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STATE-OF-THE-ART REVIEW

Genomic origin and nuclear localization of TERRA telomeric repeat-containing RNA: from Darkness to Dawn

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Long noncoding RNAs, produced from distinct regions of the chromosomes, are emerging as new key players in several important biological processes. The long noncoding RNAs add a new layer of complexity to cellular regulatory pathways, from transcription to cellular trafficking or chromatin remodeling. More than 25 years ago, the discovery of a transcriptional activity at telomeres of protozoa ended the long-lasting belief that telomeres were transcriptionally silent. Since then, progressively accumulating evidences established that production of TElomeric Repeat-containing RNA (TERRA) was a general feature of eukaryotic cells. Whether TERRA molecules always originate from the telomeres or whether they can be transcribed from internal telomeric repeats as well is however still a matter of debate. Whether TERRA transcripts always localize to telomeres and play similar roles in all eukaryotic cells is also unclear. We review the studies on TERRA localization in the cell, its composition and some aspects of its transcriptional regulation to summarize the current knowledge and controversies about the genomic origin of TERRA, with a focus on human and mouse TERRA.

Introduction

The first time telomeres were reported to be transcribed dates back to 1989 when Rudenko and van der Ploeg observed the transcription of *Trypanosoma brucei* telomeres into noncoding RNA species [1]. A few years later, similar transcriptional activity was observed at the ends of birds' lampbrush chromosomes [2] followed by the detection of TElomeric Repeat-containing RNA (TERRA) in human [3,4], mouse [4], yeast [5–7], Zebrafish [4], and plant [8]. During the past decade, it became clear that, through its ability to interact with multiple protein partners and its propensity to form RNA:DNA hybrids with telomeric repeat-containing sequences, TERRA plays a variety of roles at telomeres that include heterochromatin regulation, telomeric loop formation and telomerase recruitment. TERRA functions at telomeres have been recently reviewed [9] and will not be discussed here.

Although TERRA was initially discovered as the product of telomere transcription, it gradually emerged that not all telomeres may be transcribed or that TERRA may be transcribed from intrachromosomal telomeric repeats, at least in mouse [10,11]. Moreover,

Abbreviations
5′RACE, 5′-Rapid Amplification of cDNA Ends; ALT, alternative lengthening of telomeres; ATRX, Alpha Thalassemia/Mental Retardation Syndrome X-Linked; CHART, Capture Hybridization Analysis of RNA Targets; ChIRP, Chromatin Isolation by RNA Purification; CRISPR-cas9, Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9; CTCF, CCCTC-binding factor; ES, embryonic stem; FISH, fluorescent *in situ* hybridization; HP1α, Heterochromatin Protein 1α ; ICF, immunodeficiency, centromeric region instability, facial anomalies; PAR, pseudoautosomal region; PCR, polymerase chain reaction; qPCR, quantitative PCR; RT, reverse transcription; siRNA, silencing RNA; TAS, telomere-associated sequence; TERRA, Telomeric repeat-containing RNA; TRF2, Telomeric repeat-binding Factor 2; TSS, transcription start site; Xi, X-inactive chromosome.

a recent genome-wide mapping of TERRA-binding sites in mouse embryonic stem (ES) cells revealed the binding of TERRA at both intergenic regions and introns where the telomeric repeat-containing RNAs may regulate gene expression [12].

Here, we review the knowledge about TERRA genomic origin, its localization in the nucleus and we highlight the controversies that subsist in the field, and most specifically for mouse and human TERRA.

Human TERRA

Human TERRA colocalizes with most telomeres

Early studies using RNA-FISH approaches revealed the presence of TERRA on most telomeres of cultured human cells [3]. TERRA was further detected at telomeres of freshly isolated human muscle tissues [13], thus providing evidences for telomere-bound TERRA *in vivo*. These observations supported the view that human telomeres are transcribed, but did not prove that TERRA molecules were produced from all chromosome ends, and whether TERRA molecules remain bound to the telomeres from which they are transcribed. The *UUAGGG* repeat-containing molecules could result from the transcription of either a very limited number of telomeres or intrachromosomal *TTAGGG*-containing loci and being relocated to most telomeres after transcription (Fig. 1).

