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Irreparable telomeric DNA damage and persistent DDR signalling as a shared causative mechanism of cellular senescence and ageing

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The DNA damage response (DDR) orchestrates DNA repair and halts cell cycle. If damage is not resolved, cells can enter into an irreversible state of proliferative arrest called cellular senescence. Organismal ageing in mammals is associated with accumulation of markers of cellular senescence and DDR persistence at telomeres. Since the vast majority of the cells in mammals are non-proliferating, how do they age? Are telomeres involved? Also oncogene activation causes cellular senescence due to altered DNA replication and DDR activation in particular at the telomeres. Is there a common mechanism shared among apparently distinct types of cellular senescence? And what is the role of telomeric DNA damage?

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Introduction

Genome integrity is preserved by the DNA damage response (DDR) that, in the presence of DNA damage, arrests the cell cycle progression while coordinating DNA repair events [1]. The DDR pathway is composed of a complex protein network, regulated mainly by post-translational modifications such as phosphorylation, ubiquitylation, SUMOylation, acetylation and PARylation [1]. Recently a direct role of small non-coding RNAs in DDR modulation has also been proposed [2,3]. Among the different types of damage, DNA double-strand breaks (DSBs) are considered the most deleterious, because they can cause cell death, a permanent proliferative arrest termed cellular senescence or, in checkpoint-impaired cells, genomic instability leading to cancer development. DSBs are repaired by two major mechanisms, the homologous recombination (HR) pathway, an error-free mechanism that uses a homologous chromosome as template for repair [4], and the non-homologous end joining (NHEJ) pathway in which the two DNA ends are ligated together with no need for homologous sequences [5]. If unrepaired, DNA damage fuels persistent DDR signalling and cellular senescence establishment. Which kind of DNA damages is refractory to DNA repair and triggers a permanent cell cycle arrest was not clear until recently. An example of terminal arrest was first observed by Hayflick and Moorhead in 1961, who reported that normal human cells in culture can undergo only a limited number of population doublings and eventually stop proliferating [6]. This was later explained by the so-called end replication problem, the inability of most normal cells to completely replicate linear genomes thus causing progressive shortening of chromosome ends, the telomeres, at every cell division [7]. When telomeres become critically short, they are sensed as damaged DNA, which triggers a DDR-initiated cellular senescence [8–10]. Despite the fact that chromosomes bear ends that resemble a DNA discontinuity such as a DSB, telomeres are generally not recognized as DSBs and do not activate a DDR. This is achieved by the joint action of different telomere-binding proteins, collectively named as a shelterin complex [11,12]. It is becoming evident that there is a key role of telomeres in DDR modulation that is not restricted to their shortening. In this review we will dissect the impact of telomeric DNA damage on different types of cellular senescence.

Replicative senescence in ageing

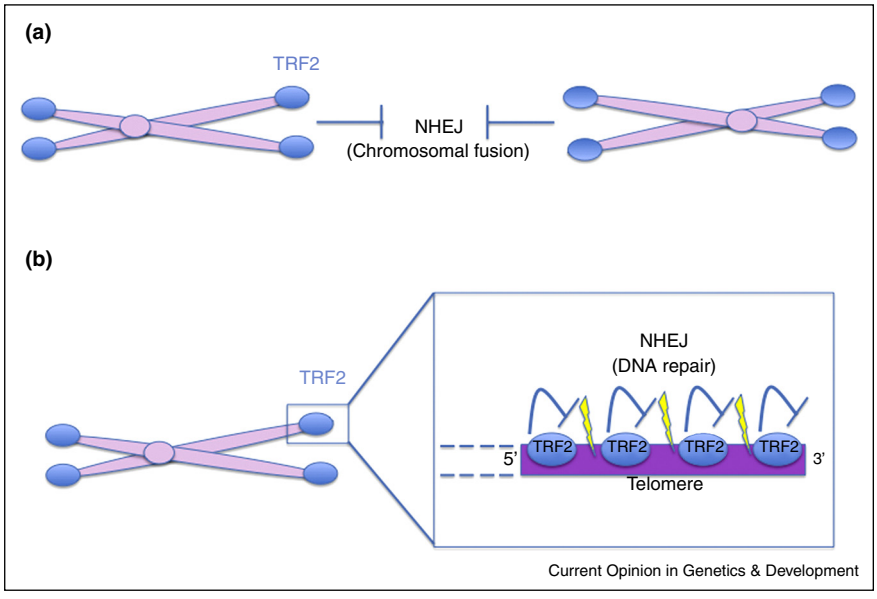
In the past years, a strong link between telomere-initiated cellular senescence and organismal ageing has emerged [13]. Evidence that cellular senescence is a biologically active response in tissue has been found

