Chapter 7
Telomeres Shortening: A Mere Replicometer?

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Abstract Telomeres are protective structures at the ends of linear chromosomes that play an important role in maintaining genomic stability. Telomere shortening, which occurs with each round of cell division, leads to a permanent proliferation arrest, also known as replicative senescence. This process has been shown to have important implications in vivo as an increase in the frequency of senescent cells occurs in mammalian tissue with age and in a variety of age-related diseases. Telomeres possess unique features, namely the presence of telomere-binding proteins collectively known as "shelterin", that equally prevent telomere end-to-end fusions and the repair of damage induced by extrinsic and intrinsic stress. This inability to repair damage contributes to the activation of a persistent DNA damage response, which has been shown to be important in the establishment of cellular senescence. Evidence suggests that telomeres not only limit the proliferative potential of cells, but also act as highly sensitive sensors of stress in cells. This may be a protective mechanism against cancer, but also contribute to tissue dysfunction observed during ageing.

Keywords Replicative senescence • Telomerase • DNA damage • Repair • Mortality • Immortality • Ageing • Anti-ageing • Longevity

7.1 Telomeres and Senescence

7.1.1 Cellular Senescence

The irreversible proliferation arrest of somatic cells, also known as cellular senescence, was first described by Leonard Hayflick in 1961, who showed that cells in vitro were only capable of undergoing a limited number of population doublings, after which they entered a state of terminal arrest (Hayflick and Moorhead 1961).

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This was later suggested to be related to the shortening of telomeres, protective caps present at the ends of linear chromosomes that decrease in length with each cell division, inducing cells to become senescent upon reaching a critical length (Hastie et al. 1990; Harley 1991; Counter et al. 1992; Chang and Harley 1995). Maintenance of telomeres is vital for genomic stability and thus cell viability; therefore, it is not surprising that cells have evolved mechanisms to ensure that these structures remain well preserved. For example, telomere length regulation can be achieved by telomerase, an enzyme that contains reverse transcriptase activity and can elongate the ends of chromosomes with the aid of an RNA template (Blackburn et al. 1989). However, this enzyme is only present in certain cell types such as those of the germline and stem cells, where genomic integrity is presumably most important (Collins and Mitchell 2002). Cells lacking telomerase activity, such as the majority of somatic cells, are subject to telomere attrition with each cell division due to the so-called "end-replication problem", which occurs due to the inability of conventional DNA polymerases to completely replicate the lagging-strand, and was first predicted to occur by Olovnikov and Watson in the early 1970s (Olovnikov 1971; Watson 1972). During the process of lagging-strand synthesis, the DNA replication machinery is required to insert RNA primers from which polymerases can initiate replication, which means that upon removal of the last primer at the 3′ end, the newly synthesised strand is shorter than the template by a few nucleotides. Moreover, the final primer required for replication is not necessarily inserted at the very 3′ end of the parent DNA, further contributing to the loss of telomeric sequences with each round of replication. Indeed, later studies demonstrated experimentally that telomere shortening occurs in human fibroblasts with age, suggesting that attrition of chromosome ends limits the replicative lifespan of proliferating cells (Harley et al. 1990; Hastie et al. 1990; Counter et al. 1992).

The discovery of cellular senescence prompted scientists to question whether this phenomenon was merely a cell culture artefact or did this permanent arrest really have a role in vivo? Indeed, since the Hayflick's discovery, a multitude of reports have shown an age-dependent increase in the frequency of senescent cells in a variety of mammalian tissues, suggesting that senescence may play a role in organismal ageing (van Deursen 2014). More recently, evidence suggests that accumulation of senescent cells with age may be a major contributing factor to the loss of tissue regeneration and function during the ageing process. This was elegantly demonstrated by the Jan van Deursen group who showed that inducible elimination of p16Ink4a-positive senescent cells was able to delay the acquisition of age-related pathologies in the BubR1 progeroid mouse (Baker et al. 2011). By eliminating senescent cells from these mice, the authors were able to not only delay age-related degeneration but also to slow down the progression of already established conditions, suggesting a causal role for cellular senescence in age-associated tissue dysfunction (Baker et al. 2011). Accumulation of senescent cells has been associated with many age-related pathologies such as type 2 diabetes (Sone and Kagawa 2005), atherosclerosis and osteoarthritis (van Deursen 2014).

Consistent with a role for telomeres in the ageing process, telomere shortening is observed in telomerase knock-out mice in a generation-dependent manner (Blasco

et al. 1997), which results in both cell senescence and apoptosis (Lee et al. 1998). These mice not only display a bone phenotype that resembles age-related osteoporosis (Brennan et al. 2014), but accelerated telomere shortening in this model also has been shown to compromise stem cell function, regeneration, organ homeostasis, and decrease lifespan (Wong et al. 2003).

