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Review

Exploring the frontier of aging: A short review of telomeres, R-loops, and their roles in the aging process

Yajun Wang (✉)

Fox Chase Cancer Center, Philadelphia, PA 19111-2497, USA

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ABSTRACT

Telomeres play a critical role in protecting chromosomes and have a profound impact on our health and longevity. While the potential to influence aging and health through telomere maintenance is tantalizing, it's a field still in its infancy, with much more to learn. From subtelomeric areas to telomeres, telomeric repeat containing RNA (TERRA) is transcribed. Telomeric dsDNA can be invaded by TERRA RNA, which then forms telomeric R-loop structures. An increasing amount of study points to the importance of TERRA-mediated R-loops in maintaining telomere length homeostasis and cell aging. Here, we provide an updated review of telomeres and R-loops, the factors that regulate telomeric R-loops, their interplay and potential contribution to the acceleration of aging, and the implications for delaying the aging process.

KEYWORDS

telomeres, telomeric repeat containing RNA (TERRA), R-loops, aging process

1 Introduction

In the pursuit of unraveling the enigma of aging, the intricate mechanisms governing cellular senescence have been extensively studied. Among the myriad factors implicated in this biological phenomenon, telomeres have emerged as captivating protagonists, offering a fascinating lens through which to understand the aging process. Recent studies have uncovered a fascinating connection between telomeres and RNA R-loops, suggesting reciprocal regulation and functional interactions between these two. The interplay between them has become a focal point of interest in aging research. It has been observed that telomeric DNA regions are particularly prone to RNA R-loop formation, and the presence of RNA R-loops at telomeres can influence telomere length regulation and stability [1]. Telomeric R-loops have been identified to be related to telomere shortening and as a potential contributor to the acceleration of the aging process [2]. These structures can exacerbate telomere shortening by hindering proper telomere replication and complicating the already complex task of telomere maintenance. Moreover, the accumulation of unresolved R-loops at telomeres can promote genomic instability, further driving the aging phenotype. Conversely, telomere dysfunction can impact RNA R-loop dynamics, potentially affecting gene expression and genome integrity [1, 3].

2 Telomeres and aging

Telomeres, the protective caps at the ends of linear chromosomes, consist of repetitive nucleotide sequences $(TTAGGG)_n$ that shield the genomic DNA from degradation and improper repair mechanisms. These structures are critical for maintaining chromosomal stability and integrity [4]. However, with each round of cell division, telomeres shorten, progressively limiting the cell's capacity to proliferate [5].

2.1 Telomere is a DNA–RNA–protein complex

A six-subunit protein complex known as shelterin protects the ends of the chromosomes [6, 7]. Telomeric DNA sequences directly contact TRF1, TRF2, and POT1, three subunits of the complex. Through protein-protein interactions, the remaining three subunits—RAP1, TIN2, and TPP1—are connected to telomeres. In mammalian cells, telomeres terminate as enormous terminal loops with a lariat-like configuration. The base of the telomere is invaded by a three-stranded G-rich overhang, which forms a displacement loop (D-loop). These structures are referred to as telomere loops (T-loops). The single-stranded terminus is wrapped back within the double-stranded DNA molecule, shielding the chromosome terminal, while the long stretches of the double-stranded telomere DNA are looped around (Fig. 1). Telomere-induced DNA damage foci (TIFs) that arise from the activation of the DNA damage

✉ Address correspondence to Yajun Wang, yajunwang696@gmail.com

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response (DDR) at telomeres are indicators of cellular senescence. Some cell types may experience autophagy or apoptosis after telomere dysfunction. The formation of TIFs can be caused by the loss of shelterin complex protection or telomere repeats. This can be measured by utilizing indirect immunofluorescence (IF) or the IF and IF-Telomere FISH assays in combination with the proximity ligation assay (TIF-PLA) to determine the colocalization of DNA damage response proteins, such as γ-H2AX and the p53 binding protein 1 (53BP1), and telomere markers, such as telomeric proteins and telomeric DNA [8].

