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TERRA and the state of the telomere

Karsten Rippe[1] & Brian Luke[2]

Long noncoding telomeric repeat–containing RNA (TERRA) has been implicated in telomere maintenance in a telomerase-dependent and a telomerase-independent manner during replicative senescence and cancer. TERRA's proposed activities are diverse, thus making it difficult to pinpoint the critical roles that TERRA may have. We propose that TERRA orchestrates different activities at chromosome ends in a manner that depends on the state of the telomere.

Long noncoding RNAs (lncRNAs) regulate genome function via a diverse set of mechanisms, including recruiting chromatin modifiers, regulating protein activity as *trans*-acting factors and performing architectural functions[1,2]. TERRA appears to integrate all of these activities into a single transcript that regulates telomere maintenance in response to a variety of cellular signals. TERRA is an RNA polymerase II–derived transcript composed of subtelomeric and telomeric repeat sequences, and it has been found in all eukaryotes tested to date[3–5]. In telomerase-containing human and mouse cells, TERRA is expressed at the G1-S transition[6,7] and appears to peak in early S phase[7]. It then decreases again toward the end of S phase as cells complete replication and pass into the G2 and M phases of the cell cycle[8,9]. Accordingly, TERRA foci colocalize with telomeres in S phase and diminish as cells pass into G2 (ref. 8). Although there have been substantial advances in understanding the biogenesis and regulation of TERRA in cells from eukaryotes including yeast and humans (recently reviewed in refs. 10–12), the functional relevance of telomere transcription remains to be determined. Specifically, does TERRA have a function that is pertinent to telomere biology, and do its proposed functions depend on the telomere state? Are the roles of TERRA different at short compared to long telomeres, and are those roles altered at recombining telomeres compared to those that are maintained by telomerase? Indeed, such questions are fundamental to the TERRA field and may also be relevant to the function of the numerous lncRNAs that have been annotated through large-scale sequencing efforts. In this Perspective, we consider selected recent publications that have interrogated TERRA function at telomeres.

TERRA levels are controlled both transcriptionally, at TERRA's subtelomeric CpG-rich promoter, and post-transcriptionally, via regulation of TERRA RNA-DNA hybrids and the stability of nontelomere-associated TERRA in the nucleoplasm. Here, we focus on the potential roles and regulation of TERRA conferred by its ability to form RNA-DNA hybrids and hence R-loop structures at telomeres, as well as its capacity to associate with and influence heterochromatin factors *in cis*. We will not discuss the regulation of TERRA promoter activity by DNA methylation[5,13–15] or by binding of CTCF or cohesin[16], as well as the proposed functions of TERRA as a *trans*-acting factor, because a host of recent reviews have provided a comprehensive overview of these aspects of TERRA activity and regulation[3,10–12,17].

TERRA and R loops at short, damaged and recombinogenic telomeres

RNA-DNA hybrids can form cotranscriptionally when the nascent RNA base-pairs with the template strand behind the advancing RNA polymerase. Alternatively, the RNA may dissociate from the DNA template and reanneal to a homologous sequence after a Rad51-dependent strand-invasion reaction[18,19]. Irrespective of the genesis of RNA-DNA hybrids, the end result entails the displacement of one strand of DNA to form a three-stranded stretch of nucleotides, referred to as an R loop (**Fig. 1**). Because GC-rich transcripts have an increased propensity to form R loops, the displaced G-rich DNA strand has been suggested to form guanosine-quadruplex (G4) structures. Indeed, TERRA R loops exist at telomeres in both yeast and human cells[20–23], and their presence at telomeres may have important implications for local DNA transactions. R loops hinder the progression of advancing replication forks, thus leading to replication stress and even double-strand break formation[24,25]. As a consequence, telomeric R loops increase the likelihood that homology-directed repair (HDR) will occur between either sister-chromatid or homologous telomeres. TERRA RNA-DNA hybrids appear to have a particularly important role at telomeres when HDR becomes essential, for example, at critically short telomeres in presenescent yeast cells[20,26]. Moreover, telomeric R loops strongly influence telomere maintenance in human alternative lengthening of telomeres (ALT) cancer cells as well as in yeast survivors that use HDR as the sole means of telomere maintenance[22,23]. TERRA levels are specifically upregulated at short or deprotected telomeres[7,27,28] and in cells using recombination to elongate their telomeres[13,14,22,23,29]. Overexpression of RNase H, which decreases TERRA levels and R loops at telomeres, prevents efficient telomere elongation via HDR in human ALT cells as well as in yeast survivors and presenescent yeast cells[20,22,23]. Consistently with this observation, accumulation of telomeric R loops increases HDR at telomeres in these cell types when RNase H is inhibited. Strikingly, neither the removal nor the accumulation of R loops has an overt effect on telomere length in telomerase-positive yeast cells[4,20,22], thus suggesting that TERRA R loops are functionally relevant only at short or deprotected and recombining

