Telomeric Chromatin and TERRA

Amandine Barral, Jérome Déjardin

Telomeric chromatin and TERRA

Amandine Barral and Jérome Déjardin

Institute of Human Genetics, UMR 9002 CNRS and Université de Montpellier
141, rue de la Cardonille, 34000 Montpellier, France.

Abstract:

Chromatin function in telomeres is poorly understood, but it is generally viewed as repressive. Yet, telomeric DNA sequences are transcribed into long non-coding RNAs named TERRA. As TERRA molecules mostly localize at telomeres, major research efforts have been made to understand their functions, and how TERRA transcription is regulated and affects telomere structure. This review describes the current state of knowledge about the nature of chromatin at telomeres, its functions, and the relation between chromatin structure and TERRA.

Introduction:

Telomeres are specialized structures at the extremity of chromosomes. They ensure genome stability [1-3] by preventing the recognition of chromosome ends as genuine double strand breaks and consequently their aberrant repair [4-6]. In most species, telomeric DNA is composed of short tandem repeats. In mammals, telomeres can be up to 50 kb-long, and are composed of head-to-tail repeats of the 6bp motif 5'-(TTAGGG/CCCTAA)-3'. Telomeres usually terminate with a single-stranded TTAGGG 3'-overhang that is up to 500 nt-long. In the absence of any maintenance mechanism, telomeres will shorten with cell division [7, 8].

Shelterin proteins are crucial telomere-binding proteins that are recruited through sequence-specific association with telomeric DNA. In this complex in mammals, TRF1 and TRF2 specifically bind to the double stranded TTAGGG sequence, while POT1 binds to the single-stranded overhang [9]. Shelterin proteins have two major functions at telomeres. They recruit other proteins to keep the DNA damage response in check [2-6, 10], and they control telomere length by regulating the recruitment of the telomerase holoenzyme complex [11-14]. When telomeres become critically short, shelterin proteins cannot bind sufficiently, and telomeres are not efficiently protected any longer. This situation is almost comparable to the occurrence of an irreparable intra-chromosomal double-strand break, and ultimately triggers permanent cell cycle exit [15].

Like any other genomic region, yeast and mammalian telomeres assemble into chromatin and therefore bear nucleosomes. Telomeric DNA sequences intrinsically disfavour nucleosome positioning, and this might explain some of the unusual chromatin structures observed at telomeres [16, 17]. Nonetheless the presence of nucleosomes implies interactions with dedicated machineries that support chromatin transactions, such as ATP-dependent chromatin remodellers and histone-modifying enzymes. As chromatin is a global determinant of eukaryotic DNA transactions, it should play an important role also at telomeres. In human cells, it has been reported that short telomeres have fewer nucleosomes than long telomeres, suggesting that telomere length is associated with distinct

chromatin features, like nucleosomal density. Historically, telomeric chromatin is viewed as generally heterochromatic, a type of chromatin that hinders DNA transactions. Yet the picture is even more complex because transcription also occurs at telomeric regions, an unusual feature in heterochromatin. This transcription leads to the production of long non-coding RNAs (lncRNAs) named TElomere Repeat-containing RNA (TERRA) [18-22]. It has been suggested that TERRA plays a role in telomerase inhibition [23-25], but also in telomerase recruitment [26, 27]. TERRA metabolism also might influence telomere maintenance by recombination [28-32].This review will describe the current state of knowledge on telomeric chromatin regulation and the potential links with TERRA and its transcription. By analogy with the well-known function of non-coding RNAs (ncRNAs) at *Schizosaccharomyces pombe* centromeric regions, or the putative role of ncRNAs in recruiting chromatin regulators, TERRA molecules are viewed as important players in telomeric heterochromatin regulation [33-39]. Telomerase RNA component, another ncRNA, also is associated with telomeres and plays an essential role in telomere elongation by telomerase [40, 41]. Currently, this ncRNA is not considered to play a role in telomeric chromatin regulation, and therefore will not be discussed in this review. Another telomere-related small RNA was described in mouse (tel s-RNA), but its function in telomere biology awaits further characterization [42].

Telomeric chromatin

For a long time, it was thought that telomeric regions assembled into heterochromatin because reporter genes placed near telomeres tend to be silenced. Moreover, silencing is positively influenced by the reporter gene proximity to telomeres and the telomere length. This phenomenon is known as Telomere Position Effect (TPE). TPE was extensively described in *Saccharomyces cerevisiae* [43] and then confirmed in *Schizosaccharomyces pombe* [44] and also in mammalian cell lines [45-47]. TPE is a classic ‘epigenetic’ metastable silencing phenomenon, and implies that telomere heterochromatinization is positively correlated with their length. Short telomeres are less heterochromatic, and in telomerase-negative cells, this might favour telomere lengthening by homologous recombination [30].

Whereas long telomeres favour DNA looping to install heterochromatin on distal region from telomeres and repress gene expression [48]. Nevertheless, yeast and mammalian telomeres rely on a specific DNA sequence for capping functions and thus, are not defined by an epigenetic phenomenon, as opposed to other repeated regions such as centromeres. In fact perturbing specific epigenetic regulators destroys centromere function, while this has not been observed at telomeres. Therefore, chromatin might not be directly linked to telomere capping function and must be playing distinct roles.