Transcription from subtelomeric promoters on most human chromosomes

It rapidly emerged that human TERRA molecules are composed of *UUAGGG* repeats preceded, at their 5′ end, by a chromosome-specific subtelomeric sequence that can be amplified by PCR [3] (Fig. 2). Hence, using subtelomere-specific primers, the vast majority of studies have so far supported the telomeric origin of human TERRA and suggested that transcription occurs from subtelomeric promoters located on at least two-thirds of chromosome ends [13–17]. The first human subtelomeric promoters that were identified comprise CpG dinucleotide-rich DNA islands shared among multiple chromosome ends [14]. These CpG islands are characterized by the presence of the so-called 61-29-37 repeats located directly upstream of TERRA Transcription Start Site (TSS) and at ~ 1 kb from the telomeric tract [14,18]. More recently, a study utilizing an RNA-seq approach in HeLa cells further

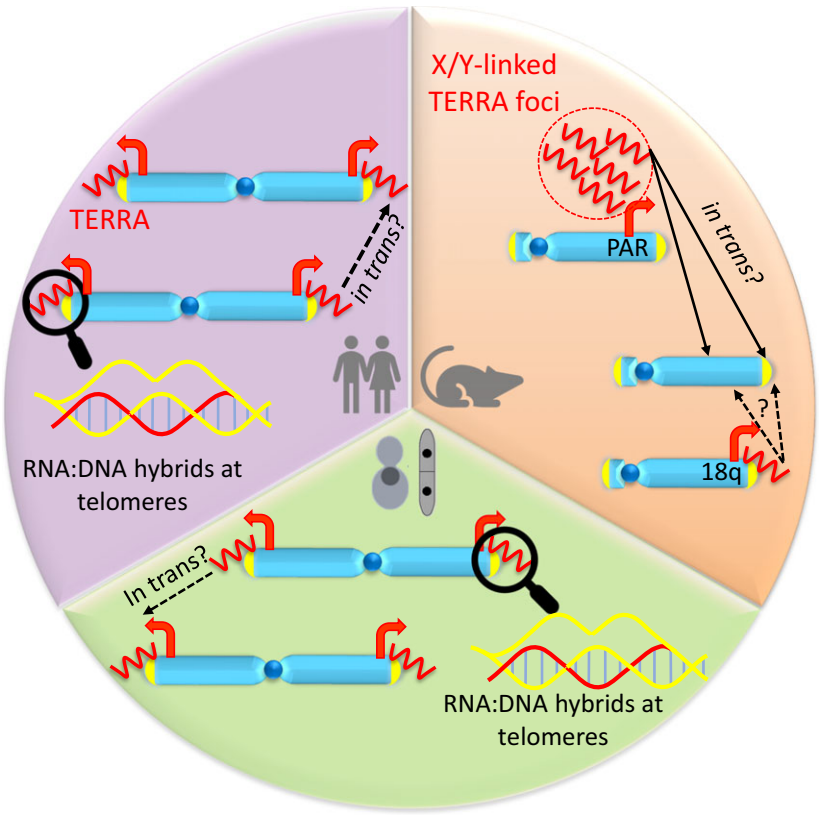

Fig. 1. Overview of TERRA genomic origin and localization in human, mouse, and yeast. The figure summarizes the main findings related to the genomic loci TERRA molecules are transcribed from, and are localized to, in the indicated species. TERRA molecules are shown in red, telomeres are in yellow and centromeres in blue. Dotted arrows indicate hypothetical movements of TERRA. PAR refers to the pseudoautosomal subtelomeric locus of sex chromosomes that has not been precisely mapped yet due to incomplete sequencing and assembly of the region.