[1]Research Group Genome Organization & Function, Deutsches Krebsforschungszentrum (DKFZ) and Bioquant Center, Heidelberg, Germany. [2]Institute of Molecular Biology (IMB), Mainz, Germany. Correspondence should be addressed to K.R. (karsten.rippe@dkfz.de) or B.L. (b.luke@imb-mainz.de).

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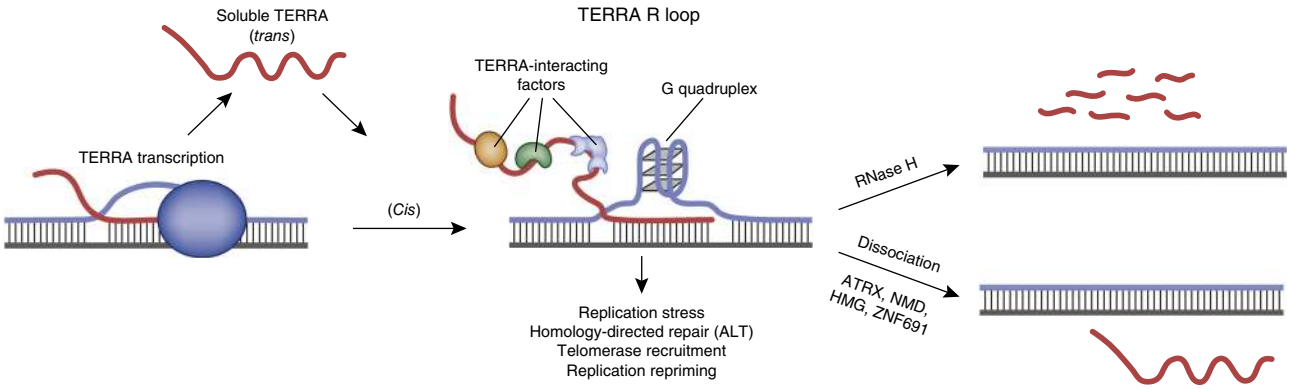

Figure 1 Formation and removal of TERRA R loops at telomeres. Through formation of an R loop, TERRA can act *in cis* on a given telomere. Its activity may occur via recruitment of TERRA-interacting factors or originate solely from the formation of an R-loop structure induced by the hybrid. The latter may be stabilized by a G quadruplex forming at the displaced G-rich DNA strand. Both RNA-DNA hybrids and G quadruplexes induce replication stress, thereby promoting either HDR or telomerase recruitment. The RNA-DNA hybrids can be resolved by RNase H–mediated degradation of TERRA or by factors that favor TERRA displacement. The latter involve the ATRX helicase[8], which could drive displacement of TERRA from the R loop by resolving G quadruplexes and/or act as an RNA-DNA helicase. Furthermore, protein components involved in nonsense-mediated RNA decay (NMD), such as the RNA-DNA heteroduplex helicase UPF1 (refs. 10,31), HMGA1 and HMGB1 high-mobility-group proteins and ZNF691 zinc-finger protein, counteract association of TERRA with telomeres[42].