7.1.2 What Are Telomeres?

Telomeres are tandem TTAGGG repeats present at the ends of linear chromosomes, ranging from 5 to 15 kb in humans and can reach up to 100 kb in rodents (O'Sullivan and Karlseder 2010). They contain a C-rich lagging strand and a G-rich leading strand, where the latter comprises 3′ single-stranded nucleotide repeats giving rise to the G-overhang (McElligott and Wellinger 1997; O'Sullivan and Karlseder 2010). Early studies by Griffith et al. suggest that this 3′ -overhang is sequestered into the upstream double-stranded telomeric region and binds to one of the DNA strands, forming a structure known as the telomere-loop (T-loop), which is thought to contribute to chromosome stability by preventing the ends of chromosomes from being recognised as double-strand breaks (DSB) (Griffith et al. 1999; Lin et al. 2014). Telomeres are also associated with a specialised set of proteins termed the shelterin complex, which play extensive roles in controlling molecular signalling from the ends of chromosomes (O'Sullivan and Karlseder 2010). In mammals, this complex is comprised of six main proteins: telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF2 interacting protein (RAP1), TRF1-interacting nuclear factor 2 (TIN2), adrenocortical dysplasia protein homolog (TPP1, also known as ACD) and protection of telomeres 1 (POT1) (O'Sullivan and Karlseder 2010; Sfeir 2012). TRF1 and TRF2 bind to double-stranded telomeric sequences, whereas the single-stranded G-overhang is coated by POT1 (Zhong et al. 1992; Bianchi et al. 1997; Bilaud et al. 1997; Baumann and Cech 2001) (Fig. 7.1).

Each of these proteins plays essential functions that help maintain telomere homeostasis within the cell. For example, studies have shown that overexpression of TRF1 or TRF2 leads to telomere shortening, thus implicating these shelterin components as negative regulators of telomere length (van Steensel and de Lange 1997; Smogorzewska et al. 2000). Moreover, TRF1 has also been shown to play roles in DNA remodelling as well as being important for efficient replication of telomeres (Bianchi et al. 1997; Griffith et al. 1998; Sfeir et al. 2009). TRF2, on the other hand, is important in telomere protection as it is involved in T-loop assembly by promoting topological changes in DNA that facilitate the formation of such structure (Stansel et al. 2001; Amiard et al. 2007). This protein also inhibits ATM-dependent DNA damage response (DDR) and DNA ligase IV-dependent non-homologous end joining (NHEJ), thus playing a critical role in preventing chromosome end-to-end fusions and chromosomal instability (van Steensel et al. 1998; Smogorzewska et al. 2002; Karlseder et al. 2004; Denchi and de Lange 2007).

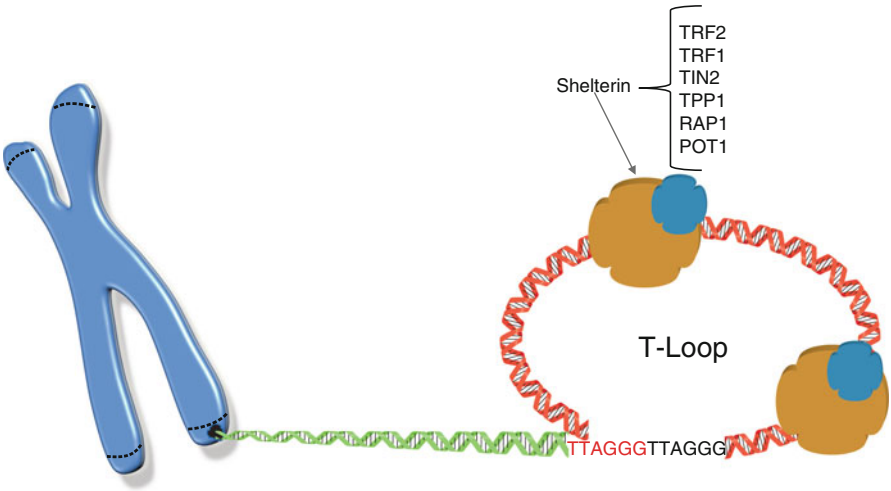

Fig. 7.1 Structure of telomeres. Telomeres are tandem TTAGGG repeats found at the ends of linear chromosomes. The 3′ end is guanidine-rich and consists of single-stranded nucleotide repeats, forming the 3′- or G-overhang, which is thought to contribute to T-loop formation by binding to upstream double-stranded telomeric regions. Telomeres are also associated with a specialized set of proteins called the shelterin complex, which are important for maintaining telomere structure and function. In mammals, this complex is composed of six main proteins, namely TRF1 and TRF2, components that bind to double-stranded telomeric regions, POT1, which coats the single-stranded G-overhang, TIN2, TPP1, and RAP1