S. cerevisiae telomeres are 250bp-long and are essentially nucleosome-free [16]. Subtelomeric regions, which are also made of distinct repeated DNA, are assembled into nucleosomes. However, the heterochromatin that forms and spreads along the subtelomeric regions depends on the binding of Rap1 (a shelterin protein) to its specific DNA motif essentially present on telomeres (**Figure 1A**) [49]. Rap1 then interacts with the Silence Information Regulation 4 (Sir4) protein that in turn recruits Sir3 and Sir2 to telomeres [49, 50]. Sir2 catalyses the removal of acetyl groups from lysine 16 of histone H4 (H4K16Ac), and leads to a hypoacetylated and repressive chromatin conformation [51, 52]. Hypoacetylation creates a binding site for Sir3 and Sir4 [53, 54] that then recruit more Sir2, providing a simple molecular explanation for hypoacetylation spreading to sub-telomeric regions and TPE enforcement in this species. Loss of Sir4 disrupts heterochromatinization and at the same time promotes telomere shortening. This is because Sir4, in addition to its hypoacetyation role, facilitates telomerase recruitment at telomeres with the help of Ku proteins [55]. Thus, *S. cerevisiae* heterochromatin partly promotes telomerase recruitment, and is at least not inhibitory to lengthening.

Similarly, in S. *pombe*, telomeres are nucleosome-free and able of TPE [44]. However, *S. pombe* heterochromatin resembles more to metazoan than to *S. cerevisiae* heterochromatin. For instance, *S. pombe* sub-telomeres harbour constitutive heterochromatin marks [56] and proteins, such as heterochromatin protein 1 (HP1), the histone methyltransferase (HMTase)

Clr4 (orthologue of the mammalian SUV39H HMTases) and the histone H9 lysine 3 trimethylation (H3K9me3) mark, all absent in *S. cerevisiae*.

In *S. pombe*, telomeric heterochromatinization also depends on a shelterin factor named Taz1 (**Figure 1B**). Taz1 tethers the SET domain-containing Clr4 to telomeres, and then Clr4 catalyses H3K9me3 deposition to neighbouring nucleosomes [57]. This histone mark in turn creates a binding site for Swi6 (an orthologue of HP1) on telomeres [58, 59]. The histone deacetylase Snf2/Hdac-containing Repressor Complex (SHREC) also is recruited by Taz1 and Swi6 to telomeres [60], and its Clr3-subunits deacetylates lysine 14 of histone H3. This contributes to the formation of repressive chromatin that spreads to sub-telomeric regions [60]. In *S. pombe*, the formation of sub-telomeric heterochromatin is promoted by the RNA interference (RNAi) machinery, as observed at centromeres [61]. Specifically, Dcr1, a subunit of the RNAi machinery, recruits Swi6 and the SHREC complex to telomeres [62]. Intriguingly, the direct involvement of the RNAi machinery in heterochromatin formation at telomeres seems to be specific to fission yeast, and is not observed at mammalian telomeres. Consistently, we never detected any RNAi pathway protein at heterochromatin purified from mammalian somatic cells [63-65].

In mammals (mouse and human cells, **Figure 1C**), the nature and function of telomeric chromatin remain unclear, and in our view, constitute a very controversial topic. Some groups reported that telomeres harbour marks of constitutive heterochromatin [38, 66-69] like most tandem repeats in the genome and consistent with TPE features [45, 47]. A more recent study showed that telomeres from most human cell lines are not heterochromatic [70] and that the heterochromatic mark H3K9me3 is enriched at telomeres only in cell lines that have activated the alternative lengthening of telomere (ALT) pathway, a recombination-based telomere lengthening mode [65, 70].

On the basis of the hypothesis that telomeres, as a source of TPE, should be heterochromatinized, it was initially proposed that telomeric heterochromatin assembly in mammalian cells is regulated by Suppressor of Variegation 3-9 homologues 1 and 2 (SUV39H1/H2) through H3K9me3 deposition on telomeres [68]. H3K9me3 acts as a landing

site for HP1α that recruits the Suppressor of Variegation 4-20 homologue 2 (SUV420H2) enzyme. SUV420H2 catalyses histone 4 lysine 20 trimethylation (H4K20me3), another mark of heterochromatin [71] with unclear functions. It was proposed that this pathway, which is very similar to what was described at pericentromeric regions, controls telomerase recruitment at telomeres, and controls the activation of telomerase-independent ALT [68, 71]. In this classical model, heterochromatin thus negatively regulates telomere lengthening. DNA methyltransferases (DNMTs) also are involved in telomere length control in mammals [72]. DNA methylation only occurs at subtelomeric regions because CpG sequences are not present in the telomere repeat motif [72-74]. As subtelomeric DNA is usually hypermethylated, it was suggested that in the mouse, this high level of DNA methylation somehow 'compacts' the telomeric fibre, rendering it less prone to damage and lengthening by recombination [72]. Intriguingly in human cells, loss of DNA methylation seems to have the opposite effect on telomere length compared with mouse cells [74-77]. In fact, subtelomeric chromatin is hypomethylated and telomeres are abnormally short in cells derived from patients with Immunodeficiency Centromeric instability and Facial anomalies (ICF) syndrome [75-77], who carry an inactivating mutation in *DNMT3B*. This shortening could be the consequence of defective telomere replication [76]. The different phenotypes observed in mouse and human cell lines might be due to the different average telomere length between the two models.

We recently analysed histone modifications at telomeres in mouse embryonic stem (ES) cells and fibroblasts [65]. We found low levels of H3K9me3 at telomeres in mouse ES cells and virtually no H3K9me3 in fibroblasts. H3K9me3 deposition mostly relies on Set domain bifurcated protein 1 (SETDB1) recruitment [65], and this heterochromatin mark is important for stimulating local nucleosome exchange, which in turn favours transcriptional processivity and recombination. Therefore, in mouse ES cells, heterochromatin indirectly promotes telomere recombination and transcription, in disagreement with the SUV39H model described above.