telomeres. Consistently with this notion, telomeric R-loop levels are not affected in telomerase-positive human cells after RNase H overexpression or depletion[17], thus indicating that RNA-DNA hybrids are efficiently inhibited though multiple redundant mechanisms, as discussed below. The combined results of these studies suggest that TERRA R loops promote HDR-mediated telomere elongation in the absence of telomerase only at telomeres that require HDR for their maintenance and not at normal-length telomeres of telomerase-positive cells. Because TERRA accumulates at short telomeres[7,27], it will be important to determine whether this is also true in ALT and/or survivor cells. It is conceivable that an increased frequency of critically short telomeres in ALT cells may account for increased TERRA levels, and thus that the increase in TERRA would occur in a similar manner in both pre- and postsenescent cells. The fraction of TERRA associated with the telomeres may also increase without apparent changes to the total cellular TERRA levels, which include soluble nucleoplasmic TERRA and TERRA present in nontelomeric foci. Such a scenario has been reported in the absence of the nonsense-mediated-decay factor Upf1 (refs. 30,31).

Why does TERRA accumulate specifically at short telomeres and ALT telomeres but not at normal-length telomeres in telomerase-positive cells? A recent study has demonstrated that the cell-cycle regulation of TERRA becomes perturbed at telomeres that are maintained by HDR[8]. In telomerase-positive cells, at telomeres with 'normal' length attributes, TERRA levels are kept low, and the RNA is removed from telomeres during G2-M. In contrast, TERRA remains telomere associated at G2-M in cells that use the ALT mechanism[8], in which total TERRA levels are greater than those found in telomerase-positive cells[13,14,22,23,29]. Interestingly, impairment of ATRX, a helicase that is frequently mutated in ALT-positive cancers[29], also leads to G2-M TERRA accumulation at telomeres even in telomerase-positive cells[8]. ATRX is a factor involved in establishing transcriptionally silenced heterochromatin by depositing the histone H3 variant H3.3 at pericentromeric and telomeric repeats[32–35]. It is not yet clear how, or whether, ATRX directly leads to TERRA displacement. One hypothesis is that ATRX interacts with and induces the resolution of G4 DNA secondary structures[36] on the displaced strand of a TERRA R loop through its helicase and ATPase activity. This would in turn promote RNA displacement by increasing base-pairing of the displaced DNA strand with the template strand (**Fig. 1**). It is thus necessary to determine whether resolving TERRA R loops is a functionally relevant mechanism of ATRX action. However, because R loops are known to accumulate at telomeres in U2OS cells[22] that are ATRX deficient, this is a plausible assumption. It will be equally important to understand whether TERRA R loops are formed *in cis* or *in trans*. A recent report has indicated that, in mouse embryonic fibroblasts, TERRA or TERRA-like molecules are largely derived from a single chromosome end even though they are found associated with other telomeres[9]. In this respect, mouse TERRA appears to differ from that of yeast and human cells, in which all telomeres are apparently transcribed. Indeed, TERRA-telomere associations in yeast occur *in cis*, because TERRA derived from one telomere preferentially reassociates with the telomere from which it was transcribed[27]. Finally, it will be interesting to determine whether the cell-cycle regulation of TERRA is also perturbed at short or deprotected telomeres, as is observed at telomeres maintained by the ALT mechanism.

A further consideration is that TERRA accumulation may be a consequence rather than a cause of replication stress at short or recombining telomeres. In this scenario, TERRA may promote replication-fork restarting by recruiting HDR mediators or even by directly priming replication in an origin-independent manner, as has recently been shown to occur at the rDNA locus in budding yeast[37]. Indeed, replication has been reported to initiate, albeit very infrequently, within the telomeric repeats. Interestingly, removal of TRF2, which also results in increased TERRA levels[28], does not lead to telomeric replication stress[38]. Thus, TERRA accumulation also originates from perturbations of the intact telomere that are independent of replication stress. Together, these findings suggest that a key regulatory step in TERRA metabolism may be the differential regulation of RNA-DNA hybrids in a telomere state–dependent manner.