In contrast to its homologue in *S. cerevisiae*, mammalian RAP1 lacks the ability to bind to telomeric repeats and relies on interactions with TRF2 for its localisation to telomeres (Li et al. 2000). It has been suggested that TRF2 inhibits NHEJ partly by recruiting RAP1, and the latter has been directly implicated in protecting telomeres from this DNA damage repair mechanism (Bae and Baumann 2007; Sarthy et al. 2009). POT1 contributes to genomic stability by inhibiting ATR-dependent DDR signalling, and it has also been shown to be an important regulator of telomere length through interactions with TRF1 (Loayza and De Lange 2003; Denchi and de Lange 2007). Studies have demonstrated that telomere length is also controlled by TPP1, which recruits telomerase to chromosome ends by directly interacting with this enzyme responsible for telomere elongation (Xin et al. 2007). Moreover, TPP1 directly interacts with POT1, increasing the affinity of the latter for single-stranded telomeric sequences, and it has been shown that a TPP1-POT1 complex increases the processivity of telomerase during the elongation process (Wang et al. 2007; Lu et al. 2013). TIN2 plays a crucial role in the assembly of the shelterin complex by bridging TRF1 and TRF2; furthermore, TPP1 was shown to stabilise this interaction and contribute to the formation of the six-protein complex that is essential for telomere protection (O'Connor et al. 2006). Additionally, TIN2 was demonstrated to be essential in stabilising TPP1/POT1 complexes on single-stranded telomeric

DNA, thus contributing to the effective inhibition of ATR signalling at telomeres (Takai et al. 2011).

These six core components of the shelterin complex also interact with other proteins to form a higher order nucleoprotein complex (Blackburn 2001). For example, protein-protein interactions have been shown to occur between TRF1 and Ku, a protein that plays a role in NHEJ (Hsu et al. 2000). However, the authors showed that at telomeres, Ku performs a capping function, since Ku80-deficient primary mouse embryonic fibroblasts (MEFs) accumulated large amounts of telomere fusions (Hsu et al. 2000). Moreover, recent studies involving genome-wide YFP fluorescence complementation screens have revealed that shelterin proteins associate with over 300 proteins with functions ranging from chromatin organisation to apoptosis and stress response sensors, reiterating the complexity underlying telomere maintenance and signalling (Lee et al. 2011).

7.1.3 The Role of Dysfunctional Telomeres in Replicative Senescence

The idea that telomere shortening is a major driver of cellular senescence was corroborated by studies demonstrating that the lifespan of normal human cells could be extended by expression of telomerase in otherwise telomerase-negative cells (Bodnar et al. 1998). These cells were not only able to undergo a higher number of population doublings but also had reduced β-galactosidase activity, a marker of senescence (Bodnar et al. 1998).

It is believed that telomere attrition may lead to loss of shelterin components, consequently compromising T-loop conformation such that the ends of chromosomes become exposed and are recognised as double-strand breaks by the DNA repair machinery (O'Sullivan and Karlseder 2010). Initial recognition of DNA damage is mediated by factors such as replication factor A (RPA) and replication factor C (RFC)-like complexes, and also by the MRN complex (MRE-RAD50-NBS1). Subsequently, upstream kinases such as ATM in the case of double-strand breaks, become activated, and trigger chromatin modifications in the vicinity of the break, namely the phosphorylation of histone H2A variant H2AX (γH2A.X being the phosphorylated form). This allows the recruitment of other proteins that may function to amplify the signal or are involved in damage repair. Adaptor proteins such as 53BP1, BRCA1, MDC1 and claspin are also involved in this response and are responsible for recruiting the downstream kinases CHK1 and CHK2, which transduce the signal to effector molecules such as p53, inducing a G1 and G2-M phase cell-cycle arrest (Campisi and d'Adda di Fagagna 2007; O'Sullivan and Karlseder 2010). Data supporting the idea that uncapping of telomeres leads to the activation of a DNA damage response (DDR) came from studies where a dominant-negative TRF2 allele was expressed in human fibroblasts, preventing the accumulation of endogenous TRF2 on chromosome ends and thus triggering

uncapping . It was shown that telomeres from these cells became associated with DNA damage response factors such as 53BP1, Mre11 complex, and phosphorylated forms of ATM, H2AX and Rad17, indicating that uncapped telomeres resemble DNA lesions (Takai et al. 2003). Moreover, treatment with PI3 kinase inhibitors that affect ATM was able to reduce the accumulation of 53BP1 at telomeres, showing that DDR proteins play a major role in the cellular response to telomere dysfunction (Takai et al. 2003). Another group showed that conditional deletion of Pot1a in mice caused telomere deprotection and consequently activation of a DDR at telomeres (Wu et al. 2006). This was also accompanied by a senescent phenotype in these cells comparable to replicative senescence and by the occurrence of aberrant homologous recombination (HR) at telomeres, supporting the idea that telomere uncapping may lead to genomic instability and acts through a DDR to induce a permanent cell-cycle arrest (Wu et al. 2006). In fact, during replicative senescence, it has been shown that human fibroblasts accumulate markers of double-strand breaks such as γH2A.X and a range of DDR proteins, including 53BP1, MC1 and NBS1 at telomeric regions (d'Adda di Fagagna et al. 2003).

