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Telomere-specific regulation of TERRA and its impact on telomere stability

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ABSTRACT

TERRA is a class of telomeric repeat-containing RNAs that are expressed from telomeres in multiple organisms. TERRA transcripts play key roles in telomere maintenance and their physiological levels are essential to maintain the integrity of telomeric DNA. Indeed, deregulated TERRA expression or its altered localization can impact telomere stability by multiple mechanisms including fueling transcription-replication conflicts, promoting resection of chromosome ends, altering the telomeric chromatin, and supporting homologous recombination. Therefore, a fine-tuned control of TERRA is important to maintain the integrity of the genome. Several studies have reported that different cell lines express substantially different levels of TERRA. Most importantly, TERRA levels markedly vary among telomeres of a given cell type, indicating the existence of telomere-specific regulatory mechanisms which may help coordinate TERRA functions. TERRA molecules contain distinct subtelomeric sequences, depending on their telomere of origin, which may instruct specific post-transcriptional modifications or mediate distinct functions. In addition, all TERRA transcripts share a repetitive G-rich sequence at their 3′ end which can form DNA:RNA hybrids and fold into G-quadruplex structures. Both structures are involved in TERRA functions and can critically affect telomere stability. In this review, we examine the mechanisms controlling TERRA levels and the impact of their telomere-specific regulation on telomere stability. We compare evidence obtained in different model organisms, discussing recent advances as well as controversies in the field. Furthermore, we discuss the importance of DNA:RNA hybrids and G-quadruplex structures in the context of TERRA biology and telomere maintenance.

1. Introduction

Transcription of repetitive sequences is associated with repeat instability in different organisms [1–3]. This effect may be due to the intrastrand secondary structures that these sequences form, such as DNA:RNA hybrids and G-quadruplexes (G4s) [4,5]. Furthermore, transcription-replication conflicts can promote replicative stress, leading to fork stalling or collapse, rendering these genomic regions particularly unstable [6]. Indeed, repetitive sequences represent a challenge to the fidelity of the DNA replication machinery, and they are often associated with common fragile sites prone to breaks, gaps, and abnormal chromosomal structures when cells experience replicative stress [4,5].

Telomeres resemble common fragile sites, and require specialized DNA binding proteins to assist the replication process [7–10]. In mammals, telomeric DNA is composed of TTAGGG repeats that range in length from 5-15 kb in humans to more than 50 kb in mice [11]. Several species, even if evolutionarily distant, share the same TTAGGG telomeric sequence, such as the protozoa *Trypanosoma brucei* [12–14]. *Caenorhabditis elegans* telomeres consist of TTAGGC repeats ranging from 2 to 7 kilobases in length [15]. In the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* telomeres consist of irregular $TG_{1–3}$ and $T_{1–2}ACA_{0–1}C_{0–1}G_{1–6}$ repeats, respectively, extending for approximately 300 bp in both species [16–18]. Telomeric DNA displays an asymmetric distribution of cytosines and guanines between the two strands. The G-rich strand has a propensity to form G4s [19] and it generally extends over the C-rich strand forming a 3′ overhang of variable size [20,21], approximately 50–300 nucleotides in mammals [22].

Transcription of the C-rich telomeric strand generates a class of telomeric repeat-containing RNAs called TERRA, which has been investigated in multiple organisms [23,24]. TERRA transcription starts from subtelomeric sequences, the genomic regions adjacent to the telomeric repeats, and proceeds towards the chromosome ends, terminating within the telomeric repeat tract [25]. Thus, any given cell expresses different populations of TERRA molecules with distinct

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subtelomeric sequences at their 5′ end and the same G-rich telomeric sequence at their 3′ end. These features are shared by TERRA molecules expressed by all the organisms studied to date [25,26].

Several lines of evidence indicate that TERRA transcription is important to telomere function and stability. TERRA transcripts have been proposed to regulate telomeric chromatin [27,28], DNA replication at telomeres [29], chromosome end processing [30], and the activity of telomerase [31–33], the reverse transcriptase enzyme that elongates telomeres during the S-phase [34,35]. The telomeric tract of TERRA interacts with numerous proteins, including telomere-binding proteins [27,36–39], enabling TERRA to act as a scaffold, reinforcing protein-protein interactions as well as promoting the recruitment of enzymes to telomeres [40,41]. Furthermore, TERRA transcripts form telomeric DNA:RNA hybrids, or R-loops, and are capable of folding into RNA G4 structures (rG4) [42–50]. Both these structures have been shown to mediate interactions with TERRA-binding proteins [42,44,51,52], and R-loops have also been proposed to facilitate homologous recombination at telomeres, promoting telomere homeostasis by alternative telomere lengthening mechanisms known as ALT [43,49,50,52,53–55]. Finally, extratelomeric functions and even extranuclear functions have been proposed for TERRA [56–59]. Supporting the many roles ascribed to TERRA, it has been observed that its levels markedly vary among telomeres of a given cell type, and different chromosome ends regulate TERRA expression by distinct mechanisms. The telomere-specific regulation of TERRA may help coordinate the numerous functions of these telomeric transcripts and prevent the potentially deleterious effects of telomeric DNA transcription on telomere stability.

Here we review the current knowledge of the mechanisms regulating TERRA levels and the impact of the telomere-specific control of TERRA on telomere function and stability. Furthermore, we discuss the importance of R-loops and G-quadruplex structures in the context of TERRA biology and telomere maintenance.

2. Telomere-specific regulation of TERRA

2.1. Human

Since the early studies on TERRA, it became clear that its levels vary between cell types. Among the human cell lines analyzed by northern blot, U2OS cells, an ALT-positive cancer cell line, displayed higher TERRA signal than HeLa cells, a telomerase-positive cancer cell line, or primary lung fibroblasts [23,24]. These analyses relied on the use of probes recognizing the G-rich telomeric repeat tract of TERRA, thereby enabling the detection of all TERRA transcripts in cells. Thus, despite the challenges posed by the quantification of these highly repetitive and heterogeneous transcripts through northern blotting [60], these observations indicated that different cell types express different levels of G-rich telomeric repeat-containing RNAs. Soon it was reported that several factors contribute to the regulation of TERRA expression in humans, primarily the methylation state of the subtelomeric regions immediately adjacent to telomeres, which inversely correlates with TERRA levels [61]. Hypomethylation of subtelomeres was proposed to act as a driving mechanism for the up-regulated TERRA levels detected in ALT cancer cell lines, as compared to telomerase-positive cells showing higher subtelomeric DNA methylation [61]. In line with these findings, TERRA levels were found elevated in lymphoblastoid cell lines from patients of immunodeficiency, centromeric instability, facial anomalies (ICF) syndrome, a rare autosomal disease caused by mutations in the DNA methyltransferase gene DNMT3b, resulting in hypomethylation of subtelomeric regions [62]. Furthermore, the telomerase-positive HCT116 cells double knock out for the DNMT1 and DNMT3b genes express elevated TERRA levels, compared to the same WT cells [63]. Intriguingly, TERRA promoter regions were identified within subtelomeric sequences called TelBam3.4 and TelSau2.0, sharing three repetitive elements consisting of 61, 29, and 37 bp-long sequences (61–29–37 repeats) and containing CpG dinucleotides [63] (Fig. 1A). These sequences are present in at least 20 chromosomes and their CpG methylation results in TERRA repression. The use of a 5′-RACE protocol that specifically amplifies 5′ capped molecules enabled the identification of a TERRA transcription start site (TSS) downstream of the 37 bp repeat, mapping at about 250 nucleotides from the telomeric repeat tract in different human cancer cell lines [63]. Recently, RT-qPCR analyses using a telomeric repeat-specific reverse transcriptase (RT) primer and chromosome-specific qPCR primer pairs, mapping within the subtelomeric sequence downstream of the TERRA TSS, confirmed transcription from several chromosome ends and that deficiency in DNA methyltransferases leads to TERRA up-regulation only from the subtelomeres containing CpG sequences [64]. This approach also enabled an estimate of the average number of TERRA molecules from specific chromosome ends per cell, confirming their different levels across cell types, and highlighting the distinct regulation of TERRA transcription between telomeres containing the 61–29–37 repeats and the ones devoid of CpG-containing promoters [64]. Over the years, numerous studies have reported mechanisms that contribute to the telomere-specific regulation of TERRA, as discussed below.

Several human subtelomeres contain binding sites for the transcriptional regulator and chromatin organizing factor CTCF overlapping with the interacting elements of the cohesin subunit Rad21 and mapping within 1–2 kb from the telomeric repeat tract [65](Fig. 1A). CTCF binding was found to contribute to the promoter activity of the 61–29–37 repeats, and shRNA-mediated downregulation of this factor or Rad21 resulted in impaired RNA polymerase II (PolII) binding to the TERRA promoters and consequent downregulation of TERRA levels from these telomeres in U2OS and HCT116 cells [65]. In glioma cell lines the binding of cohesin to subtelomeres is at least in part dependent on the chromatin remodeling complex ATRX, a member of the SNF2 family of helicase/ATPases. In these cells, ATRX is found enriched at subtelomeric regions by chromatin immunoprecipitation experiments (ChIP), and its downregulation by RNAi results in decreased recruitment of PolII and the cohesin subunit SMC1 to subtelomeres with consequent TERRA downregulation [66].

Intriguingly, RNA-seq experiments from nuclear RNA enriched for TERRA molecules also revealed the presence of TERRA TSSs mapping 5–10 kb from the telomeric repeat tract, in proximity of CTCF binding sites and CpG-rich sequences, on 10 chromosome ends in HeLa cells [40] (Fig. 1A). These findings indicate that different telomeres can initiate transcription of TERRA at various distances from the telomeric repeat tract, using proximal (61–29–37 repeats) or distal promoters (5–10 kb from telomeric repeats), in line with the high heterogeneity in length of TERRA molecules observed by northern blot [23,24].

In silico analyses revealed the presence of different families of transcription factors predicted to bind TERRA promoters [40]. The activity of transcription factors regulating TERRA expression in a telomere-specific manner has been investigated in a number of studies that highlighted the importance of TERRA transcription regulation in telomere stability. Indeed, p53 binding sites were identified on 11 subtelomeres in HCT116 cells between 2 and 12 kb distance from the telomeric repeat tract [67]. Subtelomeric sequences containing p53-responsive elements can act as promoters for TERRA in response to cellular stress upon etoposide treatment or serum starvation (Fig. 1A and B). Etoposide treatment of p53 knock-out cells showing impaired TERRA expression resulted in increased DNA damage at telomeres and telomeric DNA instability. Interestingly, deletion of a p53 binding site within subtelomere 18q prevented TERRA transcription upregulation from this subtelomere and resulted in increased DNA damage at this telomere upon treatment [67]. These findings indicate that telomere-specific regulatory mechanisms of TERRA are important to maintain the stability of the TERRA transcribing telomere. Similarly, the transcription factor heat shock factor 1 (HSF1) associates with TERRA promoters in a telomere-specific manner to regulate TERRA transcription upon heat shock [68](Fig. 1A and B). DNA damage at telomeres is

