation[35]. Deletion of *Pot1a* resulted in early embryonic lethality, but *Pot1b*-deficient mice can survive to adulthood and only show dyskeratosis congenital-like degenerative phenotypes when generated in a telomerase-haploinsufficient background[31,137,138] (BOX 2). As no effect on carcinogenesis has been reported in these mouse models, the generation of tissue-specific *Pot1a*-knockout mice, as well as the generation of mice deleted for *Pot1a*, *Pot1b* and *Trp53* will be useful for addressing the role of these proteins in neoplasic transformation.

Conditional deletion of *Terf2* induces end-to-end fusions and severe proliferative defects[2]. In marked contrast to the essential role of TRF2 for mouse embryo development, *Terf2* conditional deletion in the liver (*Mx1Terf2* mice) does not affect liver regeneration or mouse viability[34] (BOX 2). A possible explanation for this is that liver regeneration occurs in these mice without cell division by endoreduplication and cell growth, thereby overcoming the chromosome segregation problems that are associated with telomere fusions[34]. The effect of *Terf2* deletion on liver carcinogenesis was not addressed in this mouse model (BOX 2).

The observations described above support the idea that severe telomere dysfunction may be a driving force for cellular transformation. Thus, high proliferation rates in premalignant lesions cause telomere shortening and activation of the DDR, which in turn results in either end-to-end fusions or tetradiploidization, leading to genomic instability and the selection of cells lacking functional cell cycle control (FIG. 2). The eventual reactivation of telomerase or alternative mechanisms of telomere elongation during tumorigenesis[139] will allow the proliferation of cells with gross genome rearrangements, probably leading to a malignant phenotype.

Gene set enrichment analysis (GSEA)
A computational method that determines whether an a priori defined set of genes shows significant differences between two biological states.

Extra-telomeric roles for a telomeric protein

Human RAP1 was identified as a TRF2-interacting protein with homology to budding yeast Rap1 (REFS 83,140). Rap1 is the major binding activity at yeast telomeres, where it controls telomere length and the establishment of subtelomeric silencing through the recruitment of the Sir proteins[141–146]. Besides its role at telomeres, Rap1 also acts as a transcription factor by controlling the expression of glycolytic enzymes and ribosomal genes[147,148]. In contrast to Rap1, which binds telomeric DNA directly, RAP1 is thought to be recruited to telomeres by TRF2 (REF. 83). Three independent *Rap1*-knockout mouse models have recently been generated by either conditionally deleting *Rap1* exon 2 (REF. 84) and *Rap1* exon 3 (REF. 44) or by a trap insertion between *Rap1* exon 1 and exon 2 (REF. 45) (BOX 2). Complete abrogation of RAP1 was reported in one case to be embryonically lethal before embryonic day 6.5 (E6.5)[45], and in another case it was found not to affect viability or fertility[84]. Targeted *Rap1* deletion in stratified epithelia in *Rap1Δ/Δ K5-cre* mice does not affect mouse viability but leads to an early onset of skin hyperpigmentation during adulthood and to an obese phenotype in females[44].

Roles of RAP1 beyond telomeres were uncovered in two reports[44,45]. By using chromatin immunoprecipitation sequencing (ChIP–seq) technology, Martinez *et al.*[44] found that RAP1 not only binds to telomeres but also to other non-telomeric sites preferentially throughout the recognition of the $(TTAGGG)_2$ consensus motif[44]. Extra-telomeric RAP1 binding sites are enriched at subtelomeric regions, in agreement with preferential derepression of subtelomeric genes in *Rap1*-deficient cells, highlighting a conserved role for RAP1 in subtelomeric silencing[44,141,149]. In addition, RAP1 was shown to bind to non-coding regions in chromosomes 2, 11 and 17, which are enriched in TTAGGG tandem repeats, raising the possibility that RAP1 might prevent fragility and recombination at these genomic sites[44]. The fact that mammalian RAP1 seems to have diverged from Rap1 and lost its DNA-binding domain[83,150], the observation that RAP1 also binds to genomic sites lacking TTTAGGG repeats and the fact that many of these sites were also associated with genes that are deregulated on *Rap1* deletion[44], suggest that RAP1 may interact with factors other than TRF2 to help gene transcriptional regulation (FIG. 3). Gene set enrichment analysis (GSEA) on genes downregulated in *Rap1*-null MEFs revealed significant downregulation of imprinted genes, as well as downregulation of genes involved in cancer, cell adhesion and metabolism in *Rap1*-null cells. These included genes of the insulin secretion, peroxisome proliferator-activated receptor (PPAR) signalling, and growth hormone pathways. In turn, *Rap1*-null cells showed significant upregulation of ABC transporters and genes involved in type 2 diabetes, suggesting a negative effect of *Rap1* deletion on metabolism[44]. In agreement with a role of RAP1 in transcriptional regulation, RAP1-binding sites were shown to have RAP1-dependent enhancer activity[44].