in mouse stem and somatic cells as well as in baboon and human skin fibroblasts [14–19]. These senescent cells are thought to contribute to tissue ageing by at least two mechanisms. First of all intrinsically, by their inability to further proliferate and thus to replenish tissues with new cells; secondly, by up-regulating genes that encode extracellular-matrix-degrading enzymes, inflammatory cytokines and growth factors [20,21]. These secreted factors, which are responsible for the senescent-associated secretory phenotype (SASP), act also on the neighbouring cells [22,23], and fuelling DDR by still ill-defined mechanisms [24]. The association between cellular senescence and tissue ageing seems to be causative, since lack of p16, which precludes senescence establishment, prevents the age-related decline, thereby increasing healthspan [25–27]. Similarly, clearance of p16-expressing cells leads to a delay in age-related pathologies and to attenuation of established age-related disorders [28••]. Telomeres seem to play a fundamental role in senescence-mediated organismal ageing. Indeed dysfunctional telomeres have been found in senescent cells *in vivo* in primates [16,29], and loss of telomerase function in mice causes senescence and physiological impairment of many tissues [30–33]. Moreover deletion of p21 in telomerase-deficient mice with dysfunctional telomeres prolongs the lifespan [34]. Telomere shortening seems to be the driving force, since elongation of telomeres by reactivation of telomerase is sufficient to eliminate the degenerative phenotypes in multiple organs observed in telomerase knock out mice [35••].

A role for telomeres in ageing of non-proliferating compartments

Telomere-initiated cellular senescence seems to be a plausible mechanism to explain the ageing-associated functional decline of proliferating tissues *in vivo*. However, it is reasonable to assume that some other mechanisms may be in place in non-proliferating cells in which no telomeric attrition due to the end replication problem is expected to occur, either because these cells are quiescent or differentiated. Surprisingly however, we and others have shown that telomeres might have a central role in senescence establishment independently from their shortening [36••,37••]. In these reports, random DNA damage generated by ionizing radiation, genotoxic drugs, or H_2O_2, leads to DDR activation that preferentially persists at telomeres over time. Cells with persistent DDR activation show a senescent phenotype that cannot be prevented by exogenous expression of telomerase, further excluding a contribution of telomere shortening. The mechanism proposed to explain this phenomenon is the suppression of effective DNA repair at telomeres by TRF2, a telomeric DNA binding protein [36••]. Inhibition of DNA repair might reflect the evolutionary role of telomeres in preventing chromosomal fusions, illegitimate DNA repair events among chromosome ends, in order to maintain the linear structure of chromosomes. TRF2 and the associated RAP1 protein are indeed able to inhibit NHEJ *in vitro* [38,39,40] and knock out of TRF2 leads to dramatic chromosomal fusions [41,42], most of which depend on NHEJ [43•]. Similarly, TRF2 has been shown to inhibit NHEJ also when a DSB occurs within a telomere, and not only at its end (Figure 1), revealing that telomeric

Figure 1



Lack of DNA repair activity at telomeres. **(a)** Proteins belonging to shelterin complex inhibit DNA end joining (by NHEJ) at distal ends to prevent chromosomal fusions. **(b)** In the same manner, shelterin complex proteins prevent repair of DSBs (by NHEJ) occurred within telomeric repeats across the telomere length. NHEJ, non-homologous end joining.

proteins, rather than telomeric DNA, are responsible for telomere irreparability. Consistent with this model, DDR activation at telomeres is more frequent in mouse and baboon tissues from aged animals, when compared with their young counterparts [36••,37••]. This observation also suggests that having long telomeres may have an important drawback, since more telomeric DNA can offer a wider target for random DNA damage that cannot be repaired. Indeed, in different mammalian species, telomere length and lifespan are inversely correlated [44].

Telomeric DNA damage in oncogene-induced senescence

In addition to its potential role in promoting ageing and age related disorders, telomere-initiated senescence, fuelled by oncogenic signals, plays a prominent role in suppressing malignant cancer progression in humans. In cells with functional DDR, oncogene expression usually results in cellular senescence after just a few population doublings [45]. This proliferative arrest is called oncogene-induced senescence (OIS) and, depending on cell type and oncogene expression levels, is caused by activation of a number of diverse pathways [46]. Thus, by preventing cancer onset, in addition to causing impairment of regenerative capacity during ageing, cellular senescence has been considered as an example of antagonistic pleiotropy, although this has recently put to question [47]. In some human cells, oncogene expression initially causes cells to hyper-proliferate, which leads to aberrantly increased DNA replication rates causing frequent DNA replication fork stalling events. As a consequence of this, DSBs are generated in the vicinity of collapsed replication forks and this activates a DDR and forces cells to undergo senescence [48,49]. OIS not only functions as a tumour suppressing mechanism in animal model systems [50], but also cells with features of OIS, including abundant DDR foci formation, have been detected in a number of distinct benign neoplastic lesions in humans and not in the corresponding malignant cancers [51,52••,53••,54••]. Given that initiation of aberrant cell proliferation in human tissues is often associated with oncogenic events, these data are strong evidence that OIS also suppresses cancer progression in humans.