More recently, it has been proposed that Polycomb activities also regulate telomere biology. The Polycomb group protein EZH2 has been suggested to bind telomeres where it is able to catalyse histone H3 lysine 27 trimethylation (H3K27me3) and this is required to stabilize H3K9me3, H4K20me3 and HP1 telomeric enrichment [36]. While it has been suggested that H3K27me2/3 and H3K9me2/3 can coexist on the same native nucleosome [78], ChIP-sequencing approaches have found that H3K27me3 is largely non-overlapping with H3K9me3 throughout the genome, including at repeated DNA sequences [79-88]. The synergy between Polycomb activities and H3K9me3/HP1 [36, 89] is highly unusual, especially given that genetic screens aiming at identifying Polycomb regulators or constitutive heterochromatin activities usually retrieve distinct group of genes [79-81, 84-86]. Moreover, the role of Polycomb genes in telomere regulation remains unclear in the absence of described telomere phenotypes in Polycomb mutants. Using unbiased telomere chromatin proteomics, we failed to detect significant EZH2 binding or activity at telomeres in mouse ES cells [65]. This discrepancy might be explained by species- or tissue- specific differences, or different technical approaches, measuring relative versus absolute amounts of histone modifications.

Regardless of its nature at telomeres, heterochromatin is viewed as an important regulator of telomere length regulation. According to the classical view, telomeric heterochromatin is in a closed/condensed state that does not allow recombination and telomerase recruitment. Our recent results indicate that heterochromatin does not form on telomeres in all mouse cell types [65] , consistent with the study on human cell lines [70]. However, when heterochromatin forms at telomeres (like in mouse ES and in ALT cells), it promotes rather that inhibits telomere recombination and lengthening [65], a trend globally comparable, albeit mechanistically different, to what happens in *S. cerevisiae*. As heterochromatin formation is often linked to the presence of ncRNAs, we discuss below TERRA production and the possible links between TERRA and local chromatin regulation.

TERRA biogenesis

The biological function of ncRNAs is very difficult to characterize experimentally. In the absence of any identifiable coding function, such RNAs could have a structural (e.g., ribosomal RNAs), catalytic (e.g., telomerase RNA), sequence-specific recruiting, or local protecting role. In addition, one important aspect of ncRNA function is whether it acts locally, in which case its function as a ncRNA is difficult to separate from molecular events leading to its production; or whether it acts “in trans” at long distances from its production site. ncRNA production, which involves chromatin remodelling, could have a major “in cis” function in the local chromatin regulation, regardless of the RNA that is made. Hence, we first detail below what is TERRA and how it is produced.

The first evidence that telomere sequences are transcribed came from a work performed in *Trypanosoma brucei* [90]. Since then, telomere transcription has been observed in different organisms, such as yeast [21, 22, 25], mouse [18] and humans [20], which indicates conservation during evolution, an indirect sign of functional importance.

TERRA is a single-stranded lncRNA originating from the transcription of the C-rich telomeric DNA strand. Thus, TERRA is a G-rich RNA containing 5’-UUAGGG-3’ repeats [18, 20-22, 25]. TERRA length ranges from 100bp to more than 9kb in mammals [20, 91] and by consequence long TERRA transcripts are difficult to be detect by common Northern-Blot. In Yeast, TERRA range is about 400bp in yeast [22, 25] . TERRA levels are cell-cycle regulated [29, 69, 91]. Indeed, TERRA levels peak in early G1, and decrease in late G1, reaching the lowest level in late S phase, a time that roughly corresponds to telomere replication. After the G2/M phase, TERRA levels start to increase again. RNA polymerase II catalyses transcription at telomeres [18, 25, 73, 91, 92]. Subunits of the RNA polymerase I and III complexes also have been identified during the purification of telomeric chromatin [64]; however, the biological significance of these associations is unclear and might not be linked to telomere transcription.

In all species studied so far, TERRA transcription starts in the subtelomeric region and proceeds from the centromere toward the telomere direction [20, 22, 25, 73, 92]. In *S.*

pombe, other telomeric lncRNAs have been identified in both sense (αARRET) and antisense directions (ARIA and ARRET) (**Figure 2A**) [21]. Some of these anti-sense telomeric RNAs are also present in plants [93] and in human and mouse cells [18, 20], albeit at a much lower level than TERRA. Their functions are unknown. As telomere and subtelomeric sequences are made of repetitive DNA, the mechanisms involved in RNA transcription initiation, elongation and termination are difficult to address, and the characterization of TERRA species also has led to controversial findings and models. We detail below the state of knowledge about TERRA biogenesis because it is likely that the mechanisms presiding to TERRA synthesis are linked with TERRA functions.

As TERRA is transcribed by RNA polymerase II, it contains a methyl-cap at the 5' end and a poly-A tail at the 3' end, like most mRNA species [18, 25, 91]. In yeast and human cells, all TERRA species are capped. In *S.cerevisiae*, almost all TERRA RNAs also have a poly-A tail (**Figure 2B**). In contrast, only 7% of human TERRA RNAs have a poly-A tail (**Figure 2C**) like TERRA in *S.Pombe* [27, 91], whereas this fraction has not been quantified in mouse cells [18]. In *S. cerevisiae*, poly-adenylation seems to require the action of the canonical poly-A polymerase (PAP1), but the precise molecular mechanisms of termination remain unclear because TERRA does not have the canonical poly-A signal (5'-AAUAAA-3') normally found at the 3' of most class II genes [25]. Indeed, in human cell lines, TERRA-poly-A(-) terminates preferentially with the 5'-UUAGG-3' sequence, whereas TERRA-poly-A(+) terminates with 5'-UUAGGG-3' [91], a finding which supports the idea of regulated 3' end TERRA processing. Moreover, as the TERRA DNA template strand mostly ends with the ATC-5' sequence [94], TERRA transcription might not process until the end of the telomere

As is the case for most mRNA, the poly-A tail stabilizes TERRA both in yeast and in human [25, 91]. It also correlates with TERRA sub-nuclear distribution in human cells: 60% of TERRA-poly-A(-) is in the nucleoplasm, while the remaining 40% is chromatin-associated. Conversely, TERRA-poly-A(+) molecules are mostly in the nucleoplasm [91]. This suggests that chromatin-associated TERRA is not poly-adenylated, and that TERRA-poly-A(+) and TERRA-poly-A(-) might underlie distinct functions.

