

# Telomere-driven diseases and telomere-targeting therapies

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Telomeres, the protective ends of linear chromosomes, shorten throughout an individual's lifetime. Telomere shortening is proposed to be a primary molecular cause of aging. Short telomeres block the proliferative capacity of stem cells, affecting their potential to regenerate tissues, and trigger the development of age-associated diseases. Mutations in telomere maintenance genes are associated with pathologies referred to as telomere syndromes, including Hoyeraal-Hreidarsson syndrome, dyskeratosis congenita, pulmonary fibrosis, aplastic anemia, and liver fibrosis. Telomere shortening induces chromosomal instability that, in the absence of functional tumor suppressor genes, can contribute to tumorigenesis. In addition, mutations in telomere length maintenance genes and in shelterin components, the protein complex that protects telomeres, have been found to be associated with different types of cancer. These observations have encouraged the development of therapeutic strategies to treat and prevent telomere-associated diseases, namely aging-related diseases, including cancer. Here we review the molecular mechanisms underlying telomere-driven diseases and highlight recent advances in the preclinical development of telomere-targeted therapies using mouse models.

## Telomeres, telomerase, and shelterins

Telomeres form a special heterochromatic structure at the end of linear chromosomes that protects them from degradation and DNA repair and recombination activities. Thus, telomeres are essential to ensure chromosome stability (Blasco, 2005; Palm and de Lange, 2008). Mammalian telomeres comprise several kilobases, between 10 and 15 kb in humans and 25 and 50 kb in mice, of tandem TTAGGG DNA repeats (Blasco, 2005). Telomeres are characterized by the presence of a 30–400-nucleotide-long 3' overhang of a G-rich strand, known as the G-strand overhang. The G-strand overhang can fold back and invade the double-stranded telomeric region, forming the so-called T-loop and generating a displacement loop, or D-loop. The T-loop

structure has been proposed to protect chromosome ends from degradation and DNA repair activities as well as from telomerase activity (Fig. 1, A and B; Griffith et al., 1999; Doksani et al., 2013). Telomeres are bound by a specialized complex known as shelterin that has crucial functions in telomere length regulation and in the protection of telomeres from the DNA damage response (DDR) by masking the chromosome ends from the DNA repair machinery through repression of the ATM and ATR signaling pathways (Palm and de Lange, 2008; Fig. 1 C). The shelterin complex is composed of six proteins: telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1-interacting protein 2 (TIN2), protection of telomeres protein 1 (POT1), TIN2, POT1-interacting protein (TPP1), and repressor/activator protein 1 (RAP1; Fig. 1 C; de Lange, 2005; Martínez and Blasco, 2010, 2011).

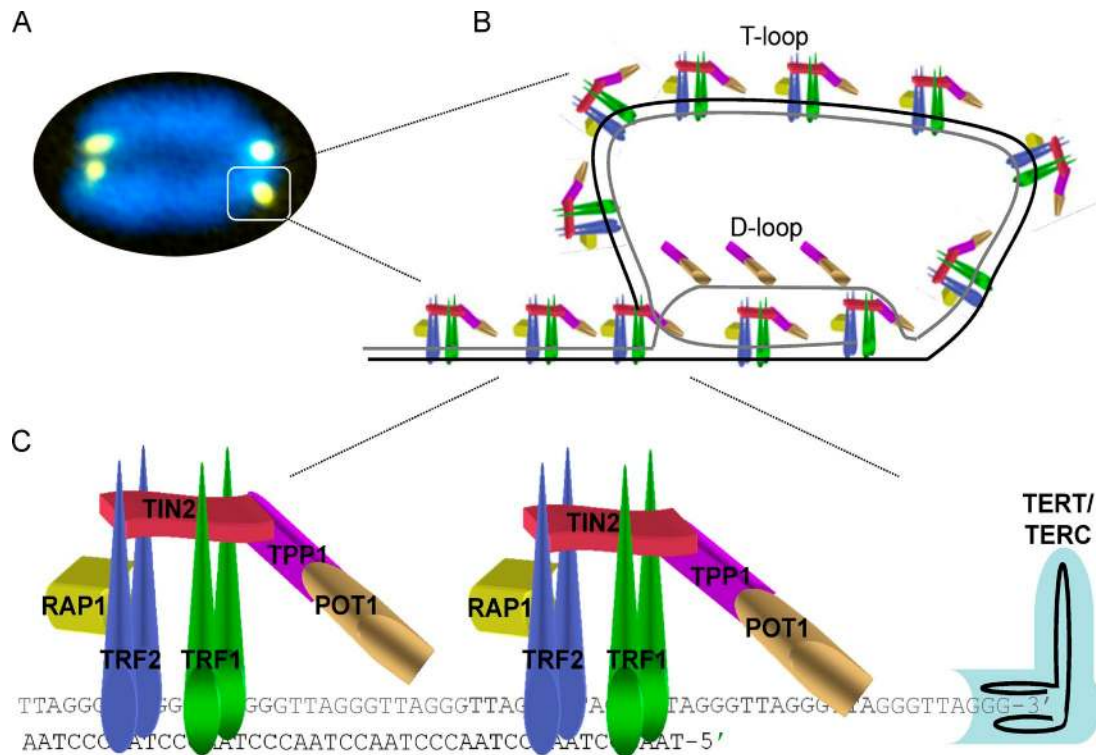
Telomeres shorten with each cell division as a result of the incomplete replication of linear DNA molecules by conventional DNA polymerases, which is called the end-replication problem (Watson, 1972; Olovnikov, 1973). Telomerase compensates for telomere attrition through de novo addition of TTA GGG repeats onto chromosome ends in those cells where it is normally expressed, such as pluripotent stem cells and adult stem cell compartments (Liu et al., 2007; Flores et al., 2008; Marion et al., 2009). Telomerase is composed of a reverse transcriptase subunit (TERT) as well as an associated RNA component (*Terc*), which is used as a template for the de novo addition of telomeric repeats (Fig. 1 C; Greider and Blackburn, 1985). Although telomerase is expressed in adult stem cell compartments, this is not sufficient to counteract telomere attrition associated with cell division throughout life, and therefore telomeres shorten with age in vitro and in vivo (Harley et al., 1990; Hastie et al., 1990; Lindsey et al., 1991; Collado et al., 2007; Liu et al., 2007; Flores et al., 2008; Marion et al., 2009). This progressive telomere shortening eventually leads to critically short telomeres that can impair the regenerative capacity of tissues and has been proposed as one of the molecular hallmarks of aging (López-Otín et al., 2013). In mice, it has been shown that the rate of increase in the percentage of short telomeres, rather than the rate of telomere shortening throughout life, is a significant predictor of life span (Vera et al., 2012). Shortened telomeres induce a DDR that leads to a growth arrest, during which cells attempt to repair the damage and, if DNA damage is irreparable, triggers replicative senescence (Zou et al., 2004; Fumagalli et al., 2012). Senescent cells progressively accumu-

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Abbreviations used: 6-thio-dG, 6-thio-2'-deoxyguanosine; AAV, adeno-associated vector; ALT, alternative lengthening of telomeres; BM, bone marrow; DC, dyskeratosis congenita; DDR, DNA damage response; HHS, Hoyeraal-Hreidarsson syndrome; HSPC, hematopoietic stem/progenitor cell; IPF, idiopathic PF; MC, mitotic catastrophe; PF, pulmonary fibrosis; TPE, telomere position effect.