TERRA as a regulator of telomeric chromatin state

The ability of TERRA to associate with telomeres provides an additional mechanism to specifically target auxiliary protein factors to chromosome ends via an RNA-mediated interaction. However, with the exception of the heterogeneous ribonucleoproteins, no clear

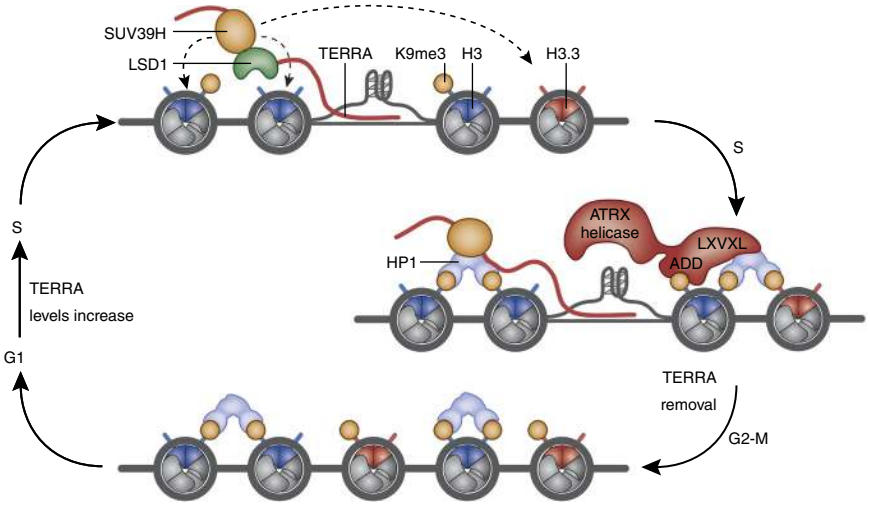

Figure 2 Cell cycle–dependent interplay of TERRA activity and H3K9me3. During S phase, TERRA transcription induces the reestablishment of the H3K9me3 heterochromatin state by interactions of TERRA with SUV39H1, HP1 and LSD1. In addition to the RNA-DNA-hybrid interaction depicted in the schematic, TERRA may also potentially associate with telomeric repeats in the absence of R-loop formation via RNA-protein interactions. The presence of HP1 and H3K9me3 and the absence of H3K4me3 due to LSD1 activity promote ATRX recruitment to telomeres during S phase[67], according to the binding mechanism described previously[50,51]. Through its various enzymatic activities, ATRX resolves G quadruplexes (possibly in conjunction with interacting factors) and displaces TERRA during the G2-M phase of the cell cycle[8]. At the same time, ATRX promotes the incorporation of H3.3, which stabilizes and enhances the H3K9me3 state. As a consequence, high H3K9me3 levels keep telomere-associated TERRA low or absent in G2-M until the trimethyl mark is diluted during replication in the subsequent S phase, and TERRA becomes retranscribed.

TERRA-interacting proteome is apparent from the literature[39–42]. Nonetheless, several lines of evidence point to functional interactions of TERRA with telomeric chromatin containing Lys9-trimethylated histone H3 (H3K9me3), which is characteristic of transcriptionally silenced regions[7,14,28,41]. H3K9me3 is enriched above the genome average at human and mouse telomeres that use telomerase as a telomere maintenance mechanism[7,14,33,43]. In human cells, H3K9me3 density is inversely related to telomeric TERRA levels[7,14]. Moreover, TERRA has been reported to interact with the histone methyltransferase SUV39H1, which sets this trimethyl mark[28], as well as with the heterochromatin proteins HP1α and HP1β, which preferentially bind to nucleosomes with the H3K9me3 modification[41].

Together, the two isoforms SUV39H1 and SUV39H2, HP1 proteins and H3K9me3 compose a circuit that mediates transcriptional silencing at pericentric repeat sequences (ref. 44 and references therein). Recently, this network has also been linked to silencing centromeric-repeat transcription involved in regulation of telomerase activity[45]. The somewhat counterintuitive situation in which active TERRA transcription induces the formation of an H3K9me3-dependent silenced chromatin state may be explained by a previously proposed negative feedback loop mechanism[7]: at telomeres of normal length, TERRA shuts down its own expression by causing SUV39H1 to set the H3K9 trimethyl mark at telomeres, thereby ensuring that further telomere transcription is kept to a minimum. Accordingly, a pioneer round of transcription would be required to prevent further transcription; this model is consistent with the relatively low levels of TERRA expression seen in telomerase-positive cells (**Fig. 2**).