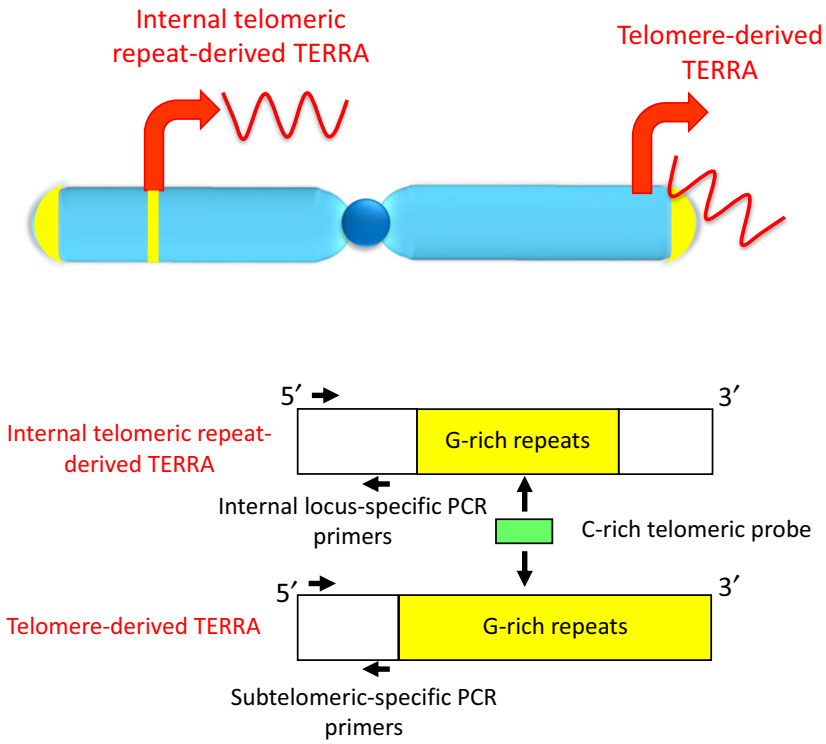

Fig. 2. Similarities and differences between telomere-derived and intrachromosomal-derived TERRA. Both telomere-derived and intrachromosomal-derived TERRA molecules display the presence of *UUAGGG* repeats that can be detected by northern blotting using C-rich telomeric probes. TERRA molecules can be analyzed distinctly by qRT-PCR using primers located either in their 5′-end subtelomeric sequence, for telomere-derived TERRA, or in the chromosomal sequences flanking the internal repeats for intrachromosomal-derived TERRA.

reported the presence of promoters located as far as 5–10 kb upstream of *TTAGGG* repeats on ten distinct chromosome ends, suggesting the existence of two different types of subtelomeric promoters [15].

Additional experimental evidences were provided that further support the existence of active human subtelomeric promoters. They include the demonstration that activated RNA polymerase II (pSer2 and pSer5) binds to both 61-29-37 repeats and telomeric repeats [14] and that CTCF transcription factor and cohesin were similarly found to bind the CpG islands at human subtelomeres to regulate TERRA transcription [17].

Human 20q subtelomere as the main source of TERRA?

On the other hand, a recent study proposed that the pool of human TERRA mostly originates from 20q subtelomere [19]. Using the CRISPR-cas9 technology to delete a 8.1-kb region from the 20q locus of U2OS osteosarcoma cells, Montero *et al.* reported an almost complete abrogation of TERRA expression [19]. It is important to note however that U2OS cells harbor numerous chromosomal abnormalities and maintain their telomeres through homologous recombination mechanisms [alternative lengthening of telomeres (ALT)] associated with unstable telomeres, dysregulated telomeric chromatin and altered TERRA expression [20,21]. However, the failure to obtain viable clones with 20q deletions in HeLa or HCT116 telomerase-expressing cancer cell lines or in normal IMR90 fibroblasts prevented Montero *et al.* from further testing the hypothesis of the nearly exclusive 20q origin of human TERRA in non-ALT cell lines [19].

Telomere length influences human TERRA length and transcription levels

From the first report on human TERRA, it rapidly emerged, based on northern blot analyses, that TERRA molecules displayed length heterogeneity [3]. It was still unclear though, at that time, whether the TERRA length heterogeneity was a result of distinct telomere lengths. The first correlation between TERRA length and telomere length was reported in 2008 when ectopic overexpression of telomerase in fibroblasts derived from patients with Immunodeficiency, Centromeric region instability, Facial anomalies (ICF) syndrome resulted in over-elongation of the telomeres accompanied with a correlative increase in the length of telomeric transcripts [22]. A few years later, using a similar approach of ectopic telomerase overexpression to over-elongate telomeres in various

human cell lines, we similarly concluded that TERRA length is dependent on telomere length [23,24].