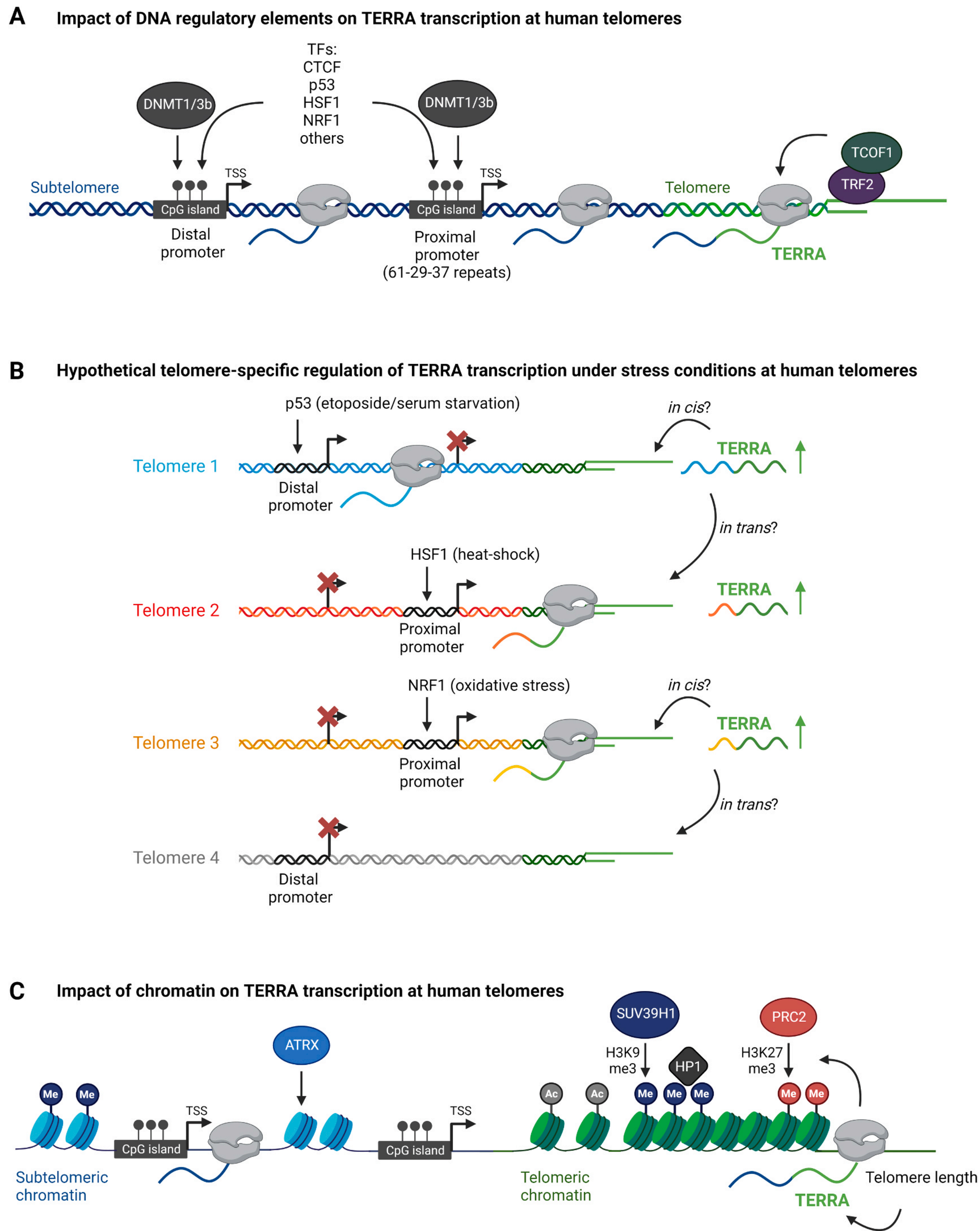


Fig. 1. Mechanisms of telomere-specific regulation of TERRA transcription in humans. **A)** Impact of DNA regulatory elements on TERRA transcription at human telomeres. A subset of human telomeres contain distal and/or proximal (61-29-37 repeats) promoters overlapping with CpG-rich sequences. TERRA promoters are regulated by DNA methyltransferases DNMT1 and DNMT3b (DNMT1/3b), and transcription factors (TFs). TRF2 and TCOF1 interact at telomeres and act as repressors of TERRA transcription. **B)** Hypothetical telomere-specific regulation of TERRA in humans under different stresses, showing the differential contribution of telomeres to the cellular pool of TERRA transcripts. Telomere 1 contains a distal promoter responsive to etoposide or serum starvation through p53 activation, telomere 2 contains a proximal promoter that is activated by HSF1 upon heat-shock, while telomere 3 presents a proximal promoter responsive to NRF1 under oxidative stress. TERRA molecules transcribed under these conditions could regulate telomere stability *in cis* at their telomere of origin or *in trans* at other telomeres. Telomere 4 does not contain TERRA promoters responsive to the abovementioned transcription factors, as a consequence, transcription is not activated and telomere 4-TERRA is not expressed under the mentioned conditions. However, TERRA transcribed from other telomeres can act *in trans*, relocating from its telomere of origin to other chromosome ends, including telomere 4. **C)** Impact of subtelomeric and telomeric chromatin on TERRA expression. The histone methyltransferase SUV39H1 regulates TERRA expression at telomeres by depositing the trimethylated H3K9 histone mark. H3K9me3 enrichment at telomeres recruits HP1 resulting in TERRA repression. Moreover, TERRA interacts with PRC2 facilitating H3K27me3 at telomeres which may participate in TERRA repression. In addition, TERRA expression is regulated by histone acetylation on telomeric chromatin. Subtelomeres are enriched in H3K9me3, which induces chromatin compaction, inhibiting TERRA transcription. The histone remodeler ATRX acts as a positive factor for TERRA transcription at subtelomeres. Telomere length was shown to regulate TERRA transcription, as short telomeres are associated with increased TERRA levels in the cell.

markedly increased upon heat shock in HSF1 deficient cells showing no induction of TERRA transcription. Furthermore, the antioxidant transcription factor nuclear respiratory factor 1 (NRF1) was found to bind several subtelomeric regions in proximity to the CpG-containing TERRA promoters to regulate TERRA transcription in human myotubes [69] and human cancer cell lines [69,70] (Fig. 1A and B). NRF1 downregulation by siRNA leads to telomeric damage in human hepatoma Huh-7 cells [69]. It was proposed that the NRF1-mediated TERRA transcription operates as an antioxidant response to protect chromosome ends from oxidative stress. Altogether, these findings highlight the importance of telomere-specific regulation of TERRA transcription to maintain telomere stability under stress conditions. TERRA promoters have been shown to be responsive to the cellular levels of other transcription factors, including ZNF48, ZFX, EGR1, and PLAG1, the binding sites of which were found in large numbers on human subtelomeres both CpG positive and negative [64]. Understanding the mechanisms that regulate the activity of the transcription factors controlling TERRA expression will be instrumental to elucidate the function of this RNA.

Transcription factors control TERRA expression by mediating changes in the chromatin state of subtelomeric regions and telomeres and by regulating PolII recruitment to chromosome ends, as reported in different studies [65,67] (Fig. 1C). In line with this evidence, the presence of heterochromatic marks within the telomeric tract of human chromosomes has been shown to regulate TERRA expression. In particular, trimethylated H3K9 and recruitment of HP1α at telomeres result in TERRA repression in telomerase-positive cells [28]. Conversely, mono-, di- and trimethylated telomeric H3K4 positively correlate with TERRA levels [26,71]. Treatment of HeLa cells with the histone deacetylase inhibitor Trichostatin A (TCA) resulted in increased TERRA levels supporting the important role of chromatin in the regulation of TERRA expression [72]. Although it should be noted that while human subtelomeric regions and telomeres were found to be enriched with the heterochromatic H3K9me3 mark, and subtelomeric sequences being highly CpG methylated [28,73–75], other observations reported that human telomeres are generally not enriched in H3K9me3, while they contain H4K20me1 and H3K27ac marks [76]. Moreover, reduced telomeric chromatin compaction has been reported in ALT cells [77]. This conflicting evidence may stem from the use of different cell lines, the different states of telomeres (long versus short), and the alternative approaches used to study the telomeric chromatin (i.e. ChIP-dot blot versus ChIP-PCR). Thus, the epigenetic nature of the human telomeres remains to some extent controversial.

Importantly, it has been shown that telomere length also influences TERRA transcription (Fig. 1C). Cells derived from ICF patients displaying high TERRA levels and hypomethylated subtelomeres show abnormally short telomeres, a condition that may contribute to the increased expression of TERRA detected in these cells [78,79]. Indeed, over-elongated telomeres by ectopic expression of the telomerase catalytic subunit hTERT or hTERT and the telomerase RNA subunit hTR repress TERRA expression in different cancer cell lines and human fibroblasts [28]. In this context, TERRA repression is for a good part due to the increased density of trimethylated H3K9 and recruitment of HP1α detected at longer telomeric repeat tracts, without spreading within subtelomeric regions. Downregulation of HP1α or of the H3K9 histone methyltransferase SUV39H1, abolished, or alleviated, the telomere length-dependent repression of TERRA. It is notable that longer telomeres were reported to express TERRA transcripts containing longer 3′ end sequences, indicating that the length heterogeneity of TERRA also stems from its 3′ end [28]. The length of the telomeric tract of the transcripts and the levels of TERRA molecules can have functional consequences on the chromatin state of telomeres. Indeed, the G-rich telomeric sequence of TERRA interacts with HP1α, H3K9me3, and SUV39H1 [27,40]. An interaction between the telomeric repeat tract of TERRA and chromatin remodeling complexes, such as MORF4L2, a component of the NuA histone acetyltransferase complex, and ARID1A, a component of the BAF-type SWI/SNF nucleosome remodeling complex has also been reported [38]. The TERRA G-rich sequences also associate with the histone methyltransferase Polycomb Repressive Complex 2 (PRC2) through direct binding with its components EZH2 and SUZ12 [80]. As TERRA transcripts localize to telomeres, these interactions can contribute to regulate chromatin at chromosome ends [81]. The telomeric localization of TERRA may also influence telomerase activity as TERRA interacts with hTERT and hTR [33]. However, TERRA function in the regulation of human telomerase remains to be defined. Indeed, TERRA-mimicking oligonucleotides were found to inhibit human telomerase activity *in vitro* [33]. The same lab also reported that this *in vitro* telomerase inhibition could be prevented by the TERRA-interacting protein hnRNPA1, belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP) family of RNA binding proteins [82]. However, in cells, inducible expression of TERRA from an engineered telomere does not affect its elongation by telomerase [83] and elevated TERRA levels can correlate with high telomerase activity, such as during the generation of human induced pluripotent stem cells (hIPSCs) [84,85]. Further studies will need to be performed in order to ascertain the mechanisms of TERRA-mediated telomerase regulation in human cells.

Importantly, TERRA transcription is also controlled by the telomere-binding protein TRF2, acting as a repressor of TERRA in human cells [40,41,71]. TERRA repression is exerted through the TRF2 homodimerization TRFH domain which has been shown to mediate telomeric DNA condensation [40,86]. Furthermore, it has been recently shown that TRF2 interacts with the nucleolar factor TCOF1, promoting its recruitment to telomeres during S-phase. At telomeres, TCOF1 can repress TERRA transcription through interaction with PolII [87] (Fig. 1A). TCOF1 downregulation results in increased levels of TERRA and telomeric R-loops, as well as RPA detection at telomeres, indicative of replication stalling. Intriguingly, TRF2 interacts with TERRA through multiple domains including its basic domain [27], an interaction that requires rG4 formation of the TERRA sequence [44]. The involvement of R-loops and rG4 in TERRA biology will be discussed in dedicated paragraphs.

2.2. Mouse

As observed in the human context, differences in TERRA levels were reported between mouse cell lines and among mouse tissues [23,24]. Despite this analogy, from early studies on TERRA it soon became apparent that the biology of these RNAs presents several distinctions between humans and mice. RNA fluorescence *in situ* hybridization (RNA FISH) experiments showed that murine TERRA molecules cluster into a few foci localizing in proximity of the X/Y chromosomes [24,88]. These findings were in contrast with the numerous nuclear TERRA RNA FISH foci detected in human cells, a subset of which localized at telomeres [23]. Surprisingly, RNA-seq experiments from TERRA-enriched RNA using biotinylated oligonucleotides complementary to UUAGGG repeats have revealed that more than 90% of TERRA transcripts are expressed from pseudoautosomal regions (PAR) mapping within subtelomeres of X/Y chromosomes in mouse embryonic stem cells (mESCs) [89]. For this reason, these transcripts were called PAR-TERRA. As PAR regions contain internal telomeric $(TTAGGG)_n$ repeats, PAR-TERRA molecules are detected by RNA FISH and northern blot using C-rich telomeric probes even if their transcription may not reach the telomere. PAR-TERRA has been shown to act *in cis*, promoting homologous X-chromosomes pairing [89]. Furthermore, in the same study, the authors used a CHIRT protocol from the combination of ChiRP (CHromatin isolation by RNA Purification) and CHART (Capture Hybridization Analysis of RNA Targets) methods to investigate PAR-TERRA genomic binding sites in mESCs and mouse embryonic fibroblasts (MEFs). This approach revealed an enrichment of PAR-TERRA to subtelomeric and telomeric regions, suggesting that these transcripts may play roles at telomeres. PAR-TERRA binding sites were also observed at extra-telomeric sites. Not surprisingly, PAR-TERRA and TERRA CHIRT profiles, obtained using PAR-specific or $CCCTAA_n$ capture probes

respectively, overlapped considerably [89]. In a different study from the same group, downregulation of TERRA in mESCs was achieved using antisense oligonucleotides (ASOs) with locked nucleic acids (LNA) chemistry and a gapmer design to promote RNase H-mediated degradation of telomeric repeat-containing RNAs [56]. Transcriptomic analyses revealed gene expression deregulation after 12 h of TERRA ASO transfection. In particular, genes overlapping or mapping nearby (± 10 kb distance) TERRA binding sites resulted up- or down-regulated, leading the authors to consider them as TERRA target genes. Mass spectrometry analyses of TERRA-interacting proteins identified several chromatin-regulating factors in mouse cells, including ATRX, as well as histone-modifying enzymes such as the EZH2 subunit of the polycomb PRC2, SETD2, and SETDB1 [56]. Interestingly, by comparing coverage densities of the TERRA CHIRT profile with available ChIP-seq data, the authors found a correlation between TERRA and ATRX binding as well as the presence of H3K27me3, a histone modification placed by PRC2, in mESCs (Fig. 2A). Furthermore, TERRA depletion was associated with the downregulation of ATRX-TERRA target genes, while ATRX knockdown resulted in the opposite effect [56]. A direct interaction between a TERRA-mimicking oligonucleotide and recombinant ATRX was confirmed *in vitro* by electrophoretic mobility shift assay (EMSA). In these experiments, TERRA oligonucleotides were found to compete with ATRX-DNA complex formation, suggesting that TERRA may antagonize ATRX-DNA interactions. These findings suggest that TERRA can control gene expression and regulate chromatin formation in mouse cells by multiple means, including influencing ATRX and PRC2 activities (Fig. 2A). Interestingly, recruitment of ATRX to chromosome ends is at least in part dependent on the telomeric H3K9me3 mark deposited by the histone methyltransferase SETDB1 in mESCs [90]. Loss of H3K9me3 in *Setdb1* knock out cells resulted in reduced telomeric binding of ATRX and significant decrease in serine 2-phosphorylated RNA PolII and H3K36me3 transcription elongation mark at telomeres, indicative of impaired transcription. Supporting these observations, northern blot analyses revealed the presence of very short TERRA species in *Setdb1* knock out cells (Fig. 2A). It will be interesting to investigate the mechanisms by which SETDB1 and H3K9me3 regulate transcription elongation at telomeres.

Given that TERRA and PAR-TERRA species share a G-rich telomeric repeat sequence, ASOs targeting these sequences are expected to downregulate both RNAs. Furthermore, TERRA and PAR-TERRA cannot be discriminated by northern blot, RNA dot blot, or RNA-FISH procedures employing C-rich telomeric probes. Yet, based on the previously discussed evidence, the vast majority of the TERRA/PAR-TERRA signal detected by these techniques is to be attributed to PAR-TERRA.

That said, a previous study detected TERRA transcribed from the mouse telomere 18q using qPCR primer pairs as well as northern blot and RNA FISH probes specific to the subtelomeric region of this chromosome [91]. Results from this work suggested that the majority of TERRA arises from telomere 18q. The detection of TERRA from this same telomere was confirmed in a recent work that reported TERRA expression from two additional intrachromosomal loci containing telomeric repeats within chromosomes 2 and X [92]. However, RNA-seq and RT-qPCR analyses indicated that PAR-TERRA levels are much higher than the other TERRA transcripts analyzed in several mouse cell lines. A different study also reported the expression of TERRA from 7 q-arm subtelomeres, including telomere 18q, in mouse induced pluripotent stem cells (mIPSCs) and mouse embryonic stem cells (mES) [93]. Also here, the relative abundance of telomere 18q TERRA was not higher than the other TERRA species detected by RT-qPCR in mESCs and mIPSCs [93]. Whether under certain conditions the expression of telomere 18q TERRA increases remains to be defined. In this regard, different mechanisms regulating the transcription of TERRA populations, including telomere 18q have been described. Indeed, RT-qPCR analyses have shown that mouse mesenchymal stem cells depleted of the transcription factor SNAIL express increased levels of TERRA from telomeres 2q, 11q, and 18q [94] (Fig. 2A). SNAIL regulates the epithelial-mesenchymal transition (EMT), a process that promotes the progression of epithelial tumors and that is induced by the upregulated levels of this transcription factor. Accordingly, TERRA levels decrease during EMT, when this process is induced in mouse mammary gland cells (NMuMG). During this condition, TERRA downregulation correlates with occurrence of DNA damage at telomeres [94]. These findings are in line with the inverse correlation between TERRA transcription and telomeric DNA damage observed in human p53, HSF1 and NRF1-depleted cells (see above).

