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The role of telomere trimming in normal telomere length dynamics

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Key words: telomere length, telomere trimming, telomere rapid deletion, telomerase, alternative lengthening of telomeres, homologous recombination

Abbreviations: TRD, telomere rapid deletion; ALT, alternative lengthening of telomeres; HR, homologous recombination; HJ, Holliday junction; t-circle, telomere-circle; 2D, two-dimensional; AT, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; ATS, abrupt telomere shortening; APB, ALT-associated PML body; PML, promyelocytic leukemia; STELA, single telomere length analysis; SSS, sudden senescence syndrome; SNP, single nucleotide polymorphism

Telomeres consist of repetitive DNA and associated proteins that protect chromosome ends from illicit DNA repair. It is well known that telomeric DNA is progressively eroded during cell division, until telomeres become too short, and the cell stops dividing. There is a second mode of telomere shortening, however, which is a regulated form of telomere rapid deletion (TRD) termed telomere trimming that is reviewed here. Telomere trimming appears to involve resolution of recombination intermediate structures, which shorten the telomere by release of extrachromosomal telomeric DNA. This has been detected in human and in mouse cells and occurs both in somatic and germline cells, where it sets an upper limit on telomere length and contributes to a length equilibrium set-point in cells that have a telomere elongation mechanism. Telomere trimming thus represents an additional mechanism of telomere length control that contributes to normal telomere dynamics and cell proliferative potential.

Linear chromosomes are "capped" at each end to avoid being recognized as double-strand breaks and subjected to DNA repair. Capping is achieved by a terminal nucleoprotein structure termed a telomere, which is composed of tandem arrays of the hexanucleotide (5'-TTA GGG-3') repeat unit and the telomere-specific shelterin complex comprising the TRF1, TRF2, POT1, TIN2, TPP1 and RAP1 proteins.[1] Integrity of the telomeric nucleoprotein complex is essential for chromosomal stability and therefore for genomic stability.

Telomeres lose their full capping function when there is insufficient telomeric DNA and/or incomplete shelterin binding, which results in a telomere-specific DNA damage response. This induces a p53-dependent cell cycle arrest known as senescence,[2] when a threshold level of DNA damage response is reached.[3] Despite inducing a DNA damage response, the telomeres of senescent cells appear to be in a functional state that is intermediate between fully capped and fully uncapped, because they are capable of preventing end-to-end fusion events.[3] It may therefore be a misnomer to refer to them as "dysfunctional" at this stage. Cells defective in their responses to DNA damage are able to evade cellular senescence and continue to divide until they reach crisis, when their telomeres become critically short and uncapped. At this point, telomeres become fusogenic, resulting in rounds of breakage-fusion-bridge cycles, which manifest as chromosome rearrangements and genomic instability.[4]

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The Importance of Telomere Length Control

Telomere length ultimately dictates the proliferative capacity of a cell. For reasons described below, telomeres undergo steady attrition during the proliferation of normal cells, which sets an upper limit on the number of times they can proliferate and thus protects against cancer. Inappropriate activation of a telomere lengthening mechanism and evasion of cellular senescence is vital for cellular immortalization (**Fig. 1**), which is one of the main hallmarks of cancer.[5] Conversely, insufficient telomere length in somatic cells results in premature proliferative failure in specific tissues, which can cause potentially fatal conditions such as aplastic anemia or pulmonary fibrosis[6,7] and possibly emphysema.[8] It has also been demonstrated that interferon-stimulated gene 15 (ISG15) is regulated by telomere length, raising the intriguing possibility that telomeres can be involved in the regulation of gene expression.[9]

The best-known telomere lengthening mechanism is the ribonucleoprotein enzyme telomerase, which, in its active form, comprises two molecules each of the reverse transcriptase hTERT, the RNA template hTR encoded by the TERC gene and the associated protein subunit dyskerin encoded by the DKC1 gene.[10] Lack of normal levels of telomerase activity resulting from mutations in TERT, TERC or DKC1 or in proteins involved in telomerase biogenesis (NHP2 or NOP10) or its recruitment to the telomere (TCAB1) result in the short telomere syndromes referred to above.[11,12]