Ultimately, telomere dysfunction leads to activation of the effector molecule p53, which may trigger cell-cycle arrest or apoptosis (Karlseder et al. 1999; Herbig et al. 2004; Campisi and d'Adda di Fagagna 2007). The transcription factor p53 is a positive regulator of p21 expression, a cyclin-dependent kinase (CDK) inhibitor that initiates growth arrest by inhibiting the action of kinases important for cell cycle progression (Campisi and d'Adda di Fagagna 2007). The importance of the p53 pathway during telomere-induced senescence has been reinforced by studies where deletion of p21 improved the regenerative capacity of intestinal crypts and hematopoietic stem cells in late-generation telomerase-deficient mice, which contain dysfunctional telomeres (Choudhury et al. 2007). Moreover, deletion of Chk2, a protein which has been shown to stabilise p53, in mice with Trf1 deficiency was shown to rescue survival and the frequency and severity of epithelial pathologies associated with telomere dysfunction (Garcia-Beccaria et al. 2014). A rescue in proliferation in the skin associated with a decrease in p53 and p21 induction was also observed (Garcia-Beccaria et al. 2014). Although the p16-pRb pathway is also important in establishing and maintaining senescence, studies have provided conflicting data on its involvement in telomere-induced senescence. It has been shown that p16 can be activated independently of telomere dysfunction in human fibroblasts (Herbig et al. 2004). However, another study demonstrated that deletion of p16 in Wrn-deficient mice, which lack a protein needed for efficient telomere replication and thus have dysfunctional telomeres, rescued proliferation of mouse embryonic fibroblasts (MEFs) when compared to Wrn-deficient MEFs (Zhang et al. 2012). Moreover, loss of p16 function either alone or in Wrn-deficient MEFs prevented these cells from undergoing senescence in response to telomere damage, implicating p16 as an important contributor to growth arrest in the presence of telomere dysfunction (Zhang et al. 2012). Induction of p16 in response to telomere damage has also been observed upon expression of a dominant negative allele of TRF2 (Jacobs and de Lange 2004). Although p16 deficiency in these cells only partially rescued growth arrest induced by telomere damage, proliferation

was almost completely restored when both p16 and p53 were inhibited, suggesting that p16 may act as a second barrier to inhibit entry into the next cell cycle following damage in chromosome ends (Jacobs and de Lange 2004). In accordance to this, another study showed that growth arrest induced by telomere dysfunction is primarily maintained by p53 and can be rescued by inhibition of this transcription factor (Beausejour et al. 2003). However, p16 was shown to provide a second barrier to cell cycle progression in some human cells, which could not be reversed even upon pRB inactivation (Beausejour et al. 2003). These studies reinforce the importance of the p53 and p16 pathways in senescence and also allude to the fact that the extent of the contribution of each pathway to the senescent phenotype may vary amongst different cell types.

Indeed, several studies have indicated that telomere-induced senescence may play an important role during organismal ageing. Telomere dysfunction, measured by the association of DDR proteins with telomeres, has been show to increase with age in vivo in the skin of primates (Herbig et al. 2006; Jeyapalan et al. 2007) and in liver and gut of mice (Hewitt et al. 2012). Moreover, it has been shown that reintroducing telomerase activity in telomerase-deficient mice was able to revert premature ageing phenotypes in tissues such as spleen, intestine and testes, suggesting that telomere attrition is an important factor affecting decline of organ function with age (Jaskelioff et al. 2011). Telomere dysfunction is also related to human diseases such as dyskeratosis congenita (DC). This condition is associated with mutations in the genes encoding telomerase components, and fibroblasts and keratinocytes derived from DC patients have very short telomeres (Buckingham and Klingelhutz 2011). DC is accompanied by a range of epidermal defects such as poor nail growth, early hair loss and skin atrophy, suggesting that telomere length maintenance is essential for epidermal homeostasis (Buckingham and Klingelhutz 2011).

7.1.4 Stress and Telomere Shortening

Although telomere attrition can occur as a result of cellular replication, other factors can influence the rate at which telomeres shorten. Indeed, many studies have shown that mild oxidative stress causes telomere shortening and reduces the replicative lifespan of cells, resulting in a phenotype similar to replicative senescence (von Zglinicki et al. 1995; Xu et al. 2000; von Zglinicki 2002; Saretzki et al. 2003). The role of reactive oxygen species (ROS) in telomere-dependent senescence has been reinforced by studies where human fibroblasts overexpressing the antioxidant enzyme superoxide dismutase (SOD3) or treated with antioxidants showed reduced levels of intracellular peroxide, a slower rate of telomere shortening and prolonged replicative lifespan (von Zglinicki et al. 2000; Serra et al. 2003). In fact, a direct relationship between telomere shortening and the levels of cellular oxidative stress has been previously proposed following observations that cells with a low antioxidant capacity and shorter lifespan displayed a higher rate of