Some chromosomal loci, called common fragile sites (CFS), appear to be hot-spots for DSB formation as a result of DNA replication stress. These sites are usually repetitive in nature and have a tendency to form secondary structures that can impede replication fork progression [55]. In addition, CFS belong to chromosomal regions poor of replication origins and thus unable to cope with stalled DNA replication forks [56]. Because of their repetitive nature, sensitivity to oxidative damage, and propensity to form secondary structures (called G quadruplexes), telomeres also pose a challenge to the replication machinery. In fact, telomeres share many other features of CFS [57,58]. Not too surprisingly, therefore, recent results demonstrated that oncogene expression leads to DNA replication stress, replication fork stalling, and formation of DDR foci at increased rates at telomeres [54••]. However, non-telomeric DDR foci are also generated but are resolved over a period of several days in arrested oncogene-expressing cells. These telomeric DDR foci persist suggesting that also oncogene-induced telomeric lesions are not efficiently repaired. Does the persistence of the telomeric DDR foci cause oncogene-expressing cells to arrest stably? In support of this, overexpression of catalytically active telomerase prevents formation of telomeric DDR foci as a result of oncogene-induced and drug-induced DNA replication stresses. Consequently, telomerase destabilizes the proliferative arrest caused by aberrant oncogene signalling [54••]. Thus, OIS is a cellular stress response that can be enforced by telomere dysfunction.

Persistent telomeric DDR foci, or dysfunctional telomeres, can also be observed in most cells of benign human neoplasias and cancer precursor lesions before telomeres have become eroded. Foci form below a critical telomere length in most cells of benign human neoplasias and cancer precursor lesions such as melanocytic nevi, ductal breast hyperplasias, and colonic adenomas [54••]. Indeed, dysfunctional telomeres in cells comprising these benign lesions on average are not shorter compared to other telomeres in the same cells, supporting this conclusion. The irreparability of telomeric DSBs in this context might therefore act as cellular sensor of hyperproliferative signals. In cells expressing telomerase, such as those of invasive human cancers, we would anticipate that replication stresses would not result in telomeric DDR activation. Rather, they would and allow continuous cell proliferation. It is therefore likely that cancer cells reactivate telomerase expression not only to prevent telomere erosion, but also to cope with telomeric replication stress that would halt cell proliferation.

Irreparability of telomeres is evolutionary conserved in eukaryotes

The inherent characteristic of telomeres to be resistant to DNA repair is conserved in the yeast *Saccharomyces cerevisiae* and *Schizoccharomyces pombe*, whose natural chromosome ends do not join with each other or with random DNA breaks [59–62]. Indeed, in a genetic system in *S. cerevisiae*, an endonuclease-induced DSB is generated immediately adjacent to a relatively short array of telomeric DNA repeats. The break inhibits the recruitment of DNA ligase IV and therefore prevents fusions by NHEJ [36••]. The presence of telomeric sequences at DNA ends can also prevent repair by HR, because it limits nucleolytic degradation and therefore the generation of single-stranded DNA (ssDNA). Moreover, it weakens the signalling activity of the Mec1 checkpoint kinase (ATR in mammals) [63,64], which is recruited to RPA-coated ssDNA [65]. Interestingly, this phenomenon

acts locally, as it inhibits checkpoint signalling from a nearby DSB devoid of telomeric repeats, but not from a DSB present on a different chromosome [63,64].