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**Figure 1. The shelterin complex and the structure of telomeres.** (A) Representative image of a metaphasic chromosome stained with DAPI (blue) and the telomeric DNA-specific peptide nucleic acid probe (yellow). (B) 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-stranded DNA overhang of a G-rich strand. The G-strand overhang (gray) invades the telomeric double-stranded DNA region to form a protective T-loop, with a displacement D-loop at the invasion site. (C) Schematic representation of telomere-bound proteins, the shelterin complex, and telomerase. The shelterin complex is composed of the telomeric repeat binding factor 1 (TRF1, also known as TERF1), TRF2 (also known as TERF2), repressor-activator protein 1 (RAP1, also known as TERF2IP1), POT1-TIN2 organizing protein (TPP1, also known as ACD), TIN2 (also known as TIFN2), and protection of telomeres protein 1 (POT1). 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' single-stranded G-overhang. TIN2 binds TRF1 and TRF2 through independent domains and recruits the TPP1–POT1 complex, constituting the bridge among the different shelterin components. Telomerase is a two-partner enzyme, the catalytic subunit (TERT) and the RNA template (TERC), that recognizes the 3'-OH at the end of the G-strand overhang and elongates the telomere.

late during life and secrete factors that influence age-associated diseases (Tchkonia et al., 2013). Indeed, senescence has been proposed as a mechanism that evolved to protect from cancer, with the drawback of promoting age-associated diseases (Shay, 2016). Therapeutic interventions based on either chemical activators of telomerase or telomerase-based gene therapy are currently being investigated in mouse models for their potential to improve health and extend life span, and as a treatment for short telomere syndromes (Bernardes de Jesus et al., 2011; Bär et al., 2014, 2015; Bär and Blasco, 2016).

### Aging-associated diseases and telomeropathies

Telomeres shorten progressively throughout an individual's lifetime (Cawthon et al., 2003; López-Otín et al., 2013; Sanders and Newman, 2013). Telomere attrition in stem cell compartments impairs their tissue and self-renewal capacity and is considered to be one of the primary molecular causes of aging and the onset of aging-associated diseases (Flores et al., 2005; Sharpless and DePinho, 2007). The first demonstration that telomere shortening is at the origin of age-related pathologies and is a determinant of longevity came from the study of telomerase-deficient mice, which show premature aging phenotypes in both low-proliferating (heart, brain) and high-proliferating compartments (bone marrow [BM], gut, skin, and testis; Blasco et al., 1997;

Lee et al., 1998; Herrera et al., 1999; Leri et al., 2003; Ferrón et al., 2004). Telomere shortening with age has also been observed in several mouse stem cell compartments from different tissues independently of high or low tissue proliferation rates (Flores et al., 2008). Telomerase-deficient mice show a worsening of phenotypes with increasing mouse generations owing to inherited progressively shorter telomeres, with the later generations showing severe phenotypes and premature death at pre-reproductive ages (Lee et al., 1998; Herrera et al., 1999; García-Cao et al., 2002). This phenomenon first indicated genetic anticipation associated with telomerase deficiency. However, and in support of critical telomere shortening being a determinant of aging and longevity, increased TERT expression in the context of cancer-resistant transgenic mice was sufficient to delay aging and extend mouse longevity by 40% (Tomás-Loba et al., 2008). More recently, these findings have been translated into a potential therapeutic strategy by using adeno-associated vectors (AAVs) to transiently activate telomerase in adult tissues (Bernardes de Jesus et al., 2012; Bär et al., 2014). Telomeropathies or telomere syndromes develop when telomere attrition occurs prematurely as a consequence of germline mutations in genes coding for factors involved in telomere maintenance and repair (Table 1). Human telomeropathies are mainly associated with Hoyerlaal-Hreidarsson syndrome (HHS), dyskeratosis congenita (DC), aplastic anemia, and pulmonary and liver disease and

### Clinical features of telomeropathies

**Aplastic anemia:** Aplastic anemia is a BM failure state characterized by low blood counts and an insufficiency of hematopoietic cells in the BM (Fogarty et al., 2003).

**Idiopathic pulmonary fibrosis (IPF):** IPF is characterized by cough, dyspnea, impaired gas exchange, and reduced lung volume. Pathologically, there is patchy fibrosis of the lungs and interstitial inflammation, normal lung alternating with fibrosis, inflammation, and collagen deposition (Armanios et al., 2007).

**Liver disease:** Liver disease associated with telomere shortening consists of mainly fibrosis with inflammation and nodular regenerative hyperplasia, a leading cause of noncirrhotic portal hypertension (Calado et al., 2009a).

**Dyskeratosis congenita (DC):** DC is classically diagnosed by the presence of the mucocutaneous triad of nail dysplasia, skin pigmentation, and oral leukoplakia or the presence of one feature of the triad in combination with BM failure and two other DC-associated findings. Patients with DC are at very high risk of BM failure, PF, liver fibrosis, stenosis, premature hair graying, leukemia, and squamous cell cancer of the head, neck, or anogenital regions (Ballew and Savage, 2013).

**Hoyeraal-Hreidarsson syndrome (HHS):** HHS is a multisystem genetic disorder characterized by very short telomeres (less than first percentile for age) and is considered a clinically severe variant of DC. Patients with HHS present in early childhood with cerebellar hypoplasia, microcephaly, immunodeficiency, BM failure, and intrauterine growth retardation. The DC-associated mucocutaneous triad is also a trait of HHS (Glousker et al., 2015).

are nowadays considered more as a spectrum disorder than distinct diseases (see Clinical features of telomeropathies; Holohan et al., 2014; Stanley and Armanios, 2015). Although these diseases show a wide and complex range of clinical symptoms, all of them are characterized by presenting with critically short telomeres. The age of onset and the severity of clinical manifestations vary among individuals. These syndromes are characterized by premature loss of the regenerative capacity of tissues, affecting tissues with both high and low proliferation rates (Armanios and Blackburn, 2012; Holohan et al., 2014). As in mice, disease anticipation is also found in human families with telomere syndromes, with the mutation generally first manifesting in adults with pulmonary fibrosis (PF), and the more severe phenotypes appearing in pediatric populations (immunodeficiency) and young adults (aplastic anemia) from the next generations (Armanios and Blackburn, 2012).

In addition to telomere-mediated replicative senescence, telomere length can have an impact on human diseases by regulating gene expression through telomere position effects (TPEs). TPEs involve the spreading of telomeric heterochromatin to silence nearby genes. In human cells, it has been shown that gene expression is affected by telomere length (Stadler et al., 2013; Robin et al., 2014). In particular, genes located near the telomere at chromosome 4q are progressively up-regulated with decreasing telomere length, and this occurs long before terminal telomere shortening would induce replicative senescence (Stadler et al., 2013). Interestingly, among the genes analyzed, the effect of TPE is most prominent with *DUX4*, the one located nearest to the 4q telomere. Expression of *DUX4*,

encoding a toxic double-homeobox protein, has been linked to facioscapulohumeral muscular dystrophy, a disease presenting with progressive atrophy and weakness of the facial, scapular, and upper arm muscles (Stadler et al., 2013). This disease has highly variable clinical manifestation, and delayed onset that can be explained by age-associated telomere shortening (Stadler et al., 2013). Thus, facioscapulohumeral muscular dystrophy constitutes the first human disease to demonstrate a potential contribution of TPE to the age-associated diseases.