In another role beyond telomeres, RAP1 has been shown to constitute an essential modulator of the nuclear factor-κB (NF-κB)-mediated pathway[45] (FIG. 3). RAP1 was identified in a genome-wide gain-of-function screen

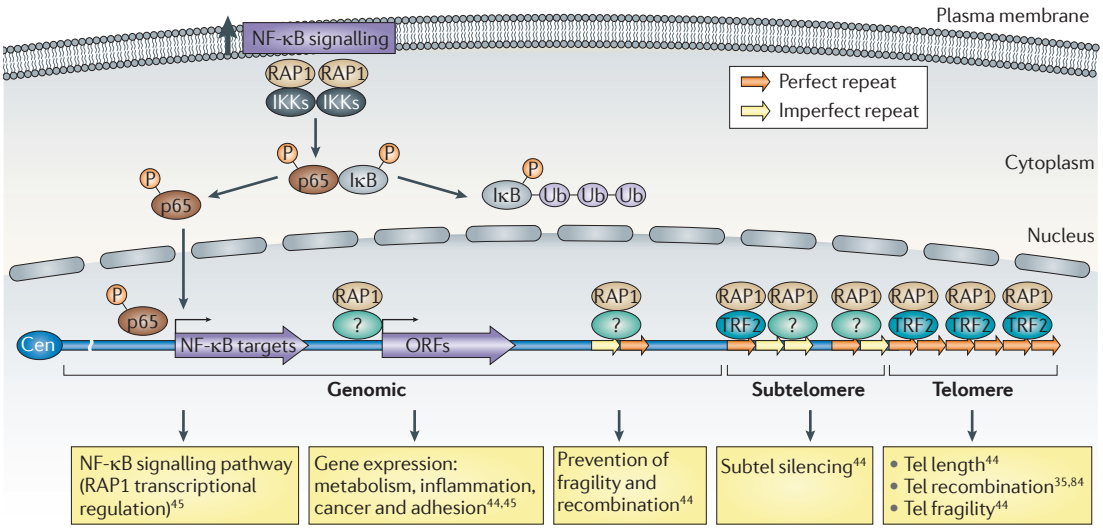


Figure 3 | **RAP1 telomeric and extra-telomeric roles.** RAP1 binds to both telomeric and non-telomeric chromatin. At telomeres and through interactions with telomeric repeat binding factor 2 (TRF2; also known as TERF2), repressor-activator protein 1 (RAP1) exerts a protective role by repressing telomeric recombination, fragility and telomere shortening. RAP1 has a transcriptional regulatory role probably through interactions with other, as yet unknown, factors (RAP1 interactors)[44,84]. At the subtelomere, RAP1 is involved in gene silencing. At other genomic positions, RAP1 regulates the expression of genes involved in different biological processes; among others, adhesion, metabolism and cancer. RAP1 also binds to non-coding regions enriched in TTAGGG repeats, where it could have a role in preventing recombination and fragility[44]. In addition, RAP1 is involved in the nuclear factor-κB (NF-κB) signalling pathway. On NF-κB activation at the plasma membrane, cytoplasmic RAP1 forms a complex with IκB kinases (IKKs) that are then recruited to the NF-κB complex (IκB–p65–p50). IKK-mediated IκB phosphorylation (P) induces IκB ubiquitylation (Ub) and its proteasome-mediated degradation. RAP1 interaction with IKKs is specifically required for IKKs to bind p65 and for subsequent p65 phosphorylation. Phosphorylated p65 is translocated to the nucleus where it controls the expression of NF-κB target genes, which includes *TERF2IP* (which encodes RAP1) itself[45]. Cen, centromere; ORF, open reading frame; Tel, telomere.