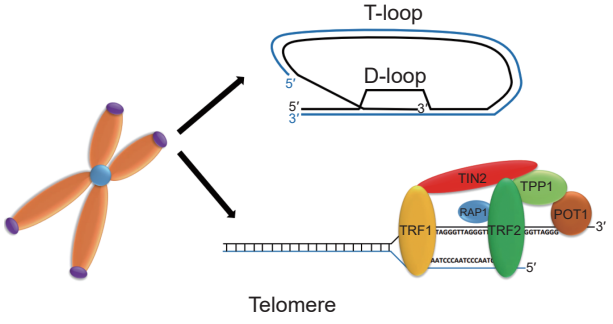

Figure 1 Illustration of telomere structure and the shelterin complex. (Top right) Telomere T-loop structure. (Bottom right) Shelterin proteins attached to the telomere. A single complex is displayed for simplicity's sake, although lots of complexes bind to the telomeric repeats.

Telomere length has been linked to various health outcomes. Short telomeres are associated with an increased risk of several age-related diseases. Moreover, they're considered markers of biological aging, as their length tends to decrease with age. Interestingly, lifestyle factors such as diet, exercise, stress, and smoking have been shown to influence telomere length and, by extension, might affect health span and longevity [9]. This connection has sparked widespread interest in telomere length as a potential biomarker for aging and disease risk.

In contrast to somatic cells, which experience telomere shortening with each division, germ cells and certain stem cell populations exhibit robust telomerase activity, maintaining telomere length and proliferative capacity. However, it's also active in many cancer cells, which helps them to proliferate indefinitely. Telomerase—a ribonucleoprotein complex comprising a catalytic subunit (TERT) and an RNA template (TERC)—has garnered considerable attention as a putative rejuvenating agent [10]. The discovery of telomerase was a breakthrough, earning the Nobel Prize in Physiology or Medicine in 2009. Preclinical studies harnessing telomerase activation have demonstrated extension of lifespan and amelioration of age-related phenotypes in various model organisms. However, the therapeutic potential of telomerase activation in humans remains contentious, with concerns regarding tumorigenesis and the balance between tissue renewal and cancer suppression. Although most cancers are dependent on telomerase, 4%–11% of cancers use one pathway called alternative lengthening of telomeres (ALT) based on homologous recombination to extend and maintain telomeres. ALT is frequently seen in malignancies with a mesenchymal origin and is typically associated with a bad prognosis. ALT is an intriguing target for cancer therapy because of its vital role in safeguarding telomeres and genomic integrity in tumor cells.

Extensive research on telomere organization reveals that this structure is multi-component and extremely dynamic, with multiple regulatory layers ensuring the telomere stays in a stable state [11–13]. Telomeric DNA, a telomeric protective protein complex, and telomeric RNA—which provides an extra scaffold for attaching telomeric proteins—compose the telomere complex. Telomeres are heterochromatic structures, yet they are also transcriptionally active. Subtelomeric promoters drive the G-rich long non-coding RNA known as telomeric repeat-containing RNA (TERRA) [14]. TERRA transcription begins at subtelomeric regions that resemble the CpG islands of eukaryotic genes and stretch toward the ends of chromosomes. TERRA has extensive tracts of UUAGGG repeats near the 3′ end and chromosome-specific subtelomeric sequences at the 5′ end. The majority of TERRA in human cells has 7-methylguanosine (m7G) cap structures at its 5′-ends, but a minority have a poly(A) tail at their 3′-end, which enhances their stability. TERRA acts as a scaffold at the telomeres by interacting with shelterin components. The most dynamic part of telomeres is TERRA, and its levels fluctuate with telomeric chromatin states and the cell cycle. Telomeric RNAs contain a great deal of regulatory potential, and the roles of TERRA are now being investigated. At the telomeres, TERRA contributes to the development of G-quadruplexes (G4) and R-loops, which are RNA: DNA hybrids with regulatory significance [15]. High levels of TERRA are thought to guarantee telomere lengthening through recombination in cancer cells of the ALT type. The discovery of TERRA, many proteins and RNP (ribonucleoprotein) partners of telomerase, and components of the shelterin complex suggests a close relationship between the telomere and a network of cellular processes.