### Mouse models for PF, aplastic anemia, DC, and HHS

The study of mouse models genetically deficient in telomerase and telomeric proteins has been crucial to understanding the role of telomere biology in human telomeropathies. In particular, the telomerase knockout, Pot1 knockout, and *Trf1* conditional knockout mouse models have recently served as proofs-of-principle of the causal role of DNA damage stemming from dysfunctional telomeres in DC, idiopathic PF (IPF), and aplastic anemia (see text box; Hockemeyer et al., 2008; He et al., 2009; Martínez et al., 2009b; Beier et al., 2012). Recently, mutant mice expressing p53 lacking the C-terminal domain, a protein that is not directly involved in telomere maintenance, have been shown to be remarkable models for human telomeropathies, in particular for HHS (see text box; Simeonova et al., 2013).

### Aplastic anemia mouse models

The aplastic anemia phenotypes in mouse (see text box) are provoked either by partially depleting TRF1 in the BM stem

Table 1. Genes known to cause telomeropathies when defective, as well as the telomere-related processes and protein complexes in which they are involved

Process/protein complex	Genes	Telomeropathies
Telomerase core components	<i>TERC</i>	DC, IPF, aplastic anemia, liver disease
	<i>TERT</i>	DC, HHS, IPF, aplastic anemia, liver disease
Telomerase biogenesis	<i>DKC1</i>	DC, HHS, IPF, aplastic anemia
	<i>NOPI0</i>	DC, IPF, aplastic anemia
	<i>NHP2</i>	DC, IPF, aplastic anemia
Telomerase trafficking	<i>TCBA1</i>	DC
Shelterin components	<i>TIN2</i>	DC, HHS
	<i>TPP1</i>	DC, HHS, aplastic anemia
Telomeric DNA synthesis	<i>RTEL1</i>	HHS, IPF
	<i>CTC1</i>	DC
TERC RNA processing	<i>PARN</i>	DC, IPF
	<i>NAF1</i>	IPF

The diseases found associated with the mutated genes are indicated (Calado et al., 2009a; Holohan et al., 2014; Glousker et al., 2015; Stanley and Armanios, 2015; Stanley et al., 2016).

cell and progenitor compartments (hematopoietic stem/progenitor cells [HSPCs]) or by transplanting irradiated wild-type mice with BM from late-generation telomerase-deficient *Tert* knockout mice presenting with short telomeres (Beier et al., 2012; Bär and Blasco, 2016). Partial depletion of the *Trf1* gene in the BM causes a persistent DDR at telomeres that leads to a fast clearance of those HSPCs lacking TRF1. Compensatory hyperproliferation of the remaining HSPCs to regenerate the BM results in a concomitant rapid attrition of telomeres (Beier et al., 2012). The short telomeres in BM of both mouse models faithfully recapitulate human acquired and congenital forms of aplastic anemia characterized by peripheral pancytopenia and marrow hypoplasia.

### PF mouse models

Among the telomere syndromes, IPF is the most common condition associated with telomere dysfunction in humans (see text box; Armanios and Blackburn, 2012). Mutations in telomere genes have been found in up to 25% of familial and 1–3% of sporadic IPF patients (Armanios et al., 2007; Alder et al., 2008; Cogan et al., 2015; Kannengiesser et al., 2015; Stuart et al., 2015; Stanley et al., 2016). Sporadic cases of IPF, not associated with mutations in telomere maintenance genes, also show shorter telomeres compared with age-matched controls, with 10% of the patients showing telomeres as short as those of the telomerase mutation carriers (Alder et al., 2008). In spite of PF and emphysema being the most frequent manifestations of telomere defects in humans, telomerase-deficient mice with critically short telomeres do not spontaneously develop pulmonary disease. Telomere dysfunction in telomerase-deficient mice induces senescence in alveolar progenitor cells and recapitulates the regenerative defects, inflammatory responses, and susceptibility to injury that are characteristic of human telomere-mediated lung disease, although no IPF was reported in these mouse models (Alder et al., 2015a). Exposure to cigarette smoke, which is known to accelerate PF onset in humans, induces emphysema but not PF in telomerase-deficient mice (Alder et al., 2011). These findings suggested that additional damages, or a telomere damage of higher severity, might be required for PF onset in mice. This was demonstrated in work from our laboratory, whereby we generated two independent mouse models that develop PF. In one of them, treatment of late-generation telomerase-deficient mice presenting short telomeres with low doses of bleomycin, which normally does not lead to PF in wild-type mice, results in full-blown progressive PF in telomerase-deficient mice (Povedano et al., 2015). In the second mouse model, severe PF was developed by induction of telomere dysfunction in the absence of telomere shortening specifically in the lungs, by genetically deleting the shelterin component *Trf1* from epithelial type II alveolar cells (Povedano et al., 2015). In both models, we observed activation of the p53/p21 and p19ARF pathways, resulting in cell senescence and apoptosis. Both TERT and TRF1 deficiencies were previously shown to lead to a decreased ability of stem cells to regenerate tissues (Flores et al., 2005; Schneider et al., 2013). Thus, increased lung cell loss associated with severe telomere dysfunction triggers aberrant lung healing by fibroblasts, eventually leading to scar formation and PF. We showed that a DNA-damaged burden above a certain threshold, combined with defective stem cell regeneration ability, lead to the development of PF (Povedano et al., 2015). The TRF1-interacting shelterin protein TIN2 has also been found to be mutated in telomere syn-

dromes (Savage et al., 2008; Alder et al., 2015b; Hoffman et al., 2016). Interestingly, a *TIN2* mutation in its TRF1-interacting domain has recently been identified in a patient with sporadic PF who showed normal telomere length in the peripheral blood compartment, underlining the importance of telomere protection proteins and not only telomere length in human telomere syndromes (Hoffman et al., 2016).

### DC and HHS mouse models

DC and HHS share a common etiology, are typically caused by germline mutations in telomere biology genes, and are characterized by very short telomeres (see text box; Glousker et al., 2015). In mice, double deletion of *Pot1b* and *Terc* gives rise to some DC features such as hyperpigmentation and BM failure (Hockemeyer et al., 2008; He et al., 2009). HHS is considered a clinically severe variant of DC (Glousker et al., 2015). Telomeres in HHS patients are shorter than those of age-matched patients with classic DC (Alter et al., 2012). Although exceedingly short telomeres are considered the main cause of HHS, it is likely that other telomeric or nontelomeric defects also contribute to the pathology of HHS, perhaps distinguishing it from DC (Glousker et al., 2015). Indeed, to our knowledge, the only mouse mutant that faithfully models human HHS does not harbor mutations in any of the known telomere maintenance genes associated with these diseases, but rather a mutation in *TP53* gene (Simeonova et al., 2013). *p53<sup>Δ31/Δ31</sup>* mice, homozygous mutant mice expressing a truncated p53 protein lacking the C-terminal domain, suffer from aplastic anemia and PF as well as other phenotypic traits associated with DC and its severe variant, HHS (Simeonova et al., 2013). Thus, *p53<sup>Δ31/Δ31</sup>* mice are born at the expected Mendelian ratio, but most die 14–43 d after birth because of aplastic anemia and heart failure, develop PF, and present cutaneous hyperpigmentation, nail dystrophy, and oral leukoplakia as in DC. In addition, some mice also present HHS features such as small size, hypogonadism, and cerebellar hypoplasia. The p53<sup>Δ31</sup> protein shows increased p53 activity leading to down-regulation of genes involved in telomere metabolism such as *Dkc1*, *Rtel1*, *Tin2*, and *Trf1*, thereby explaining the appearance of phenotypes related to human telomere syndromes (Simeonova et al., 2013).