for regulators of the NF-κB signalling pathway, which is involved in the transcriptional response to various cellular and developmental signals[151]. Ectopic expression of RAP1 induces NF-κB, whereas RAP1-depletion inhibits NF-κB activity. RAP1 was not only localized at telomeres but some RAP1 molecules were also associated with macromolecular complexes in the cytoplasm of human cells. In particular, RAP1 associates with the inhibitor of NF-κB kinase (IKK) complex (IKKA–IKKB–IKKG), which is essential for the phosphorylation of both the p65 subunit of NF-κB and the IKKB inhibitor, enabling NF-κB to recruit chromatin-remodelling proteins that are necessary for the transcriptional activation of target genes. RAP1 is required for the recruitment of the IKK complex and p65 phosphorylation in order to render NF-κB transcriptionally competent. This finding provides additional evidence supporting a role for RAP1 in transcriptional regulation. Interestingly, RAP1 levels are regulated by NF-κB signalling, and two conserved NF-κB binding sites were found at the *RAP1* promoter[45]. In addition, RAP1 binding peaks were found at the *RAP1* locus[44]. These results strongly suggest a feedback regulatory loop for RAP1 selfactivation[44,45]. Deregulation of the NF-κB pathway has been linked to human diseases. Constitutive NF-κB signalling has been implicated in the development and progression of breast cancers[152,153]. In this regard, Teo and co-workers[45] found that RAP1 levels were significantly higher in tumour samples than in neighbouring healthy cells, and a positive correlation between increasing RAP1 levels with the malignant grade of the tumour was also found. Interestingly, TERT has been shown to directly interact with NF-κB p65 *in vivo*, and this interaction mediates the nuclear translocation of telomerase[154], further linking telomere biology with inflammatory processes. The issue of whether TERT interaction with p65 is RAP1-dependent remains to be addressed.

In light of these discoveries, the identification of mammalian RAP1-interacting partners constitutes a very interesting area of research that would certainly provide information on the molecular mechanisms behind RAP1 telomeric-independent functions. It also remains unknown in which cellular compartment, cytoplasm or nucleus, RAP1 interacts with its potential partners. As RAP1 recruitment to telomeres is TRF2-dependent, and because the amount of telomere-bound TRF2 may vary between cell types, differentiation degree, embryonic development and age, the amount of telomere-unbound RAP1 might be a way to regulate transcriptional networks that are involved in several biological processes such as inflammation and metabolism. As the organism ages, the level of telomere-bound RAP1 may decrease, thereby affecting gene expression changes, which in turn may favour age-related phenotypes.

The multitasking jobs of telomerase

Telomerase is essential for the long-term proliferation potential of stem cells and cancer cells, and for normal tissue renewal. Increasing evidence suggests that ageing

and cancer share many common aspects of biology and are considered as stem cell diseases resulting from the decline of, or defects in, regenerative capacity and organ homeostasis[36].

Telomerase activation is able to extend the lifespan of cells in culture by maintaining telomeres[155], and it is activated in the vast majority of human cancers[139]. Indeed, constitutive telomerase expression in several independent *Tert*-transgenic mouse models results in an increased incidence of spontaneous tumours[21,23,156–158] (BOX 2). In the cancer cell scenario, it is conceivable that, on oncogenic stress, cells accelerate their proliferative rate, and telomere length is a limiting factor to their cell division capacity. Indeed, telomeres are usually shorter in tumour cells than in the surrounding healthy tissue[91–96]. As discussed above, in the absence of the appropriate checkpoints, short telomeres potentiate the occurrence of mutations. In support of this idea, sequence variants at the cisplatin resistance-related protein CRR9P (*CPTM1L*)-*TERT* locus on chromosome 5p.15.33 have been associated with many cancers, including cancers of the lung, brain, urinary bladder, prostate, cervix and pancreas, and acute myelogenous leukaemia[159–163]. Reactivation of telomerase would then provide the mutated precancerous cell with the capacity to divide indefinitely, impinging on tumorigenesis. Indeed, this genomic region has also been found to be frequently amplified in many types of cancer, such as lung and cervical cancers[164,165]. Together, these observations suggest that telomerase activation is common to many cancers and that its targeted inhibition could potentially be an effective anticancer therapy by triggering critically short telomeres and loss of cell viability in the tumour.