The observation that telomere length influences TERRA length strongly supported the telomeric origin of human TERRA. However, based on the technical challenges linked to northern blot analyses of TERRA — possibly because these molecules adopt secondary structures that impede their migration properties in agarose gels — a proper evaluation tool of TERRA length is still lacking [24]. On the other hand, Porro *et al*. reported that the majority of TERRA *UUAGGG* tracts are shorter than 400 bases and that the length of TERRA is mostly dependent on the heterogeneity of subtelomeric TERRA 5′ ends [25]. This conclusion however was based on an experimental approach assuming that human telomeric tracts are made of pure *TTAGGG* repeats, which may not be a general feature of telomeres. This should be investigated further in the future.

The demonstration that telomere length also impacts on TERRA expression levels was yet another evidence in favor of human telomere transcription. Indeed, we reported that short telomeres display an increased transcriptional activity that correlates with a reduced density of repressive histone marks at telomeric repeats [23]. Although it is tempting to speculate that the up-regulation of telomere transcription occurs *in cis*, from the short telomere itself, an effect *in trans* is not excluded as it was recently reported that, in p53-positive cells, activation of the DNA damage response globally up-regulates telomere transcription [26]. We acknowledge the possibility that both types of regulation, *in cis* and *in trans*, may coexist.

Additional evidences supporting human telomere transcription

The detection of telomeric RNA:DNA hybrids (Fig. 1) and the ATRX-dependent regulation of telomere transcription further supports the human chromosome ends as being the source of TERRA transcripts. Accordingly, the transcriptional activity of a telomere was reported to be correlated with the abundance of RNA:DNA hybrids at the same locus [27,28].

Concomitantly, ATRX (Alpha Thalassemia/Mental Retardation Syndrome X-Linked), a chromatin remodeling protein that catalyzes the insertion of H3.3 histone variant at human telomeres [29], was found to bind subtelomeric promoters to promote RNA Polymerase II recruitment and telomere transcription [30]. Accordingly, depletion of ATRX was associated with a compaction of telomeres and reduced transcription from all telomeres tested [20].

Mouse TERRA

Mouse TERRA localizes mostly outside telomeres

Although mouse TERRA transcripts were first reported in 2008 [4], their intranuclear localization is still a matter of debate as the interpretation of RNA-FISH experiments has been — and remains — challenging. While TERRA was reported to form a few large nuclear aggregates mostly colocalizing with the X-inactive (Xi) chromosome, in the same study, the noncoding RNA appeared to colocalize with telomeres [4]. Shortly after, a second study confirmed the enrichment of TERRA on both sex chromosomes of mouse ES cells and reported an alteration of TERRA distribution during differentiation as only heterochromatic sex chromosomes (Y or Xi) appeared to be marked by TERRA in differentiated cells [31]. Concomitantly, a new study from Maria Blasco's group confirmed the association between TERRA and Xi in mouse keratinocytes but failed to detect any colocalization with telomeres in interphase nuclei [32]. This study further revealed that, in response to telomere shortening, the interaction between *UUAGGG*-containing RNAs and Xi is suppressed, supporting a close link between Xi and TERRA [32]. In 2012, using RNA-FISH in mouse tissues, Deng *et al*. confirmed the accumulation of TERRA in 1–2 large nuclear foci *in vivo* [33].

In a very recent study, additional genomic-binding sites of mouse TERRA were unraveled through a combination of ChIRP (Chromatin Isolation by RNA Purification) and CHART (Capture Hybridization Analysis of RNA Targets) [12]. The vast majority of TERRA-binding sites were found outside of telomeres, mostly in distal intergenic and intronic regions of the genome where TERRA regulates gene expression. Importantly however, TERRA depletion in ES cells was also associated with telomere deprotection, suggesting that TERRA is nevertheless important for mouse telomeric integrity [12].

Mouse 18q subtelomere as the main source of TERRA?