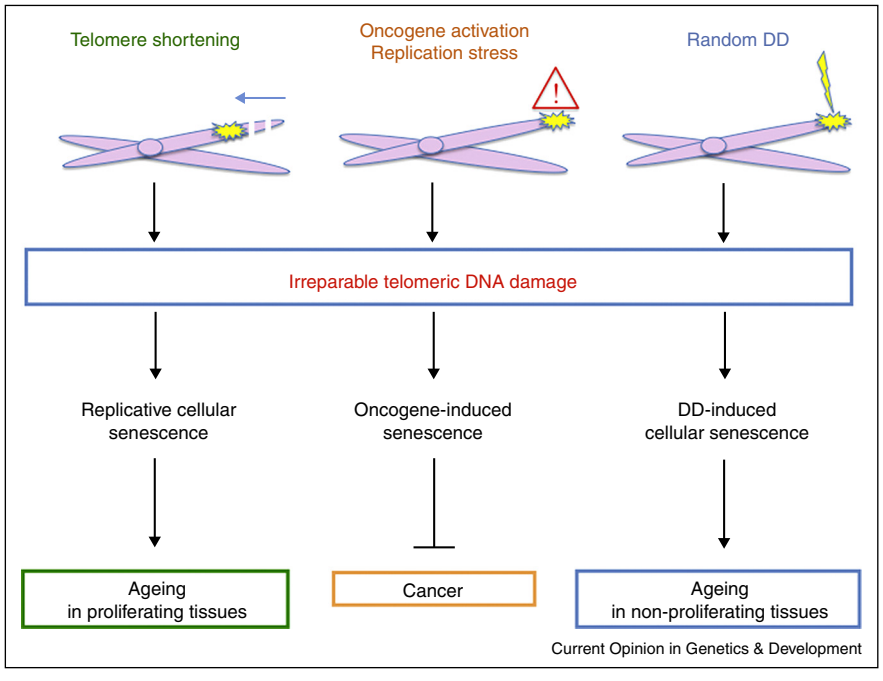
In budding yeast, the ability of telomeric ends to resist NHEJ-mediated repair and nucleolytic degradation depends on at least three different protein complexes, which are conserved from yeast to mammals. One of them is the CST (Cdc13–Stn1–Ten1) complex, which binds to the telomeric single-stranded overhang and prevents nucleolytic degradation and therefore checkpoint activation at telomeres [66,67]. A second complex, the Ku70-Ku80 heterodimer, blocks ssDNA formation specifically in the G1 phase of the cell cycle by inhibiting the action of the exonuclease Exo1 [68–70]. Finally, NHEJ inhibition at telomeres is controlled primarily by the Rap1 protein, which binds to the telomeric double-stranded DNA [71]. Rap1 prevents NHEJ by establishing two parallel inhibitory pathways through its interacting proteins Rif2 and Sir4 [72]. While it is currently unclear how these proteins prevent NHEJ, the observations that DSBs flanked by telomeric repeats show reduced DNA ligase IV binding [36••] suggest that they might function by counteracting the loading of NHEJ proteins. It has been recently shown that maintenance of NHEJ inhibition by Rap1 requires Uls1, which is both a Swi2/Snf2-related translocase and a Small Ubiquitin-related Modifier (SUMO)-Targeted Ubiquitin Ligase [73•]. Uls1 requirement is alleviated by inhibiting formation of SUMO chains and by *rap1* mutations altering SUMOylation sites. Furthermore, Uls1 limits the accumulation of Rap1 poly-SUMO conjugates, suggesting that Uls1 ensures the efficiency of NHEJ inhibition by eliminating non-functional poly-SUMOylated Rap1 molecules from telomeres.

Removal of telomerase causes replicative senescence also in *S. cerevisiae* [74]. Interestingly, the presence of a single critically short telomere accelerates senescence in a telomerase-negative context [75,76], suggesting that the length of the shortest telomere is a major determinant of the onset of senescence in this organism. The Mec1 checkpoint kinase is required for the accelerated loss of viability in the presence of a short telomere [75], indicating that, like in human fibroblasts, DDR is activated at the shortest telomere in cells undergoing senescence.

Conclusions

On the basis of the results described in this review, we can propose a unifying model, according to which telomeres play an essential role not only in replicative but also in DNA damage-induced and oncogene-induced cellular senescence (Figure 2). This provides a mechanism for DDR-mediated and senescence-mediated ageing of non-

Figure 2



Unifying model explaining the role of telomeres in replicative, DNA damage-induced and oncogene-induced senescence both at cellular and organismal levels. DNA damage at telomeres cannot be repaired, independently from the source that generated it, both endogenously (i.e. telomere shortening, replication stress) or exogenously (i.e. X-rays). Irreparable telomeres are, therefore, associated not only with replicative cellular senescence but also with oncogene-induced and DNA damage-induced cellular senescence. These events prevent cancer onset on the one side, but on the other side, cause impairment of regenerative capacity during ageing both in proliferating and non-proliferating tissues at organismal level. DD, DNA damage.

proliferating tissues, which could not be explained solely by telomeric shortening.

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