Telomerase activity is upregulated inappropriately in the majority of cancers.[13] A smaller proportion of tumors, representing a broad spectrum of cancer types but predominantly from mesenchymal or neuroepithelial precursors, activate an alternative lengthening of

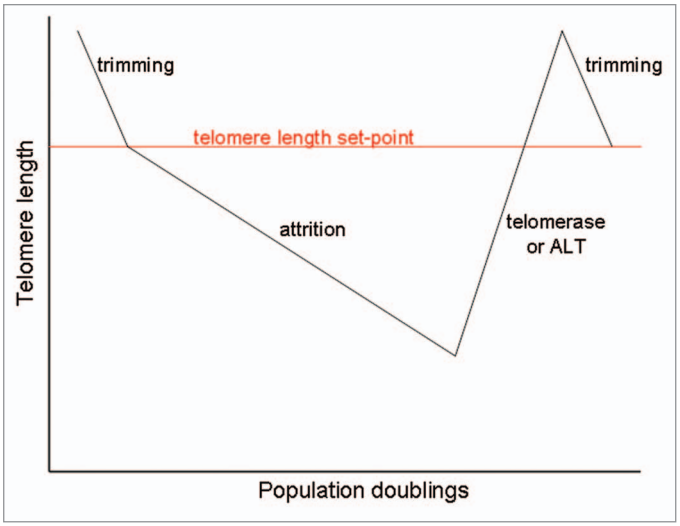

Figure 1. Telomere length regulation in mammalian cells. Telomere length is subject to regulated rapid deletion events known as telomere trimming, gradual telomere attrition and elongation by activation of telomerase or ALT.

telomeres (ALT) mechanism.[14] ALT cells use homologous recombination (HR)-mediated replication of telomeric DNA to maintain their telomeres. ALT cells typically display very long and heterogeneous telomere lengths, whereas telomerase-positive cancer cells predominantly maintain substantially shorter telomeres.[15-17]

It is currently not known whether excessive telomere lengths are detrimental to cells. Long telomeres may simply enable increased cell proliferative capacity prior to telomere length-mediated senescence. However, it can also be speculated that very long telomeres may become prone to replication slippage, stalled replication forks or the formation of secondary structures such as G-quadruplexes. Excessively long repeat tracts are also likely to impinge on cell cycle progression by presenting a replicative burden, which may slow down cell cycling and ultimately result in increased chromosome instability. It is possible that the short telomere lengths typical of telomerase-positive cancer cells fall below the threshold required for telomere trimming, and that this may allow the cancer cells a proliferative advantage.

Paradoxically, it is becoming increasingly clear that inadequate telomere length is associated with risk of cancer, including hematological malignancies, such as acute myeloid leukemia.[18,19] The mechanisms underlying risk of malignancy appear complex; however, it has been speculated that shorter telomere lengths may favor the acquisition of genomic instability and, thus, oncogenesis. Consequently, targeted manipulation of telomere maintenance mechanisms not only provides a promising strategy for treating cancer, but in specific circumstance may also be useful for cancer prevention.

Telomere Length Regulation in Normal Mammalian Cells

In normal mammalian cells, telomere length is ultimately determined by the balance between lengthening and shortening processes[20] (**Fig. 1**). Telomere repeat synthesis can occur in normal human cells in response to increased proliferative requirements by the stringent control of telomerase. Telomerase is activated in the germline, early in embryogenesis, in progenitor cells and in activated lymphocytes as part of the immune response. Telomeres progressively shorten in replicating cells as a result of the limitations of RNA-primed DNA synthesis of the lagging strand, known as the end-replication problem.[21-23] The action of cellular nucleases also contributes to gradual telomere erosion.[24,25] Thus, telomere attrition acts to limit the cellular proliferative lifespan[26] (**Fig. 1**).