Telomerase activity is absent or very low in most adult tissues. Studies from telomerase-deficient and telomerase-overexpressing mouse models, as well as from human age-related diseases caused by mutations in telomerase components (BOX 1) have documented that critical telomere shortening owing to telomerase deficiency causes premature loss of stem cell function[11,22–24,36,56–60,166–168]. This led to the idea that increased telomerase activity in stem cell compartments could maintain tissue functionality for longer times, thus increasing longevity. However, the effect of telomerase overexpression on ageing in the adult organism has been hampered owing to the cancer-promoting activity of telomerase. This has recently been addressed by telomerase overexpression in mice that are genetically engineered to be cancer-resistant by means of increased expression of the p53, p16 and ARF tumour suppressors (BOX 2). These mice show a 40% increase in the median lifespan and delayed ageing, as indicated by improved neuromuscular coordination, increased glucose tolerance and a better fitness of epithelial barriers, demonstrating the anti-ageing activity of TERT[24].

Emerging evidence suggests non-telomeric roles for telomerase in stem cell biology, which in turn could impinge on tissue homeostasis and ageing. The study of TERT-overexpressing mouse models, led to the demonstration that enhanced TERT expression activates mobilization of quiescent hair-follicle stem cells, increases keratinocyte proliferation, stimulates hair growth and augments skin hyperplasia[22,28]. However, some controversy has arisen from these studies. Thus, although Sarin and co-workers conclude that these effects can also occur in a *Terc*-deficient background or by expressing a *Tert* catalytic mutant allele, and are therefore independent of TERT-dependent telomere synthesis[28], other authors found that the absence of *Terc* abolishes the proliferative effect of TERT overexpression[21,22]. Artandi and co-workers investigated the genome-wide transcriptional response to acute changes in TERT levels in mouse skin and found that TERT depletion resulted in expression changes of genes involved in epithelia development, signal transduction, the cytoskeleton and adhesion[25]. Pattern-matching algorithms further revealed that the TERT transcriptional response strongly resembled the responses mediated by the MYC and Wnt signalling pathways, which are of key importance for cancer and stem cell biology[25]. Indeed, TERT can function as a transcriptional modulator of the Wnt–β-catenin signalling pathway[27,169] (FIG. 4). Thus, TERT functions as a cofactor in a β-catenin transcriptional complex through interactions with BRG1 (also known as SMARCA4), which is a SWI/SNF-related chromatin remodelling protein. The ability of TERT to enhance Wnt signalling explains how TERT activates bulge stem cells, as overexpression of β-catenin in mouse skin results in a very similar stem cell activation phenotype[170,171]. TERT physically occupies the gene promoters of Wnt-target genes and activates Wnt reporter genes in culture and *in vivo*. Indeed, TERT was shown to be essential for proper embryonic development in *Xenopus laevis* and important for homeotic transformation in mice[27].

In addition, TERT in a complex with *RMRP* can act as an RNA-dependent RNA polymerase (RDRP)[26] (FIG. 4). The TERT–*RMRP* complex acts as an RDRP and processes *RMRP* into double-stranded RNA (dsRNA), which is then processed by the endoribonuclease Dicer into small interfering RNA (siRNA), which controls *RMRP* endogenous levels. Thus, TERT–*RMRP*–RDRP regulates *RMRP* levels by a negative-feedback control mechanism. Other RNAs that act as templates for the TERT–*RMRP*–RDRP remain to be identified. However, it was proposed that the TERT–*RMRP* complex may amplify other small non-coding RNAs and thereby regulate the expression of other genes by generating specific siRNAs. In support of a role of TERT in controlling gene expression, several independent transcriptional analyses with different human cell lines have revealed that TERT ectopic expression seriously affects gene expression patterns (reviewed in REF. 172). The RNAase MRP complex is involved in diverse cellular and mitochondrial functions, and mutations in *RMRP* have been linked to cartilage hair hypoplasia, a syndrome that is characterized by premature multi-organ failure mainly in highly proliferative organs and which consists of stem cell dysfunction[173] (BOX 1). Thus, it could be expected that alterations in the TERT–*RMRP* complex could be involved in the pathogenesis of this disorder. Therefore, two different ribonucleoprotein complexes containing TERT, TERT–*TERC* and TERT–*RMRP* have so far been identified. Both are implicated in stem cell biology by regulating telomere maintenance