Another potentially important factor in H3K9me3-dependent transcriptional silencing of telomeric repeats is the above-mentioned ATRX helicase and ATPase, which has a number of diverse roles. ATRX, a component of silenced pericentric heterochromatin, deposits histone variant H3.3 from the histone chaperone DAXX[32–34] downstream of SUV39H and HP1 binding[46]. Notably, H3.3 incorporation by ATRX and DAXX also occurs at telomeric repeats[35]. ATRX has been linked to H3K9me3-mediated silencing of endogenous retroviral element repeats[47,48] and imprinted loci[49], and deletion of ATRX leads to loss of H3.3 and the H3K9me3 modification within these regions. These findings suggest that ATRX and H3.3 are needed to maintain a transcriptionally silenced heterochromatin state.

The ADD domain of ATRX and the chromodomain of HP1 both specifically bind to H3K9me3-containing nucleosomes, and ATRX also interacts with HP1 via its LXVXL motif[50,51]. However, direct recognition of H3K9me3 by ATRX is inhibited by the co-occurrence of Lys4-trimethylated histone H3 (H3K4me3). The H3K4 trimethyl mark is set by the mixed-lineage leukemia (MLL) methyltransferase, which has been shown to associate with telomeres and to promote TERRA transcription[52]. Removal of the H3K4 trimethyl mark is catalyzed by the histone demethylase LSD1, which has been identified as a direct TERRA-interacting partner both *in vivo* and *in vitro*[53]. Accordingly, TERRA may prevent the occurrence of H3K4me3 via LSD1 recruitment, which promotes binding of ATRX to H3K9me3-modified nucleosomes. That said, the upregulation of TERRA at damaged telomeres has not yet been linked to changes in the density of telomeric trimethylated or dimethylated H3K4 but rather to the MRE11 nucleases[53]. It will therefore also be important to investigate the potential effects of TERRA on H3K4me3 levels at nondamaged telomeres. We conclude that, at telomeres, H3K9me3 and TERRA are linked by TERRA's interaction with SUV39H1 and HP1, and, in addition, ATRX interacts with HP1 and binds H3K9me3 in the absence of H3K4me3, owing to TERRA's recruitment of LSD1 (**Fig. 2**). ATRX could in turn promote further accumulation of H3K9me3 at telomeres by depositing H3.3 by the mechanism reported for transcriptional silencing at other genomic loci[47–49]. ATRX helicase and ATPase activity could also remove TERRA, either directly or by resolving G4 structures, and thus counteract formation of TERRA-DNA hybrids. In summary, we speculate that ATRX is targeted to telomeres by an initial TERRA-induced accumulation of H3K9me3 and HP1, which subsequently promotes a repressive chromatin configuration that counteracts TERRA-telomere association (**Fig. 2**). This model proposes that partial loss of this silencing mechanism during replication triggers TERRA reexpression and R-loop formation during S phase, thus inducing silencing.

Histone modifications that could potentially counteract H3K9me3-dependent silencing include phosphorylation of Ser10, Thr11 and Ser31 of histone H3 and the H3.3 variant[45,54–57]. In particular, Ser10-phosphorylated H3 is indicative of an active chromatin state that impedes HP1 binding[45,54,55]; its accumulation also coincides with the presence of R loops and could thus favor a chromatin environment that promotes formation of RNA-DNA hybrids[58].