telomere shortening than strains with a higher antioxidant capacity (Richter and von Zglinicki 2007). The involvement of ROS in telomere dysfunction is not only limited to in vitro studies. Indeed, oxidative stress has been suggested to mediate demyelination and axonal damage in multiple sclerosis (MS) patients (Gilgun-Sherki et al. 2004), who have been recently reported to have increased markers of oxidative stress and shorter telomeres when compared to healthy controls (Guan et al. 2015). Although it is difficult to delineate the major source of ROS responsible for telomere attrition, studies have suggested that oxidative stress originating from mitochondria is an important contributor to telomere shortening. For example, when fibroblasts were treated with MitoQ, an antioxidant which is targeted directly to the mitochondria, the rate of telomere shortening was significantly reduced, and this was also sufficient to extend replicative lifespan under conditions of hyperoxia (Saretzki et al. 2003). The importance of mitochondrial-derived ROS has also been reiterated by studies showing that mild uncoupling of mitochondria, which reduces the accumulation of superoxide in the latter, extended the lifespan of human fibroblasts and also decreased the rate of telomere shortening in these cells (Passos et al. 2007). Moreover, inducing mitochondrial dysfunction by severe depolarization using FCCP was shown to increase ROS production and lead to telomere attrition, telomere loss and chromosome fusions in mouse embryos (Liu et al. 2002). The suggestion that mitochondrial dysfunction can accelerate telomere shortening is further supported by studies showing that patients with mitochondrial diseases such as MELAS and LHON, which are characterised by respiratory chain dysfunction, contain on average shorter telomeres in white blood cells when compared to healthy subjects (Oexle and Zwirner 1997). Importantly, increased ROS production as a result of mitochondrial dysfunction has been reported in many age-related disorders such as Alzheimer's and cardiovascular disease (Dai et al. 2014), both of which have also been associated with telomere shortening (Fyhrquist and Saijonmaa 2012; Cai et al. 2013).

Telomeres are thought to be particularly susceptible to oxidative stress relative to the rest of the genome due to their high content of guanine triplets, which are highly sensitive to oxidative modifications (Oikawa and Kawanishi 1999). In fact, mild oxidative stress has been shown to cause single-stranded breaks to preferentially accumulate at telomeres, and it has been suggested that such lesions contribute to accelerated telomere shortening by stalling the replication fork, leading to incomplete replication of chromosome ends (Petersen et al. 1998; von Zglinicki 2000) (Fig. 7.2). Further studies have also shown that oxidative damage at telomeres disrupts the binding of TRF1 and TRF2, possibly offering another mechanism by which oxidative stress may contribute to telomere dysfunction (Opresko et al. 2005). However, a more recent report has provided evidence that stress-induced telomere damage can still occur in the presence of TRF2, suggesting that telomere dysfunction may also occur via alternative mechanisms other than loss of shelterin components (Fumagalli et al. 2012). In addition, telomeric damage has been shown to be less efficiently repaired when compared to non-telomeric lesions, a characteristic mainly owed to the presence of shelterin proteins (Kruk et al. 1995). Components such as TRF2 and RAP1 have been shown to prevent

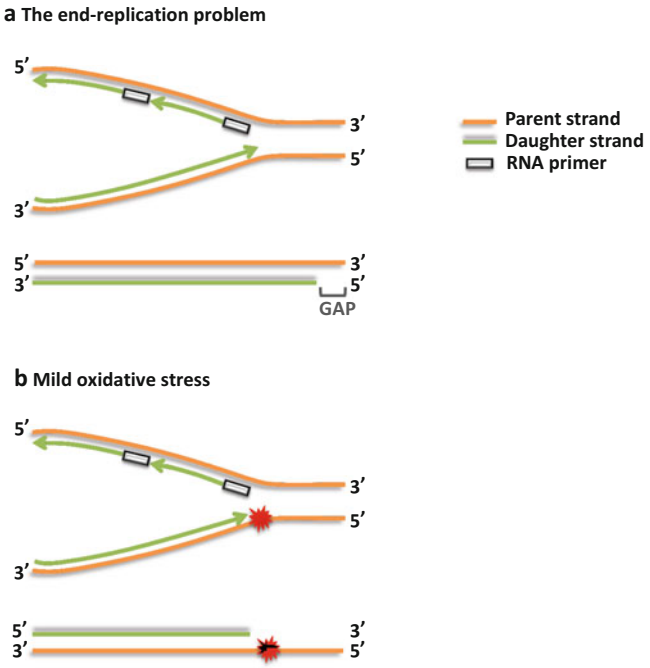

Fig. 7.2 Mechanisms that contribute to telomere shortening. **a** The end replication problem. Replication of the lagging-strand (*top strand*) occurs in short fragments, known as Okazaki fragments, each starting from a different RNA primer (shown in *grey*). When DNA replication is complete, the primers are removed, and the gaps between Okazaki fragments are filled; however, the gap left from the removal of the primer at the most distal position cannot be filled, leading to shortening of the newly-synthesised strand (*bottom*). **b** Mild oxidative stress. Oxidative damage has been shown to preferentially accumulate at telomeres. It is suggested that damage occurring in telomeric regions (*bottom*) causes stalling of the DNA polymerase on that strand, whilst not necessarily affecting replication of the opposite strand. This may cause the replication fork to collapse before replication is fully completed, resulting in a shorter daughter strand