TERRA might also be regulated at the initiation step in the subtelomeric region. While globally heterochromatic, subtelomeres can locally bear euchromatin marks, with an enrichment in histone H3 lysine 4 trimethylation (H3K4me3) [92, 95, 96], deposited by the Mixed Lineage Leukemia protein (MLL) [97], and histone H3 lysine 27 acetylation (H3K27Ac) enrichment [95] and RNA polymerase II binding [92, 95, 96], indicative of transcriptional initiation. However, many aspects regarding the initiation mechanisms remain unclear because TERRA initiation sites lack canonical promoter sequences.

In yeast, the subtelomere sequence is made of X-elements and Y'-elements, bound by the Sir silencing complex and also Rif 1 and Rif2 (**Figure 2B**). While X-element sequences are strongly repressed by the Sir silencing complex and Rif1 and Rif2, the Y' elements are weakly repressed, only by Rif 1 and Rif 2. Y' element are enriched in H4K16Ac and harbor transcribed open reading frames and and TERRA initiation sites [19, 98].

In mammals, several groups identified TERRA production at many (if not all) telomeres [20, 33, 73, 74, 92, 99], whereas others suggested that the bulk of TERRA is produced only from one or a very limited number of (sub)telomeric regions [100-102]. These two models have fundamentally different implication on the function of TERRA. The seminal study on TERRA showed transcription from several human telomeres [20], suggesting the presence of a transcription start site (TSS) on each of them. RNA fluorescent in situ hybridization indicates that most telomeres can be found associated with TERRA but this does not prove that TERRA is transcribed from each telomere, as TERRA could be made from a limited number of loci then addressed to other telomeres. Transcription from multiple telomeres was also demonstrated by the same group [74, 99]. Two subtelomeric promoter types,Type-I and Type-II, were identified at 1Kb and 5-10 Kb from the subtelomere-telomere boundary, respectively (**Figures 2C and 2D**) [33, 73, 92]. Both promoter types include CCCTC-binding factor (CTCF) binding sites and CpG island elements [73, 92]. At type I promoters, CpG islands are composed of three distinct repetitive tracts of 61bp, 29bp and 37bp in length. These elements are referred as "61-29-37 repeats" and are present at 13 distinct human chromosome ends [73]. These 61-29-37 repeats are bound by RNA polymerase II

and have intrinsic promoter activity [73, 92, 95]. Moreover, the 61-29-37 repeats have high CpG content and are methylated by DNMT3B and DNMT1, unlike most CpG islands elsewhere in the genome, which usually escape DNA methylation. In fact, TERRA initiation at these elements is controlled by DNA methylation [73, 76]. In addition, two tandem DNA binding motifs are present upstream of the 61-26-37 repeats. They are bound by CTCF and Cohesin (Rad21) [73, 92, 96]. These two chromatin-organizing factors cooperate to promote TERRA correct orientation [96]. Type-II promoters were identified by RNA-seq analysis on ten other chromosomes, and are located 5-10 kb upstream of the telomere tracts. TERRA production at different telomeres has been measured also in mouse embryonic fibroblasts and during early developmental stages [33].

The other model proposes that TERRA molecules are produced at a single or a limited number of telomeres [100-102]. Then, TERRA molecules can travel (via uncharacterized mechanisms) through the nucleus to other telomeres and also to a subset of genes to regulate the local expression and chromatin composition. One implication of this model is that telomere transcription, and therefore the inherent local chromatin remodelling, plays a limited role in telomere biology, while TERRA molecules are important. The other implication is that the status of one telomere has the potential to govern that of all the other telomeres. It was proposed that in U2OS human cancer cell lines, which maintain their telomeres with ALT, chromosomes 20 and X are the only TERRA producers [102]. U2OS ALT telomeres harbor heterochromatin features [65, 70] and non-physiological TERRA expression [24]. Moreover these observations were not made in telomerase-positive cell lines [20]. Similarly, in mouse ES cells, the TERRA FISH signal forms two main dots that co-localize with the X chromosomes, and drive X chromosome pairing during X inactivation [101]. In Mouse Embryonic Fibroblasts (MEFs), the chromosome 18 telomere is the main source of TERRA [100]. In both cell types, TERRA signals are also found associated with other telomeres by FISH and with non-telomeric regions by CHIRT-seq [37, 103], suggesting a function in trans. Like the X telomere transcript in mouse ES cells, the chromosome 18 TERRA seems thus to travel to other genomic regions. It was proposed that in these cells, this interaction in trans

protects telomeres from the DNA damage response [100], therefore, presumably supporting an entirely different function from X chromosome pairing described in mouse ES cells [101]. Live-cell imaging in a human cancer cell line, using MS2 knock-ins to track endogenous TERRA from one telomere, shows TERRA molecules diffusing into the nucleus [104], sometimes co-localizing as a cluster with one telomere. This result could reflect TERRA binding to another telomere in trans, or it could reflect the actual TERRA production from the modified telomere. Similar to the mouse situation, disrupting this single telomere TERRA transcript leads to the activation of the DNA damage response throughout the genome.