The expression levels of TERRA from telomeres 18q and 10q were also found reduced in mouse osteoblasts haploinsufficient for the transcription factor Rb1 ($Rb1^{+/\Delta 19}$), a well-known tumor suppressor gene (Fig. 2A). Rb1 haploinsufficiency was accompanied by altered chromatin at telomeres, including decreased levels of acetylated H3K9 (H3K9Ac), H4K16Ac, and H4K20me3, and increased H3K9me3 [95]. Interestingly, RB1-mediated regulation of TERRA may also be conserved in humans [95].

Northern blot and RNA dot blot experiments revealed that the TERRA/PAR-TERRA signal is higher in miPSCs and mESCs, as compared to MEFs [96,91,93]. This difference correlated with decreased detection of the heterochromatic marks H3K9me3, H4K20me3, and HP1 at telomeres of IPSCs and ESCs [96]. Accordingly, mouse cells knock out for *Suv39h* or *Suv4–20 h* histone methyltransferase genes display elevated PAR-TERRA/TERRA levels [24] (Fig. 2A). Although, it has been recently suggested that SUV39H1 preferentially methylates H3K9 at pericentromeric regions and that the effects of *Suv39h* loss may be due to a relocalization of HP1 from pericentromeres to telomeric regions stimulating SETDB1 recruitment and H3K9me3 formation at chromosome ends [90]. Thus, the molecular mechanisms regulating H3K9 formation at telomeres and how it controls TERRA expression may need further investigations. Altogether this evidence suggests that the chromatin state at telomeres regulates TERRA transcription in mice, as observed in human cells. Despite these findings, several differences are observed in the TERRA regulation between these two species. First, while TRF2-depleted telomeres express higher TERRA levels in human cells, transcription of mouse telomeres is not increased upon TRF2 depletion in immortalized MEF [92]. Furthermore, in contrast to humans, mouse TERRA levels are reduced in cells deficient for DNMT1 and DNMT3a/3b [93]. These observations suggest that the mechanisms of TERRA regulation are not fully conserved between species. The distinct regulation of TERRA may be influenced by the different lengths of telomeres, which are much longer in the *Mus musculus* strains most frequently used in laboratory experiments, or their different heterochromatic nature, with mouse telomeres enriched with H3K9me3 and H4K20me3 and recruiting high levels of HP1 [97,98]. Furthermore, while human subtelomeres are enriched in CpG dinucleotides, mouse subtelomeres are relatively CpG poor and differences in their methylation levels do not correlate with TERRA expression [93].

Despite the differences in the regulation of TERRA transcription and the distinct genomic origins between mice and humans, the conservation in the G-rich telomeric sequences may underline similar roles in both species. Indeed, several TERRA binding proteins are conserved between mice and humans, including hnRNPs, the BLM helicase, and the paraspeckle component NONO [27,36,92].

2.3. Yeast

The telomere-specific regulation of TERRA transcription has been well described in yeast. In *S. cerevisiae*, TERRA transcription is repressed by the activity of the histone deacetylase Sir2 (Silent Information Regulator 2) as well as Sir3 and Sir4 and by the Rap1-interacting factor 1 and 2 (Rif1 and Rif2) complexes [99]. Sir2 mediates the removal of acetyl groups from lysine 16 of histone H4 (H4K16Ac) and lysines 9 and 14 of histone H3 (H3K9Ac and H3K14Ac) resulting in a repressive chromatin state [100] (Fig. 2B). Histone deacetylation occurs within subtelomeric regions as the *S. cerevisiae* telomeric repeats are not

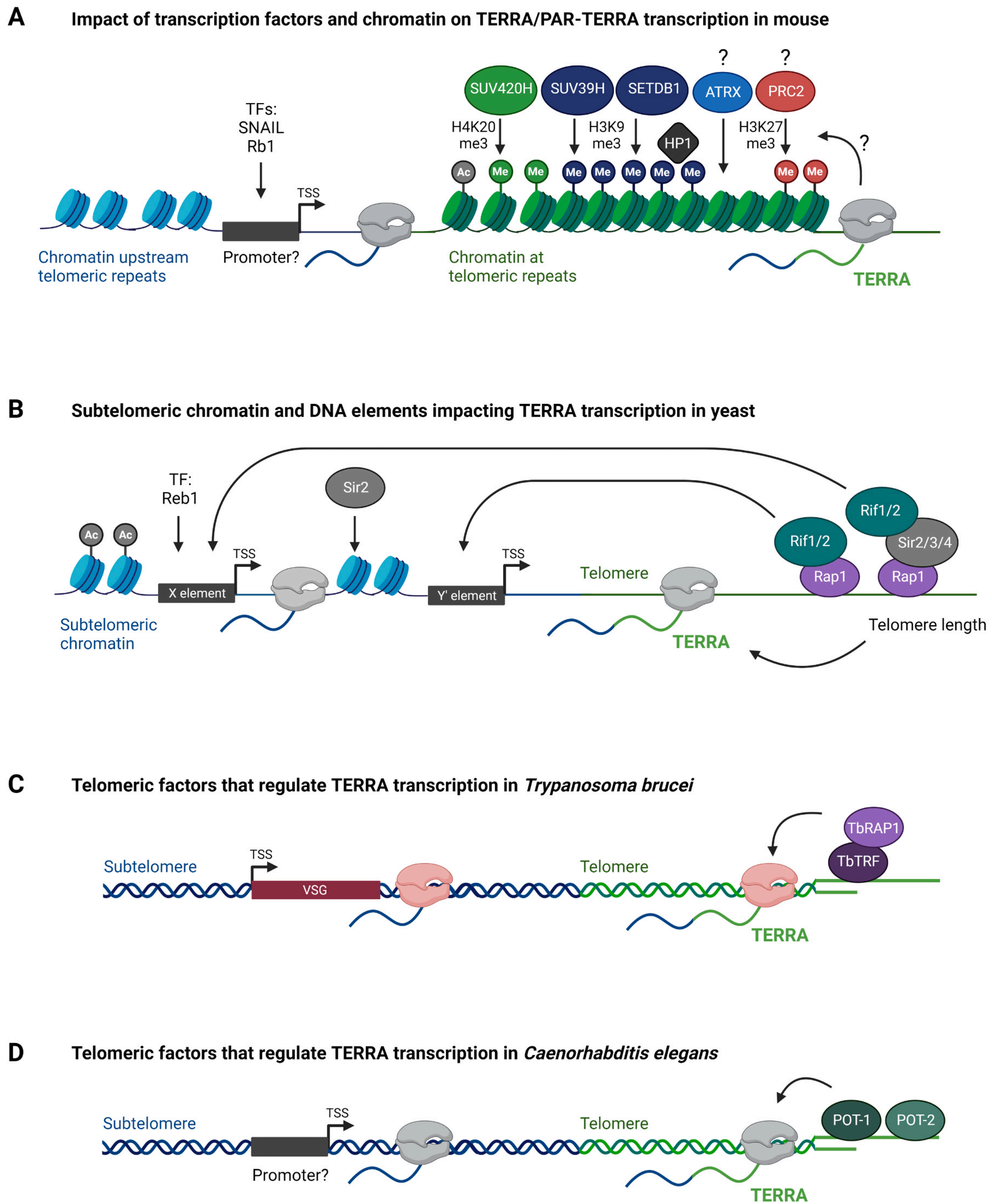


Fig. 2. Mechanisms controlling TERRA transcription in different model organisms. **A)** Impact of transcription factors and chromatin on TERRA/PAR-TERRA transcription in mouse. The transcription factors SNAIL and Rb1 have opposing roles on the regulation of TERRA transcription, acting as a repressor and activator of TERRA, respectively. SUV39H and SUV420H repress TERRA transcription through H3K9me3 and H4K20me3 histone marks respectively at mouse telomeres. A different study reported that SETDB1 is responsible of H3K9me3 deposition at telomeres and acts as a TERRA activator. Telomeres are enriched in H3K27me3 marks, deposited by PRC2, a TERRA-interacting factor. TERRA may thus contribute to PRC2 activity at telomeres resulting in a feedback regulatory loop. ATRX, another TERRA-interacting factor, may play a role in TERRA transcription regulation. In addition, evidence suggests that histone acetylation contribute to TERRA transcription regulation. **B)** Subtelomeric chromatin and DNA elements impacting TERRA transcription in budding yeast *S. cerevisiae*. TERRA transcription is repressed at telomeres containing only X-elements by Sir2/3/4 and Rif1/2 complexes interacting with Rap1; at Y'-element-containing telomeres, TERRA is repressed by Rap1-interacting Rif1 and Rif2. Sir2 can repress TERRA by deacetylating histones and stabilizing the +1 nucleosome downstream TERRA TSS. The transcription factor Reb1 binds to X-elements and contributes to TERRA transcription termination. Telomere shortening associates with increased TERRA levels in both budding and fission yeasts (only budding yeast is shown). **C)** Telomeric factors that regulate TERRA transcription in *Trypanosoma brucei. Tb*TERRA is transcribed as a result of transcription readthrough of the subtelomeric variant surface glycoprotein (VSG) gene transcription by RNA PolI. TbTRF and TbRAP1 repress TERRA expression. **D)** Telomeric factors that regulate TERRA transcription in *Caenorhabditis elegans*. TERRA is repressed by POT-1 and POT-2 that bind the C-rich and G-rich telomeric overhang respectively.

organized in nucleosomes [101,102]. These factors are all recruited to telomeres through their interaction with the carboxy-terminal domain of the repressor/activator protein Rap1, a major telomere-binding protein in budding yeast [103,104].

Nevertheless, intriguingly, while Rap1 associates with double-stranded telomeric repeats at all chromosome ends [104,105], regulation of TERRA transcription occurs in a telomere-specific manner and it depends on the subtelomeric sequences of chromosomes. At telomeres that contain only X-elements, subtelomeric repetitive sequences composed of X-core and associated elements found at all chromosome ends [106], Rap1 recruits Sir2/3/4 and Rif1/2 complexes to repress transcription (Fig. 2B). Conversely, at telomeres that contain the Y′-element, a repetitive sequence found in approximately 50% of the subtelomeric regions [16], Rap1 represses TERRA transcription through the recruitment of Rif1 and Rif2 [99,107] (Fig. 2B).

Mechanistic insights into TERRA transcription regulation recently came from Micrococcal nuclease (MNase) analyses showing that Sir2 stabilizes the + 1 nucleosome in the X-core element downstream of the TSS of TERRA on different chromosome ends. Sir2 mutant cells show nucleosome destabilization that contributes to TERRA upregulation, without altering TERRA TSS, as observed by 5′-RACE experiments [108]. Interestingly, binding of the transcription factor Reb1 to the X-core elements was proposed to have the opposite role, promoting nucleosome destabilization, a process that can contribute to the transcription termination of TERRA (Fig. 2B). Thus, Sir2 and Reb1 may repress TERRA expression by opposing functions [108].

The identification of the TERRA TSS within the X-core element of telomere 1L, mapping 346 nt upstream of the telomeric tract, enabled the generation of an engineered telomere containing a doxycycline-responsive promoter to induce TERRA expression [30]. Forced TERRA transcription led to resection of the transcribing telomere through the activity of the 5′– 3′ exonuclease Exo1. TERRA interacts with the Exo1-inhibiting Ku70/80 complex, and it was proposed that unscheduled TERRA transcription may impair Ku70/80 function unleashing Exo1-mediated resection of the engineered telomere and its consequent shortening. These findings underline the importance of fine-tuning TERRA transcription in order to maintain telomere stability. Importantly, telomere shortening upon TERRA induction occurred *in cis* as the length of the other chromosome ends remained unaltered [30].

As in humans, telomere length has been observed to regulate TERRA expression also in budding yeast, where upregulated TERRA levels are detected during telomere shortening [31,109] (Fig. 2B). Increased expression of TERRA from short telomeres may be due to a limiting number of Rap1, Sir, and Rif proteins recruited at these chromosome ends [104]. In this scenario, telomere-specific regulation of TERRA transcription is controlled *in cis*, by the length of the telomeric tract. When a chromosome end erodes to a certain threshold due to the end replication problem [110], TERRA transcription is de-repressed. Yet, additional mechanisms are most likely involved. Indeed, the magnitude of TERRA upregulation in telomerase-negative cells displaying shortened telomeres varies across different chromosome ends, suggesting the involvement of subtelomeric sequences in TERRA induction during telomere erosion [109]. Furthermore, transcription is not just a byproduct of telomere erosion. Instead, TERRA transcripts expressed from shortened telomeres were proposed to promote the re-elongation of these chromosome ends by telomerase. Indeed, TERRA interacts with the yeast telomerase, and it may positively regulate telomerase by nucleating its clustering and favoring its consequent recruitment to the TERRA-transcribing chromosome end [31]. How telomerase is targeted to the TERRA-transcribing telomere remains to be defined. R-loop structures, possibly involving the subtelomeric regions of the chromosome, or the state of the telomere (short) being more attractive to a TERRA-telomerase cluster due to the absence of telomerase repressive proteins (Rif2) may contribute to this mechanism.

Telomere shortening has been shown to induce TERRA also in fission yeast, where a TERRA-induced mechanism of telomerase-mediated elongation of the eroded telomeres has also been described [32]. In fission yeast, TERRA levels are upregulated and PolII is enriched at telomeres in Rap1 mutant strains [111]. mRNA splicing and protein stability of Rap1 have been shown to be regulated by Cay1, a member of the family of Cactins, which is thus indirectly involved in TERRA transcription regulation [112]. Interestingly, TERRA expression is also repressed by the double-stranded telomeric DNA binding protein and TRF2 homolog Taz1 [113]. 5′ RACE experiments identified transcription start sites for TERRA within subtelomeric regions of fission yeast chromosomes I and II positioned 211 nucleotides upstream of the telomeric repeat tract [111]. The 5′ RACE protocol used in this study enabled the amplification of only RNA molecules containing a methylated cap, indicating that at least a fraction of TERRA is 7-methyl capped in fission yeast, as previously observed in humans [114] . Surprisingly, loss of the heterochromatic factor Swi6, a fission yeast HP1 homolog, or of the histone H3K9 methyltransferase Clr4 did not alter TERRA levels [113], suggesting that these chromatin regulators are not involved in TERRA expression. Furthermore, it has been observed that cells maintained in a quiescent state express higher levels of TERRA than cycling cells. This effect was particularly evident in telomerase-negative cells with eroded telomeres [115]. In these cells, increased TERRA levels correlated with subtelomeric rearrangement and telomere instability. In a subsequent study, the same group reported that eroded telomeres in quiescent cells detach from the nuclear periphery, a process that associates with increased TERRA transcription [116]. The findings presented by the study indicate that during quiescence TERRA transcription is repressed by the telomere positioning to the nuclear envelope, which relies on the interaction between Rap1 and the inner nuclear membrane protein Bqt4. Repression of TERRA at the nuclear periphery may represent a mechanism to prevent telomere instability [116,117]. Further investigations will be required to determine the actors involved in the transcription regulation of TERRA in quiescence cells and whether it involves changes in the chromatin state.