Telomeric DNA exhibits a G/C strand bias in nearly all species, and the G-rich strand's short tandem repeats can form secondary structures like four-stranded G-quadruplexes in vitro. There is some indication that G-quadruplexes at telomeres, at least in yeast, may serve as protective capping structures and that they may have a positive effect on telomere maintenance through recombination-based mechanisms or the telomerase enzyme. However, G-quadruplex formation in telomeric DNA, as well as other regions of the genome, can also cause instability in the genome and act as a barrier to DNA replication. Telomere shortening can result from unresolved telomeric G-quadruplexes inhibiting telomerase action [16]. However, POT1 increases telomerase activity by moving or substituting a G-quadruplex structure. And it is hypothesized that G-loops, special structures produced by the co-formation of G-quadruplexes and R-loops, aid in the ALT recombination process.

2.2 Age-related dysfunction of telomeres

Senescent cells are accumulated by aging organisms and are known to be caused by telomere damage. Accelerated telomere dysfunction causes a number of illnesses linked to the aging process in humans. The reduction of telomere length below a threshold is the cause of replicative cellular senesce [17]. Telomere shortening in animal models gradually triggers cellular senescence and DDR at telomeres activation, which reflects aspects of aging and age-related disorders. A

recent research on telomere length in human tissues indicated age-dependent telomere shortening [18]. The limited telomeres, however, do not always indicate a limited lifetime across species. For example, humans have shorter telomeres than rats, yet also live longer. Large sample sizes have revealed that the correlation between chronological age and telomere length in the species of non-human vertebrates and humans is poor and depends on the tissue type and telomere measuring technique. Alternatively, it has been suggested that telomere shortening rates and the rise in short telomeres can predict lifespan. Regardless of the bulk of generally long telomeres, one or a few critically short telomeres are consistently sufficient to cause DDR and impose cellular senescence *in vivo*. Thus, the destiny of cells and the aging process of an organism are largely determined by DDR signaling events at telomeres. In fact, in various mammalian tissues, the amounts of TIFs and DDR markers increase during aging. And older baboons have more damaged telomeres in their skin, liver, and brain [19]. Mice exhibit an age-dependent increase in defective telomeres in both non-proliferating and proliferating tissues, despite with long telomeres and ubiquitous telomerase expression [20]. TIFs, along with other senescence markers, rise with age in humans in the absence of telomere shortening in skin melanocytes and $CD8^+$ T cells [21, 22]. Long but faulty telomeres also contribute to age-related illnesses and cellular senescence, which are especially evident in post-mitotic tissues, in addition to severely short telomeres. POT1 mutations linked to long telomeres predispose to a familial clonal hematopoietic syndrome with a variety of benign and malignant solid tumors. Risk for these abnormalities is mediated by the prolonged cellular lifespan and the ability to sustain telomeres throughout time [23].

Lots of evidence point to telomere DDR's involvement in the aging process and age-related illnesses. The frequency of cells with TIFs can be decreased by implementing therapies known to lengthen health span, such as exercise, diet restriction, and rapamycin. TIF levels are decreased in vivo by using senolytic techniques to eliminate senescent cells [24]. On the other hand, TIFs levels increase following conditions that are known to hasten aging, such as obesity, mitochondrial dysfunction, chronic inflammation, and decreased autophagy. It's most possible that telomere dysfunction-activated DDR, rather than telomere dysfunction per se, is what causes cellular senescence, which in turn promotes the age-related loss of tissue functions [22, 25].

Research into telomere biology has opened up potential therapeutic avenues, including strategies to activate telomerase for anti-aging purposes or inhibit it to fight cancer. Yet, the manipulation of telomere length and telomerase activity in humans remains highly experimental and is not without risks, including the potential for increased cancer risk. The insights into the intricate interplay between telomere dysfunction and the aging process could serve as an indispensable resource for navigating the frontiers of aging biology.