In a model of a single telomere-producing TERRA [100-102], addressing TERRA function by disrupting the unique TERRA promoter should be straightforward. This was attempted, but TERRA RNA production was not entirely abrogated [100, 102], complicating the interpretation and clear conclusions about TERRA roles in trans.

The distinction between the two models is critical to understand TERRA in cis and/or in trans functions. As there is currently no clear explanation about the discrepancies between laboratories, more quantitative approaches might be required to clarify this important question.

TERRA RNA functions:

It has been proposed that lncRNAs regulate genome functions [105]. Specifically, they work by interacting with chromatin-modifying enzymes and nucleosome-remodelling factors to modulate chromatin structure. For this, they might recruit chromatin factors to specific genomic regions, or they might anchor chromatin factors away from target regions. LncRNAs could also act as scaffolds to build chromatin-modifying complexes, without necessarily targeting them to specific loci. As the lncRNA TERRA is associated with telomeres, it might play an important function in telomeric chromatin regulation. TERRA cannot be genetically inactivated easily, and consequently its physiological functions remain largely unknown. Several proteins that interact with this lncRNA were identified *in vitro* by affinity pull-downs [37, 38, 106, 107], including TRF1 and TRF2, HP1, the Origin Recognition Complex (ORC)

[38], several heterogeneous nuclear ribonucleoproteins (HnRNPs) [107], and also various interactors that need to be further characterized to determine whether TERRA acts as a local recruiter of biologically relevant activities. Since many relevant chromatin proteins promiscuously interact with long RNA *in vitro*, it will be important to determine whether *in vitro* interactors are recruited, and if the case, whether TERRA targets specific chromatin functions to telomeres. Nevertheless, some TERRA-related regulations are starting to emerge.

In *S. cerevisiae*, forced TERRA expression from one telomere induces exonuclease 1-mediated shortening of that telomere without any measurable change in the length of the other telomeres [108, 109]. This key observation suggests that at least in this artificial setting, TERRA or the process of telomere transcription mostly has an effect in cis, and that an appropriate level of TERRA or transcription is required for telomere integrity.

Telomeric transcription has the potential to generate local TERRA-telomere DNA hybrids that create R-loop structures and a displaced single-stranded G-rich telomeric DNA. As these structures generate replication stress, TERRA must be cleared from telomeres during S phase to ensure complete telomere replication [29, 110] (**Figure 3A**). R-loop clearance is ensured by an RNase H activity. In human ALT-positive cells, RNase H1 is highly enriched at telomeres and prevents RNA-DNA hybrid accumulation, which would otherwise promote increased homologous recombination (**Figure 2C**) [28]. In telomerase-negative yeast, RNase H1 and RNase H2 also act to limit RNA-DNA hybrid accumulation at telomeres (**Figure 2B**), thus controlling their elongation through homologous recombination (**Figure 3A**), [29, 30]. In yeast, RNA-DNA hybrid accumulation is regulated also by the THO complex, which is normally involved in mRNA export. Indeed, inactivation of Tho2p (a THO complex subunit) leads to RNA-DNA hybrid accumulation (**Figure 2C**) [30-32] and exonuclease 1-dependent telomere shortening [30]. These findings indicate that two different RNA processing pathways regulate telomere RNA-DNA hybrid levels [30, 32], with an apparently stronger action for RNaseH [32], but the connection between the two pathways are not known. In human ALT-positive cells, FANCM was identified has a new regulator of telomeric R-loops

363 [111, 112], by limiting R-loop accumulation to control replicative stress (**Figure 2C**),
364 representing another indication that TERRA processing is a highly regulated process.
365 In addition to forming potentially toxic local R-loop structures, TERRA might also fold into G-
366 quadruplex structures (G4) [113, 114] which can be bound by TRF2. It seems that TRF2 is
367 able to simultaneously bind to TERRA-RNA-G4 and to telomere-DNA-G4, forming a tri-
368 complex, and helping TERRA association with telomeric DNA [115] (**Figure 3C**), providing a
369 potential mechanism for TERRA recruitment to telomeres in trans.

370 Another potentially relevant TERRA function is the ability to associate with the RNA
371 component of the telomerase holoenzyme (**Figure3B**). TERRA could inhibit telomerase
372 activity by competing for binding to the single-stranded telomeric DNA overhang, which is the
373 normal substrate for this enzyme. This inhibition was observed *in vitro* [23]. Conversely, in
374 yeast, TERRA seems to stimulate telomerase (**Figure3B**) [26, 27]. It could be that TERRA
375 positively regulates telomerase accessibility to telomeres by displacing inhibitory proteins
376 from telomeres [116]. TERRA can also anchor hnRNPA1 proteins away from the telomere to
377 allow proper telomere replication [35, 117].

378 TERRA might also regulate heterochromatin formation at telomeres because TERRA
379 downregulation correlates with reduced heterochromatin marks at telomeres [36, 38],
380 whereas higher TERRA levels, as in G1 phase [69], or longer TERRA correlates with
381 enrichment of these marks [33, 69]. TERRA has been shown to bind HP1α [38, 118],
382 suggesting a role in heterochromatin formation. This might seem counterintuitive because
383 heterochromatin generally correlates with transcriptional silencing. The mechanisms by
384 which TERRA correlates with heterochromatin is poorly understood, but TERRA, like several
385 other lncRNAs, can also directly interact with different enzymes involved in heterochromatin
386 formation [33, 34, 119-121] (**Figure 3D**). However, it must be noted that this interaction
387 generally appears to be RNA sequence-independent, suggesting that locus-specific
388 recruitment might depend on other factors or on the ability of the heterochromatin factor to
389 identify nascent RNA and associate with it. Upon telomere deprotection, increased TERRA