### Therapeutic strategies in the treatment of telomere syndromes

Despite the growing understanding of the molecular defects associated with telomeropathies, the only current interventions to treat these diseases involve organ transplantation; i.e., BM, liver, and lung (Holohan et al., 2014; Stanley and Armanios, 2015). Although organ transplantation improves the physical condition of patients, it does not address the underlying cause of the symptoms, which is short telomeres. Patients diagnosed with interstitial lung disease presenting with short telomeres also showed subclinical BM and liver abnormalities, in some cases in the absence of clinical manifestations in peripheral blood count and liver dysfunction (George et al., 2015). These observations clearly favor a telomerase-activating therapy against other alternatives, such as organ transplant for the treatment of telomeropathies, because it also addresses the molecular defects of other affected organs. Indeed, the use of telomerase activators in the treatment of aging-associated conditions has raised commercial interest. Among telomerase chemical activators, TA-65, a small molecule derived from *Astragalus membranaceus* extracts, is the most widely studied. The telomerase activator



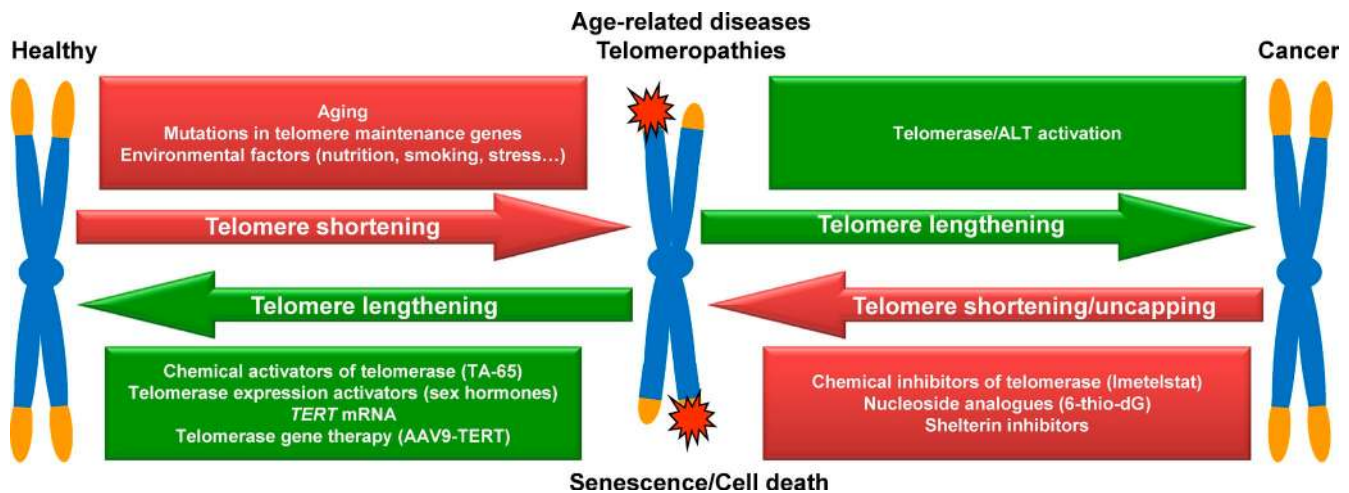


Figure 2. **Natural factors and therapeutic interventions affecting telomere-mediated diseases.** Telomere shortening naturally occurs as a consequence of cell division throughout life, whose pace can be influenced by genetic and environmental factors. Shortened unprotected telomeres elicit a DDR that induces cellular senescence, impacting the regenerative capacity of tissues and giving rise to a whole range of age-associated diseases as well as the so-called telomeropathies, in which tissue degeneration occurs prematurely as a consequence of inherited defects in telomere maintenance. Several therapeutic interventions are being assessed to counteract telomere shortening: among others, chemical activators of telomerase (TA-65), activators of the telomerase reverse transcription (*TERT*) transcription (sex hormones), intracellular administration of *TERT* mRNA, and telomerase gene therapy (AAV9-TERT). Spontaneous mutations that activate telomerase expression or ALT result in telomere lengthening that, if occurring in genetically unstable checkpoint deficient cells, allows them to divide unlimitedly and eventually become cancer cells. Several therapeutic strategies based on chemical induction of telomere dysfunction have been assessed as anticancer therapies. Among others, we highlight the use of a chemical inhibitor of telomerase (imetelstat), a nucleoside analogue (6-thio-dG), and the use of molecules that inhibit shelterin components.

TA-65 has been shown to lead to moderate telomere lengthening and improvement of some aging-related parameters in mice and humans, although no effect on longevity has been observed (Fig. 2; Bernardes de Jesus et al., 2011; Harley et al., 2013). In addition, androgen therapy has been applied as a treatment for aplastic anemia for many years without knowing its mechanism of action (Fig. 2; Shahidi and Diamond, 1961; Jaime-Pérez et al., 2011). It was subsequently reported that sex hormones activate *TERT* transcription (Calado et al., 2009b). Indeed, testosterone therapy in mice suffering from aplastic anemia was shown to up-regulate telomerase expression, rescue telomere attrition, and extend the life span of these mice (Fig. 2; Bär et al., 2015). In humans, administration of a synthetic androgen, danazol, to patients with telomeropathies resulted in telomere elongation in circulating leukocytes in association with hematologic improvement (Townsend et al., 2016). However, endogenous telomerase reactivation strategies are applicable only in those clinical cases not associated with mutations in telomere maintenance genes such as *TERT* or *TERC*. In this regard, therapeutic interventions based on telomerase-based gene therapy are currently being investigated in mouse models for their potential to improve health and extend life span, and as a treatment for short telomere syndromes (Bär and Blasco, 2016).