3 RNA R-Loops and aging

3.1 R-loop is a three-stranded nucleic acid structure

R-loops, also known as RNA: DNA hybrids, are tripartite structures created when homology-directed base pairing unites a single-stranded RNA molecule with a double-stranded DNA duplex (Fig. 2). R-loops were originally observed in 1976 as a three-stranded nucleic acid structure with a displaced single-stranded DNA and an RNA: DNA hybrid using electron microscopy [26]. R-loops are hypothesized to mediate a wide range of biological processes, such as chromatin patterning, DNA replication, gene regulation, and DNA repair. According to genome-wide profiling of R-loop sites via DNA: RNA immunoprecipitation sequencing (DRIP-seq) using the S9.6 antibody (recognizing RNA: DNA hybrid), R-loops are found as the dynamic structures that make up about 5% of mammalian genomes [27–30].

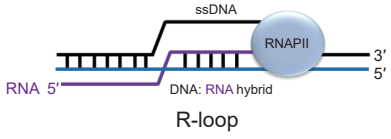

Figure 2 The schematic of R-loop structure. The R-loop is a three-stranded nucleic acid structure containing a displacing non-template single-stranded DNA (ssDNA) and a DNA: RNA hybrid.

Cells have evolved a number of strategies to balance the generation and resolution of RNA: DNA hybrids because of their quick resolution and short lifespan [31–33]. The first-layer mechanism controls the spontaneous/transient R-loops formation. Prior research utilizing THO-complex mutants in yeast revealed that RNA export and processing factors are crucial in limiting the accumulation of hybrids [34, 35]. Moreover, it has been demonstrated that DNA topoisomerases are necessary for the resolution of torsional stress on DNA resulting from negative supercoiling [36, 37]. RNA: DNA helicases and RNase enzymes are used in the second-layer mechanism to eliminate stable R-loops that have already formed. It has been observed that several helicases may directly unwind and eliminate RNA: DNA hybrids, including Sen1 in yeast, senataxin (SETX) in humans, DXH9, and DDX5 [38–40]. The most famous RNase enzyme among the nucleases is RNase H. RNase H1 and H2 are essential enzymes in eukaryotes that remove R-loops by selectively breaking down the hybrid's RNA moiety [41]. Repair factors that identify ssDNA in R-loops or RNA: DNA hybrids are part of the third-layer mechanism. It is known that unscheduled or prolonged R-loops frequently endanger DNA transcription and cause ssDNA and dsDNA lesions in the genome as well as replication stress and transcription-replication conflicts [42–44].

3.2 R-loops are a double-edged sword

R-loops play essential roles in various biological processes, including transcription, replication, DNA repair and so on. R-loops can influence gene expression by promoting or inhibiting transcription [45]. They are involved in transcription termination in certain genes and can affect the processivity of RNA polymerase. Besides, proper R-loop management is essential for maintaining genomic integrity. While controlled R-loop formation is part of normal cell function, dysregulated R-loops can lead to DNA damage, mutations, and chromosomal rearrangements. R-loops can also impact the epigenetic landscape by influencing DNA

methylation patterns [46]. This can alter gene expression without changing the DNA sequence, affecting cellular function and identity. Moreover, R-loops are implicated in the initiation of DNA replication in certain origins and play a role in various DNA repair mechanisms, highlighting their importance in genome duplication and maintenance.

Despite their functional importance, R-loops are a double-edged sword. Unscheduled or unresolved R-loops can lead to replication stress, double-strand breaks, and genomic instability, contributing to the development of cancer and other diseases [47]. Mutations affecting proteins involved in R-loop processing are linked to several genetic disorders and neurodegenerative diseases, underscoring the need for precise R-loop regulation.

