

Telomeric RNAs as a novel player in telomeric integrity

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Abstract

Telomeres protect linear chromosome ends from being recognized and processed as double-strand breaks by DNA repair activities. This protective function of telomeres is essential for chromosome stability. Until recently, telomeres have been considered to be transcriptionally silent. This notion was overturned in a series of recent papers that describe the existence of telomeric repeat-containing RNAs (TERRAs) in vertebrates and yeast. Here, we summarize recent developments in this field of telomere research, in particular the possible mechanisms that control TERRA expression.

Introduction and context

Telomerase is a specialized ribonucleoprotein complex that maintains the integrity and stability of eukaryotic chromosome ends [1-3]. Since conventional DNA replication enzymes lack the ability to replicate telomere ends completely, telomere length homeostasis is crucial to avoid premature cellular senescence and age-related disease [2]. Telomere length homeostasis is mediated by telomerase, a cellular telomere-specific reverse transcriptase that uses an internal RNA moiety as a template for the synthesis of telomere repeats. Mammalian telomeres consist of tandem arrays of TTAGGG repeats, and the G-rich strand extends beyond its complement to form a 3' overhang named the G-tail. The G-tail is a substrate of telomerase, which elongates the 3' ends of chromosomes by adding TTAGGG repeats. The complementary strand is filled in by conventional DNA polymerases using primase-derived short RNA oligonucleotides. The TTAGGG repeats are recognized by subunits of a protein complex called shelterin [4]. The shelterin complex, which is specific for telomere ends, allows the cell to distinguish telomeres from sites of DNA damage, thereby protecting the telomeres from inappropriate processing by DNA repair pathways [4].

In mammalian cells, the telomeres and subtelomeric regions are organized into tightly packed nucleosomes

with several markers (e.g., trimethylation of histone H3 at lysine 9 [H3K9me3] and dimethylation or trimethylation of histone H4 at lysine 20), similar to constitutively expressed heterochromatin [5]. Another characteristic heterochromatic mark at mammalian telomeres consists of subtelomeric cytosine DNA methylation within CpG dinucleotides. These modifications may contribute to a heterochromatin-like conformation and the observed telomere position effect, which manifests as an ability to silence the transcription of experimentally inserted subtelomeric reporter genes [6-8].

Major recent advances

The heavily methylated state of subtelomeric regions, the gene-less nature of telomeres, and the observed telomere position effect led to the notion that telomeres are transcriptionally silent [5-8]. This hypothesis was recently challenged when several groups independently demonstrated that subtelomeric and telomeric regions, although devoid of genes, have the potential to be transcribed into telomeric repeat-containing RNA (TERRA) [9,10]. TERRA molecules have been identified in human, mouse, fish, and recently baker's yeast, indicating that TERRAs are conserved among eukaryotes. TERRA transcripts are synthesized from the C-rich strand and polyadenylated, and their synthesis is α -amanitin-sensitive, suggesting that they are transcripts of RNA

polymerase II (RNA Pol II) [9-11]. Further support of a role for RNA Pol II in TERRA transcription derives from the observation that RNA Pol II is enriched at telomeres and associates with the telomeric DNA repeat-binding protein TRF1. TERRA transcripts can be found throughout the different stages of the cell cycle, and their levels are affected by several factors that include telomere length, tumor stage, cellular stress, developmental stage, and telomeric chromatin structure. TERRA most likely regulates telomere length directly since longer telomeres produce more TERRA molecules and thereby create a negative feedback by blocking telomere access to telomerase [10]. However, it is largely unclear how the expression of TERRA and the amount of TERRA transcripts are regulated in the cell.

Insights into the possible regulation of TERRA synthesis came from experiments demonstrating that TERRA synthesis is upregulated by the mixed lineage leukemia (MLL) protein, which is the mammalian homologue of the *Drosophila melanogaster* trithorax protein, and p53 after the induction of telomere uncapping [12]. MLL is cleaved into two fragments by taspase 1: the N-terminal fragment associates the MLL complex to chromosomal sites, whereas the C-terminal fragment contains a SET (Suvar3-9, Enhancer-of-zeste, Trithorax) domain that manifests H3/K4 histone methyltransferase (HMT) activity. Besides its established role as an HMT and general transcriptional regulator, MLL binds to telomeres. The binding of MLL to telomeres has been shown to affect telomere epigenetic status by methylating telomeric H3K4 [12]. MLL also interacts with p53 to increase TERRA synthesis in response to progressive telomere uncapping [12]. Therefore, MLL-dependent TERRA synthesis seems to represent a cellular response to telomere uncapping, thereby preventing DNA damage responses and the induction of cellular senescence.

Besides MLL, other proteins have been suggested to play a role in regulating TERRA levels either directly or indirectly [9,10]. For instance, downregulating the cellular abundance of TRF1 has been shown to decrease the cellular abundance of TERRA without affecting the binding of RNA Pol II to telomeres [9]. Furthermore, TERRA levels are increased in cells that are deficient for certain HMTs, whereas TERRA levels are decreased in cells lacking DNA methyl transferase (DNMT) proteins or Dicer [10]. A role for these proteins in regulating TERRA levels suggests that the heterochromatic state of telomeric regions is important for the regulation of TERRA synthesis.