Intriguingly, fission yeast telomeres also produce other species of telomeric transcripts: ARRET, subtelomeric RNAs transcribed in a telomere-to-centromere direction, C-rich telomeric repeat-containing transcripts (ARIA) and subtelomeric transcripts complementary to ARRET (α-ARRET) [111,113]. These RNAs may reveal important players in telomere biology

2.4. *Trypanosoma brucei*

In *Trypanosoma brucei*, a protozoan parasite causing human African trypanosomiasis, transcription of TERRA is resistant to α-amanitin treatment, suggesting that in this organism TERRA is not transcribed by PolII, as instead observed in other organisms [118]. Subsequent studies have shown that in *T. brucei* TERRA is transcribed by RNA polymerase I, and mainly from a single telomere [119,120]. Furthermore, in contrast to the evidence obtained in other species, *T. brucei* TERRA was found to be transcribed as a result of readthrough of the subtelomeric variant surface glycoprotein (VSG) gene transcription, which codes for a protein that coats the entire surface of trypanosomes [119,120]. TERRA expression is negatively regulated by the telomere-binding protein *Tb*TRF, that binds double stranded telomeric DNA, and by its interacting factor *Tb*RAP1, which also represses VSG transcription (Fig. 2C). *Tb*RAP1-depleted cells display increased TERRA levels and telomeric DNA:RNA hybrids, which associate with double-strand breaks (DSBs) at telomeres and at the subtelomeric VSG locus [120]. Expression of an ectopic allele of *Tb*RNase H1 in *Tb*RAP1-depleted cells reduced telomeric DNA:RNA hybrid and DSB formation. Similarly, *Tb*TRF-depleted cells display elevated TERRA levels and increased formation of telomeric R-loops, a condition that associates with increased amount of DNA damage at telomeres [119]. Ectopic expression of RNase H1 in *Tb*TRF-depleted cells partially suppressed the increase in telomeric R-loops and attenuated the induction in telomeric DNA damage detected in these cells. These findings indicate that proper maintenance of TERRA

and telomeric R-loop levels is essential to telomere integrity in *Trypanosoma brucei*.

2.5. Leishmania major

Northern blot analyses revealed the expression of TERRA also in the protozoa *Leishmania major* [121]. In this organism, TERRA transcripts are processed by trans-splicing, a process consisting in the addition of a splice leader sequence at the 5' end of the RNA [122], and by polyadenylation at their 3' end. RT-qPCR and Splice Leader RNA-Seq (SL-RNA-Seq) analyses [123] indicate that TERRA molecules are expressed from multiple telomeres, differently from the single locus transcription detected in *T. brucei*. Interestingly, *L. major* TERRA expression varied with the parasite's life stage and continuous passages, being more abundant in the infective forms, as detected by northern blot and RNA FISH. Furthermore, R-loops were detected at different chromosome ends also in this organism, in particular in its infective forms [121]. It will be interesting to investigate how telomere transcription and telomeric R-loops influence the life cycle and infective stage of this parasite that causes cutaneous leishmaniasis in humans.

2.6. Zebrafish

Early investigations on TERRA revealed its expression also in zebrafish [24]. In this study, TERRA transcripts were detected by northern blot as UUAGGG repeat-containing RNAs, heterogenous in length and expressed during both juvenile and adult stages. In a subsequent study, TERRA levels were found to be upregulated in a model of juvenile zebrafish brain tumor, as estimated by RNA dot blot and RT-qPCR [124]. RNA FISH experiments also revealed a higher number of TERRA foci and more intense foci in tumor cells as compared to controls. This model of zebrafish brain tumor resembles human pediatric glioblastomas of mesenchymal origins [125] and displays ALT features [124,126]. Interestingly, in these tumors Rad21 haploinsufficiency results in a lower TERRA signal by RNA dot blot and prevents ALT-associated phenotypes [127]. The use of this model may thus reveal important to study the role of TERRA and its mechanisms of transcription regulation in ALT.

2.7. Caenorhabditis elegans

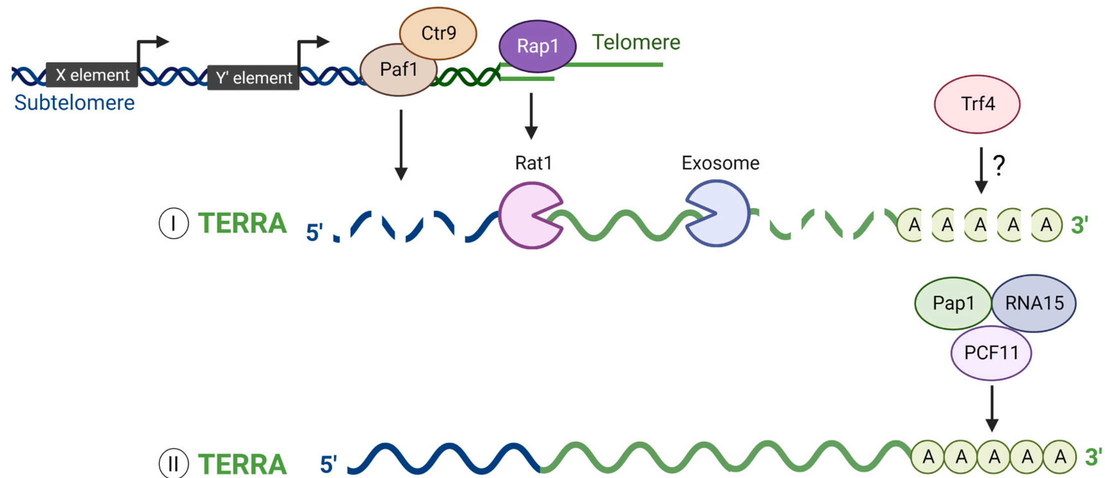
The expression of TERRA in *C. elegans* is regulated by the telomere binding proteins POT-1 and POT-2 [128] (Fig. 2D). Total TERRA levels were found elevated in *pot-1* and *pot-2* mutant strains, as detected by northern blot, while RT-qPCR analyses revealed a telomere-specific regulation, with TERRA levels increasing only from a subset of telomeres analyzed. These findings indicate that POT-1 and POT-2 repress TERRA expression in a telomere-specific manner. *C. elegans pot-1* and *pot-2* genes are homologous to the human *POT-1,* and interact with the single stranded telomeric overhang that in this organism is formed by the C-rich rich and the G-rich strands [129]. However, depletion of human POT-1 by shRNAs does not influence TERRA expression [40], suggesting that this mechanism of TERRA regulation is not conserved. RNA FISH experiments enabled *C. elegans* TERRA detection both in germ cells and post-mitotic cells. Interestingly, in the germline, a higher TERRA signal was detected during pachytene, a stage in meiosis when homologous recombination is ongoing [128]. While the presence of R-loops was not investigated, a fraction of TERRA was reported to localize at telomeres in the *pot-2* mutant strain. As opposed to the observations in yeasts, telomere shortening did not induce TERRA in *C. elegans*, as observed by northern blot and RT-qPCR analyses of a telomerase-deficient *trt-1* strain harboring eroded telomeres. Conversely, TERRA levels were found to rise in *trt-1*; *pot-2* double mutant animals, a strain prone to ALT induction [130–132]. In this strain, TERRA upregulation was detected at early generations, before the onset of ALT, with TERRA levels remaining higher than WT also upon selection of ALT-like survivors [128]. *C. elegans* may thus represent a useful model to study TERRA in ALT.

3. Mechanisms of TERRA stability

The mechanisms regulating TERRA transcript stability have been less investigated compared to the processes controlling their transcription. The first indications of a decay pathway targeting TERRA came from budding yeast where TERRA detection could be achieved by northern blot upon inactivation of the $5'-3'$ RNA exonuclease Rat1, by shifting a *rat1–1* temperature-sensitive mutant strain to nonpermissive conditions [133] (Fig. 3A). Rat1 is involved in the termination of transcription by PolII which transcribes past the polyadenylation site, enabling the recruitment of the cleavage and polyadenylation factor (CPF) and the cleavage factor I (CFI) complex to the nascent RNA [134]. Cleavage of the nascent transcript downstream of the poly(A) site by these complexes provides an access point for Rat1 that degrades the nascent RNA until it encounters the RNA polymerase, helping to disengage it from DNA. According to this "torpedo model" of transcription termination, Rat1-targeted RNAs can represent readthrough transcripts from upstream transcription units. However, several observations indicate that TERRA transcripts are instead generated independently from the transcription of the upstream subtelomeric genes in budding yeast. Indeed, northern blot analyses using probes specific for the transcripts generated by the subtelomeric Y′ elements confirmed that these RNAs are different from TERRA [133]. Furthermore, the substitution of the subtelomeric YFR057w open reading frame, mapping in proximity of the telomeric repeats on chromosome 6R, with a kanamycin cassette in two different orientations to allow transcription toward the telomere or in a centromeric direction, did not affect TERRA detection from chromosome 6R by RT-qPCR. Identification of TERRA TSSs on different chromosome ends upstream of the telomeric tract confirmed that at least for a subset of telomeres TERRA is regulated by its own core promoter [30,135,108]. Then, why would Rat1 inactivation upregulate TERRA levels?

Rat1 is enriched at subtelomeres and the stability of a galactose-induced TERRA species increased in *rat1–1* mutant cells, compared to WT cells [133], suggesting that Rat1 is indeed involved in the stability of endogenous TERRA transcripts. Furthermore, RT-qPCR experiments revealed that TERRA levels are increased from all telomeres analyzed in *rat1–1* mutant cells and genetic interactions indicate that the telomere binding protein Rap1 can promote the Rat1-mediated decay of TERRA [99] (Fig. 3A). Consistently, telomere shortening in a telomerase negative *tlc1* mutant strain, defective for the expression of the RNA subunit of telomerase (TLC1), a condition associated with lower number of telomere-bound Rap1 [104], also results in a reduced amount of Rat1 at telomeres and increased TERRA levels [109]. Intriguingly, recent evidence indicates that Rat1 can also regulate transcription [136], and control cotranscriptional splicing [137]. It will be interesting to investigate whether these processes may participate in the regulation of TERRA levels by Rat1.

RNA dot blot analyses of TERRA from poly(A) enriched RNA indicated that approximately 90% of TERRA transcripts are polyadenylated in *rat1–1* mutant cells, an observation that also argues against the possibility of TERRA consisting of readthrough transcripts from upstream transcription units [133]. Interestingly, reverse transcription (RT) of total RNA from WT cells using an oligo dT primer followed by PCR using subtelomere-specific primer pairs enabled the detection of TERRA from telomere 1L and not telomere 6R in WT yeasts, suggesting that telomere-specific mechanisms of TERRA polyadenylation may be present [133]. Furthermore, oligo dT RT followed by qPCR employing a forward primer specific for subtelomeres 1L or 15L paired with a telomeric repeat-specific reverse primer indicated that only about 7% of TERRA expressed from these subtelomeres is polyadenylated in WT cells [138]. Altogether, these findings suggest that TERRA polyadenylation may be differently regulated in WT versus *rat1–1* cells, and the expression of mutant Rat1 may upregulate TERRA polyadenylation.



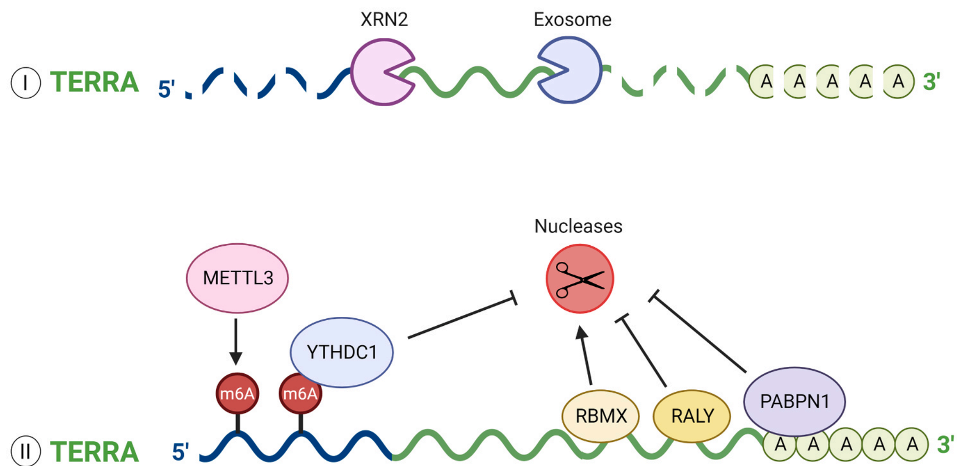


Fig. 3. Processes regulating TERRA stability. **A)** Regulation of TERRA stability in budding yeast *S. cerevisiae*. I) TERRA transcripts are degraded by the 5'-3' RNA exonuclease Rat1. The telomere binding protein Rap1 can promote the Rat1-mediated decay of TERRA. The poly(A) polymerase Trf4 may oligoadenylate TERRA, targeting the transcripts for exosome degradation. Paf1 and Ctr9, components of the transcription elongation complex PAF1, can promote TERRA degradation through Rat1- and exosome-independent mechanisms. II) The poly(A) polymerase Pap1 and the cleavage factor I complex (CFI) subunits RNA15 and PCF11 are involved in TERRA polyadenylation, a process that stabilizes TERRA transcripts in budding yeast. **B)** Regulation of TERRA stability in humans. I) TERRA is degraded by the 5'-3' RNA exonuclease XRN2. The exosome complex can contribute to TERRA decay. II) TERRA-interacting factor PABPN1 binds both poly(A)+ and poly(A)- TERRA molecules and contributes to poly(A)+ TERRA stabilization. PABPN1 also participates in poly(A)- TERRA decay possibly by targeting the transcripts for exosome degradation (not shown). The hnRNP RALY binds both poly(A)$^+$ and poly(A)$^-$ TERRA participating to their stability. The TERRA interacting hnRNP RBMX may promote exosome-directed decay of TERRA. TERRA m^{6}A modification catalyzed by the m^{6}A methyltransferase METTL3 and recognized by the m^{6}A reader YTHDC1 stabilizes TERRA transcripts.