The dual nature of R-loops presents both opportunities and challenges for therapeutic intervention. Targeting the pathological aspects of R-loops could offer new avenues for treating diseases associated with their dysregulation. However, the essential roles of R-loops in normal cell function necessitate a nuanced approach to modulate their activity without disrupting vital processes. Future advancements in detection methods and a deeper understanding of R-loop biology may pave the way for innovative treatments for diseases associated with R-loop dysregulation.

3.3 Role of RNA R-Loops in aging

Aging is characterized by a decrease of cellular function and an increase in cell death. Recent studies have uncovered intriguing links between RNA R-loops and the aging process. It has been demonstrated that R-loop levels tend to increase with age, correlating with a decline in cellular function and tissue homeostasis. Elevated R-loop formation has been implicated in age-related genomic instability, contributing to the accumulation of DNA damage and mutations commonly observed in aging cells [48, 49]. Several factors contribute to the age-dependent accumulation of RNA R-loops. Dysregulation of RNA processing and clearance mechanisms, including RNA decay pathways and RNA-binding proteins, may lead to the persistence of R-loops in aging cells. Moreover, age-associated changes in chromatin structure and epigenetic modifications can influence R-loop formation and resolution dynamics, exacerbating genomic instability over time.

Significantly, mounting data connects R-loop accumulation to cellular dysfunction, elevated apoptosis, and the beginning of chronic illness. In neurodegeneration, R-loop-mediated DNA damage has been linked to neuronal dysfunction and cell death, highlighting the detrimental consequences of R-loop accumulation in the aging brain. Similarly, in cancer, dysregulated R-loop dynamics can fuel genomic instability and oncogenic transformation, driving tumor progression in aged individuals. Age-related human neuropathies have been associated with transcriptional impairment, genomic instability, and R-loop homeostasis. The study in aged *Drosophila melanogaster* photoreceptor neurons demonstrated that bulk R-loop levels rose with age. Furthermore, transcribed genes accumulated R-loops over gene bodies during aging, which linked with decreasing expression of long and highly expressed genes, according to genome-wide mapping of R-loops. Crucially, although photoreceptor-specific down-regulation of Top3β, a DNA/RNA topoisomerase involved with R-loop resolution, leads to impaired visual function, over-expression of Top3β or nuclear-localized RNase H1, results in greater positive light response with aging [50].

4 Intersecting pathways: R-Loops at telomeres and aging

Recent research has uncovered an intriguing intersection between telomeres and RNA R-loops, shedding light on their interplay and potential implications in health and disease. Here, we delve into the intricacies of telomeric R-loops and aging, exploring the emerging understanding of their interconnectedness.

4.1 R-loops at chromosome ends

The tight connection between RNAs and R-loops suggests that TERRA is critical for the creation of telomeric R-loops. Since telomeres include nearly all of the favorable characteristics for R-loop formation, such as high GC skew, G-quadruplex secondary structure, and repetitive sequences, TERRA can invade telomeric dsDNA and generate telomeric RNA: DNA hybrids (Fig. 3). Higher TERRA levels are correlated with higher R-loop levels in both yeast and human cells [51–54]. Lots of proteins have been discovered by proteomic investigations for R-loop binding partners [55]. About 200 overlapping proteins were found, despite the fact that the investigations were carried out in various cell types and using various techniques, such as *in vitro* pull-down using biotinylated synthetic hybrids and *in vivo* affinity purification with the S9.6 antibody. Helicases, chromatin-associated proteins, and RNA processing proteins are among the proteins that are shared by both strategies. It has been