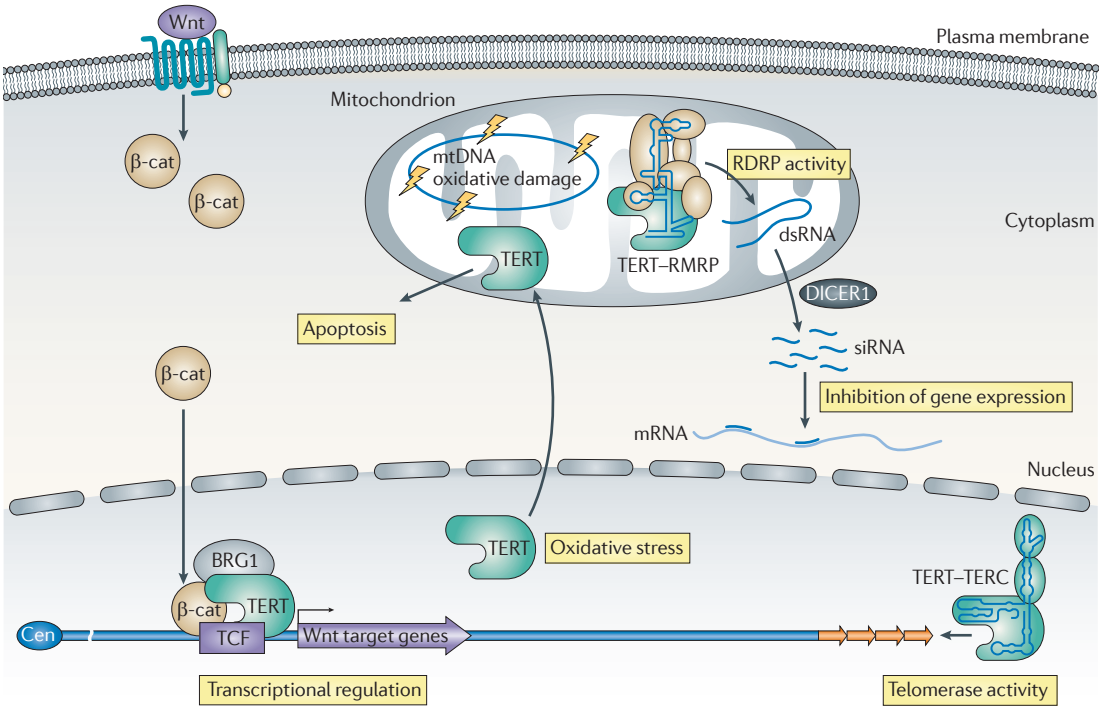


Figure 4 | **Telomerase, a master in coping with multiple jobs.** The telomerase catalytic subunit (TERT) in association with the telomerase RNA component (*TERC*) forms an enzyme, the telomerase (TERT–*TERC*), with reverse transcriptase activity that recognizes the 3′-OH at the end of the G-strand overhang and elongates the telomere using *TERC* as the template[10]. TERT has a role as a transcriptional modulator of the Wnt–β-catenin (β-cat) signalling pathway. On stimulation of Wnt receptors at the plasma membrane, TERT forms a complex with the Wnt transcription factor BRG1 (also known as SMARCA4) and binds to the promoters of Wnt-target genes, regulating their expression[25]. In the mitochondria, TERT associates with the RNA component of mitochondrial RNA processing endoribonuclease *RMRP*, and this complex shows an RNA-dependent RNA polymerase (RDRP) activity. TERT–*RMRP* RDRP produces *RMRP*-derived double-stranded RNAs (dsRNAs) that are further processed into small interfering RNAs (siRNAs) in a Dicer-dependent manner that controls the endogenous levels of *RMRP*[26]. It has been speculated that TERT association with other, as yet unidentified, RNAs may regulate gene expression by generating specific siRNAs. In the mitochondria, TERT has also been proposed to have a role in regulating oxidative damage-induced apoptosis. Oxidative stress triggers nuclear export of TERT to the mitochondria[172]. Cen, centromere.

and by altering gene expression programmes that are related to stem cell homeostasis. Identification of novel TERT–RNA partners merits further research and would certainly provide further links between telomere biology and other relevant pathways.