production induces the recruitment of SUV39H1 to human telomeres and triggers local H3K9me3 deposition [33]. SUV39H1 N-terminus contains a chromodomain that directly interacts with TERRA [33], providing a molecular mechanism for SUV39H1 presence at deprotected telomeres. Along this line, SUV39H1 recruitment to pericentromeric regions is stabilized by interaction with local nascent RNA [122, 123], suggesting that RNA interaction might be a recruiter of molecules involved in heterochromatin activities [123], or might at least contribute to their function by stabilizing enzyme binding to chromatin [122]. Upon telomere deprotection, TERRA also promotes the recruitment of the histone demethylase LSD1 and G-quadruplex RNA binding [39, 121]. The consequence of LSD1 presence at telomeric nucleosomes was not fully explored, but it was shown that LSD1 recruitment promotes 3' telomere overhang processing though stimulation of the double-strand break repair protein MRE11 [39].

Moreover, a protein named translocated in liposarcoma (TLS/FUS) binds to TERRA-RNA-G4 structures and to telomeric DNA, and forms a ternary complex proposed to anchor TERRA to telomeres [48, 119, 124]. Tethered TLS/FUS in turn promotes H3K9me3 deposition at telomeres by an unknown mechanism [48, 119].

Finally, on the basis of TERRA interaction with Polycomb repressive complex 2 (PRC2) subunits *in vitro* [120], it was proposed that TERRA recruit Polycomb activities to telomeres [36]. It should be noted that in some cases the interaction between a chromatin enzyme and RNA inhibits the enzymatic activity. This is true for LSD1 [121], and EZH2 [125], suggesting that a simple model where TERRA mediates the recruitment of enzymes such as EZH2 to work at telomeres is probably incomplete.

It was also suggested that TERRA modulates the telomeric chromatin structure through ATRX eviction from telomeres after a direct interaction with this chromatin remodelling enzyme [37]. ATRX associates with G-rich DNA sequences [126, 127], interacts with the DAXX histone chaperone [128], and promotes heterochromatin formation [129]. While the potential mechanisms were not explored, the same authors reported that TERRA knock-down led to telomerase stimulation and, counterintuitively, to telomere damage [37]. Using

the same mouse ES cell line, we found that ATRX recruitment to telomeres largely depends on SETDB1 and does not seem to correlate negatively with TERRA [65]. The loss of ATRX in the context of heterochromatinized telomeres stimulates telomere recombination, while the loss of ATRX in the absence of heterochromatin at telomeres has no measurable impact. These effects occur without any strong change in TERRA levels, suggesting that TERRA functions are unlikely to be strictly linked to heterochromatin formation at telomeres in ES cells. However, detailed insights await more mechanistic characterizations.

Conclusions:

Telomeric chromatin features and telomeric transcripts actively participate in telomere stability that is required for ensuring genome stability. From yeast to human cells, telomeric chromatin has been defined as silent heterochromatin, due to TPE. Typical constitutive heterochromatin marks are enriched at chromosome ends: Sir4-Sir3-Sir2 in *S. cerevisiae* [49, 51, 52, 54], and H3K9me3 and Swi6/HP1 in *S. pombe* and mammals [57, 58, 65]. However, in most cells, telomeres are nucleosome-poor or have poorly positioned nucleosomes, a situation which generally disfavours constitutive heterochromatin formation. The organization of telomeric heterochromatin remains unclear and controversial, which is why its function is difficult to define. Mechanisms of TERRA mediated functions is challenging to address due to the telomeric RNA repeated sequence and the different producing genomic loci, and therefore we are facing major technical challenges. Moreover, in order to fully understand the interplay between TERRA and chromatin regulation, measuring TERRA steady-state levels will not be sufficient. We will need to detail the TERRA transcription mechanisms with more dedicated methods such as nuclear run-on, studying the RNA polymerase II phosphorylation state and common mechanisms linked to transcriptional elongation and termination, which are determined by chromatin. Discovering a significant function for TERRA in regulating telomeric chromatin will thus require robust and quantitative tools that await further development.

Acknowledgements

Work in the Dejardin lab is supported by grants from ARC equipe labelisee 2016, the ERC CoG METACHROM, from INCA, from the Fondation Schlumberger pour l'education et la recherche and from Merck (MSD). A.B. is supported by the University of Montpellier and by The Fondation pour la Recherche Medicale.

References:

[1] Sfeir A, de Lange T. Removal of shelterin reveals the telomere end-protection problem. Science. 2012;336:593-7.
[2] van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. Cell. 1998;92:401-13.
[3] Yang Q, Zheng YL, Harris CC. POT1 and TRF2 cooperate to maintain telomeric integrity. Mol Cell Biol. 2005;25:1070-80.
[4] Bae NS, Baumann P. A RAP1/TRF2 complex inhibits nonhomologous end-joining at human telomeric DNA ends. Mol Cell. 2007;26:323-34.
[5] Denchi EL, de Lange T. Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature. 2007;448:1068-71.
[6] Karlseder J, Hoke K, Mirzoeva OK, Bakkenist C, Kastan MB, Petrini JH, et al. The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. PLoS Biol. 2004;2:E240.
[7] Loayza D, De Lange T. POT1 as a terminal transducer of TRF1 telomere length control. Nature. 2003;423:1013-8.
[8] Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, et al. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci U S A. 1992;89:10114-8.
[9] de Lange T. Shelterin-Mediated Telomere Protection. Annu Rev Genet. 2018;52:223-47.
[10] Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science. 1999;283:1321-5.
[11] Kelleher C, Kurth I, Lingner J. Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. Mol Cell Biol. 2005;25:808-18.
[12] Ye JZ, Hockemeyer D, Krutchinsky AN, Loayza D, Hooper SM, Chait BT, et al. POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev. 2004;18:1649-54.
[13] Wang F, Podell ER, Zaug AJ, Yang Y, Baciu P, Cech TR, et al. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature. 2007;445:506-10.
[14] Li B, de Lange T. Rap1 affects the length and heterogeneity of human telomeres. Mol Biol Cell. 2003;14:5060-8.
[15] Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, et al. Extension of life-span by introduction of telomerase into normal human cells. Science. 1998;279:349-52.
[16] Wright JH, Gottschling DE, Zakian VA. Saccharomyces telomeres assume a non-nucleosomal chromatin structure. Genes Dev. 1992;6:197-210.