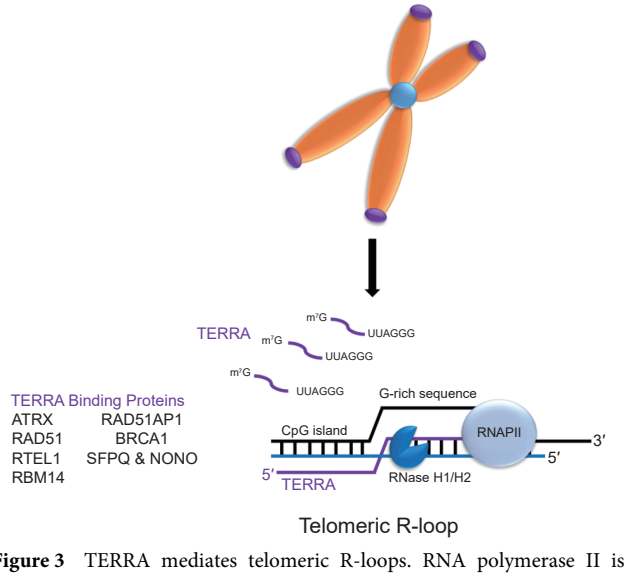

Figure 3 TERRA mediates telomeric R-loops. RNA polymerase II is responsible for the transcription of TERRAs, which begins at CpG-rich promoters in the subtelomeric region and proceeds toward telomeres. TERRA is bound by many proteins, which control its level. Through direct base pairing, TERRA RNA can infiltrate telomeric dsDNA and create R-loop structures. Telomeric R-loop formation is favored by G-rich telomeric repeats and G-quadruplex structures in telomere sequences. RNase H1/H2 and helicases are the general R-loop regulatory factors that control R-loop stabilization.

established that these influence telomeric R-loop function as well. The THO complex represses telomeric R-loops in budding yeast. RNase H enzymes are implicated in the elimination of R-loops at yeast and mammalian telomeres in addition to being known to digest the hybrid's RNA moiety at genome-wide R-loop locations [56]. On the other hand, helicases, which belong to a different class of common factors and include Upf1, Sen1, and DDX5, can either promote hybrid structures based on the status of telomere length or limit the accumulation of telomeric R-loops through their unwinding activity. The TERRA Binding Proteins, such as ATRX, SFPQ and NONO, RAD51, BRCA1, RTEL1, RAD51AP1, and RBM14, can also control telomeric R-Loop Levels [57]. Shelterin proteins play important functions at telomeres and that deletion of TRF1 and TRF2 has been shown to change TERRA levels. The first shelterin member shown to be connected to R-loops is RAP1 [58–60].

TERRA-mediated R-loop development can happen post-transcriptionally in trans, which is different from the traditional theory of co-transcriptional-mediated R-loop formation in cis. In the past ten years, it has become evident that TERRA R-loops are crucial for end protection and telomere length management. The dynamic maintenance of telomeric R-loops is one of their most significant features. Telomere integrity depends on the R-loop level that is precisely adjusted. In addition to causing replication stress, the temporary creation of R-loops in ALT cancer cells also indicates the beginning of DNA repair by attracting repair proteins like as BRCA1 and RAD51 and starting HR-mediated telomere synthesis, which is triggered by breaks in DNA strands. It is necessary to effectively eliminate the already existing R loops in order to finish HR-mediated telomere formation. Therefore, a dynamic transition between R-loop creation and resolution at telomeres is necessary for ALT survival. Since telomeric R-loop levels in non-ALT cancer cells have a stronger correlation with cell cycle regulation, telomere-associated R-loops can be resolved early to reduce the risk of transcription and replication collisions. As demonstrated in yeast cells and human ICF patient cells, lack of dynamic regulation leads to the accumulation of unscheduled or persistent R-loops at telomeres, which becomes one of the main drivers of genome and telomere instability [1]. Given their close relationship to human diseases and the possibility of creating R-loop-based therapies to specifically target ALT tumors, the balanced creation and resolution of telomeric R-loops represent an important field for further study.