We have developed a therapeutic strategy by using AAVs to transiently activate telomerase in adult tissues (Fig. 2; Bernardes de Jesus et al., 2012; Bär et al., 2014). Treatment with *Tert* gene therapy using nonintegrative, replication-incompetent AAV9 vectors of adult mice was able to delay aging and increase longevity by decreasing age-related pathologies such as osteoporosis and glucose intolerance, as well as neuromuscular and cognitive decline. Furthermore, the onset of cancer was delayed in the *TERT*-treated mice (Bernardes de Jesus et al., 2012). Interestingly, AAV9-*Tert* delivery specifically to the heart was sufficient to significantly increase mouse survival and heart function upon myocardial infarction. AAV9-*Tert* after in-

farct treatment led to lower fibrotic scarring of the heart and increased cardiac myocyte proliferation concomitant with transcriptional changes suggestive of a regenerative signature (Bär et al., 2014). These findings support the notion that telomere shortening is at the origin of age-related diseases and that, by reverting this process with telomerase expression, it is possible to delay and more effectively treat age-associated pathologies, such as cardiovascular diseases. In addition, *TERT* gene therapy is particularly attractive for the clinical treatment of human telomere syndromes associated with telomerase mutations and short telomeres. Indeed, by using the mouse preclinical model of aplastic anemia provoked by short telomeres in the BM, we demonstrate that AVV9-*Tert* rescues aplastic anemia and mouse survival by inducing telomere lengthening in peripheral blood and BM cells as well as increasing blood counts (Bär et al., 2016). We are currently investigating the feasibility of AVV9-*Tert* therapy in the treatment of PF, with positive results so far (unpublished data). An alternative nonintegrative method to transiently express *TERT* based on *TERT* mRNA delivery into human cells in culture has been developed, although it has not yet been tested in vivo (Fig. 2; Ramunas et al., 2015).

Telomerase activation strategies should be taken with caution given their potential off-target effects and their ability to promote cancer (Bär and Blasco, 2016). Nevertheless, recent data from our group demonstrated that chimeric mice presenting hyperlong telomeres showed neither disadvantageous effects nor enhanced predisposition for cancer development compared with control chimeric mice presenting normal telomere length, indicating that long telomeres do not cause negative consequences to the organism (Varela et al., 2016). Indeed, in chimeric mice with hyperlong telomeres, cells in compartments with high rates of renewal retain longer telomeres and accumulate fewer short telomeres, less DNA damage burden, and lower levels of p53 with age (Varela et al., 2016). Thus, up to date experimental data in preclinical settings provide proof

of concept for the viability of telomerase activation strategies to counteract telomere shortening and its associated consequences. In particular, the use of *TERT* gene therapy constitutes a promising candidate in the prevention and treatment of human telomeropathies mediated by *TERT* mutations and deserves further research efforts for clinical implementation. It should be noted that *TERT* gene therapy in the treatment of telomeropathies mediated by mutations in other genes associated with telomere metabolism (*TERC*, *DKC1*, *NOP10*, *NHP2*, *TCAB1*, *TIN2*, *TPP1*, *RTEL1*, *CTC1*, *PARN*, and *NAF1*) may, however, not be effective.

### Telomeres and malignant transformation

Telomere shortening throughout life is a natural consequence of cell division because of the end replication problem, replication fork collapse, oxidative stress, and nucleolytic processing. Critically shortened telomeres trigger replicative senescence that causes stem cell dysfunction and inflammation, in turn causing aging-associated diseases (Fig. 3 A; Jafri et al., 2016). In the situation of acquired germline mutations in genes coding for factors involved in telomere maintenance and repair, telomere shortening is accelerated, and replicative senescence occurs prematurely, giving rise to telomeropathies (Fig. 3 B; Holohan et al., 2014). In mammalian cells, replicative senescence can be bypassed by acquisition of loss-of-function mutations in tumor suppressor genes that permit further proliferation during a period of time that has been named the extended life span period (Fig. 3 C; Wright and Shay, 1992; Shay and Wright, 2005; Shay, 2016). Proliferation of premalignant cells during the extended life span period results in further telomere shortening. Eventually, cells enter the so-called crisis state, during which short telomeres contribute to genomic instability. Recent work has unraveled how dysfunctional telomeres govern cell fate during senescence, crisis, and transformation in human cells. Replicative senescence is triggered when at least five telomeres become dysfunctional, a critical damage threshold to elicit a DDR characterized by p53 activation (Fig. 3, A and B; Kaul et al., 2011). At that point, named the intermediate telomere state, telomeres are too short to be fully functional and are presumably unable to form the T-loop structure but retain sufficient protective shelterin to inhibit end-to-end fusions (Cesare et al., 2009, 2013; Kaul et al., 2011; Cesare and Karlseder, 2012). Upon loss of p53 and retinoblastoma protein (Rb), cells bypass senescence and continue to grow despite having more than five G1-phase intermediate state telomeres, experiencing further telomere shortening and entering the uncapped state, when they do not retain any protective properties and fuse (Fig. 3 C). A few fused telomeres is sufficient to lead to spindle assembly checkpoint-independent mitotic arrest, during which telomere dysfunction is amplified through Aurora B-dependent removal of TRF2, causing cell death during crisis, a second proliferative barrier (Fig. 3 D; Hayashi et al., 2012, 2015). Cell death in mitosis, also known as mitotic catastrophe (MC), occurs as a consequence of failure to complete mitosis. MC is driven by a complex and poorly understood signaling cascade that antecedes apoptosis, necrosis, senescence, or autophagy (Levine and Kroemer, 2008; Vitale et al., 2011). The molecular mechanisms that underlie MC, especially those that sense mitotic failure to engage the apoptotic, autophagy, or necrotic machinery, remain to be elucidated. It is tempting to speculate that structural and epigenetic changes experienced by multicentric chromosomes could serve as the signal for MC induction. Cell death during crisis thereby

constitutes the second blockade against transformation in cells that bypassed the first barrier of senescence (Fig. 3 D). Reactivation of either telomerase activity or the alternative lengthening of telomeres (ALT) allows these premalignant cells to escape crisis, undergo unlimited divisions (immortalization), and evolve into a fully transformed cancerous state (Fig. 3 E; Pickett and Reddel, 2015; Shay, 2016). Indeed, ~90% of human cancers present telomerase activity (Kim et al., 1994). To date, several mechanisms underlying reactivation of telomerase in cancer cells have been described: notably, mutations in *TERT* promoter, alterations in the alternative splicing of *TERT* pre-mRNA, gene amplification, epigenetic changes, alterations in regulatory factors, and/or disruption of TPE (Jafri et al., 2016). Whether telomerase reactivation is an early or late event during tumorigenesis is still a debated question. Recent data showing that *TERT* promoter mutations are found at early stages of carcinogenesis in most tumor types support the hypothesis that telomerase reactivation occurs at early time points during neoplastic transformation (Kinde et al., 2013; Allory et al., 2014; Hurst et al., 2014; Baerlocher et al., 2015; Chan et al., 2015; Huang et al., 2015; Tefferi et al., 2015; Jafri et al., 2016). However, it is still under debate whether telomerase or ALT reactivation is the driver or simply a facilitator of postcrisis cell growth. It is possible that other molecular events may be required to facilitate the escape from crisis. Studies on telomere dynamics and karyotype analyses in a broad range of human tumor types at early stages underpin telomere crisis as a key event driving genomic instability and clonal evolution during progression to malignancy (Shih et al., 2001; Meeker et al., 2004; Lin et al., 2010; Davoli and de Lange, 2011; Roger et al., 2013). Fused chromosomes in postcrisis cells lead to large-scale genomic rearrangements through breakage-fusion-bridges cycles (B-F-B), aneuploidy, tetraploidization, nonreciprocal translocations, chromothripsis, and kataegis, which promote acquisition of oncogenic mutations and malignant traits required for a fully malignant phenotype (Artandi and DePinho, 2010; Martínez and Blasco, 2010; Davoli and de Lange, 2012; Maciejowski et al., 2015).