NHEJ at telomeres by inhibiting DNA-PK, a double-stranded break repair complex protein, and also by preventing ligase-IV-mediated end joining (Bombarde et al. 2010). In accordance to this, inducing DNA double-stranded breaks adjacent to telomeric repeats in budding yeast results in impaired recruitment of ligase IV to the site of damage (Fumagalli et al. 2012). Moreover, expression of TRF2 in the vicinity of a DSB in mammalian cells leads to persistent DNA damage, reinforcing the irreparability of telomeres (Fumagalli et al. 2012). ROS are not only involved in the induction of senescence via telomere dysfunction, but also are believed to act as an effector mechanism during senescence. Our group has shown that mitochondrial ROS can be induced downstream of a DNA damage response and is partially dependent on the p53 and p21 pathways (Passos et al. 2010). Consistently, mice with dysfunctional telomeres (late generation $TERC^{-/-}$ mice) show increased markers of oxidative damage in tissues which can be significantly decreased by deletion of p21

(Passos et al. 2010). Moreover, a separate study has shown that short telomeres activate p53 which in turn binds and represses PGC-1α and PGC-1β promoters inducing mitochondrial dysfunction (Sahin et al. 2011). Evidence suggests that ROS emanating from dysfunctional mitochondria may contribute to the stabilisation of the senescent phenotype by further increasing DNA damage and maintaining the DDR (Passos et al. 2010).

Recently, studies have pointed towards an association between psychological stress and reduced telomere length. It was shown that both chronic stress, such as that experienced by mothers caring for critically ill children, and perceived stress were linked to shorter telomeres, decreased telomerase activity and increased oxidative stress in leukocytes of healthy women (Epel et al. 2004). A relationship between chronic psychological stress and reduced telomere length has also been observed in caregivers of Alzheimer's disease patients who also displayed higher levels of depressive symptoms when compared to controls (Damjanovic et al. 2007). Furthermore, many reports have now suggested that early life stresses may have an impact on the rate of telomere shortening. Indeed, it has been shown that adults with a history of childhood maltreatment, including physical and emotional neglect, had shorter telomeres in leukocytes when compared to participants who did not report maltreatment (Tyrka et al. 2010). Further support is given by a recent longitudinal study where children exposed to two or more types of violence displayed accelerated rate of telomere shortening in buccal cells between the ages of 5 and 10 years (Shalev et al. 2013). These associations raise questions about how early life stresses that affect telomere length may be related to the risk of disease development in later life. Although further research is necessary to establish the mechanisms involved, one possibility is that inflammation may mediate the relationship between early life stresses and telomere shortening (Shalev 2012). Whilst the link between stress and increased inflammation is not mechanistically clear, studies have shown that a history of childhood stress is associated with enhanced inflammation in adulthood (Danese et al. 2007). Indeed, increased inflammation has been linked to the development of conditions such as cardiovascular diseases (Pearson et al. 2003) and depression (Dantzer et al. 2008). Moreover, inflammation has been shown to stimulate proliferation of immune cells, thus leading to accelerated telomere shortening in circulating leukocytes (Goronzy et al. 2006). Consistent with a link between inflammation and telomere damage, chronic inflammation in mice has been shown to lead to telomere dysfunction in a variety of tissues, such as gut and liver, and also impair tissue regenerative capacity, a phenotype ameliorated by anti-inflammatory ibuprofen treatment (Jurk et al. 2014). Another plausible mediator may be cortisol, the main stress hormone. It has been shown that cortisol increases the susceptibility of neurons to oxidative stress (Behl et al. 1997), and it also reduces telomerase activity in human T lymphocytes (Choi et al. 2008). Therefore, it is possible that increased oxidative stress and decreased telomerase activity triggered by stress-induced cortisol release may contribute to accelerated telomere shortening, although further studies are necessary to confirm these associations (Shalev 2012).