In addition, several factors (UPF1, EST1A/SMG6, and SMG1) of the mammalian-cell nonsense-mediated

mRNA decay (NMD) machinery are enriched at telomeres and negatively regulate TERRA association with telomeric chromatin [9,13], suggesting a link between TERRA, NMD factors, and telomere stability. Consistent with a role for these NMD factors in telomerase regulation, depletion of any one of these factors from cells not only increases TERRA binding to telomeres but also results in telomere damage and telomere loss [9].

A first clue to how TERRA transcripts might regulate telomerase function came from experiments demonstrating that TERRA-mimetic oligonucleotides are able to inhibit human telomerase *in vitro* [10]. This result suggests that TERRA might directly inhibit telomerase function by duplexing with the RNA moiety of the telomerase.

The finding that TERRA is also present in the genetically tractable *Saccharomyces cerevisiae* was instrumental to further dissecting the processes that regulate TERRA synthesis and TERRA cellular abundance [11]. Using yeast, Lingner and colleagues [11] demonstrated that, in *rat1-1* mutant cells, which are deficient in the Rat1p 5'-to-3' exoribonuclease, much higher levels of TERRA could be detected. This result indicates that Rat1p, which functions in the decay of normal mRNAs and NMD targets, degrades TERRA RNAs [11]. The association of Rat1p with telomeres suggests that Rat1p might directly degrade TERRA RNAs and provides the first evidence for a role of RNA degradation in the regulation of the TERRA transcriptome.

Like mammalian TERRAs, yeast TERRAs are transcribed by RNA Pol II since they (a) fail to accumulate in *rat1-1* mutant cells in which RNA Pol II has been co-inactivated [11] and (b) are polyadenylated by the poly(A) polymerase Pap1p [11]. Since Pap1p adds poly(A) tails to pre-mRNAs during the process of mRNA biogenesis, a possible role during TERRA biogenesis for the same endonucleolytic cleavage factors that generate substrates for poly(A) addition during mRNA biogenesis was tested. Interestingly, TERRA levels were indeed decreased in *rat1-1* cells harboring co-inactivated subunits of the endonucleolytic cleavage factor CF1A, demonstrating the importance of this factor in TERRA biogenesis [11]. These results suggest that TERRA processing involves the Pap1p-mediated addition of poly(A) to TERRA 3' ends that are generated by the same activities that generate pre-mRNA 3' ends. Alternatively, the 5' ends of TERRA that are generated by these activities could be degraded by the 5'-to-3' exonucleolytic activity of Rat1p [11]. Together, these results suggest an intimate interplay between mRNA-processing pathways and TERRA turnover. Further support for the regulation of telomerase

function by Rat1p and TERRA comes from the observation that *rat1-1* mutant cells have short telomeres. TERRA seems to inhibit telomerase activity directly since telomeres were shown to shorten at the same rate in telomerase-deficient cells that did or did not express Rat1p.

How could TERRA regulate telomerase activity? Two scenarios are conceivable. In one, TERRA could duplex with the RNA moiety of telomerase and directly inhibit telomerase activity. In the other, TERRA could associate with telomeric DNA repeats and block telomerase access to the telomeres. Support for the first mechanism comes from experiments described above demonstrating that TERRA-mimetic oligonucleotides inhibit telomerase activity [10]. Support for the second scenario comes from the finding that overexpressing ribonuclease H in *rat1-1* cells results in telomere lengthening. This observation suggests that telomerase function in *rat1-1* cells is inhibited by RNA/DNA hybrids formed between TERRA and telomeric DNA [11]. However, inhibition was less pronounced in cells with endogenous levels of ribonuclease H or in wild-type cells that overexpress ribonuclease H [11]. In theory, both mechanisms could regulate telomerase activity at telomeric DNA ends, and future experiments will undoubtedly address existing uncertainties.

Together, these observations are of medical relevance because abnormal levels of TERRA and the resulting abnormal telomeric phenotypes are likely to affect processes such as hematopoiesis, embryonic development, and stem cell biology. Indeed, increased TERRA levels have already been associated with human disease [14]. As a case in point, mutations within the DNA methyltransferase 3b (DNMT3b) gene cause hypomethylation of satellite and non-satellite repeat regions, including subtelomeric regions, which leads to an autosomal-recessive disease called ICF (immunodeficiency, centromeric region instability, facial abnormalities) syndrome [15]. In cells from ICF patients, hypomethylation of subtelomeric regions was recently associated with increased levels of TERRA synthesis and accelerated telomere shortening [14]. These findings provide a first mechanistic explanation for the observed telomeric phenotype in ICF patients. However, these observations are in contrast to the finding that TERRA levels are decreased in cells deficient for DNMT1, DNMT3a, and DNMT3b [10]. The use of different cell lines (mouse embryonic stem cells versus somatic cells from ICF patients) might account for the discrepancy.