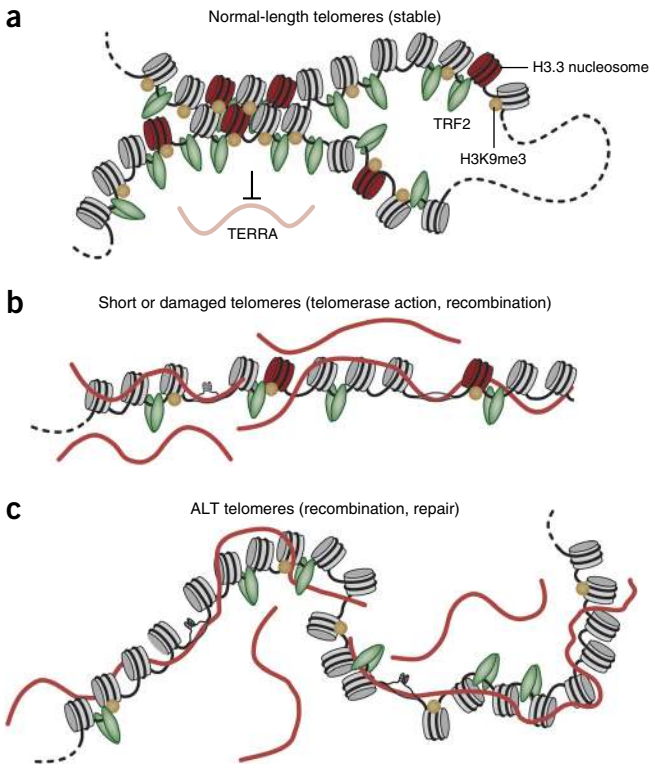

Figure 3 TERRA activity and telomere state. (**a**) At normal telomeres in telomerase-positive cells, the H3K9me3 modification (also shown in **Fig. 2**) and TRF2 are abundant, and the histone variant H3.3 (red) is incorporated by ATRX. Long-range telomere back-folding and physical interaction with other chromosome domains (TPE-OLD)[59] are possible. Together, these mechanisms ensure that TERRA is maintained at a low level. (**b**) At short or damaged telomeres, TERRA is upregulated, and the amounts of associated and soluble TERRA are increased. This might be related to loss of TPE-OLD[59], decreased H3K9me3 levels[7] or depletion of TRF2 (ref. 28). In the latter case, H3K9me3 levels are increased, thus pointing to a difference between short versus long TRF2-depleted telomeres. At these telomeres, the likelihood may increase that they will be extended by either HDR or telomerase, respectively. (**c**) ALT telomeres are frequently associated with loss of functional ATRX and H3.3 incorporation[29], lower H3K9me3 levels and increased sensitivity to micrococcal nuclease[14], and PML-dependent TRF2 depletion[66]. These features of ALT telomeres are correlated with high TERRA expression and increased telomere association. The latter may promote HDR through an R-loop intermediate.

TERRA and telomere status

Although many open questions remain regarding how TERRA affects telomere biology, it is important to consider three distinct scenarios for TERRA function: at normal-length telomeres, at shortened or damaged telomeres and at telomeres maintained by the ALT pathway (**Fig. 3**).

Normal telomeres. TERRA association with telomeres of normal length leads to recruitment of factors that promote a repressive chromatin state via the H3K9me3-SUV39H-HP1 transcription-silencing network (**Fig. 2**). In this manner, TERRA appears to inhibit its own expression[7,14]. Additionally, ATRX functions together with other factors for removal or degradation of TERRA and probably limits the lifetime of TERRA and associated R loops. The relatively long repeat length at normal-length telomeres permits the telomere to fold back and to organize itself into distinct topological structures. This ability to fold back and physically interact with other chromosome domains (referred to as the telomere position effect over long distances (TPE-OLD))[59] may contribute to maintenance of the heterochromatic telomere state, which keeps TERRA expression and R loops to a minimum, presumably to prevent replication stress (**Fig. 3a**). It remains to be seen whether TERRA negatively regulates itself through the establishment of an inhibitory fold-back structure, as has been described for other lncRNAs at gene loops[60,61]. In summary, at normal-length telomeres, a myriad of measures appear to be in place to ensure that TERRA and R loops are kept to an absolute minimum.