Furthermore, TERRA polyadenylation may be regulated differently at distinct telomeres.

Inactivation of the poly(A) polymerase Pap1 or RNA15 and PCF11, subunits of the CFI complex which is required for Pap1-mediated RNA polyadenylation, markedly reduced TERRA levels in *rat1–1* mutant cells, suggesting that a Pap1-mediated TERRA polyadenylation stabilizes TERRA transcripts [133] (Fig. 3A). Notably, conversely, inactivation of the poly(A) polymerase Trf4, a subunit of the Trf4/Air2/Mtr4p Poly-adenylation (TRAMP) complex that oligoadenylates RNAs for subsequent degradation by the exosome [139], further increased TERRA levels in *trf4*Δ *rat1–1* double mutant cells, compared to a *rat1–1* mutant strain [133] (Fig. 3A). These findings suggest that a fraction of TERRA may be oligoadenylated by the TRAMP complex and degraded by the exosome. To date, it has not been verified whether TERRA molecules are oligoadenylated, probably due to the difficulties in sequencing these highly repetitive RNAs or to the possibly low abundance of the modified TERRA transcripts. In this regard, *trf4*Δ and *rrp6*Δ single mutants, the latter gene codifying for an essential subunit of the exosome, did not

result in increased TERRA levels compared to the WT strain, as detected by northern blot [133]. Similarly, a low increase in TERRA levels from telomeres 1L and 15L was detected by RT-qPCR in *trf4*Δ mutant cells [138]. These findings suggest that the exosome may have a minor role in TERRA degradation compared to Rat1. The involvement of the exosome in TERRA decay in yeast will require further investigation.

Additional regulators of TERRA stability were shown to be Paf1 and Ctr9, components of the PAF1 (RNA polymerase-associated factor) transcription elongation complex that binds PolII in budding yeast [140] (Fig. 3A). Interestingly, Paf1 and Ctr9 were found to localize to telomeres, and cells mutant for either of the two genes displayed augmented levels of TERRA with a magnitude of increase varying across telomeres. Indeed, RT-qPCR analyses on *paf1*Δ and *ctr9*Δ mutant strains revealed significant upregulation of TERRA expressed from telomeres 10R and 15L, while a milder effect on telomere 1L and no significant differences in TERRA levels from telomere 13R were detected [138]. A marked increase in the stability of TERRA expressed from telomeres 15L and 1L was observed in *paf1*Δ cells, with the transcripts half-life shifting from 21 to 25 minutes, in WT cells, to 316 minutes and further higher for telomere 15L TERRA in *paf1*Δ mutant cells. Interestingly, the effect of Paf1 in reducing TERRA levels was independent on Rat-1 and the TRAMP complex [138]. Indeed, *rat1–1 paf1*Δ, *paf1*Δ *trf4*Δ, and *paf1*Δ *rrp6*Δ double mutant strains displayed increased TERRA levels than paf1Δ mutant cells from different telomeres. These findings suggest that multiple RNA decay pathways can act independently on TERRA transcripts to control their degradation.

A similar scenario depicting multiple RNA decay pathways regulating TERRA levels most likely occurs also in human cells. Indeed, recent evidence indicates that the 5′– 3′ RNA exonuclease XRN2, the human homolog of the yeast Rat1, is involved in the regulation of TERRA [141] (Fig. 3B). TERRA levels increased after 6 h from XRN2 degradation using an auxin-inducible degron (AID), an effect that could be rescued by re-expression of the WT XRN2 but not the catalytically inactive mutant protein. Given the rapid effect of XRN2 degradation, and of its rescue, on TERRA levels, these findings indicate that TERRA is a direct target of this nuclease [141]. Thus 5′– 3′ RNA degradation represents a decay pathway regulating TERRA levels both in yeasts and humans.

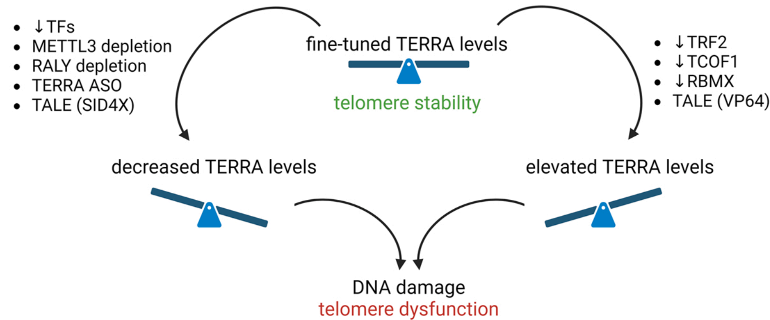
An estimated 7–8% of TERRA has been reported to be polyadenylated in human HeLa cells [72]. Northern blot and RNA dot blot analyses of TERRA from actinomycin D-treated cells revealed similar half-life of TERRA transcripts between HeLa and U2OS cell lines, estimated to approximately 3 h and 2.2 h, respectively [23,24]. Subsequent RNA fractionation studies indicated that polyadenylated TERRA transcripts are more stable than non-polyadenylated TERRA molecules, as detected by northern blot analyses revealing a half-life of approximately 8 h for poly(A)$^+$ TERRA and 3 h for poly(A)$^-$ TERRA molecules in HeLa cells [114]. Cell fractionation experiments reported that poly(A)$^+$ TERRA transcripts are nucleoplasmic while poly(A)$^-$ TERRA are both nucleoplasmic and chromatin-associated molecules, suggesting that these two TERRA species are regulated by different mechanisms and may exert distinct functions [114]. Adding to these findings, recent evidence indicates that human TERRA polyadenylation occurs preferentially at distinct telomeres, with a pattern of telomere-specific TERRA polyadenylation conserved between different human cell lines, including HeLa and U2OS cells [142]. In these experiments, TERRA polyadenylation was reported from three out of eight telomeres analyzed, telomeres 17q, 20q, and XpYp. It will be interesting to expand the analyses to other TERRA species.

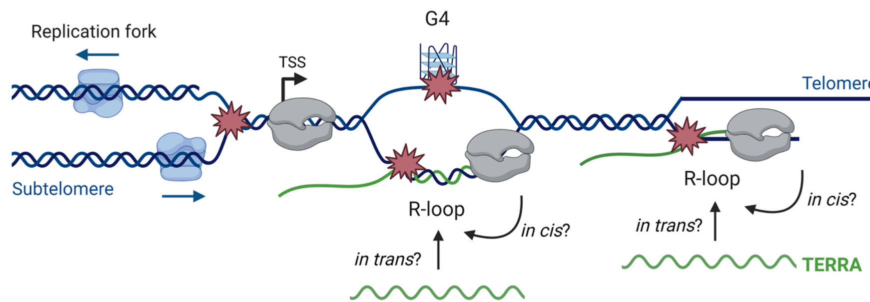
Although the poly(A) polymerase mediating human TERRA polyadenylation remains unknown, this process is not influenced by the poly (A) binding protein nuclear 1 (PABPN1), previously found to stimulate poly(A) Polymerase PAP activity [143]. Conversely, PABPN1 was found to interact with both polyadenylated and non-polyadenylated TERRA transcripts and to mediate a decay pathway of poly(A)$^-$ TERRA (Fig. 3B). PABPN1 was previously shown to promote RNA decay by targeting transcripts for exosome degradation [144]. In a recent study, it was shown that the downregulation of PABPN1 or EXOSC3, an essential subunit of the exosome, results in increased TERRA levels from different telomeres in HeLa cells, as detected by RT-qPCR [142]. In this regard, it should be noted that TERRA levels were also reported to not change after 6 h from degron-mediated degradation of the DIS3 and EXOSC10 subunits of the exosome in HCT116 cells, as detected by RNA dot blot [141]. The different cell lines used, different times of TERRA detection from exosome inactivation, and the distinct approaches used to quantify TERRA may explain the conflicting results between these studies. Notably, PABPN1 depletion also resulted in increased stability of three out of four non-polyadenylated TERRA species analyzed, while no effect on TERRA half-life was observed for polyadenylated TERRA molecules [142]. These findings further suggest that poly(A)$^+$ and poly(A)$^-$ TERRA are regulated by different mechanisms and that TERRA decay pathways may operate in a telomere-specific manner. Importantly, TERRA levels and stability in HeLa cells are also dependent on the hnRNP RALY, which binds both poly(A)$^+$ and poly(A)$^-$ TERRA (Fig. 3B). RALY downregulation impacts the stability of poly(A)$^-$ TERRA molecules while depletion of PABPN1 in RALY KO cells impairs the stability of both poly (A)$^-$ and poly(A)$^+$ TERRA, as compared to PABPN1-depleted HeLa cells [142]. These findings suggest that the interplay between RALY and PABPN1 represents an important mechanism for regulating TERRA transcript stability in human cells. How poly(A) polymerases can be recruited and act on TERRA molecules remains mysterious since no polyadenylation sites are expected in the telomeric repeat tract. Nuclear run-on (NRO) experiments indicate that TERRA polyadenylation occurs co-transcriptionally for two out of three TERRA subpopulations analyzed, suggesting that this process may also be regulated differently at distinct telomeres [142]. Other hnRNPs have been shown to regulate TERRA stability. Indeed, downregulation of the TERRA-interacting hnRNP RBMX results in increased TERRA signal in U2OS and HeLa cells, as detected by RNA dot blot (Fig. 3B). Transcripts arising from telomere 10q and 12p were found to be stabilized by RBMX depletion in U2OS cells and it was proposed that RBMX promotes exosome-directed decay of TERRA [145]. TERRA-binding hnRNPs have been found to impair TERRA stability also in mouse cells [37]. Increased levels of TERRA upon depletion of hnRNPs results in telomere dysfunction in both human and mouse cells, in line with the importance of a tight control of TERRA expression in both species [37,145].

In addition to its 3′ end, the 5′ subtelomeric sequence of TERRA is involved in the regulation of its stability. Indeed, it has been recently reported that the subtelomeric regions of TERRA are N6-methyladenosine (m^{6}A) modified in ALT cell lines [146]. TERRA m^{6}A modification is catalyzed by the m^{6}A methyltransferase METTL3 and recognized by the m^{6}A reader YTHDC1. Knockdown of either METTL3 or YTHDC1 enhances TERRA degradation (Fig. 3B). Numerous m^{6}A consensus motifs (RRACH) were found within several subtelomeric regions, downstream of TERRA TSS, suggesting that this modification can occur pervasively on TERRA transcripts [146]. However, not all the TERRA RRACH motifs were found to be functional in regulating TERRA stability and experimental validations will be required in order to investigate their impact on TERRA biology. These findings indicate that the m^{6}A modification of TERRA subtelomeric regions contributes to the telomere-specific regulation of TERRA stability in human cells.

A minor fraction of TERRA was estimated to be polyadenylated also in the fission yeast *S. pombe* [111]. TERRA levels increased in strains expressing mutant Rap1 as well as mutant Cid12 or Cid14, two noncanonical poly(A) polymerases [147], the latter showing the highest effect on TERRA. As Cid14 is the fission yeast homolog of Trf4, these findings suggest that the TRAMP complex may be involved in TERRA decay in fission yeasts. Interestingly, 3′ RACE approaches followed by sequencing revealed that polyadenylated TERRA molecules are either devoid of telomeric repeats or contain a very short telomeric tract [32]. These findings were obtained in cells containing short telomeres due to the deletion of the *ter1* gene encoding for the RNA subunit of telomerase, a

condition resulting in increased levels of TERRA. Intriguingly, poly(A)$^+$ TERRA species were found to interact with a myc-tagged Trt1, the catalytic subunit of telomerase, in RNA immunoprecipitation experiments (RIP). This interaction was not influenced by DNase I treatment of the immunoprecipitated samples before RNA extraction, suggesting that it is not mediated by DNA. In line with these findings, poly(A)$^+$ TERRA was detected in the nucleoplasmic fraction of cells, similar to the human condition [32]. In this same study, a strain carrying a transcriptionally inducible telomere was generated in order to investigate the consequences of TERRA expression on telomerase activity. Interestingly, induced transcription of the engineered telomere resulted in increased interaction between TERRA molecules and telomerase, with concomitant recruitment of telomerase to the induced telomere. As a consequence, transcription of the inducible telomere promoted elongation of





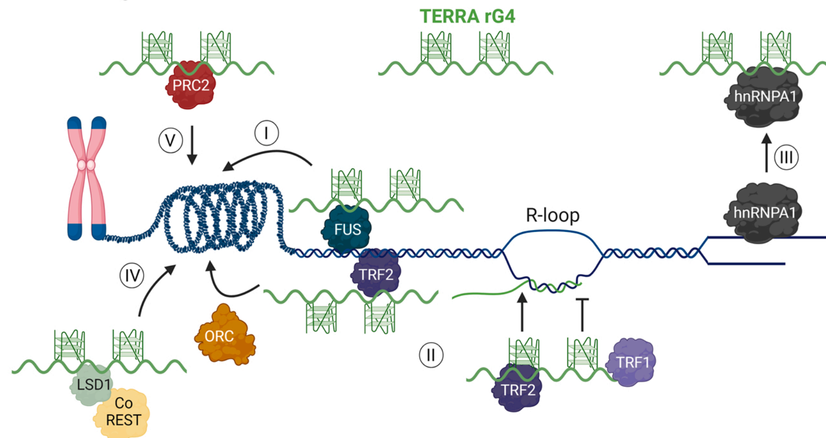


Fig. 4. How TERRA transcripts can regulate telomere stability. **A)** Impact of TERRA deregulation on telomere stability. Alteration of TERRA levels—both up- and downregulation—leads to DNA damage at telomeres and telomere dysfunction, compromising telomere stability. TF: transcription factors, SIDX4: transcription repressor, VP64: transcription activator. More details and references in the main text. **B)** Impact of telomeric TERRA R-loops on telomere stability. TERRA can form telomeric R-loops both at subtelomeres and telomeres. TERRA transcripts could invade telomeric DNA *in cis* or *in trans*. Accumulation of R-loops may favor telomeric G4 formation, DNA breaks and replicative stress. **C)** TERRA G4 binding factors. TERRA RNA G-quadruplexes (rG4) interact with a number of proteins at telomeres and possibly take part in heterochromatin formation, R-loop regulation and influence terminal capping. I) The ternary complex of telomeric DNA (teloDNA), TERRA rG4 and TLS/FUS stimulates H3K9me3 deposition and chromatin compaction at telomeres. II) The ternary complex of teloDNA, TERRA rG4 and TRF2 recruits ORC to telomeres and drives heterochromatin formation. Additionally, TRF2 binds TERRA helping to invade telomere duplex, with TRF1 antagonizing the invasion. III) The binding of hnRNPA1 to TERRA rG4 may mediate the hnRNPA1 displacement that allows for POT1 telomere capping (not shown). IV) The demethylase activity of LSD1-CoREST is inhibited by its TERRA rG4 binding. V) PRC2 transcription repressor is recruited to telomeres through TERRA rG4 binding.

this chromosome end, without influencing the length of the other telomeres [32]. These findings represented the first evidence of the role of TERRA in the regulation of telomerase in fission yeast, proposing a potential role of poly(A)$^{+}$ TERRA molecules as positive regulators of telomerase.