4.2 Telomeric R-loops, cellular senescence, and aging

To stop cellular senescence, telomeres must be maintained at a minimum length. In order to delay the beginning of premature senescence, homology-directed repair (HDR) can be used to repair critically short telomeres that develop in the absence of telomerase. At very short telomeres, the non-coding RNA TERRA preferentially accumulates as HDR-promoting RNA: DNA hybrids in budding yeast. A local deficiency in the RNase H2 and Rat1 nucleases' ability to degrade RNA is the cause of the elevated levels of TERRA and R-loops, which are only present at short telomeres. As a result, at shortened telomeres, the way that TERRA degradation and telomere replication coordinate is changed. The retention of R-loops at short telomeres facilitates the recruitment of Rad51 recombinase and activates the DDR. Thus, a key factor in determining the rate of replicative senescence is the telomere length-dependent regulation of TERRA and TERRA R-loops [3]. Similar findings have also been reported recently in human cells, showing that TERRA R-loops preferentially associate with short telomeres [53]. Nevertheless, it is still unknown how short telomeres attract additional TERRA R-loops.

ICF syndrome (Immunodeficiency, Centromeric Instability, and Facial Anomalies) is a rare human illness linked to genetic abnormalities in DNA methylation. With about half of all known ICF patients, type I ICF has the DNMT3B mutation and is also most relevant for TERRA R-loops. TERRA transcription dramatically increases as a result of hypomethylation brought on by DNMT3B deficiency. Total TERRA levels have been demonstrated to be 6–10 times greater in ICF cells compared to healthy control cells, and at certain chromosomal ends, may be 100 times higher [61]. It is confirmed that higher TERRA levels lead to enhanced R-loop formation at telomeres in ICF cells. Compared to their parental controls, the mean telomere length of ICF patients is much shorter. Additionally, the chromosomal ends with telomeric signal loss and DNA damage foci are more common in ICF patients. Accelerated telomere shortening causes early replicative senescence in primary cells from ICF patients, resulting in unusually low population doublings [62]. It is hypothesized that these symptoms are associated with loss of HR repair and TERRA R-loop-mediated DNA damage because of the markedly elevated levels of TERRAs.

TERRA R-loops can connect and protect sister telomeres in mitosis. The recruitment of polyADP-ribosyltransferase tankyrase to telomeres by TRF1 is necessary for the resolution of cohesion between sister telomeres in human cells. In human aging cells, sisters maintain cohesion during mitosis because TRF1/tankyrase is not sufficiently recruited to shorter telomeres. There is a protective aspect to this ongoing coherence, but it is unclear how sisters manage to maintain their cohesiveness. The study revealed how sister telomeres are held together by TERRA through RNA: DNA hybrid complexes. The RNA-binding protein C19orf43, a tankyrase-interacting partner, is necessary for the regulation of TERRA R-loops. RNaseH1 counteracts the persistent telomere cohesion in C19orf43-depleted cells, indicating that RNA: DNA hybrids keep sisters together. According to its protective function for continuous telomere cohesion, C19orf43 reduction in aged cells prevents DNA damage and postpones replicative senescence. The shortened telomeres unable to recruit R-loop-repressing machinery could control the onset of senescence [63].

5 Therapeutic implications and future directions

In a number of model organisms, it has been demonstrated that maintaining telomere length via genetic modifications, antioxidants and anti-inflammatory agent, or telomerase activity modulation delays cellular aging and lengthens healthspan [64, 65]. By focusing on the aging process itself, therapeutic approaches aimed at postponing cellular and

tissue aging may be possible through the regulation of telomeres through genetic manipulation or pharmacological techniques [66]. Understanding the nuanced relationship between telomeric R-loops and aging opens new avenues for therapeutic interventions aimed at promoting longevity and mitigating age-associated diseases. Strategies to modulate telomerase activity, enhance telomere protection, and appropriately resolve R-loops are under investigation, with the potential to delay the onset of cellular senescence and extend healthy lifespan.

However, significant challenges remain. The mechanisms governing the formation and resolution of R-loops at telomeres are not fully understood, necessitating further research. Additionally, interventions must be finely tuned to avoid unintended consequences, such as the potential for increased cancer risk associated with telomerase activation. As research in this area continues, we will hopefully unlock the mysteries of aging and potentially change the way we approach age-related diseases and longevity. Continuing to explore these fundamental aspects of cell biology will undoubtedly help us understand life, offering hope for extending healthspan and improving quality of later life.

Conflict of interests

The author declares no competing interests.

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