Evidence has been provided for a role for telomerase in the regulation of apoptosis in a telomere maintenance-independent manner (reviewed in REF. 172). TERT contains a mitochondrial localization signal peptide at its N-terminal that targets TERT to mitochondria where it was shown to be active by the telomeric repeat amplification protocol (TRAP)[174]. In addition, it was shown that telomerase sensitizes mitochondrial DNA to hydrogen peroxide-induced oxidative damage, probably through the modulation of metal homeostasis[174]. The mitochondrial localization of telomerase also has an important role in apoptosis[175]. In support of a pro-apoptotic role of telomerase is the observation that oxidative stress triggers nuclear export of TERT[176]. In addition, overexpression of TERT suppresses apoptosis, and its down-regulation sensitizes mitochondrial apoptotic pathways not by telomere attrition, but rather by post-translational activation of BAX, which triggers a CD90-independent mitochondrial pathway of apoptosis[177,178]. In mice, expression of TERT has been linked with increased resistance to apoptosis that is induced by both staurosporin and *N*-methyl-*D*-aspartic treatment, and this effect was also independent of telomerase activity[179]. Although the exact molecular mechanisms by which TERT regulates apoptosis in mitochondria are still largely unknown, the results described above suggest that TERT might promote apoptosis not only by altering mitochondrial membrane potential or metal homeostasis in mitochondria but also by controlling the expression of genes that are involved in apoptosis through siRNA generation by the mitochondrial TERT–*RMRP* complex.

Results from other laboratories, however, are in marked contrast to the finding that telomerase possesses telomere length maintenance-independent roles[180,181]. Gene expression comparisons among G1 *Terc*$^{-/-}$ mice, G1 *Tert*$^{-/-}$ mice and wild-type mice, all of which had similar telomere length, showed no differences in transcriptional profiles[180]. In contrast to the experiments described above in which telomerase was overexpressed[22,25–28,174,177,179], these studies were carried out in knockout mice, and so under physiological conditions[180]. The observed effect of

TERT overexpression in transcriptional regulation and apoptosis may be due to the generation of hypermorphs or neomorphs that display an excess of the given activity or a new phenotype that is not associated with the original gene[180,182]. It should also be kept in mind that inappropriate activation of the Wnt pathway owing to experimental conditions has been reported[183]. Further validation of the TERT 'extracurricular' roles under conditions in which TERT is expressed at endogenous levels are needed to clarify this apparent controversy.

Telomerase and anticancer treatment

The link between the inability to maintain telomeres with age and consequent declining health, including the increased risk of degenerative diseases and cancer, has suggested that telomerase is an appealing target for the treatment of these diseases. Several factors make telomerase inhibition as an anticancer treatment a safe and rather specific therapy. First, telomerase is expressed in >85% of tumours from all types of cancers and so it would be widely applicable[184]. The likelihood of developing resistance mechanisms is low, as telomerase is encoded by a non-redundant gene; nevertheless, alternative mechanisms to maintain telomeres (such as ALT) may be potentially selected. In addition, the different telomerase expression levels, as well as the generally longer telomeres in healthy cells versus tumour cells, suggest a high degree of tumour specificity and a low risk of toxicity to normal tissues[185]. Different approaches have been designed in the search for telomerase inhibitors: drugs that inhibit telomerase enzymatic activity, active immunotherapy, gene therapy using telomerase promoter-driven expression of a suicide gene, agents that block telomerase biogenesis and G-quadruplex-stabilizing molecules as telomere-disrupting agents. However, only drugs that inhibit telomerase enzymatic activity and immunotherapy-based drugs have so-far reached clinical trials (reviewed in REF. 185).