Thus, an increasing amount of evidence indicates that in addition to the transcription regulation, mechanisms involved in TERRA stability exert a critical role in controlling the total levels of TERRA and tightly regulating TERRA expression at single telomeres. For the observations discussed above, regulation of TERRA expression is important to telomere length homeostasis and telomere stability in multiple organisms.

4. Roles of TERRA in telomere stability

The importance of fine-tuning TERRA levels by transcription regulation and RNA decay mechanisms is highlighted by the consequences that the deregulation of these mechanisms has in various organisms. As discussed in the previous paragraphs, inhibition of TERRA transcription by downregulation of transcription factors regulating TERRA associates with DNA damage at telomeres [68,69,87](Fig. 4A). In line with these findings, depletion of TERRA using ASOs results in activation of DNA damage response (DDR) pathways at telomeres and telomere dysfunction in humans as in mice [56,148]. Upregulation of TERRA levels is also associated with telomere dysfunction, as observed upon depletion of TRF2 or TCOF1 which repress TERRA expression [40,41,71,87]. The impact of deregulated TERRA in telomere stability is exemplified by recent studies using modified transcription activator-like effectors (TALEs) binding to TERRA promoters and showing that both upregulation and repression of TERRA transcription are associated with telomere instability in ALT cancer cells [149,150]. Notably, decreased TERRA levels due to impaired stability of the transcripts also resulted in telomere damage as observed in METTL3-depleted U2OS cells and RALY-depleted HeLa cells [142,146]. Similarly, stabilization of TERRA transcripts upon depletion of TERRA-interacting hnRNPs is associated with activation of DDR at telomeres in U2OS and HeLa cells [145] as well as MEFs [37]. Thus, physiological levels of TERRA are essential to the maintenance of telomeres, and altered TERRA levels are deleterious to telomere integrity (Fig. 4A).

By which mechanisms can TERRA transcripts regulate telomere stability? The chromatin state of telomeres and subtelomeric regions is important to telomere stability [151]. As TERRA participates in chromatin maintenance at chromosome ends, the impact on chromatin of its deregulated levels may expose telomeres to damage [27,28]. In addition, it has been proposed that increased TERRA levels upon TRF2 depletion can contribute to telomere dysfunction by different mechanisms. In TRF2-depleted HeLa cells, derepressed TERRA transcription can sustain DDR at telomeres by promoting the recruitment of the TERRA-interacting factors lysine-specific demethylase (LSD1) and SUV39H1 to telomeres [40,41]. LSD1 would then assist the recruitment of the MRN (Mre11/Rad50/NBS2) complex, a sensor of DNA damage, at telomeres, while SUV39H1 activity can promote activation of ATM, an apical kinase of DDR, by mediating histone H3K9 trimethylation at chromosome ends [40,41,152,153].

Genome editing of TERRA promoters provided important insights into the role of TERRA in telomere stability. Indeed, deletion of CTCF-binding sites within the TERRA promoter at the subtelomere 17p in HCT116 cells resulted in impaired H3K4 trimethylation at the same subtelomere and decreased telomere 17p TERRA transcription. These cells were sensitive to replicative stress and the TERRA-lacking telomere exhibited impaired DNA replication, with the formation of ultra-fine anaphase bridges during replicative stress conditions, suggesting that TERRA transcripts may facilitate DNA replication *in cis* [29]. The function of TERRA in regulating chromatin may contribute to assist DNA replication at telomeres [154]. Interestingly, by using a nascent chromatin capture (NCC) method optimized for the detection of RNAs at active replication forks, it was reported that telomeric repeat-containing RNAs detected by RNA-seq are associated with replicated DNA in HeLa cells [155]. These RNAs, which may include TERRA transcripts, were found to be transcribed soon after DNA replication and remained associated with newly replicated DNA for several hours after fork passage. In line with these findings, it may be hypothesized that TERRA facilitates chromatin formation after fork passage or recovery from DNA replication pausing or stalling, by promoting the recruitment of its interacting factors to telomeres, such as the helicases BLM and RTEL1 or the origin replication complexes [38,56,152]. The association of chromatin-modifying enzymes to TERRA promoters may also assist replication through telomeres [156].

It must be noted that live imaging studies reported that TERRA molecules only transiently localize to telomeres [31,148,157], and RNA FISH IF experiments have shown that not all telomeres display TERRA localization [23,88]. Furthermore, the total TERRA levels decrease during S-phase in human cells [114], a process that is at least in part regulated by the recruitment of TCOF1 to telomeres through its binding to TRF2 [87]. TCOF1 depletion and consequent abnormally elevated TERRA levels lead to telomere replication defects. Despite this evidence, it cannot be excluded that low levels of TERRA transcription may transiently occur during/after replication at telomeres. In this case, it will be important to test the length of these transcripts and whether they contain subtelomeric sequences. Indeed, intriguingly, human chromosome ends have been shown to replicate within distinct time windows which are influenced by their subtelomeric sequences [158]. While the involvement of TERRA in telomere-specific regulation of DNA replication needs to be further defined, transcription could positively impact the replication timing of telomeres by modulating their chromatin structure [159,160] or promoting the sliding of the replicative helicases [161]. In budding yeasts, forced transcription of TERRA through an inducible promoter integrated within subtelomere 7L leads to a replication-dependent loss of telomeric sequence and shortening of the TERRA transcribing telomere [162]. These findings underline the need to tightly regulate TERRA transcription and most likely to coordinate it with DNA replication at telomeres in order to preserve telomere integrity (Fig. 4A).

In the following paragraphs, we will discuss how telomere stability is impacted by R-loop and rG4 structures that TERRA form at chromosome ends.

5. The impact of telomeric R-loops on telomere stability

5.1. Telomere-specific regulation of R-loops

R-loops are three-stranded structures generated when the RNA anneals to the DNA template forming a DNA:RNA hybrid, displacing the non-template strand as a single-stranded DNA loop. Although R-loops play physiological roles in cells, such as immunoglobulin class-switch recombination, DNA replication and transcription regulation, unscheduled R-loops represent a source of DNA damage and genome instability [163]. One source of DNA damage in the R-loop is the displaced ssDNA, which is exposed to mutagenic agents and breaks, and can adopt secondary structures prone to breakage [164]. Furthermore, R-loops can stall replication fork progression resulting in DNA lesions. In this regard, R-loops can directly block the replisome, or alternatively, promote PolII pausing, transcription-generated topological stress, or recruitment of R-loop-binding proteins, that in turn collide with the replication fork [164,165]. Because of the coexistence of these processes, it is challenging to dissect the exact mechanism by which R-loops induce replication stress.

R-loop formation is favored by GC-skewed sequences [166], CpG islands [167], active transcription [168], G-rich transcripts [169], G4 formation [170] and repetitive sequences [171–173]. Thus, telomeres represent genomic sites susceptible to harbour R-loops that form by the base-pairing of TERRA with its DNA template strand and the displacement of the TTAGGG DNA repeat-containing strand. Studies using

genome-wide approaches have shown the presence of R-loops at telomeres in yeast and in human cells [174,175], and it has been demonstrated that transcription of telomeric tracts in the direction producing TERRA-like molecules results in accumulation of R-loops [42,175].

Three early studies reported the presence of RNase H-sensitive R-loops at specific yeast telomeres using immunoprecipitation with the S9.6 antibody combined with qPCR [43,50,176]. S9.6-based methods rely on the recognition of DNA:RNA hybrids by the S9.6 antibody [177] and the exogenous use of recombinant RNase H to validate the specificity of the antibody. RNase H enzymes are the best characterized factors that remove R-loops in cells, degrading the RNA strand of the hybrid [178]. In two different studies it was shown that in the absence of endogenous RNase H1 and RNase H2 enzymes, telomeric R-loops drastically increased, suggesting that these enzymes control R-loops levels also at telomeres [43,176]. Interestingly, mutants of the RNA processing THO complex also accumulate telomeric R-loops and show enhanced telomere recombination [43,50,176], in line with previous evidence indicating that THO regulates co-transcriptional R-loops in yeast [179]. Balk and colleagues demonstrated that in telomerase-negative senescent yeast cells, telomeric R-loops correlate with recombination-dependent telomere elongation. When removing RNase H enzymes, stabilization of R-loops led to increased recombination, while overexpression of RNase H1 resulted in reduced telomere recombination [43]. Thus, this work introduced the concept that a tight regulation of telomeric R-loops formation and resolution is required for telomere length maintenance in telomerase-negative cells [49]. Moreover, Balk and colleagues showed that reduced TERRA transcription and telomeric R-loops at a single telomere result in decreased telomere recombination only at that single telomere, suggesting an effect *in cis* [43,49].

The first reports on telomeric R-loop formation, regulation and impact on telomere lengthening in budding yeast set up the stage for a series of studies in human cells aiming at deciphering whether the role of TERRA on telomere biology in cancer and other diseases was mediated by the formation of R-loops. Arora and colleagues reported accumulation of telomeric R-loops by DNA immunoprecipitation (DIP) using the S9.6 antibody followed by qPCR in both telomerase-positive and ALT-positive cells [42]. Interestingly, telomeric R-loops were found most enriched at CpG-island containing subtelomeres (10q and 15q), compared to XpYp telomeres, suggesting the presence of a telomere-specific regulation of R-loop formation. siRNA-mediated depletion of RNase H1 led to an increase of telomeric R-loops specifically at CpG-containing promoters, phosphorylated RPA at telomeres (a marker of replication stress), C-circles (a marker of ALT) and telomere loss in ALT-positive U2OS cells but not in telomerase-positive HeLa cells. Conversely, overexpression of RNase H1 resulted in telomere shortening as observed in the reduction of telomere signal intensity and incidence of fragile telomeres in metaphase spreads from ALT cells 13 days post infection with retroviruses expressing RNase H1 [42]. Using cell lines carrying a transcriptionally inducible telomere, the authors showed that increased transcription of a single telomere leads to enhanced telomere instability and recombination at the engineered chromosome end in U2OS cells but not in HeLa cells, suggesting that R-loops can form co-transcriptionally and impact telomere stability *in cis*. This work highlights the importance of maintaining a balanced amount of telomeric R-loops to ensure telomere homeostasis of ALT cells as both excess and deprivation of telomeric R-loops result in telomere loss [180]. Recent studies provided further evidence supporting these observations by developing a unique system to downregulate [150] or upregulate [149] TERRA expression at a transcriptional level from CpG-containing TERRA promoters using TALEs in U2OS cells. Apart from the effect on the telomere stability described above, the authors showed that downregulation of TERRA levels alleviates ALT activity, leading to the shortening of telomeres [150]. Conversely, upregulation of TERRA levels resulted in enhanced ALT activity and telomere instability with rapid loss of telomeres in a mechanism dependent on the nuclease Mus81 [149]. It would be interesting to assess the changes in telomeric R-loop levels under down and upregulation of TERRA in this context to better define their contribution to DNA damage and telomere instability. Understanding whether downregulation of TERRA from one telomere impacts the ALT phenotype only at its telomere of origin or also at other telomeres, will also be important to gain further insights on the role of TERRA in the ALT mechanism.

The influence of R-loops on telomere maintenance of telomerase-positive cancer cells remains to be defined, however, a study by Shiromoto and colleagues has provided key elements to understand telomeric R-loop resolution in these cells [181]. In this work it was reported that telomerase-positive cells, but not ALT cells, depleted for the Adenosine deaminase acting on RNA (ADAR) factor, the enzyme involved in adenosine-to-inosine (A-to-I) RNA editing [182], accumulate telomeric R-loops that lead to telomere DNA damage, as measured by DNA:RNA immunoprecipitation combined with dot-blot (DRIP-dot blot) experiments. Interestingly, the authors have shown *in vitro* that the ADAR isoform ADAR1p110 can efficiently edit RNA and DNA strands of telomeric R-loops formed between canonical and variant telomeric repeats [181]. Even if ALT cells contain higher levels of telomere variants compared to telomerase-positive cells, the authors have shown that telomeric R-loops accumulated in telomerase-positive cells depleted for ADAR1 present RNA and DNA variant repeats. Interestingly, by using a combination of *in vitro* and *in vivo* studies, the authors have shown that editing of A-C mismatches in telomeric R-loops by ADAR1, facilitates their resolution by RNase H2, specifically in telomerase-positive cells. This and previous studies [42] highlight a differential regulation of telomeric R-loops in ALT versus non-ALT cells, dependent on RNAse H1 and RNase H2 respectively.

The impact of telomeric R-loops in telomere homeostasis of ALT cells raised the interest in investigating factors associated with their correct regulation. Many R-loop-binding proteins have been reported to date, including ATRX [183], the RNA-binding proteins SFPQ and NONO [36], the translocase FANCM [51,52], the DNA repair factor BRCA1 [184], the recombinase Rad51 [185], the RAD51 associated protein 1 RAD51AP1 [186,187] and shelterin proteins [188]. These proteins have been shown to regulate telomeric R-loops by binding or sequestering TERRA, regulating its stability, its retention or recruitment to telomeres, or by directly binding and modulating the telomeric R-loop. A recent review has comprehensively summarized and discussed the main R-loop regulating factors and TERRA binding proteins controlling telomeric R-loops formation [189].