In 2014, whole-genome RNA sequencing approaches carried out by Lopez de Silanes *et al*. [10] identified the subtelomere of mouse chromosome 18 as the possible main source of TERRA production. Mouse 18q subtelomere is characterized by the presence of a gene coding for a zinc finger-like protein whose transcription is predicted to end only 8 kb upstream of the telomeric tract. However, since the open reading frame of the gene displays a ~ 300 bp-long region containing

both degenerate and canonical telomeric repeats — as potential source of *UUAGGG*-like repeats — it was unclear, whether the *UUAGGG* repeats of this Chr18 TERRA transcript were coming from the transcription of 18q telomere (in which case transcription would proceed until the telomere) or from these internal telomeric repeats (in which case transcription would stop before the telomeric tract). Since the subtelomeric Chr18 probe that was used for the northern blot overlapped with the ~ 300 bp-long region containing the intrachromosomal degenerate telomeric repeats, this question remained unanswered and is still awaiting clarification (Fig. 2). On the other hand, using different probes that originate from transcribed 18q subtelomeric regions but do not contain the telomere hexameric repeat, the RNA-FISH approach revealed that subtelomeric Chr18 RNAs were enriched into 2–3 big foci in the nucleus, a profile highly reminiscent to the one observed for mouse *UUAGGG* repeats [10]. Since subtelomeric Chr18 RNA-FISH signals partially colocalized with TERRA foci in the interphase nuclei and appeared to colocalize with some telomeres on metaphase chromosomes, the authors proposed that subtelomeric Chr18 RNAs may be the primary source of mouse TERRA and may act *in trans* on other chromosome ends [10] (Fig. 1).

Transcription mostly from the pseudoautosomal regions of sex chromosomes in mouse embryonic stem cells?

Recently, a study added to the prevalent controversy by showing that, at least in ES cells, the so-called PAR-TERRA, transcribed from the pseudoautosomal subtelomeric regions of sex chromosomes, is > 200-fold more abundant than subtelomeric Chr18 RNA [11] (Fig. 1). The subtelomeric region harboring the pseudoautosomal region (PAR) locus, although located at the distal ends of ChrX and ChrY, is incompletely sequenced and assembled, making the precise distance from the telomere unknown [11]. Moreover, the PAR locus is characterized by the presence of internal (*TTAGGG*)n tracts, making it difficult, in this case as well, to determine whether the PAR-TERRA transcripts extend to the very ends of chromosomes or contain *UUAGGG* tracts produced from the transcription of these internal (*TTAGGG*)n repeats [11]. Presuming that the signals observed by RNA-FISH reflect, at least partially, nascent TERRA transcripts, the observation that the majority of TERRA transcripts originate from the PAR locus is in agreement with the previously observed association of TERRA with sex chromosomes [4,31,32]. According to Chu *et al.*, PAR-TERRA transcripts contribute to more than 99% of TERRA molecules in mouse ES cells and their expression levels are reduced by about sixfold in mouse embryonic fibroblasts [11]. It remains to be established whether, upon differentiation, alternative mouse TERRA transcripts are produced.

RNA:DNA hybrids at mouse telomeres?

To our knowledge, there has been no report so far on the presence of RNA:DNA hybrids at mouse telomeres. In future, investigating the genome-wide maps of RNA:DNA hybrids would provide clues to trace back the origins of mouse TERRA transcripts.

Impact of ATRX on telomere transcription

Mouse ATRX is required for the incorporation of H3.3 histone variant at (*TTAGGG*)n repeats of ES cells [34]. As ATRX depletion in ES cells was associated with a 1.5-fold induction of TERRA transcripts, this led to propose that the modifications of histone composition at mouse telomeres may impact on telomere transcription [34]. However, data from this study do not exclude the possibility that ATRX may regulate the transcription of other loci, such as the PAR pseudoautosomal subtelomeric locus of sex chromosomes, as TERRA transcripts were detected by northern blot and not by qPCR [34]. In the future, it would be interesting to test this further.

TERRA in other species

Saccharomyces cerevisiae

The first indication that yeast telomeres are transcribed was provided through northern blot analyses of total yeast RNA using either telomeric or subtelomeric probes [5]. In the same study, qRT-PCR analyses with subtelomeric primers confirmed the production of TERRA from all the telomeres tested and ChIP experiments showed the recruitment of RNA polymerase II at the yeast subtelomeres [5]. A few years later, using 5′-Rapid Amplification of cDNA Ends (5′RACE), the TSS of TERRA at chromosome 1L was mapped 346 nucleotides upstream the TG-telomeric repeats [35]. TERRA levels were also found to be negatively regulated by Rap1/Rif and Rap1/Sir telomere-binding complexes in a chromosome-end-specific manner [36]. In addition, the demonstration that yeast mutants defective for the function of Rat1p 5′–3′ exonuclease were accumulating TERRA and RNA:DNA hybrids at telomeres further demonstrated the existence of

transcriptional activity at yeast telomeres [5] (Fig. 1). Formation of RNaseH-sensitive DNA:RNA hybrids at budding yeast telomeres was next shown to induce the formation of so-called R-loops which, if left unresolved, induce fork replication stalling [37,38]. R-loops appear to be regulated by telomere length and their accumulation at short telomeres promotes homologous recombination-dependent repair events that ultimately help delay senescence of yeast cells [39]. An additional manner by which TERRA aids in lengthening very short telomeres is by participating in the telomerase-dependent re-elongation of the highly transcribing very short telomeres [40].