Telomere trimming is a mechanistically distinct process of negative telomere length regulation, which results in the deletion of large segments of telomeric DNA[27] (**Fig. 2**). Importantly, telomere trimming is a regulated mechanism that does not produce telomere signal-free ends by FISH analysis and does not induce telomere dysfunction.[27] For this reason, telomere trimming is compatible with continued cell growth and appears to play a role in normal telomere biology (**Fig. 1**). It is possible that telomere trimming is of equal importance to gradual telomere attrition and the de novo synthesis of telomeric DNA by telomerase or ALT.

The term telomere trimming was coined in response to a reviewer who requested a name for this process that would not be interpreted as meaning that the whole telomere is lost.[27] The underlying mechanism appears to involve homologous recombination (HR)-mediated resolution of telomeric structures called t-loops. These structures are able to form, because telomeric DNA, although mostly double-stranded, terminates in a single-stranded G-rich 3' overhang that is able to fold back on itself and invade the proximal duplex telomere repeats.[25,28-31] At the point of insertion, a recombination intermediate is formed, and although this is generally stable, its resolution can result in sudden shortening equivalent to the length of the t-loop and generate extrachromosomal telomeric DNA, which is predominantly detectable in the form of telomere circles (t-circles),[27,32] (**Fig. 2**). Although some linear telomeric DNA is also likely to be generated by this process, t-circles are less prone to experimental artifacts and are, consequently, a more useful marker of telomere trimming.

Experimental Model Systems of Telomere Rapid Deletion

Regulated and coordinated decreases in telomere length were first observed during macronuclear development in the ciliates *Euplotes crassus* and *Tetrahymena thermophila*.[33,34] Several years later, TRD events were identified and characterized in *Saccharomyces cerevisiae*, following expression of the telomere-binding protein Rap1 containing a deletion mutation.[35] Ultimately, this work by the Lustig group demonstrated that a mechanism of TRD, distinct from gradual telomere attrition, was able to reduce elongated telomeres in a single resolution event.[36] Elegant studies involving the insertion of HaeIII restriction enzyme sites into yeast telomeres found no evidence of inter-telomeric movement, but a deletion/retention pattern consistent with intra-telomeric deletion, which was dependent on wild-type telomere length.[37] In this context, TRD is analogous to telomere trimming.