[17] Tommerup H, Dousmanis A, de Lange T. Unusual chromatin in human telomeres. Mol Cell Biol. 1994;14:5777-85.
[18] Schoeftner S, Blasco MA. Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. Nat Cell Biol. 2008;10:228-36.
[19] Iglesias N, Redon S, Pfeiffer V, Dees M, Lingner J, Luke B. Subtelomeric repetitive elements determine TERRA regulation by Rap1/Rif and Rap1/Sir complexes in yeast. EMBO Rep. 2011;12:587-93.
[20] Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, Lingner J. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science. 2007;318:798-801.
[21] Bah A, Wischnewski H, Shchepachev V, Azzalin CM. The telomeric transcriptome of Schizosaccharomyces pombe. Nucleic Acids Res. 2012;40:2995-3005.
[22] Greenwood J, Cooper JP. Non-coding telomeric and subtelomeric transcripts are differentially regulated by telomeric and heterochromatin assembly factors in fission yeast. Nucleic Acids Res. 2012;40:2956-63.
[23] Redon S, Reichenbach P, Lingner J. The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. Nucleic Acids Res. 2010;38:5797-806.
[24] Ng LJ, Cropley JE, Pickett HA, Reddel RR, Suter CM. Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription. Nucleic Acids Res. 2009;37:1152-9.
[25] Luke B, Panza A, Redon S, Iglesias N, Li Z, Lingner J. The Rat1p 5' to 3' exonuclease degrades telomeric repeat-containing RNA and promotes telomere elongation in Saccharomyces cerevisiae. Mol Cell. 2008;32:465-77.
[26] Cusanelli E, Romero CA, Chartrand P. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. Mol Cell. 2013;51:780-91.
[27] Moravec M, Wischnewski H, Bah A, Hu Y, Liu N, Lafranchi L, et al. TERRA promotes telomerase-mediated telomere elongation in Schizosaccharomyces pombe. EMBO Rep. 2016;17:999-1012.
[28] Arora R, Lee Y, Wischnewski H, Brun CM, Schwarz T, Azzalin CM. RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells. Nat Commun. 2014;5:5220.
[29] Graf M, Bonetti D, Lockhart A, Serhal K, Kellner V, Maicher A, et al. Telomere Length Determines TERRA and R-Loop Regulation through the Cell Cycle. Cell. 2017;170:72-85.e14.
[30] Balk B, Maicher A, Dees M, Klermund J, Luke-Glaser S, Bender K, et al. Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence. Nat Struct Mol Biol. 2013;20:1199-205.
[31] Yu TY, Kao YW, Lin JJ. Telomeric transcripts stimulate telomere recombination to suppress senescence in cells lacking telomerase. Proc Natl Acad Sci U S A. 2014;111:3377-82.
[32] Pfeiffer V, Crittin J, Grolimund L, Lingner J. The THO complex component Thp2 counteracts telomeric R-loops and telomere shortening. Embo j. 2013;32:2861-71.
[33] Porro A, Feuerhahn S, Delafontaine J, Riethman H, Rougemont J, Lingner J. Functional characterization of the TERRA transcriptome at damaged telomeres. Nat Commun. 2014;5:5379.
[34] Takahama K, Takada A, Tada S, Shimizu M, Sayama K, Kurokawa R, et al. Regulation of telomere length by G-quadruplex telomere DNA- and TERRA-binding protein TLS/FUS. Chem Biol. 2013;20:341-50.