Schizosaccharomyces pombe

RNA polymerase II-dependent transcription of fission yeast telomeres was documented in 2012 by two complementary studies. In both reports, northern blot experiments revealed the presence of both G- and C-rich telomeric RNAs [6,7]. 5′RACE experiments then showed that TERRA TSS lie within the TAS1 telomere-associated sequences present on *Schizosaccharomyces pombe* Chromosomes I and II and therefore represent *bona fide* telomere transcription products [6]. RNA-FISH experiments revealed the presence of 1–3 discrete TERRA foci in the nucleus of fission yeast cells that could correspond to telomeres although, due to technical challenges linked to antibodies, this was not formally proven [6]. Similar to the observations made in budding yeast for Rap1/Rif telomere-binding proteins [36] or human TRF2 [15], *S. pombe* telomere-associated proteins Taz1 and Rap1 were reported to regulate telomeric transcript production [7]. Evidences in favor of fission yeast telomere transcription have thus accumulated over the last five years. In addition, *S. pombe* TERRA was also reported to promote telomerase-dependent elongation of telomeres *in cis* [41].

The discovery of C-rich telomeric RNAs, dubbed ARIA and absent from budding yeast transcriptome, was surprising and suggested that the telomeric repeats themselves may serve as promoters [6]. Interestingly, a third type of transcript emanating from chromosome ends was reported in fission yeast and corresponds to subtelomeric RNA species, called ARRET (for telomere-to-centromere subtelomeric transcripts) or αARRET (for centromere-to-telomere subtelomeric transcripts) [6]. Their function is still unknown.

Trypanosoma brucei

Trypanosoma brucei was the first species in which telomeric transcription was reported [1]. Telomeres of *T. brucei* are transcribed unidirectionally toward the ends of the chromosomes and transcription was proposed to be a consequence of the read-through of subtelomeric genes located directly upstream the repeats [1].

Although in mouse, human and yeast cells, primarily RNA Polymerase II is involved in telomere transcription, TERRA production appears to be resistant to α-amanitin in *T. brucei*, suggesting an involvement of RNA Polymerase I [1]. More recently, the detection of telomeric RNA:DNA hybrids at *T. brucei* telomeres [42] further supported telomere transcription.

In the future, it would be interesting to study whether more downstream TERRA promoters exist in this eukaryote.

Arabidopsis thaliana

In *Arabidopsis thaliana*, telomeric repeat-containing transcripts appear to be produced from telomeres as well as from intrachromosomal telomeric sequences, the latter being abundant in plants [8]. Interestingly, *A. thaliana* TERRA molecules are processed into ~ 24 nt siRNA species and, through the plant-specific RNA-dependent DNA methylation pathway, are involved in the recruitment of DNA methyltransferases to sequences containing asymmetric cytosines, like telomeres [8].

Conclusions: is mouse an appropriate model for the study of the biological roles of TERRA?

Human and mouse TERRA: from a telomere point-of-view

To date, studies on TERRA seem to indicate that the number of mouse telomeres that are transcribed is distinct from that of human cells. The reason why telomere transcription appears to be differently regulated in human and mouse cells is still unknown. It is also undetermined whether this distinct regulation is an exclusive feature of *Mus musculus* laboratory mice or may be shared by other mouse species living in the wild. This will undoubtedly be an interesting question to address in the future.