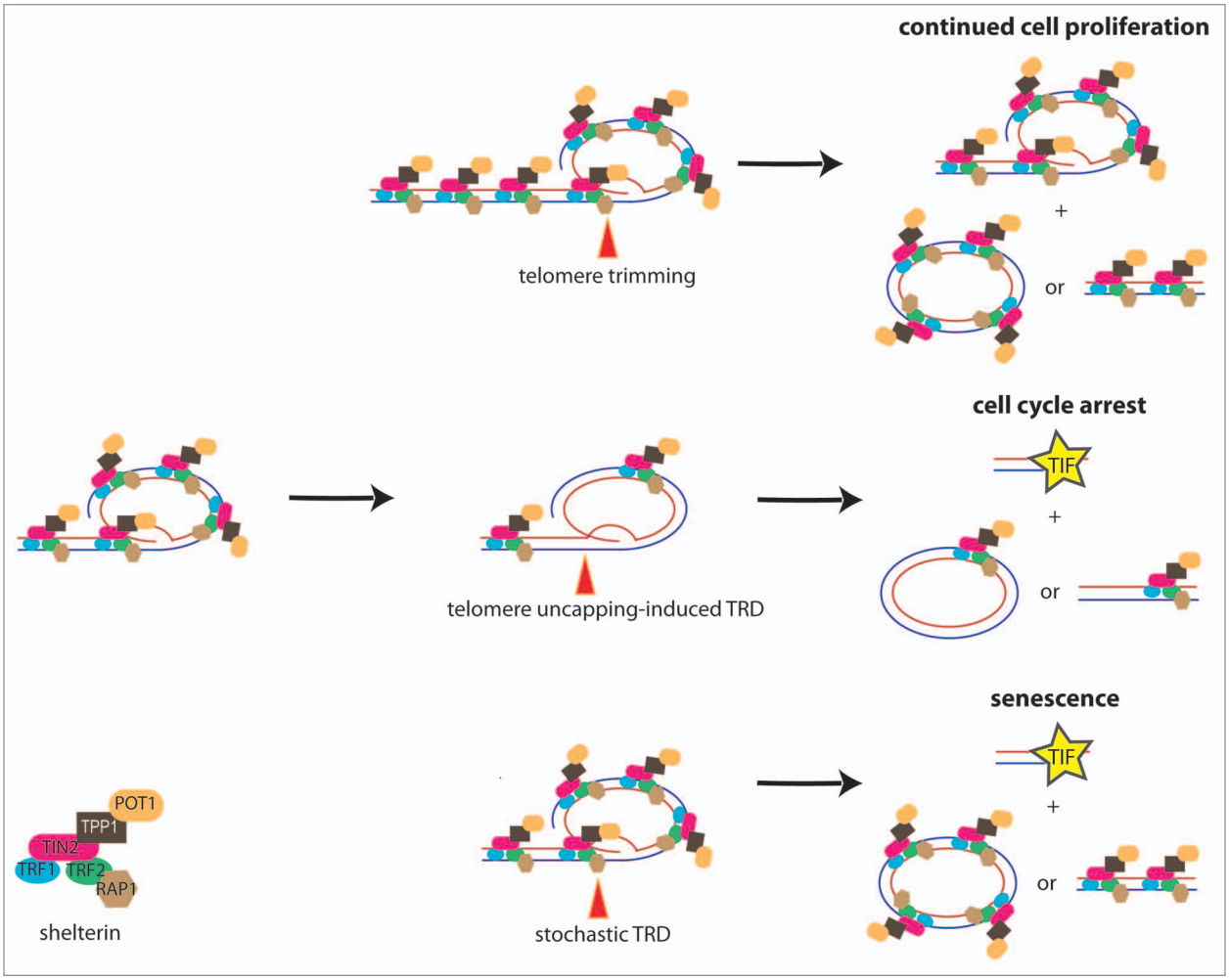

Figure 2. Distinct pathways of telomere rapid deletion (TRD) in mammalian cells. Highly regulated and moderate telomere trimming events occur in response to telomere elongation and are compatible with continued cell proliferation. Telomere uncapping causes catastrophic TRD, and a DNA damage response and ultimately leads to cell cycle arrest. Stochastic TRD events occur at low frequency in response to unknown cellular triggers in cycling cell populations to induce senescence.

TRD in yeast requires the recombination proteins Rad52, Mre11 and Rad50 and can be enhanced by mutations in hyper-recombination protein Hpr1 and in the absence of yKu70.[36-38] Consequently, t-loop formation and subsequent resolution by HR has been implicated as the underlying mechanism.[39] TRD is also responsible for the resetting of telomere length during yeast meiosis.[40] Meiotic deletion occurs similarly to mitotic TRD by an intra-telomeric recombination pathway and is dependent on the meiotic telomere binding protein Ndj1p, which facilitates bouquet structure formation and homologous pairing.[41]

Following the comprehensive characterization of TRD in yeast, TRD has been found to occur in *Arabidopsis thaliana*[42] as well as in mammalian cells, most notably in response to experimentally induced telomere deprotection.[43] Importantly, extrachromosomal t-circles were identified in these studies, providing evidence that HR-mediated resolution of the t-loop contributes to telomere-shortening events.[43] T-circles specifically comprise telomeric repeats and lack subtelomeric sequences.[43] Electron microscopy in conjunction with two-dimensional (2D) gel electrophoresis has demonstrated that telomere length and the size of t-loops and t-circles correlate,[31,43,44] consistent with the notion that t-circles are derived from t-loop deletions.