### Mouse models for telomere-driven cancer development

In vivo studies with transgenic mouse models of telomere dysfunction in combination with loss-of-function mutations in tumor suppressor genes strongly underscore the statement that telomere dysfunction leads to genomic rearrangements that are permissive for cancer initiation and progression (Table 2; Martínez and Blasco, 2011). Thus, mice doubly deficient for p53 and telomerase, the *Terc*<sup>-/-</sup> *p53*<sup>-/-</sup> mouse model, present a reduced tumor latency compared with single *p53*<sup>-/-</sup> mice, and *Terc*<sup>-/-</sup> *p53*<sup>+/-</sup> mice are prone to develop epithelial cancers typically observed in older humans (Chin et al., 1999). In addition, deficiency in the mismatch repair protein MSH2 abolishes the tumor-suppressor effects of short telomeres and prevents degenerative pathologies in *Terc*<sup>-/-</sup> *Msh2*<sup>-/-</sup> mice (Martínez et al., 2009a). Similarly, and despite the fact that constitutive deletion of either *Tf1* or *Pot1a* results in embryonic lethality, tissue-specific conditional deletion of these genes in combination with p53-null mutation leads to tumorigenesis (Martínez et al., 2009b; Pinzaru et al., 2016). Thus, *Tf1*<sup>lox/lox</sup> *K5-cre* *p53*<sup>-/-</sup> mice lacking TRF1 in stratified epithelia spontaneously develop invasive and genetically unstable squamous cell carcinomas in the tail and ear skin (Martínez et al., 2009b). Depletion of POT1a

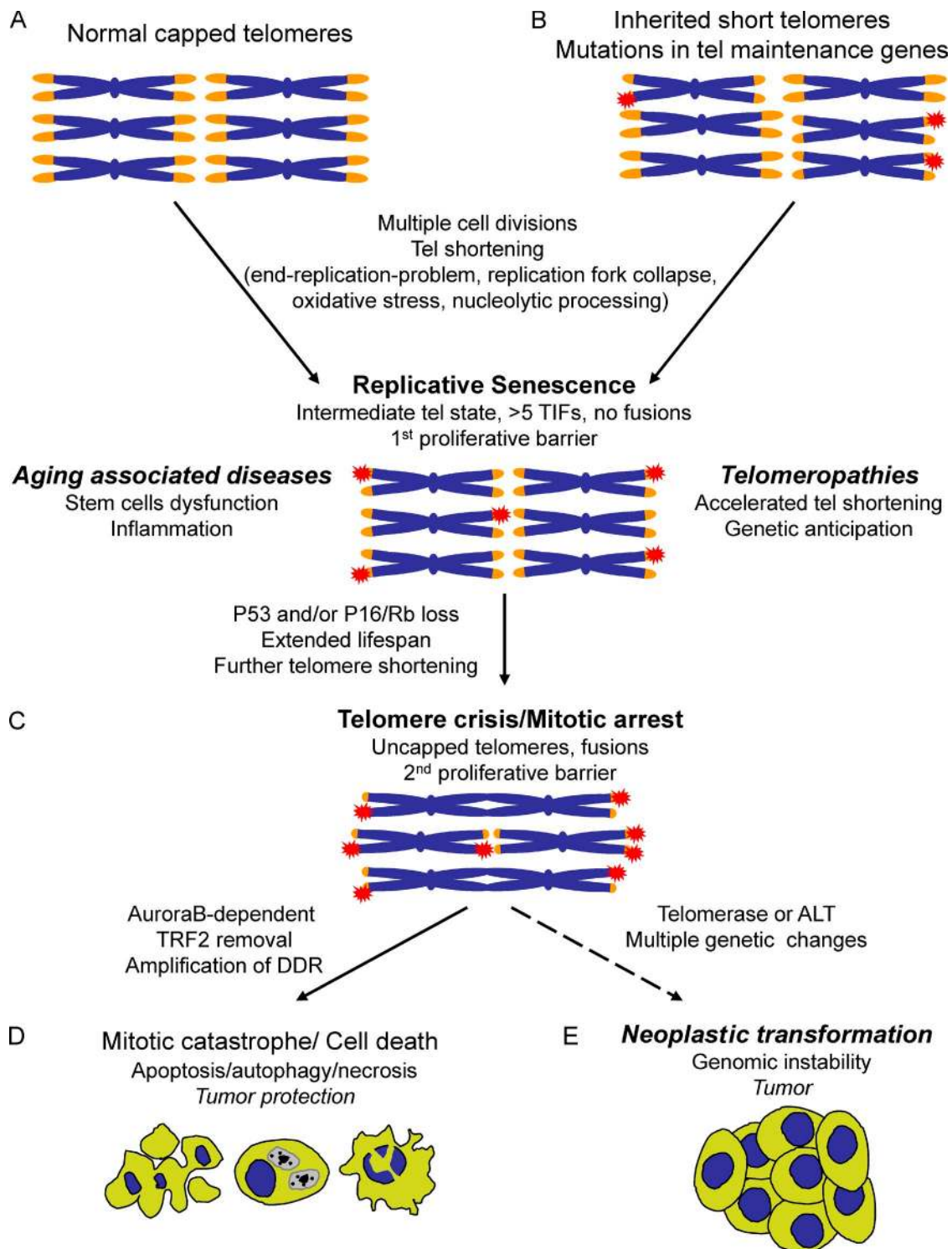


Figure 3. **Impact of telomere shortening on aging-associated diseases, telomeropathies, and cancer.** (A) Young, healthy cells contain long, fully protected telomeres that progressively shorten with increased cell divisions because of the end replication problem, replication fork collapse, nucleolytic processing, and oxidative stress. This progressive telomere shortening eventually leads to some critically short deprotected telomeres that have been termed intermediate-state telomeres. In this intermediate state of deprotection, telomeres retain enough shelterin to inhibit fusions but induce a DDR characterized by formation of the so-called telomere-induced focus (TIF). Replicative senescence is triggered when at least five telomeres become dysfunctional (more than five telomere-induced focuses), a critical damage threshold to elicit a DDR characterized by p53 activation. Telomere attrition in stem cell compartments impairs their tissue and self-renewal capacity and is considered to be one of the primary molecular causes of aging and the onset of aging-associated diseases. (B) Telomeropathies or telomere syndromes develop when telomere attrition occurs prematurely as a consequence of germline mutations in genes coding for factors involved in telomere maintenance and repair. Successive telomere shortening across generations exhibits genetic anticipation, whereby diseases show a progressively earlier age of onset and an aggravation of symptoms. (C) Senescence can be bypassed by acquisition of loss-of-function mutations in p53 and p16/Rb tumor suppressor genes that permit further proliferation during a period of time that has been named the extended life span period, during which cells experience further telomere shortening, eventually entering the uncapped state, when they do not retain any protective properties and



and p53 in common lymphoid progenitor cells, *Pot1a*<sup>lox/lox</sup> *p53*<sup>lox/lox</sup> *hCD2-iCre* mice, induces genetic instability, accelerates the onset, and increases the severity of T cell lymphomas (Table 2; Pinzaru et al., 2016). Similarly, simultaneous inactivation of *Pot1a* and *p53* in endometrial epithelium, *Pot1a*<sup>lox/lox</sup> *p53*<sup>lox/lox</sup> *Spr2f-Cre* mice, results in invasive metastatic endometrial adenocarcinomas presenting nuclear atypia and tetraploid genomes (Akbay et al., 2013). In line with defects in shelterin components as drivers of genome instability, mice harboring a hypomorphic mutation in *TPP1* in a *p53*<sup>-/-</sup> background show increased carcinomas (Else et al., 2009). Interestingly, complete *TPP1* and *p53* abrogation in stratified epithelia, the *Tpp1*<sup>lox/lox</sup> *K5-cre p53*<sup>-/-</sup> mouse model, does not lead to epithelial carcinomas, most likely because *TPP1* is required to recruit telomerase to chromosome ends, mimicking the tumor-resistant phenotype of telomerase-deficient mice (Table 2; Tejera et al., 2010).