We previously reported an inverse correlation between, on one hand, telomeric chromatin compaction and H3K9me3 repressive histone mark density and, on the other hand, human TERRA levels [20,23]. Our data also suggested that Heterochromatin Protein 1α (HP1α), recruited to telomeres through binding to H3K9me3, was involved in TERRA down-regulation

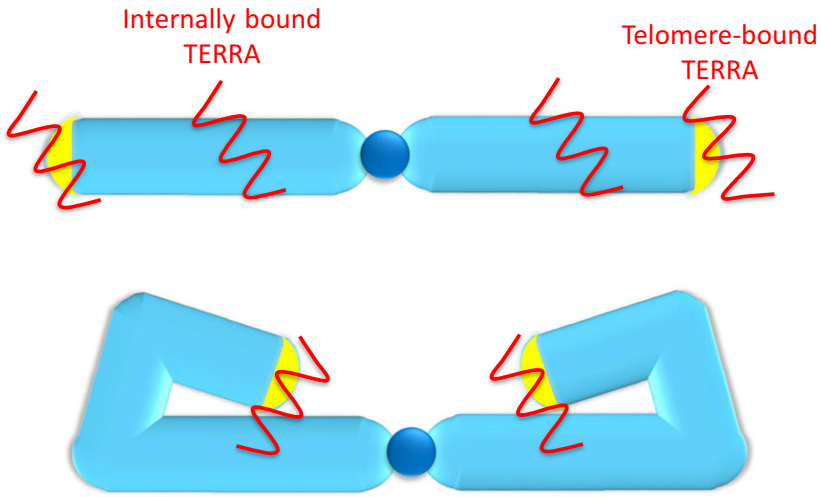

Fig. 3. Hypothetical models for human TERRA localization outside telomeres. Some evidences indicate that human TERRA may also be involved in the transcriptional regulation of genes inside chromosomes. To account for this, telomere-derived TERRA transcripts may either relocate to internal loci to perform functions *in trans* (upper panel) or be in contact with these internal loci through telomere looping events (lower panel).

[23]. In that respect, the density of H3K9me3, H4K20me3, and H3K27me3 repressive histone marks appears to be much less abundant at human telomeres than at mouse chromosome ends [43–45]. Therefore, it is possible that the distinct heterochromatin properties of human and *M. musculus* telomeres account for the observed differences in their transcriptional activity. Befitting this hypothesis, the H3K4me3 euchromatin mark, associated with active transcription and detected at human telomeres, appears to be absent from mouse telomeres [44]. Moreover, the association of mouse short arm telomeres with the pericentromeric heterochromatin condensed into large chromocenters, highly enriched in H3K9me3 repressive histone mark and in HP1α [46], is yet another typical feature of mouse nuclei that may possibly contribute to repression of transcription from the p-arm telomeres. The possible lack of transcriptional activity at most telomeres of *M. musculus* does not, however, exclude the possibility that TERRA is globally involved in mouse telomere biology. TERRA may play important roles *in trans*, probably independently of RNA:DNA hybrid formation, but possibly through protein–RNA interactions [12,47]. An important role for TERRA at mouse telomeres, is supported by the finding that TERRA depletion induced telomeric DNA damages as well as telomere abnormalities, including duplication events, telomere losses or insertions [12].

Human and mouse TERRA: from a genome-wide point-of-view

As discussed above, recent ChIRP experiments suggested that mouse TERRA binds to many genomic loci outside telomeres where the noncoding RNA appears to play important regulatory functions related to gene expression [11,12]. In the future, it would be interesting to perform similar ChIRP experiments in human cells. First evidences in favor of TERRA-dependent genome-wide alterations in human cells were provided by the discovery that increasing intracellular levels of *UUAGGG* repeats — through ectopic lengthening of telomeres — was reducing the expression level of innate immunity genes [48]. Since several studies recently reported the existence of a telomere position effect over long distances in human cells [49–51], it remains to be established however whether these telomere length-dependent effects are the consequence of TERRA binding *in trans* to these loci or may be mediated through the looping of telomeres that could bring TERRA and/or heterochromatin-promoting factors in vicinity to an internal locus (Fig. 3). Since similar effects on innate immune gene expression were observed upon transfection of TERRA-like oligonucleotides, the study by Hirashima and Seimiya suggests however that binding of TERRA to nontelomeric human loci is possible [48].

In summary, although the sun has not reached the zenith yet in the TERRA field, differences between mouse and human TERRA point toward a distinct regulation of telomeric RNA between these two species. However, a lot of light still needs to be shed to fully understand both similarities and differences regarding TERRA origin, localization and function in mouse and human cells.

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