Telomere shortening has also been associated with increased risk of morbidity and mortality. For example, a recent study has suggested that longer telomeres

in early life strongly correlates with a longer lifespan in birds (Heidinger et al. 2012). A relationship between decreased telomere length and higher mortality risk has also been observed in humans, where individuals over 60 years old who had shorter telomeres in blood cells were more likely to die from conditions such as heart disease and infectious diseases (Cawthon et al. 2003). Following these observations, researchers in the field began to question whether telomere length may be used as a good biomarker of ageing, in other words, can telomeres be used as diagnostic tools to predict lifespan and age-related diseases? This hypothesis is contradicted by other reports, such as a study involving a large cohort of healthy participants that failed to identify any correlations between telomere length and lifespan or increased mortality risk, although they showed that individuals with longer telomeres generally had more years of healthy life (Njajou et al. 2009). These disparities can be partly explained by issues involved in different methods used to measure telomere length by different laboratories (Martin-Ruiz et al. 2014). A recent study revealed a large inter-laboratory variation of telomere length estimates (Martin-Ruiz et al. 2014), challenging the possibility of using telomere length measurements as a prognostic tool (von Zglinicki 2012). Moreover, normative reference values for telomere length are not available, limiting its use as a predictor of morbidity and mortality (von Zglinicki 2012).

7.2 Persistent DNA Damage Is Important for Stabilising Senescence

Normally, DNA damage acquired during the lifespan of a cell becomes repaired relatively rapidly; however, research now indicates that telomeric lesions persist for several months both in vitro and in vivo (Fumagalli et al. 2012; Hewitt et al. 2012). In fact, live-cell imaging experiments have shown that in stress-induced senescence, the majority of long-lived foci are associated with telomeres (Hewitt et al. 2012). Moreover, persistent DDR signalling at telomeres has also been observed in vivo in mouse hippocampal neurons even 3 months after exposure to genotoxic stress (Fumagalli et al. 2012). Telomere dysfunction has also been shown to be important in oncogene-induced senescence and play a role in preventing human cancer progression. Recent evidence indicates that oncogene activation in human cells leads to persistent telomeric DDR foci, stalling at telomeric replication forks and DNA replication stress (Suram et al. 2012). Stable growth arrest caused by oncogene activation was not achieved in cells overexpressing telomerase, which prevents telomeric DDR foci formation in response to oncogene-induced replication stress, indicating that telomere dysfunction is important to reinforce oncogene-induced senescence (Suram et al. 2012). Moreover, cancer precursor lesions, such as human melanocytic nevi, ductal breast hyperplasias, and colonic adenomas display features of telomere dysfunction-induced senescence, such as the accumulation of damage at telomeres, suggesting that this is also important in vivo as a tumour-suppressor mechanism. It has been suggested that oncogene activation causes

cells to undergo replication stress, leading to telomere erosion and dysfunction in telomerase-negative cells. This in turn induces senescence in normal somatic cells, and prevents formation of malignant cancers (Suram et al. 2012).

Given that activation of a DDR at telomeres is a key player in the initiation of replicative senescence, and that persistent telomeric DNA damage is observed in both stress- and oncogene-induced senescence, it has been proposed that persistent DDR signalling at unrepaired telomeres may be a major contributor to the establishment of senescence (Fumagalli et al. 2012). However, non-telomeric damage has also been shown to play a role in the senescent phenotype. In replicative and stress-induced senescence, half of the DNA damage foci have been shown to be located in non-telomeric regions, and although these are short-lived, they are constantly renewed during senescence (Passos et al. 2010; Hewitt et al. 2012). This occurs possibly as a result of increased ROS production during senescence, as inhibition of ROS has been shown to rescue the proliferation arrest in a proportion of cells (Passos et al. 2010). Therefore, DNA damage signalling occurring at both telomeric and non-telomeric regions plays important roles in the senescent phenotype, although distinguishing how each of these contributes to senescence may prove experimentally difficult.

7.3 Dysfunction in Telomeres Irrespective of Length

So far, emphasis of numerous studies has been given to senescence induced by telomere shortening. However, recent amounting evidence now indicates that telomere dysfunction can also occur independently of shortening (Fig. 7.3). Studies have reported that genotoxic stress leads to persistent DDR signalling at telomeres irrespectively of length in human fibroblasts in vitro and in mouse neurons in vivo (Fumagalli et al. 2012). DNA damage proteins have also been shown to associate with longer telomeres in vivo during the ageing process. Early observations showing that mouse embryonic fibroblasts could replicate indefinitely when cultured at low oxygen conditions, coupled with the fact that mice have extremely long telomeres in comparison to humans and express telomerase ubiquitously, led to the belief murine cell senescence occurred in a telomere-independent manner, and was generally mediated by oxidative damage (Parrinello et al. 2003). However, more recently, evidence suggests that this is not the case, as an age-dependent increase in telomeric DNA damage has been shown in the gut and liver of mice, which occurred independently of length (Hewitt et al. 2012; Jurk et al. 2014). Length-independent telomere dysfunction has also been reported in hippocampal neurons and liver of baboons with age, suggesting that this possibly contributes to tissue dysfunction during ageing by leading to cellular senescence (Fumagalli et al. 2012). Furthermore, in benign lesions such as melanocytic nevi, telomeres signalling a DDR were not shorter compared to functional ones, showing that this phenomenon is also a feature of oncogene-induced senescence (Suram et al. 2012). In fact, the authors showed that a fraction of dysfunctional telomeres still