Telomere uncapping instigates substantial and catastrophic TRD events, sometimes involving the majority of the telomere and resulting in frequent signal-free ends and telomere dysfunction (**Fig. 2**). Despite mechanistic similarities, in this context, TRD events are distinct from regulated telomere trimming and are not compatible with continued cell growth. Expression of a mutant TRF2, TRF2$^{\Delta B}$, which lacks the basic domain required to bind to DNA four-way junctions but retains the ability to bind to the telomere and prevent telomere fusions,[45] resulted in sudden catastrophic telomere deletion events, characterized by the onset of cellular senescence and a telomere DNA damage response.[43] TRD events occurred stochastically, were proportional to telomere length and appeared to be post-replicative, with preferential deletion of the leading strand. Deletion products were visualized as t-circles and were dependent on the HR proteins XRCC3 and NBS1.[43]

Several other functionally related proteins have been demonstrated to play a role in t-circle generation. The origin recognition complex, ORC, localizes to telomere repeats and associates with TRF2 but not TRF2$^{\Delta B}$. Depletion of the ORC subunit ORC2 resulted in loss of telomeric DNA and an increase in dysfunctional telomeres and t-circle formation, comparable to the TRF2$^{\Delta B}$ overexpression phenotype, implicating ORC recruitment by TRF2 in t-loop stability.[46] The Werner syndrome RecQ helicase, WRN, also interacts with TRF2[47,48] and is able to bind to Holliday junction (HJ) recombination intermediates. WRN is required for TRF2$^{\Delta B}$-mediated telomere shortening and also represses the formation of spontaneous t-circles, requiring both its exonuclease and helicase activities.[49]

Deletion of the NHEJ factor Ku86 in human somatic cells results in cell death and is accompanied by massive telomere loss in the form of t-circles, affecting leading and lagging strands equally.[50] This is likely to be the result of decreased chromatin binding of TRF2.[51] Inhibition of TRD by the Ku heterodimer has also been demonstrated in *A. thaliana* and in yeast.[38,52] Extensively shortened telomeres and extrachromosomal telomeric DNA have also been observed in ataxia-telangiectasia (AT) mutated (ATM)-deficient mice and AT patients, suggesting a role for the ATM kinase in TRD.[53] Consequently, proteins involved in telomere capping, t-loop stabilization and telomere length have all been functionally implicated in TRD.

Detection of Regulated Telomere Trimming in Mammalian Cells

Initial indications of a telomere trimming mechanism came from the detection of low levels of t-circles in human cell lines,[43] and, more recently, abrupt telomere shortening (ATS) was identified in normal human fibroblasts.[54] We have demonstrated and characterized telomere trimming in normal human cells of both germline and somatic origin. T-circles were detected first in sperm DNA and second in response to telomerase activation and telomere lengthening in stimulated lymphocytes.[32] In stimulated lymphocytes, the disappearance of elongated telomeric products coincided with the appearance of t-circles. It remains to be determined whether telomere trimming also occurs in response to telomere lengthening in stem cell populations, which also activate telomerase to engage in increased proliferation. The detection of t-circles in the brain, liver, kidney and testes from *Mus musculus castaneus* and *Mus musculus musculus* mice indicates that telomere trimming is conserved across mammalian species.[32]

Extrachromosomal t-circles were found to accumulate in telomerase-positive cancer cell lines following progressive telomere lengthening by exogenous telomerase activity.[27] T-circles were also prevalent in cells utilizing the ALT mechanism of telomere length maintenance.[55] Other ALT markers also overlap with the telomere trimming phenotype, including the presence of long and heterogeneous telomere lengths and nuclear ALT-associated PML bodies (APBs). Nevertheless, these mechanisms appear distinct.[27] Telomerase-positive cells undergoing telomere trimming of artificially elongated telomeres did not display elevated telomere sister-chromatid exchange events and were not permissive to copying of a telomere tag, which is perhaps the most definitive assay by which to determine ALT activity.[27]

It has been proposed that abundant t-circles may enable rolling-circle amplification of telomeric DNA, and that this may contribute to ALT telomere length maintenance;[56] however, to date, no direct experimental data exist to support this hypothesis. Consequently, it seems most likely that the prominent t-circles observed in ALT cells are the product of telomere trimming counteracting extensive recombination-mediated telomere lengthening that occurs in these cells. The detection of APB-like colocalizations between PML protein and telomeric DNA in cells undergoing telomere trimming in the absence of ALT implicates these nuclear foci as potential sites for intra-telomeric length resolution by TRD, or for the accumulation and processing of extrachromosomal telomeric DNA.