Short or damaged telomeres. Shortening of telomeres leads to increased TERRA levels[7,27,28], and this increase may be due in part to the inability of short telomeres to fold back and exert the TPE-OLD effect, as recently described[59] (**Fig. 3b**). In addition, DNA damage and loss of TRF2 also induce an upregulation of TERRA[28], thus suggesting that at short or damaged telomeres the autorepression mechanisms (depicted in **Figs. 2** and **3a**) are no longer active. Consistently with this view, an inverse relationship between telomere length and H3K9me3 levels has been reported[7]. However, the high TERRA levels observed upon TRF2 depletion are associated with an increase in H3K9me3 modification levels, thus indicating that H3K9me3 alone is unable to trigger TERRA silencing in the absence of TRF2 (ref. 28). Upon TERRA accumulation, R loops may persist because of the inability of TERRA-removal or TERRA-degradation factors (for example, ATRX and RNase H) to associate with shortened or TRF2-depleted telomeres. R loop–mediated replication stress may trigger HDR at short or damaged telomeres in telomerase-negative cells. In telomerase-positive cells, an increase in soluble TERRA promotes recruitment of telomerase to short telomeres[27].

ALT telomeres. Mammalian cell lines or yeast survivors, in which ALT is active, have higher TERRA levels than those in telomerase-positive cells[13,14,22,23,29]. Moreover, loss of ATRX shows a high correlation with human ALT[29] and is considered to be a suppressor of the ALT mechanism[62,63]. Although no clear correlation between ATRX and global TERRA expression levels is apparent[14,29,35,64,65], ATRX may affect the number of TERRA RNA-DNA hybrids. Interestingly, a recent RNA–fluorescence *in situ* hybridization analysis has revealed that short interfering RNA–mediated knockdown of ATRX in HeLa cells increases the number of TERRA foci during the G2-M phase of the cell cycle; this increase reflects bona fide TERRA accumulation at telomeres. Hence, ATRX depletion may stabilize TERRA's association with telomeres, thus leading to eventual replication stress and increased replication-fork stalling. ATRX is also linked to TERRA via the H3K9me3 modification, as discussed above (**Fig. 2**). Accordingly, loss of ATRX and an associated decrease in H3.3 incorporation may contribute to the decrease of the H3K9me3 heterochromatin modification reported for ALT telomeres[14] as well as for other loci[47–49]. In addition, TERRA levels within ALT cells may be influenced by complexes of telomeres with promyelocytic leukemia (PML) protein in so-called ALT-associated PML bodies (APBs). These complexes might provide a structure for TERRA-telomere association[22] (perhaps in an R loop–independent manner) that is absent in telomerase-positive cells and may also affect telomeric-repeat and TRF2 density[66]. The decreased TRF2 density in APBs[66] may further contribute to raising TERRA levels, because it would relieve the TRF2-dependent TERRA silencing described previously[28]. Loss of telomeric repeat–bound TRF2 in APBs may also contribute to the observed increased micrococcal nuclease sensitivity of ALT cells[14], because a decrease in TRF2 would increase the accessibility of linker DNA, thereby facilitating TERRA

R-loop formation. We propose that an interlinked network of TERRA, ATRX, H3K9me3 and TRF2 controls the association of TERRA with telomeres in ALT cells. The individual contributions of these various factors remain to be further explored. However, it appears likely that neither the H3K9me3-mediated TERRA-autorepression mechanism nor the ATRX TERRA-removal pathways function in ALT cells, thus leading to a state mimicking that of short telomeres (**Fig. 3c**).

In summary, current data suggest that TERRA does indeed have a purpose at telomeres, but surprisingly its function appears to be regulated in a telomere state–dependent manner. This differential regulation may represent a cellular signal of telomere state that reflects the altered access of TERRA regulators to different telomere types. Accordingly, it is important to consider telomere context–dependent functions for future studies of TERRA. Critical outstanding questions include how TERRA specifically accumulates at short or damaged telomeres, and in cells using ALT, rather than at normal-length telomeres. Furthermore, how do TERRA levels and R-loop formation impinge on telomere replication: do they promote or respond to replication stress? How R loops support the ALT mechanism by promoting telomere recombination and whether TERRA removal from telomeres is a critical function of ATRX remain to be determined. Finally, how TERRA R loops are regulated and how this regulation varies with telomere state is a future priority. The road to understanding TERRA function seems to have branched according to telomere state, and each new road will have to be further explored before a general map can be drawn.

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COMPETING FINANCIAL INTERESTS

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