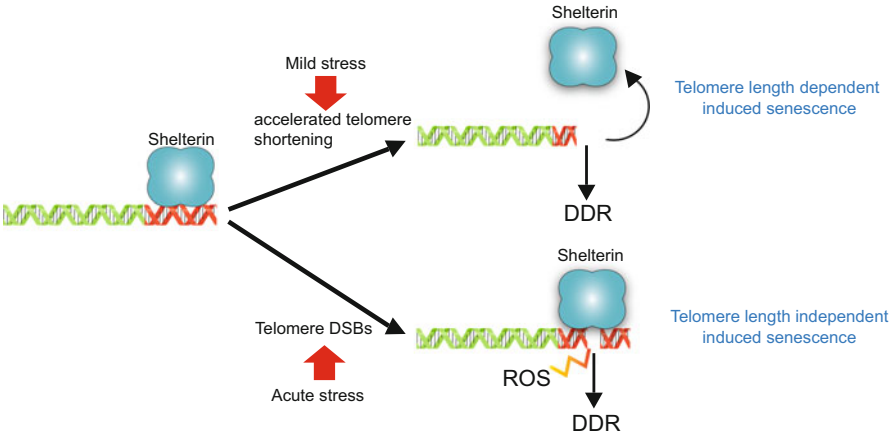

Fig. 7.3 Telomeres as sensors of stress. Recent evidence suggests that telomeres play an intricate role within cells, not only limiting their replicative lifespan by "counting" the number of cell divisions, but they may also serve as sensors of both extrinsic and intrinsic sources of stress. It is possible that cells that acquire telomere damage (*double and single-stranded breaks*) caused from mild stress are still able to replicate; however, this may result in accelerated telomere shortening, leading cells to a premature proliferative arrest, or "telomere length dependent induced senescence". However, acute stresses may result in double-strand breaks occurring at telomere regions which cannot be efficiently repaired by cells, preventing them from undergoing any further rounds of replication regardless of telomere length status (as shown in Hewitt et al. 2012; Fumagalli et al. 2012), which can also be termed "telomere length independent induced senescence"

retained TRF2, suggesting that a DDR may be activated due to factors other than shortening and uncapping (i.e. loss of shelterin proteins) (Suram et al. 2012). In support of this, dysfunction independent of telomere length has also been reported in cells undergoing replicative senescence, where some DDR-positive telomeres were still associated with TRF2 and RAP1, suggesting that displacement of shelterin components does not necessarily precede DDR activation at telomeres (Kaul et al. 2012).

It has been suggested that long telomeres represent a more abundant target for DNA damage to occur, making them more susceptible to damage and explaining the occurrence of DDR activation independently of length in replicative-, stress-, and oncogene-induced senescence (Fumagalli et al. 2012). However, so far evidence does not unequivocally indicate that longer telomeres are particularly susceptible to damage when compared to shorter ones. In fact, analysis of individual telomere length in melanocytic nevi and in mice revealed no significant length difference between damaged and undamaged telomeres (Hewitt et al. 2012; Suram et al. 2012). It is possible that the current methods to analyse individual telomeres in tissues may not be good enough to detect the shortest of telomeres, masking any significant differences in length that may exist between dysfunctional and functional telomeres.

In order to explain how telomeres can signal a DDR in the presence of shelterin components Roger Reddel's lab has proposed that telomeres may exist in three

different states. Firstly, telomeres may maintain an intact t-loop conformation in a length-dependent manner, thus preventing DDR activation, and inhibiting NHEJ through the presence of shelterin proteins. This is also known as the fully capped or closed state. However, if telomeres lose the T-loop conformation they may adopt an intermediate state, where a DDR is activated but shelterin proteins such as TRF2 are maintained at sufficient levels such that prevention of end-to-end fusions still occurs. This may occur in a length-dependent and – independent manner, possibly offering an explanation to the occurrence of damage at telomeres that are not critically short and still contain shelterin proteins. Lastly, the fully uncapped state occurs when extreme telomere erosion leads to loss of a protective structure, activating a DDR. Extensive loss of telomeric repeats also means that shelterin components can no longer bind to chromosome ends and inhibition of NHEJ is compromised, causing end-to-end fusions to occur (Cesare et al. 2009). Based on recent data from our lab and others we propose that telomeres may also exist in a fourth state. Telomeres may still maintain the T-loop conformation and shelterin components, but activate a persistent DDR within telomere repeats.

Overall, it appears that telomeres play a much more intricate role in modulating DNA damage responses in senescence, and do not only simply act as a replicometers, limiting the number of DNA replication cycles cells are capable of undergoing. Instead, evidence suggests that telomeres act as sensors of stress and damage, and prevent genomic instability from occurring by inhibiting division of cells that have accumulated genomic damage.

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