Extrachromosomal t-circles are used as a marker of telomere trimming and are conventionally detected by 1D and 2D gel electrophoresis techniques,[54,57] which provide limited sensitivity. A sufficiently quantitative and reproducible alternative assay to analyze telomere trimming is currently lacking. Electron microscopy has been used to identify the existence of t-circles,[44] although this technique is technically challenging and hard to quantitate. Truncated telomeres have been observed by single telomere length analysis (STELA) at multiple telomeres in normal human fibroblasts, at high frequency in the male germline and to a lesser extent in human stem cell populations.[58-60] This technique allows detailed analysis of individual telomere lengths but is unable to characterize deletion products. Future developments in the detection of telomere trimming events will undoubtedly improve our understanding of this mechanism.

It is also pertinent to distinguish the different extrachromosomal telomere DNA species. T-circles are open-circular and predominantly double-stranded entities, which contain nicks and, consequently, cannot undergo rolling-circle replication without prior fill-in processing. Partially single-stranded C-rich or G-rich closed circular telomeric DNA molecules (referred to as C- or G-circles, respectively) have also been identified, and C-circles are particularly abundant in ALT cells. Because these DNA species are partly double stranded, they can be used as self-priming substrates for rolling-circle replication.[61] C-circles are not generated by telomere trimming.[61]

Mechanistic Insights and Speculations Regarding Telomere Trimming under Normal Cellular Circumstances

Telomere trimming appears to be triggered by telomere length, and it is possible that shelterin binding could contribute to telomere length sensing in this scenario. Human telomerase-positive cells maintain a balance between telomere loss and telomere elongation in part by negative feedback of telomere length on telomerase activity, achieved by the binding of TRF1 along the telomere tract.[62-65] Elongation of telomeres by overexpression of exogenous telomerase results in a corresponding increase in telomere-bound TRF1 and TRF2.[66] However, despite the in cis

regulation of telomerase extension, it is currently unclear whether binding of the shelterin complex can similarly regulate telomere length by telomere trimming.

Formation and resolution of the t-loop structure is also an important mechanistic component of telomere trimming. TRF2 is able to enhance strand invasion by inducing DNA untwisting and displays a high affinity for telomere branched or chickenfoot structures.[67,68] TRF2 stimulates the formation and prevents the resolution of telomeric HJs that form at the base of the t-loop, thus stabilizing the telomere secondary structure.[68] Formation of the t-loop intermediate is essential for its subsequent resolution, which involves the HR proteins XRCC3 and NBS1.[43,69] We have shown that XRCC3 is required for the generation of t-circles in cells undergoing telomere trimming, and that in its absence, telomeres are able to further elongate, thus implicating intra-telomeric HR as the underlying mechanism.[32]

The abundance of t-circles in cells undergoing telomere trimming raises questions as to the functional significance and processing of these by-products. T-circles appear to be relatively stable but are unable to self-replicate, meaning that inhibition of telomere trimming results in dilution of t-circles with each successive cell division.[32] It is likely that extrachromosomal telomeric DNA is degraded or processed by the cell, but the molecular details of t-circle metabolism are currently unknown. This also raises the intriguing possibility that t-circles may fulfil a signaling role, potentially providing feedback control of telomerase activity.

Additional mechanistic components of telomere trimming encompass the cell signaling pathways that are likely to be involved in the recognition of overlengthened telomeres and the recruitment of processing factors, for instance HR proteins. Deleted telomere trimming products were identified as extrachromosomal doublets as well as single signals in metaphase cells.[27] This implies that the telomere deletion event takes place during G_2/M of the cell cycle, after DNA synthesis, but prior to sister-chromatid separation, when the two sister-chromatids remain associated with cohesins. It is therefore possible that telomere length is subject to G_2/M checkpoint control. Short or uncapped telomeres are subject to checkpoint control, initiating ATM-mediated phosphorylation of the G_2/M checkpoint kinase, Chk2 and induction of replicative senescence through the activation of p53.[70] However, little is known regarding the downstream cellular responses to overlengthened telomeres. In the absence of DNA damage, TRF2 has been shown to physically interact with Chk2 to inhibit its activation,[71] and further analysis of this association may identify additional regulatory factors underlying telomere trimming.