Telomerase recruitment to telomeres. A central question in telomere biology concerns how telomerase is recruited to telomeres. The shelterin components have been known to have important roles in telomerase recruitment and in telomerase regulation; however, the molecular mechanisms controlling this have remained largely unknown. Recently, a role for TPP1 in telomerase recruitment has been proposed. The POT1–TPP1 heterodimer augments telomerase processivity through specific interactions with telomerase[46,81,186,187]. In addition, human cells depleted for TPP1 fail to recruit telomerase to telomeres[43]. Furthermore, conditional *Tpp1* deletion in mice results in decreased TERT binding to telomeres and in accelerated telomere shortening. Moreover, *Tpp1*-null cells fail to elongate telomeres when reprogrammed into pluripotent stem cells by using defined factors[39], the iPS cells[68], a process that is dependent on telomerase activity[53,188] (BOX 3). Together, these results suggest a telomere-capping model in which TPP1 not only prevents the induction of a DDR at telomeres by preventing fusions and telomere breakage but it is also required for telomere elongation by telomerase[39].

It should be noted that in contrast to *Terf1* deficiency, simultaneous *Tpp1* and *Trp53* abrogation does not increase the frequency of epithelial carcinomas[37,39] (A.M. Tejera and M.A.B., unpublished observations). This may be explained by the fact that TPP1 is required to recruit telomerase to chromosome ends, and therefore mimics the tumour-resistant phenotype of telomerase-deficient mice, supporting the proposal that TPP1 is a potential anticancer target[39]. In addition, *Tpp1*-null cells show less chromosomal instability than *Terf1*-null cells, which could also contribute to their lower tumorigenecity. By contrast, ACD mice lacking p53 show increased carcinoma development, showing that the *ACD* allele resulted in hypomorphic TPP1 protein expression.

Perspectives

Research over the past two decades has revealed that telomere dysfunction, whether owing to the loss of telomeric sequence or owing to the loss of telomere structure, and telomerase regulation have an important role in tissue regeneration during ageing, as well as during tumour initiation and progression. There is mounting evidence that components of the telomere-maintenance machinery, in particular TERT and RAP1,

Box 3 | **Role of shelterins in stem cell biology and nuclear reprogramming**

Research over the past few years has unequivocally demonstrated the importance of telomere length and telomerase activity in stem cell biology. Telomerase expression is restricted to adult stem cell compartments, which have the longest telomeres[7,50–52]. Differentiated somatic cells can be reverted to a more pluripotent state through a mechanism known as nuclear reprogramming: the induced pluripotent stem (iPS) cells[68]. During iPS generation, telomerase is reactivated and telomere elongation occurs until reaching embryonic stem (ES) cell telomere length[53,68,188,197,198]. Reprogramming efficiency of mouse embryonic fibroblasts (MEFs) with damaged or uncapped telomeres is dramatically decreased, indicating the existence of 'reprogramming barriers' that abort the reprogramming of cells with dysfunctional telomeres. Abrogation of p53 rescues reprogramming defects of cells with dysfunctional telomeres, demonstrating that p53 is crucial for preventing the generation of pluripotent cells from suboptimal parental cells[188]. As shelterin components are mediators of telomere length and required for proper telomere capping, it is conceivable that the expression or function of these proteins is differentially regulated during different developmental stages, as well as during nuclear reprogramming to contribute to telomere rejuvenation. Although the regulation of shelterin components in stem cells and during nuclear reprogramming is still unexplored, some recent evidence underscores shelterin components as key factors in 'stemness'. Indeed, some cases of premature ageing in human syndromes have been linked to shelterin mutations, such as in telomeric repeat binding factor 1 (TRF1; also known as TERF1), TRF2 and TRF1-interacting protein 2 (TIN2; also known as TINF2)[114,115,191,199]. Further research is needed to fully establish the molecular roles and regulation of shelterin components during embryonic development, ageing and nuclear reprogramming.

when associated with different factors and targeted to different locations away from the telomere, do exert other cellular functions independently of their canonical telomere-maintenance role. The novel findings discussed in this Review link telomeres with a plethora of biological processes, such as mitochondrial function, inflammation, embryonic development, regulation of gene expression, metabolism, stem cell homeostasis, adhesion and cancer. One major future challenge is to identify new interacting factors of telomerase and shelterin components, and to understand their biological function and how their activities are controlled in more detail. Such knowledge would not only enhance our appreciation of the molecular mechanisms underlying telomere maintenance but would also provide valuable insights into human genetic disease, ageing and cancer, and thereby provide opportunities for the better management of human health and disease.

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