A recent study reported that the THO complex also plays a role in regulating R-loops at human telomeres [190], as initially described in yeast [43,50,176]. By DNA:RNA immunoprecipitation (DRIP) combined with dot blot detecting telomeric DNA repeats, the authors showed that the THOC1 and THOC2 subunits of the complex counteract telomeric R-loop accumulation. Interestingly, the THO complex suppressed telomere instability and recombination, as assessed by telomeric sister chromatid exchange and C-circle levels respectively, in U2OS cells but not in HeLa cells [190]. ALT cancer cells may be more prone to telomeric R-loop formation, compared to telomerase-positive cancer cells, due to the higher levels of TERRA detected in these telomerase-negative cells, but also to the different telomeric context, including chromatin dynamics [191,192], telomerase inhibition [193], and clustering of telomeres to ALT-associated PML bodies (APBs) which are enriched in recombination factors [194–196].

As mentioned at the beginning of this review, transcription-replication conflicts can pose an obstacle to DNA replication leading to replicative stress and DNA damage. R-loops can exacerbate these conflicts (Fig. 4B). One of the strategies to avoid transcription-replication collisions is the temporal separation of the processes. TERRA expression is cell-cycle regulated, with TERRA levels peaking in G1 and decreasing during S-phase in human HeLa cells [114]. This regulation may facilitate telomere replication. In this regard, the cell cycle regulation of TERRA is impaired in ICF cells, showing upregulated TERRA and abundant telomeric R-loops during all stages of the cell

cycle, including S-phase [175]. These cells also display high DNA damage at telomeres, which is to some extent sensitive to RNase H1 overexpression, suggesting that telomeric R-loop deregulation contributes to the telomere dysfunction reported in ICF cells [175]. The cell-cycle regulation of TERRA is also lost in ALT cancer cells in which TERRA remains high in S-phase leading to replicative stress [197]. Thus, while physiological levels of TERRA may assist telomeric DNA replication, as discussed in the previous paragraph, altered TERRA transcription and accumulation of telomeric R-loops can lead to replicative stress and telomere dysfunction (Fig. 4B). Interestingly, replication stress at telomeres has been proposed to facilitate the ALT mechanism. Indeed, accumulation of telomeric R-loops upon depletion of RNase H1 in the ALT cell line Saos2 caused an increase in the ALT hallmarks, including γH2Ax at telomeres, telomere fragility, APB formation and telomeric EdU incorporation on metaphase chromosomes as a measure of mitotic DNA synthesis (MiDAS) [198]. These findings are in line with the evidence supporting the important function of telomeric R-loops in ALT mechanism [36,42,51,149,150].

Interestingly, in budding yeast elevated TERRA levels and telomeric R-loops were reported in G1/early S-phase cells, as detected by RT-qPCR and by DRIP combined with qPCR (DRIP-qPCR) experiments, respectively [109]. TERRA levels and telomeric R-loops were found to decrease as the cells proceed during the S-phase, a mechanism that may facilitate DNA replication. Notably, R-loop degradation was found to be mediated by the activity of the RNase H2, which is recruited to telomeres through the interaction with the telomere-binding protein Rif2. Consistently, telomeric R-loops accumulate at critically short telomeres that lose Rif2. The buildup of telomeric R-loops leads to DDR activation, possibly through replicative stress, that can trigger telomere re-elongation by homologous recombination processes [109]. Interestingly, a similar process has been described in the fission yeast *S. pombe* [54]. Recently, telomeric R-loops were reported to prevent telomere resection by the Exo-1 nuclease upon inactivation of the temperature sensitive allele of the single-stranded telomeric DNA binding protein Cdc13 in *S. cerevisiae* [199]. In addition, the same laboratory recently described an important function of TERRA in budding yeast type II "survivors", cells that escaped replicative senescence due to telomerase depletion by elongating telomeres through homologous recombination processes relying on the Mre11, Rad50, Xrs2 (MRX) complex. The authors reported that type II survivors experience telomere attrition over time and subsequent decrease of replicative potential. In these cells, homologous recombination occurs only at critically short telomeres through formation of TERRA DNA:RNA hybrids. Accordingly, the replicative potential of these cells was found to be regulated by telomeric DNA:RNA hybrid formation [200]. In a different study on type II survivors, it was reported that telomeric DNA:RNA hybrids are degraded by the Rad27 endonuclease that cleaves the RNA component of the R-loop structure [201]. This study proposed the function of Rad27 in maintaining genome stability of type II survivors by preventing accumulation of R-loops. Thus, under certain conditions, telomere instability resulting from deregulation of telomeric R-loops can contribute to telomere length homeostasis in the absence of telomerase, in both yeast and human cells.

5.2. TERRA can form telomeric R-loops both in cis and in trans

A still unresolved question is whether telomeric TERRA R-loops only form *in cis* at their telomere of origin or also *in trans* at other telomeres. Studies in yeast [43,54] and in ALT cancer cells [42,149,150], in which TERRA levels from specific telomeres were modulated, suggest that at least in part telomeric R-loops form co-transcriptionally, impacting replication fork progression and telomere stability *in cis*. However, TERRA ectopically expressed from a plasmid has been shown to localize to telomeres and form R-loops *in trans* in HeLa cells [185,190] (Fig. 4B). Interestingly, telomeric R-loops formed by ectopic TERRA required the presence of the telomeric repeat tract of TERRA and were stabilized under RNase H1 and THO depletion leading to telomere instability [185,190]. Conversely, telomeric R-loop structures were promoted by the recombinase RAD51, which catalyzes R-loop formation *in vitro* through physical interaction with TERRA [185].

It is important to note that changes in cellular TERRA levels do not always induce changes in the levels of telomeric R-loops. For example, increased TERRA transcripts due to depletion of TRF1 and TRF2 in U2OS cells does not result in increased telomeric R-loops [42]. Moreover, decreased TERRA transcripts due to reduced stability in the absence of RALY does not lead to changes in telomeric R-loops in HeLa cells [142]. One could speculate that TERRA molecules invade telomeric DNA, but the successful formation of a stable R-loop would depend on the state of chromatin, telomere occupancy by the shelterin proteins, and/or on the properties of TERRA molecules such as their length, polyadenylation and m6A modification.

5.3. TERRA-mediated R-loops form at both subtelomeres and telomeres

Current models in the R-loop field suggest that differences in the sequence, length, frequency of formation, 3D structure and half-life could account for the toxicity and the different outcomes of R-loops on genome stability [202,203]. Therefore, a better characterization of telomeric R-loops will be important to predict their impact on telomere stability.

With the techniques currently used to detect telomeric R-loops it is challenging to dissect their nature and structure. Indeed, care should be exercised when using the S9.6 antibody in immunofluorescence experiments since although the antibody recognizes predominantly DNA:RNA hybrids, it also has affinity for double-stranded RNAs [204]. A strategy to overcome this problem is the treatment with RNase T1 and RNase III to degrade RNA while preserving DNA:RNA hybrids [205].

As discussed earlier, the S9.6 antibody has been widely used to immunoprecipitate hybrids by DRIP [206]. R-loops are then visualized either with a telomeric probe by dot blot [51,185,190], to assess their formation at telomeres, or by qPCR using subtelomere-specific primers [42,43,109,176,185] to verify their formation at subtelomeres. To define the region involved in R-loop formation, it is important to take into account the resolution reached during the sonication process and the distance of the primers to the telomeric repetitive tract. By analysing DRIP-sequencing data from fibroblast and human embryonal carcinoma cell lines, Sagie and colleagues identified significant enrichment of DRIP-seq signals at subtelomere regions, suggesting the presence of R-loops at subtelomeres in cancer cells and normal primary human cells [175]. The authors then demonstrated by DRIP-qPCR that lymphoblastoid ICF cells show increased levels of telomeric R-loops compared to WT cells. Interestingly, by separating the subtelomere from the telomeric tract through enzymatic digestion prior to DRIP-qPCR, the authors showed that R-loops formed at certain subtelomeres extend into the telomeric repeats [175]. Altogether, these findings suggest that telomeric R-loop could potentially form at both subtelomeres and telomeric repeats (Fig. 4B).

RNase H1-based methods rely on the use of the catalytically inactive RNase H1-D210N, which recognizes but does not process R-loops, such as RNase H1-D210N-ChIP (R-ChIP). R-ChIP is able to specifically bind native R-loops while the S9.6 antibody captures R-loops *ex vivo* [205]. To date, only a few studies have applied R-ChIP for the detection of telomeric R-loops [142]. Catalytically inactive human RNase H1 tagged with GFP appears as a more specific method to visualize R-loops, avoiding the detection of dsRNA [207].

The development of new tools to detect or modulate R-loops specifically at telomeres would be essential to deepen our understanding of these structures. In this regard, the hybrid-binding domain (HBD) of RNase H1, has been recently used to visualize R-loops in live-cell microscopy and immunofluorescence experiments [208] and to map R-loops [209].

The formation of R-loops is interconnected with the folding of telomeric DNA G4s with possibly important implications for telomere

stability. The unwinding of DNA duplex by RNA polymerase exposes the G-rich ssDNA, which may be an *in vivo* prerequisite for the formation of the G4 structure [210]. In line with this scenario, the depletion of TERRA leads to decreased levels of telomeric DNA G4s [211]. The formation of telomeric DNA G4s at TERRA R-loops was shown to stimulate the RAD52-independent ALT pathway of telomere maintenance [187]. Vice versa, the formation of DNA G4 at the non-template DNA strand of R-loops may enhance transcription [212]. TERRA molecules were also described to form RNA G4s (rG4s). In the following paragraph, we will thus discuss the implications of telomeric G4s and TERRA G4s in telomere stability.

6. G-quadruplex structures (G4s) in TERRA and telomere biology

6.1. DNA G4s at telomeres

G4 is a secondary structure formed by Hoogsteen-pairing between guanines arranged into planar quartets in DNA or RNA with runs of guanines with non-guanine spacers. This structure is stabilized by cations, such as K^+ or Na^+ (much less by Li^+), and exhibits diverse topologies (parallel, antiparallel or hybrid), depending on the primary sequence and environmental conditions [213]. Telomeric sequence has a high G-content, and its propensity to form both intramolecular and intermolecular G4 structures *in vitro* has been recognized early after telomeric sequence identification [214–217]. *In vivo*, telomeric G4s have been detected by G4-specific antibodies or ligands in protists, humans, yeasts and other models [218-220].

Telomeric DNA G4s have been shown to interact with an array of telomere-associated proteins, including a number of helicases capable of their unwinding [221,222]. Telomeric G4 interaction partners include telomerase which is able to bind, unwind and extend telomeric DNA folded in a G4 structure [223]. However, telomeric DNA G4 stabilization through a range of ligands inhibits telomerase activity and the replication of telomeres [224,225], which makes the G4 ligands a promising candidate for cancer therapeutics [226].

6.2. TERRA RNA G4 structure and function

Like its DNA counterpart, the telomeric portion of TERRA sequence is capable of folding into RNA G4 structure (rG4). With the widely employed toolkit of nuclear magnetic resonance (NMR), circular dichroism, mass spectrometry and EMSA, TERRA-mimicking telomeric repeat-containing RNAs were shown to assume a parallel G4 topology in both Na^+ and K^+ buffer, and to form intramolecular rG4s as well as mediate multimerization. Interestingly, the formation of rG4 may protect TERRA from RNase degradation [45–48,227].

The rG4 structure of TERRA-mimicking RNAs has also been detected *in vivo*: Xu and colleagues developed a TERRA rG4-binding pyrene probe forming a fluorescent dimer upon rG4 formation. With this approach, they have demonstrated the presence of rG4s at the TERRA-mimicking oligonucleotide transfected into live HeLa cells. The TERRA rG4 specifically co-localized with telomeres [228]. Furthermore, the *in-cell* NMR spectroscopy of ^{19}F-labeled oligonucleotides transfected in *Xenopus laevis* oocytes confirmed the formation of rG4 structure at TERRA *in cellulo*, indicating its multimerization under molecular crowding conditions [229].

It has been suggested that under steady-state conditions, living cells do not contain folded rG4s [230], with their unfolding being promoted by a number of helicases [231]. However, more recent results challenge these findings and show that rG4s may play a role in stress response [232]. Indeed, using an rG4-specific sequencing method developed in the above mentioned study [230], Kharel and colleagues have demonstrated that hundreds of cellular RNAs form rG4s in U2OS cells under starvation, oxidative stress and cold stress [232]. Given the impact of various cellular stresses, including serum starvation, oxidative stress, etoposide treatment, and heat shock, on TERRA regulation [152] it will be interesting to investigate whether these conditions influence TERRA rG4s formation and the contribution of these structures to the biology of TERRA under cellular stress. Indeed, a number of studies suggest that TERRA rG4 structures may play various roles in telomere biology. A study focusing on cancer cells with shortened telomeres explored the role of TERRA rG4s in innate immunity signaling [233]. The authors observed that in several cancer cell lines, the overexpression of telomerase leads to elongated telomeres, increased levels of TERRA, and repression of innate immunity signaling genes. Further focusing on this genome-wide expression regulation, the authors reported that synthetic TERRA, as well as another rG4-forming oligonucleotide, attenuate the innate immunity genes in 3D cancer cell cultures with short telomeres, mimicking the effect of telomere elongation [233]. While the mechanism would require further elucidation, this observation suggests that TERRA rG4s may have a signaling role in cells with perturbed telomere maintenance; however, these results are at odds with the recent observation that in fibroblasts undergoing replicative crisis, increased levels of TERRA participate in triggering the innate immune response to telomere shortening through the interaction with the innate immune sensor Z-DNA binding protein 1 (ZBP1) [59]. TERRA-bound ZBP1 can activate the innate immune adapter protein mitochondrial antiviral-signaling protein (MAVS) leading to interferon response and cell death [59]. Furthermore, telomere elongation by overexpression of telomerase was previously reported to decrease TERRA levels in human cancer cells and fibroblasts [28]. The conflicting evidence reported in these studies may be explained by the different cell lines used.

In other works, the physiological functions of TERRA rG4s have been explored mainly with the emphasis on its protein interaction partners, as summarized in the next part of the paragraph.