It should be noted that similar mutations in a different genetic context give rise to opposing outcomes resulting in cancer phenotype suppression. For example, late generation of mice doubly deficient for telomerase and the tumor suppressor *INK4a/ARF*, *Terc*<sup>-/-</sup> *Ink4a/Arf*<sup>-/-</sup> mice, show a cancer suppression phenotype and increase survival (Khoo et al., 2007). *Trf1*<sup>lox/lox</sup> *p53*<sup>-/-</sup> *K-Ras*<sup>LSLG12V</sup> mice constitute an example of shelterin depletion as a cancer protective mechanism. Thus, simultaneous expression of *K-RAS*<sup>G12V</sup> and *TRF1* depletion upon Cre recombinase expression in the lungs impairs the growth of lung carcinomas and increases mouse survival independently of telomere length (Table 2; García-Beccaria et al., 2015). These studies clearly revealed that the neoplastic outcome of telomere dysfunction depends on the status of the DNA damage surveillance mechanisms, acting oncogenes, as well as on tissue-specific factors.

### Role of shelterins in human cancer development and progression

Mutations in shelterin components have also been found in cancer. Several studies have reported up-regulation of the shelterin complex *TRF1* and *TRF2* in lung, gastric, breast, and renal cancers, suggesting that their overexpression might confer proliferative advantages to tumor cells (Miyachi et al., 2002; Saito et al., 2002; Nakanishi et al., 2003; Diehl et al., 2011; Pal et al., 2015). However, the role of *TRF1* and *TRF2* in cancer development and progression is still unknown. One possible explanation could be that high level of *TRF2* in tumor cells decreases their ability to recruit and activate natural killer cells, thereby contributing to the bypass of innate immunosurveillance (Biroccio et al., 2013). Interestingly, *TRF1* is highly expressed in pluripotent stem cells and adult stem cells (Boué et al., 2010; Varela et al., 2011; Schneider et al., 2013). *TRF1* is essential for both induction and maintenance of pluripotency in a telomere length-independent manner (Schneider et al., 2013). *TRF1* is a direct transcriptional target of the pluripotency factor *OCT3/4*, which binds the *TRF1* promoter to up-regulate *TRF1* expression, providing a mechanistic link between *TRF1* and pluripotency (Schneider et al., 2013). Although *TRF1* is clearly essential

for IPSC generation and the maintenance of pluripotency, the mechanisms by which *TRF1* impacts stemness and carcinogenesis have yet to be elucidated. It is tempting to speculate that *TRF1* favors the proliferative capacity of stem and cancer cells.

Mutations in the shelterin component *POT1* that disrupt proper telomere capping and result in telomere aberrations have been linked to familial melanoma, familial glioma, Li-Fraumeni-like syndrome, mantle cell lymphoma, parathyroid adenoma, and chronic lymphocytic leukemia (Newey et al., 2012; Ramsay et al., 2013; Bainbridge et al., 2014; Robles-Espinoza et al., 2014; Shi et al., 2014; Zhang et al., 2014; Calvete et al., 2015). Importantly, all the identified *POT1* variants conferred a telomere-lengthening effect, suggesting that telomere elongation in combination with the telomere aberrations induced by these mutations favors tumor development. Introduction of *POT1* mutations found in cutaneous T cell lymphoma in human and mouse cells showed that inhibition of *POT1* results in defective telomere replication caused by impaired CST (*CTC1-STN1-TEN1*) function at telomeres and activates ATR-dependent DNA damage signaling (Pinzaru et al., 2016). Attenuation of the ATR kinase pathway allows cancer cells lacking *POT1* to proliferate, suggesting that *POT1*-mediated tumorigenesis may be accompanied by concomitant compensatory mechanisms that down-regulate the ATR-dependent *DDR* (Pinzaru et al., 2016). It is intriguing that specific *POT1* variants give rise to different cancer types. Future work warrants elucidation of the molecular mechanisms that determine the specificity of certain specific disease-associated mutations in shelterin components.

Mutations in *TPP1* and *RAP1* have also been found to be associated with familial melanoma (Aoude et al., 2014). In addition, families carrying *TPP1* and *RAP1* mutations were enriched with other cancer types, suggesting that these variants also cause predisposition to a broader spectrum of tumors than just melanoma. Although the telomeric phenotypes induced by these mutations have not been addressed, the observation that four of the five mutations found in *TPP1* clustered in the *POT1*-binding domain suggests that *POT1* recruitment to telomeres is disrupted, thereby causing similar phenotypes as observed in *POT1* variants discussed earlier (Liu et al., 2004). More intriguing is the finding of *RAP1* variants co-segregating with melanoma because *RAP1* is dispensable for telomere function (Martinez et al., 2010; Sfeir et al., 2010). A possible explanation for the role of *RAP1* in cancer could be either through its role in repressing homology-directed repair at telomeres (Martinez et al., 2010; Sfeir et al., 2010) or through its role in telomere maintenance in the absence of telomerase (Martínez et al., 2016).

### Telomeres as anticancer targets

Given that most tumors (85–90%) present telomerase activity, whereas telomerase is absent in most normal tissues, or is strictly regulated in transient-amplifying stem cells, inhibition of telomerase is an attractive target for cancer therapy (Kim et al., 1994; Shay, 2016). Different approaches have been designed in the search for telomerase inhibitors: small-molecule inhibitors, antisense oligonucleotides, G-quadruplex stabilizers, immu-

fuse. (D) Fused telomeres lead to a mitotic arrest checkpoint during which telomere dysfunction is amplified by Aurora B-dependent *TRF2* removal, causing cell death through apoptosis, necrosis, or autophagy in crisis, a second proliferative barrier that protects against tumor development. (E) Reactivation of either telomerase activity or ALT in some very rare crisis cells allows these premalignant cells to escape crisis and divide unlimitedly (immortalization). Fused chromosomes in postcrisis cells lead to large-scale genomic rearrangements that promote acquisition of oncogenic mutations and malignant traits required for a fully malignant phenotype, cancer.