Telomere trimming generates 5' single-stranded C-rich telomeric DNA at the chromosome termini.[27,32] Similar telomeric overhangs have also been identified in the nematode *Caenorhabditis elegans* and at low levels in human and mouse telomeres.[72,73] C-rich overhangs are prevalent in ALT cells and appear to be a mark of both intra-telomeric HR by telomere trimming as well as ALT-mediated HR, although it is currently unclear whether ss C-rich telomeric DNA is of mechanistic significance or occurs as a recombination intermediate or by-product.[32,73] Nevertheless, this unusual telomeric entity does not appear to instigate a DNA damage response during telomere trimming, further demonstrating the regulated nature of this mechanism.[27]

Potential Implications of Telomere Trimming

Critically short telomeres are recognized as DNA damage sites, triggering a DNA damage response and the onset of senescence.[2] Senescence is determined by the presence of a sufficient number of critically short telomeres, rather than the average telomere length.[2,74,75] Over a decade ago, a theoretical model of TRD was proposed as the mechanism underlying stochastic cellular senescence.[76] TRD was proposed to occur through DNA recombination or nuclease digestion, causing rapid onset of senescence, referred to as sudden senescence syndrome (SSS).[76] In support of this model, low levels of extrachromosomal t-circles have been identified in cycling fibroblast populations, indicative of TRD.[54] Further evidence has been provided by STELA of young and senescent human fibroblast cell populations, which identified large stochastic TRD events superimposed on progressive telomere attrition that increased in frequency in senescent cells.[77,78]

The detection of TRD products in cycling and, more prevalently, in senescent cell types, implicates a dysregulated form of telomere trimming at shortened rather than overlengthened telomeres as an underlying mechanism of stochastic cellular senescence[54,77] (**Fig. 2**). Critical TRD events are likely to trigger a DNA damage response. The accumulation of cellular damage will eventually exceed tenability,[3] and it can be speculated that TRD may act in specific cells as a programmed response to cellular signals such as DNA damage or viral assault, to cause cell cycle exit by rapid and critical telomere shortening. Similarities between senescence-associated TRD and TRD following artificially induced telomere uncapping indicate that telomere uncapping may provide a potential trigger for stochastic cellular senescence. More globally, this may prevent the detrimental accumulation of tumorigenic mutations, viral infections, or the persistence of overlengthened telomeres.

Mechanistic insights into both regulated telomere trimming in the absence of DNA damage and stochastic telomere deletion events, which result in a rapid DNA damage response and cell cycle exit, raise the possibility that telomere trimming could be exploited for therapeutic purposes. First, TRD has the potential to be used in cancer cells to rapidly induce telomere shortening and thus telomere length-mediated cell cycle exit. Second, the telomere length set-point could be manipulated in both cancer cells and in patients suffering from premature aging disorders or short telomere syndromes to control cell proliferative capacity. It is possible that single nucleotide polymorphisms (SNPs) in genes involved in telomere trimming may affect the telomere length set-point in different individuals. Mutations in these genes may be an additional underlying cause of short telomere syndromes. In addition, cancer cells with uncharacteristically long telomeres, such as HeLa1.2.11,[31] may have altered expression of telomere trimming mechanistic factors and may provide further insight into length regulation processes.

Conclusion

Telomere trimming is a conserved mechanism of rapid telomere length resolution that represents an additional factor of telomere length control, alongside gradual telomere attrition and activation of a telomere maintenance mechanism. Telomere trimming is well-regulated, compatible with continued cell growth and takes place in the absence of a DNA damage response. Telomere trimming can be distinguished from aberrant TRD, which, in contrast, triggers a DNA damage response and ultimately initiates cell cycle arrest.

The mechanistic details underlying telomere trimming remain largely elusive. Nevertheless, HR-mediated resolution of the terminal t-loop structure represents a conserved component and in human cells involves the HR protein XRCC3. Further characterization of telomere trimming will provide intriguing possibilities for the manipulation of cell proliferation through modulation of telomere length.

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