6.3. TLS/FUS interaction with telomeric DNA and TERRA may impact telomere homeostasis

TLS/FUS (translocated in liposarcoma/fused in sarcoma) is a protein with many functions including roles in RNA processing, DNA damage, and stress response [234] that also localizes to telomeres [235]. It has been demonstrated that TLS/FUS forms a ternary complex with telomeric G4 and TERRA rG4 in K^+ buffer, and the interaction is disrupted both by G-to-U/T mutations in the RNA and DNA oligonucleotide, respectively, and the replacement of potassium with lithium, indicating that both DNA and RNA G4 structure is required for the binding [236] (Fig. 4C). The RGG3 domain of the protein mediates the interaction, and if deleted, the *in vivo* interaction of TLS/FUS with telomeric DNA or TERRA is lost [236]. Searching for the physiological relevance, the overexpression of TLS/FUS was found to lead to H3K9me3 accumulation and telomere shortening in an RGG-dependent manner, leading the authors to propose that the interaction between TLS/FUS, TERRA rG4, and telomeric DNA G4 is important for histone modification at telomeres that has previously been connected to telomere maintenance [236].

6.4. TRF2 & TRF1 binding to TERRA rG4 can influence telomere stability

TERRA and TRF2 have been shown to form a ternary complex with origin recognition complex (ORC) that promotes the heterochromatin formation at telomeres and possibly DNA replication [27]. As outlined in paragraph 2.1, *in vitro*, it has been shown that it is the rG4 structure of TERRA, rather than its sequence, that is required for the interaction with TRF2, and that TRF2 binds TERRA rG4 and telomeric DNA duplex simultaneously [44] (Fig. 4C).

The interaction with TERRA rG4 is mediated by the N-terminal GAR domain of TRF2, while the Myb/SANT domain binds telomeric dsDNA [27,39,237,238]. The loss of TRF2 interaction with TERRA rG4, induced either by TERRA G4 ligand NMM or deletion of the TRF2 GAR domain, leads to a drop in TERRA nascent transcription, telomere shortening, accumulation of telomeric DNA damage, and telomere fragility. While

the authors point out that NMM also inhibits telomerase, they also suggest that the effect on telomere stability is very fast, likely reflecting telomerase-unrelated changes in telomere structure and replication stress [237]. As TRF2 was reported to act as a repressor of TERRA expression [40,41], it will be interesting to investigate the molecular mechanisms by which it may facilitate TERRA nascent transcription.

In vitro, TERRA rG4s are also required for the interaction with TRF1, the other main dsDNA-binding telomeric protein in humans [39]. As the authors have previously demonstrated that TRF2 aids TERRA in telomeric DNA duplex invasion and TRF1 suppresses this process [239] (Fig. 4C), these observations suggest that TERRA rG4 formation may be a crucial player in the regulation of R-loop formation at telomeres with potential consequences on telomere stability.

6.5. hnRNPA1-rG4 TERRA binding may impact telomerase activity and telomere capping

Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) is a protein with a number of roles exerted through RNA binding, also capable of interaction with telomeric DNA. It can also stimulate telomerase activity in a cell cycle-dependent manner [240,241]. hnRNPA1 was shown to bind telomerase RNA [242] and TERRA [243], with the latter work suggesting that TERRA displaces hnRNPA1 from telomeric DNA to allow for POT1 binding, promoting telomere capping. Moreover, as discussed in paragraph 2.1, a fine balance between TERRA and hnRNPA1 was proposed to be required for telomerase activity, as hnRNPA1 binding of TERRA may prevent telomerase inhibition by TERRA, but hnRNPA1 in excess was also observed to function as telomerase inhibitor in a direct telomerase activity assay [82]. As discussed in paragraph 2.1, the role of TERRA in the regulation of telomerase in human cells remains to be defined [244].

The interaction between hnRNPA1 and TERRA was found to be mediated by rG4 structures. Liu and colleagues have demonstrated that hnRNPA1 binds to rG4s species bearing loops in their structure (intramolecular or TERRA dimer rG4), not to the single-stranded or tetramer rG4 structure [245]. The authors further developed an rG4-specific fluorescent ligand (Cy5-linked macrocyclic heptaoxazole) and confirmed the co-localization of hnRNPA1 and rG4 *in vivo* [245] (Fig. 4C). Both RGG and UP1 domains of hnRNPA1 were later shown to be involved in the binding, with RGG specifically enhancing hnRNPA1 affinity towards loop-containing rG4s [246]. The UP1 domain also exhibits the capacity to unfold the loop-containing TERRA rG4 structure [246]. Similarly, the RGG box was previously shown to mediate binding to telomeric DNA G4 and aid the UP1 DNA G4 unwinding activity [247]. An emerging picture for several of these proteins is thus that TERRA and telomeric ssDNA or telomeric DNA G4s may both bind to proteins involved in telomere stability *in vivo*, which can lead to their telomeric recruitment, or a competition between the two interaction partners.

6.6. LSD1-rG4 TERRA interaction can influence DDR at telomeres and regulate CoREST enzymatic activity

As mentioned in paragraph 4, another protein connected to telomere biology and previously identified to bind TERRA is LSD1. This enzyme removes methyl groups on H3K4/H3K9 in cooperation with CoREST protein, remodeling chromatin and regulating gene expression [248]. As discussed in the previous paragraph, its interaction with TERRA in TRF2-depleted HeLa cells, has been previously proposed to promote Mre11-mediated resection of the 3’ G-rich overhang of uncapped telomeres [41], a process that precedes chromosome end recognition by the nonhomologous end-joining (NHEJ) machinery and telomere fusions [249]. *In vitro*, the LSD1-CoREST demethylation complex interacts with TERRA rG4 which is capable of negatively regulating its demethylation activity as a noncompetitive inhibitor [153] (Fig. 4C).

6.7. PRC2 may be regulated by TERRA through rG4

The polycomb PRC2 complex is another transcription repressor involved in the chromatin remodeling of chromosome ends that is capable of TERRA rG4 binding. *In vitro* studies involving circular dichroism and EMSA experiments demonstrated that PRC2 has a binding preference for rG4-forming TERRA (and G-tract containing motifs in general), with controls involving a scrambled-sequence oligonucleotide and K^+ vs. Li^+ binding buffer [80] (Fig. 4C). Previously, the TERRA-driven telomeric recruitment of PRC2 was shown to be important for heterochromatin establishment at telomeres in human cells [81].

In vitro, TERRA rG4s have been observed to interact with other proteins that may influence telomere stability. Nucleolin, a phosphoprotein component of nucleoli, was shown to bind telomerase and this interaction can be important for the nucleolar localization of telomerase [250]. In a recent study, nucleolin was reported to bind both TERRA and telomeric DNA G4s, destabilizing the latter, opening a possibility that it plays a role in the G4 regulation at telomeres [251]. The authors speculate that TERRA rG4 binding may influence telomerase recruitment to telomeres. Overall, these studies demonstrate that TERRA rG4 protein interaction partners identified both *in vitro* and *in vivo*, some of them recruited by TERRA to telomeres, play roles in the establishment of telomeric heterochromatin (TLS/FUS, PRC2, TRF2, LSD1) [252], telomere capping (TRF2, LSD1, hnRNPA1) and may impinge on telomerase recruitment and activity (hnRNPA1, nucleolin), together proposing a picture of multifaceted involvement of TERRA rG4s in maintaining telomere stability.

More studies will be required to identify under which conditions TERRA forms rG4s *in vivo*, and what roles of TERRA are connected to the formation of intramolecular, but also intermolecular G4s. Interestingly, the G4 ligand NMM was found to have a higher affinity for TERRA than for telomeric DNA G4 [237], and a widely used G4 stabilizer TMPyP4 also binds TERRA [253], opening a possibility that the impact of G4 ligands on telomere biology may be at least partially exerted through binding to TERRA rG4s. TERRA rG4s have indeed been explored as a potential therapeutic target. Two chromene compounds were identified as selective stabilizers of TERRA rG4 (as compared to telomeric DNA G4s) and were found to have anti-proliferative effects on lung and colorectal adenocarcinoma [254]. A quindoline derivative capable of TERRA rG4 stabilization strengthens its binding with TRF2 and this induces a dissociation of TRF2 from telomeres, leading to DNA damage and cell cycle arrest in ALT osteosarcoma cell line U2OS [255]. Similarly, in multiple myeloma, a newly identified TERRA rG4 stabilizing drug with undisclosed structure also induces TRF2 displacement from telomeres, DNA damage response and exhibits anti-proliferative activity [256].

Adding to the structural space of telomeric secondary structures, the existence of a chimeric DNA:RNA G4 structure has also been reported. It has been studied both *in vitro* and *in vivo* and proposed to play a role in telomere capping [257–259].

7. Concluding remarks

An increasing amount of evidence indicates that TERRA expression is controlled in a telomere-specific manner. This regulation relies for a good part on the subtelomeric sequences of chromosomes presenting binding sites for different transcription factors and TERRA promoter regions laying at different distances from the telomeric repeat tract, containing or not CpG dinucleotide sequences. The telomeric regions of chromosomes also participate to the telomere-specific regulation of TERRA, as telomere length can significantly influence TERRA expression, possibly by impinging on the local chromatin.

Several findings indicate that these regulatory mechanisms have been developed to enable proper telomere maintenance, in particular in response to stress conditions. Telomeres can react to oxidative stress,

heat shock, DNA damaging agents, or chromosome end erosion by upregulating TERRA levels. Under these circumstances, failure in promoting TERRA transcription may precipitate telomere dysfunction. Thus, given the importance of TERRA in telomere stability, its telomere-specific regulation poses an obvious question, which is *why would defined stimuli induce TERRA only from a subset of telomeres instead from all chromosome ends?* One possibility is that the total levels of TERRA in the nucleus is what matters to allow telomere homeostasis. This hypothesis would fit with the observations that TERRA molecules only transiently localize to telomeres, and as a non-stable constituent of telomeres, TERRA transcripts expressed from a single chromosome may in principle act on multiple chromosome ends. This scenario is also suggested by the evidence that TERRA molecules can relocate from their transcription site to multiple telomeres. In addition, having different regulatory sequences across subtelomeres may enable a cell to respond to a higher number of stimuli as compared to a scenario where all subtelomeres contain the same TERRA promoter. However, these hypotheses are at odds with the observations that removal of TERRA transcription factor binding sites from a single subtelomere exposes this engineered chromosome end, and no other telomeres, to activation of DDR under replication stress conditions or etoposide treatment, suggesting that the proposed protective function of TERRA is exerted *in cis* at its telomere of origin. Similarly, an *in cis* function of TERRA as positive regulator of telomerase has been observed in yeasts. The fine-tuned regulation of TERRA transcription is indeed important to telomere homeostasis as induced transcription from a single telomere promotes exonuclease activity at this chromosome end and consequent shortening of the same telomere. In this regard, in future studies it will be important to attempt to tease out the impact of the transcription process from the roles that TERRA molecules may play at their telomere of origin. Further insights into this matter may be revealed by live imaging studies on TERRA that enable tracking TERRA molecules expressed from a single telomere.

Following this reasoning on the importance of telomere-specific regulation of TERRA expression in telomere stability, emerging evidence in the field places us in front of an additional dilemma. Intriguingly, it was reported that TERRA regulation also relies on its post-transcriptional modifications. Indeed, the subtelomeric-derived sequence of TERRA can be m^{6}A modified, depending on the presence of METTL3 functional motifs in these regions, and this modification regulates the stability of the transcripts. Moreover, the 3′ end of TERRA can be polyadenylated in a telomere-specific manner. The poly(A) tail addition can also influence TERRA transcript half-life and their subnuclear localization. These findings imply that different telomeres can express TERRA species which are different not only in their subtelomeric sequence or their length, but also for the presence of m^{6}A modifications and the poly(A) tail. Thus, expressing TERRA from one chromosome end may have different implications in terms of its impact in telomere stability and more general TERRA functions, than regulating TERRA from another telomere. It is difficult to reconcile these findings. Increasing the number of TERRA regulatory mechanisms may allow a more combinatorial effect in response to the abovementioned stimuli and/or increasing the number of functions that TERRA can exert at telomeres, extratelomeric sites and even extranuclear or extracellular sites. The use of long-read sequencing technologies may reveal a key asset to better understand these aspects of TERRA biology by enabling researchers to evaluate the sequence and post-transcriptional modifications of full-length TERRA transcripts [260–262].

Somewhat reassuring, all TERRA transcripts can fold into rG4s and form telomeric R-loops by virtue of their shared G-rich 3′ end sequence. These structures can significantly impact telomere stability and mediate TERRA functions, by promoting TERRA-protein interactions or impacting processes such as DNA replication. Thus, in principle, all TERRA molecules expressed from any telomere may synergically act in promoting rG4- and R-loop-mediated processes or acting as scaffold for TERRA-binding factors. Although it must be considered that longer TERRA transcripts, which can be expressed from longer telomeres, may be more prone to rG4 and R-loop formation or in scaffolding binding partners than the TERRA species consisting of shorter 3′ end sequences. Furthermore, evidence suggests that R-loops can occur both co-transcriptionally and *in trans*, and they can be enriched at specific (short) telomeres. In order to gain further insights into these processes and on the impact of TERRA secondary structures in telomere biology, it will be important to develop tools that enable the study of telomeric R-loops at single telomere resolution and the discrimination of rG4 from DNA G4.

These observations also have practical implications for the study of TERRA. First, TERRA detection by northern blot is clearly a key approach to quantify the total levels of TERRA in cells. However, investigating TERRA expression from single telomeres by RT-qPCR, or single-molecule sequencing, can provide further information such as the fraction of post-transcriptionally modified TERRA molecules over the total TERRA population and their telomere of origin. In addition, an important information is the extent of TERRA localization at telomeres, since this does not necessarily correlate with TERRA levels. Finally, importantly, TERRA depletion by ASOs and more in general RNAi-based approaches, may result in a different outcome compared to repression of TERRA transcription, since persistent telomeric R-loops may result more impervious to ASO depletion than nucleoplasmic TERRA molecules, and due to the impact that the process of transcription can have on telomere stability, including transcription-replication conflicts which are exacerbated by R-loop formation. It may be useful to consider this prospect in particular in contexts where R-loops are persistent at telomeres, such as in ALT cells.

The recent findings on TERRA post-transcriptional modifications, its role in innate immune response and on its possible translation giving rise to dipeptide repeat proteins [263] highlight that novel unexpected features of TERRA are incumbent and further investigations in the field will be required in order to define the mechanisms of TERRA regulation and function. Given the conservation of TERRA expression across different organisms, but also the species-specific features of TERRA reported to date, investigations of these telomeric repeat-containing RNAs in different model organisms may set the stage to further unanticipated scenarios on the mechanisms of TERRA regulation and the roles that these RNAs can play in telomere biology.

Declaration of Competing Interest

none.

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