notherapy, gene therapy using telomerase promoter-driven expression of a suicide gene, and chemicals that block telomerase biogenesis (Harley, 2008; Agrawal et al., 2012; Buseman et al., 2012; Jafri et al., 2016). Among all antitelomerase compounds developed, the oligonucleotide imetelstat (GRN163L) appears to be the most promising telomerase inhibitor and the one most extensively evaluated in clinical trials. Imetelstat sequence binds to a complementary oligonucleotide region of hTR at the active site of telomerase holoenzyme, leading to complete inhibition of enzyme activity (Fig. 2; Jafri et al., 2016). However, inhibition of telomere maintenance by targeting telomerase in cancer has showed effectiveness in only some myeloid tumors but has largely failed in solid tumors (Baerlocher et al., 2015; Tefferi et al., 2015; Jafri et al., 2016). The reasons underlying the lack of success are likely that therapeutic strategies based on telomerase inhibition to treat cancer will be effective only when telomeres shorten below a minimum length, implying a long lag period before cell death. Indeed, telomerase activity is dispensable for transformation of cells with long telomeres (Seger et al., 2002), and studies with telomerase inhibitors indicate that they are effective preferentially in cells with short telomeres (Wang et al., 2004; Buseman et al., 2012). In line with this idea, telomerase abrogation in the context of cancer-prone mouse models, including the *K-Ras<sup>G12D</sup>* lung tumorigenesis mouse model, showed antitumorigenic activity only after several mouse generations in the absence of telomerase when telomeres reached a critically short length (Chin et al., 1999; Greenberg et al., 1999; González-Suárez et al., 2000; Perera et al., 2008). Moreover, these antitumorigenic effects of short telomeres owing to telomerase deficiency are abrogated in the absence of p53 (Chin et al., 1999).

Alternative therapeutic approaches that target telomeres in a telomere length-independent manner are starting to emerge. In contrast to telomerase inhibition, telomere uncapping has been shown to cause rapid induction of cell death and/or senescence in a manner that is independent of telomerase activity and telomere length (Karlseder et al., 1999; Martínez et al., 2009b). Thus, given that telomere dysfunction can be achieved independently of telomere length, telomere uncapping strategies emerge as a more universal way to rapidly impair the growth of dividing cells. One of these alternative approaches consists of a telomere-targeted telomerase-dependent potential anticancer therapy that uses a nucleoside analogue, 6-thio-2'-deoxyguanosine (6-thio-dG). 6-Thio-dG is recognized by telomerase and incorporated into de novo synthesized telomeres, leading to telomere dysfunction solely in telomerase-expressing cells (Fig. 2). Treatment with 6-thio-dG leads to rapid cell death in

most of the cancer cell lines assayed and to telomere dysfunction in in vivo xenograft models (Mender et al., 2015). Targeting telomerase recruitment to telomeres has also been proposed as a potential anticancer treatment (Nakanishi et al., 2003).

We have recently developed a novel telomerase-independent and telomere length-independent strategy to induce telomere dysfunction by targeting TRF1 (Fig. 2; García-Beccaria et al., 2015). Genetic *Trf1* deletion impaired the growth of p53-null *KRas(G12V)*-induced lung carcinomas and increased mouse survival independently of telomere length. Chemical inhibition of TRF1 binding to telomeres by small molecules blocked the growth of already established lung carcinomas by inducing a rapid DDR and without affecting mouse survival or tissue function, supporting induction of acute telomere uncapping as a promising therapeutic target for lung cancer and very likely for many other cancer types (García-Beccaria et al., 2015). Chemical inhibition of TRF2 dimerization has also been achieved by targeted peptide synthesis that directly binds to the TRFH dimerization domain of TRF2 (Di Maro et al., 2014). Treatment of HeLa cells with TRF2-binding chemotypes did not affect TRF2 telomeric localization but induced rapid DDR activation, end-to-end fusions, and cell death indicative of telomere dysfunction (Di Maro et al., 2014). Although further clinical research is needed to ultimately address the efficacy of telomere-uncapping chemical inhibition in human cancer, these strategies provide novel opportunities for the development of anticancer agents.

#### Outstanding questions in telomere-related diseases and cancer

Although telomere biology has been extensively studied, our knowledge of the implications of telomeres and telomere proteins in human disease has considerably improved in recent years. Current research efforts focus on how our knowledge of telomere biology and its connection with human disease can be translated into the clinic to improve human health. Although some telomere-based therapies have already reached clinical trials, the path is still long. The fact that telomere dysfunction may have different and even opposing outcomes in aging and cancer makes the goal of developing telomere-based anti-aging and anticancer therapies a delicate challenge that requires a highly controllable technology. Results obtained in mouse models with telomerase gene therapy as a treatment for aging-associated diseases and telomere syndromes, as well as shelterin chemical inhibition as anticancer therapy treatment, constitute promising opportunities for further clinical development. However, additional studies are needed to determine the

Table 2. Examples of mouse models underscoring telomere dysfunction as either a driving force in cancer development or as a cancer suppressor

Genotype	Cancer phenotype	Reference
<i>Terc</i> <sup>-/-</sup> <i>p53</i> <sup>-/-</sup>	Earlier onset lymphoma/sarcoma, epithelial carcinoma	Chin et al., 1999
<i>Terc</i> <sup>-/-</sup> <i>Msh2</i> <sup>-/-</sup>	Lymphoma/carcinoma	Martínez et al., 2009a
<i>Trf1</i> <sup>lox/lox</sup> <i>K5-cre</i> <i>p53</i> <sup>-/-</sup>	Squamous cell carcinoma	Martínez et al., 2009b
<i>Pot1</i> <sup>lox/lox</sup> <i>p53</i> <sup>lox/lox</sup> <i>hCD2-iCre</i>	T cell lymphomas	Pinzaru et al., 2016
<i>Pot1</i> <sup>lox/lox</sup> <i>p53</i> <sup>lox/lox</sup> <i>Sprr2f-Cre</i>	Endometrial adenocarcinomas	Akbay et al., 2013
<i>Tpp1</i> <sup>Acid</sup> <i>p53</i> <sup>-/-</sup>	Carcinomas	Else et al., 2009
<i>Tpp1</i> <sup>lox/lox</sup> <i>K5-cre</i> <i>p53</i> <sup>-/-</sup>	Cancer suppressor	Tejera et al., 2010
<i>Terc</i> <sup>-/-</sup> <i>Ink4a/Arf</i> <sup>-/-</sup>	Delayed lymphoma/fibrosarcoma onset	Khoo et al., 2007
<i>Trf1</i> <sup>lox/lox</sup> <i>p53</i> <sup>-/-</sup> <i>K-Ras</i> <sup>LSIG12V</sup>	Growth impairment of lung carcinoma	García-Beccaria et al., 2015

The effects of telomere dysfunction in cancer-prone mouse models depend on the status of DNA damage surveillance mechanisms, acting oncogenes, and tissue-specific factors.

undesired long-term effects associated with exogenous reexpression of telomerase and shelterin inhibition.

The molecular mechanisms underlying the specificity of certain specific disease-associated mutations/variants in shelterins are yet an open field of research. For example, specific POT1 variants give rise to chronic lymphocytic leukemia, whereas other amino acid substitutions predispose to familial melanoma, cardiac angiosarcomas, or gliomas. Several shelterin mutations associated with different tumor types have recently been found, and presumably many more shelterin mutations are yet to be identified in human cancer. These observations further support the use of shelterins as cancer targets and raise the question of the potential translational applications of shelterin gene therapy as preventive treatment in mutation carriers.

In summary, the works discussed in this review provide proof of concept for the feasibility of telomere-targeted therapeutic approaches for the prevention and treatment of telomere dysfunction-mediated diseases in animal models and pave the way for their development and implementation in the clinic.

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