Further support for TERRA RNA regulating telomerase activity comes from the observation that tumor-derived

cell lines that rely on telomerase to maintain telomeres manifest a decreased level of TERRA RNA when compared with tumor-derived cells using the ALT (alternative lengthening of telomeres) mechanism [16]. This decrease correlates mainly with increased cytosine DNA methylation at positions that reside adjacent to telomeres. Therefore, telomerase activity seemed to be tightly linked to subtelomeric DNA methylation, TERRA levels, and a telomeric heterochromatin state that represses telomeric transcription.

Future directions

An important issue that has yet to be addressed is how TERRA is specifically recruited to the telomeric regions. One attractive scenario is that TERRA-specific binding proteins assist in tethering TERRA RNA to telomeric DNA. Good candidates would be telomere-associated proteins that manifest RNA- or DNA-binding activity or both, including the shelterin components TRF1 and TRF2 [17]. Indeed, TERRA is recruited to the telomere via direct interactions with TRF1 and TRF2. TERRA also mediates interactions between TRF2 and the ORC1 subunit of the origin recognition complex (ORC), thereby promoting the recruitment of ORC to telomeres. This association was demonstrated to be important for the enrichment of H3K9me3 and for HP1-mediated heterochromatin formation at telomeres [17]. Therefore, the TRF2-TERRA-ORC1 interactions seem to be critical for telomere structural stability and heterochromatin formation [10,17].

Defining possible roles for other TERRA-regulating proteins such as UPF1, SMG1, or MLL will lead to a deeper understanding of the function of TERRA in telomere biology and the mechanism of TERRA biogenesis. Furthermore, the mode of TERRA-mediated regulation of telomerase function – either via RNA/RNA hybrid formation between TERRA and the telomerase RNA moiety or via blocking telomerase access to the telomeric repeat region – remains to be elucidated. Since certain NMD factors, including UPF1, are able to promote TERRA dissociation from telomeres, UPF1, which is an RNA and DNA helicase, would be an attractive candidate to regulate RNA/DNA hybrid formation. This would directly link NMD factors, TERRA expression, and telomere length homeostasis.

The identification of TERRA promoter regions and transcription start sites will increase our understanding of TERRA biogenesis. This information will help us to understand whether TERRA RNA is required for the heterochromatinization of eukaryotic telomeres, similar to what has been observed for X-chromosome inactivation in females via Xist RNA (X inactive-specific

transcript), another long non-coding RNA [18]. From the patho-physiological perspective, unravelling the role of TERRA at telomeres will enhance our insight into processes such as aging and cancer.

The elucidation of the structures of TERRA RNAs will provide an additional understanding of the functions of these non-coding RNAs. In a recent report, Randall and Griffith [19] proposed a beads-on-a-string model for long telomeric RNA folding, in which individual (UUAGGG)₄ units form quadruplex beads that are connected by UUA linkers. Future studies will undoubtedly examine the structure of RNA-protein complexes formed with the G-rich telomeric RNA.

It will be interesting to see whether further insights into TERRA regulation will advance our understanding of mechanisms that are central to stem cell biology. Along this line, promoting TERRA synthesis might be advantageous during stem cell expansion protocols. Lastly, since telomerase is viewed as an almost universal marker for human cancer, the inhibition of telomerase is an attractive strategy for new cancer therapeutics. The finding that TERRA-mimetic oligonucleotides regulate telomerase activity will hopefully soon translate basic research on TERRA function and regulation into new telomerase-directed therapeutic approaches in cancer.

Notably, TERRA RNA is present in cells without detectable telomerase activity [12,16]. Therefore, it will be important to elucidate roles for TERRA RNA other than in regulating telomerase activity (e.g., in cells that are devoid of any telomere maintenance mechanism). For one, TERRA has been proposed to play an important role in heterochromatin formation and maintenance [17]. Additionally, TERRA could help to regulate telomere DNA replication via its interaction with ORC. Furthermore, although TERRA localizes mainly to telomeric repeats, it is possible that TERRA binds to other chromosomal locations to exert yet-undiscovered functions.

Abbreviations

ALT, alternative lengthening of telomeres; DNMT, DNA methyl transferase; EST1A, mammalian-cell homologue of *Saccharomyces cerevisiae* ever shorter telomeres 1A; H3K9me3, histone H3 lysine 9 trimethylation; HMT, histone methyltransferase; ICF, immunodeficiency, centromeric region instability, facial abnormalities; MLL, mixed lineage leukemia; NMD, nonsense-mediated mRNA decay; ORC, origin recognition complex; RNA Pol II, RNA polymerase II; SET, Su(var)3-9, enhancer-of-zeste, trithorax; SMG1, mammalian-cell homologue of *Caenorhabditis elegans* phosphatidylinositol 3-kinase-related

protein kinase; SMG6, mammalian-cell homologue of *Caenorhabditis elegans* suppressor with morphogenetic defects in genitalia; TERRA, telomeric repeat-containing RNA; TRF1, telomeric repeat-binding factor 1; UPF1, mammalian-cell homologue of *Saccharomyces cerevisiae* up-frameshift mutation 1.

Competing interests

The authors declare that they have no competing interests.

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