[35] Flynn RL, Centore RC, O'Sullivan RJ, Rai R, Tse A, Songyang Z, et al. TERRA and hnRNPA1 orchestrate an RPA-to-POT1 switch on telomeric single-stranded DNA. Nature. 2011;471:532-6.
[36] Montero JJ, Lopez-Silanes I, Megias D, M FF, Castells-Garcia A, Blasco MA. TERRA recruitment of polycomb to telomeres is essential for histone trymethylation marks at telomeric heterochromatin. Nat Commun. 2018;9:1548.
[37] Chu HP, Cifuentes-Rojas C, Kesner B, Aeby E, Lee HG, Wei C, et al. TERRA RNA Antagonizes ATRX and Protects Telomeres. Cell. 2017;170:86-101.e16.
[38] Deng Z, Norseen J, Wiedmer A, Riethman H, Lieberman PM. TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. Mol Cell. 2009;35:403-13.
[39] Porro A, Feuerhahn S, Lingner J. TERRA-reinforced association of LSD1 with MRE11 promotes processing of uncapped telomeres. Cell Rep. 2014;6:765-76.
[40] Armstrong CA, Tomita K. Fundamental mechanisms of telomerase action in yeasts and mammals: understanding telomeres and telomerase in cancer cells. Open Biol. 2017;7.
[41] Wu RA, Upton HE, Vogan JM, Collins K. Telomerase Mechanism of Telomere Synthesis. Annu Rev Biochem. 2017;86:439-60.
[42] Cao F, Li X, Hiew S, Brady H, Liu Y, Dou Y. Dicer independent small RNAs associate with telomeric heterochromatin. Rna. 2009;15:1274-81.
[43] Gottschling DE, Aparicio OM, Billington BL, Zakian VA. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell. 1990;63:751-62.
[44] Nimmo ER, Cranston G, Allshire RC. Telomere-associated chromosome breakage in fission yeast results in variegated expression of adjacent genes. Embo j. 1994;13:3801-11.
[45] Koering CE, Pollice A, Zibella MP, Bauwens S, Puisieux A, Brunori M, et al. Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity. EMBO Rep. 2002;3:1055-61.
[46] Pedram M, Sprung CN, Gao Q, Lo AW, Reynolds GE, Murnane JP. Telomere position effect and silencing of transgenes near telomeres in the mouse. Mol Cell Biol. 2006;26:1865-78.
[47] Baur JA, Zou Y, Shay JW, Wright WE. Telomere position effect in human cells. Science. 2001;292:2075-7.
[48] Kim W, Ludlow AT, Min J, Robin JD, Stadler G, Mender I, et al. Regulation of the Human Telomerase Gene TERT by Telomere Position Effect-Over Long Distances (TPE-OLD): Implications for Aging and Cancer. PLoS Biol. 2016;14:e2000016.
[49] Luo K, Vega-Palas MA, Grunstein M. Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. Genes Dev. 2002;16:1528-39.
[50] Hecht A, Laroche T, Strahl-Bolsinger S, Gasser SM, Grunstein M. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell. 1995;80:583-92.
[51] Tanny JC, Dowd GJ, Huang J, Hilz H, Moazed D. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. Cell. 1999;99:735-45.
[52] Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature. 2000;403:795-800.
[53] Carmen AA, Milne L, Grunstein M. Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J Biol Chem. 2002;277:4778-81.
[54] Hoppe GJ, Tanny JC, Rudner AD, Gerber SA, Danaie S, Gygi SP, et al. Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. Mol Cell Biol. 2002;22:4167-80.

[55] Hass EP, Zappulla DC. The Ku subunit of telomerase binds Sir4 to recruit telomerase to lengthen telomeres in S. cerevisiae. Elife. 2015;4.
[56] Greenwood J, Patel H, Cech TR, Cooper JP. Fission yeast telosomes: non-canonical histone-containing chromatin structures dependent on shelterin and RNA. Nucleic Acids Res. 2018;46:8865-75.
[57] Zhang K, Mosch K, Fischle W, Grewal SI. Roles of the Clr4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin. Nat Struct Mol Biol. 2008;15:381-8.
[58] Kanoh J, Sadaie M, Urano T, Ishikawa F. Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. Curr Biol. 2005;15:1808-19.
[59] Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, et al. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature. 2001;410:120-4.
[60] Sugiyama T, Cam HP, Sugiyama R, Noma K, Zofall M, Kobayashi R, et al. SHREC, an effector complex for heterochromatic transcriptional silencing. Cell. 2007;128:491-504.
[61] Zofall M, Grewal SI. RNAi-mediated heterochromatin assembly in fission yeast. Cold Spring Harb Symp Quant Biol. 2006;71:487-96.
[62] Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science. 2002;297:1833-7.
[63] Saksouk N, Barth TK, Ziegler-Birling C, Olova N, Nowak A, Rey E, et al. Redundant mechanisms to form silent chromatin at pericentromeric regions rely on BEND3 and DNA methylation. Mol Cell. 2014;56:580-94.
[64] Dejardin J, Kingston RE. Purification of proteins associated with specific genomic Loci. Cell. 2009;136:175-86.
[65] Gauchier M, Kan S, Barral A, Sauzet S, Agirre E, Bonnell E, et al. SETDB1-dependent heterochromatin stimulates alternative lengthening of telomeres. Sci Adv. 2019;5:eaav3673.
[66] Episkopou H, Draskovic I, Van Beneden A, Tilman G, Mattiussi M, Gobin M, et al. Alternative Lengthening of Telomeres is characterized by reduced compaction of telomeric chromatin. Nucleic Acids Res. 2014;42:4391-405.
[67] Wong LH, McGhie JD, Sim M, Anderson MA, Ahn S, Hannan RD, et al. ATRX interacts with H3.3 in maintaining telomere structural integrity in pluripotent embryonic stem cells. Genome Res. 2010;20:351-60.
[68] Garcia-Cao M, O'Sullivan R, Peters AH, Jenuwein T, Blasco MA. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. Nat Genet. 2004;36:94-9.
[69] Arnoult N, Van Beneden A, Decottignies A. Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1alpha. Nat Struct Mol Biol. 2012;19:948-56.
[70] Cubiles MD, Barroso S, Vaquero-Sedas MI, Enguix A, Aguilera A, Vega-Palas MA. Epigenetic features of human telomeres. Nucleic Acids Res. 2018;46:2347-55.
[71] Benetti R, Gonzalo S, Jaco I, Schotta G, Klatt P, Jenuwein T, et al. Suv4-20h deficiency results in telomere elongation and derepression of telomere recombination. J Cell Biol. 2007;178:925-36.
[72] Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, et al. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol. 2006;8:416-24.
[73] Nergadze SG, Farnung BO, Wischnewski H, Khoriauli L, Vitelli V, Chawla R, et al. CpG-island promoters drive transcription of human telomeres. Rna. 2009;15:2186-94.

[74] Feretzaki M, Renck Nunes P, Lingner J. Expression and differential regulation of human TERRA at several chromosome ends. Rna. 2019;25:1470-80.
[75] Yehezkel S, Shaked R, Sagie S, Berkovitz R, Shachar-Bener H, Segev Y, et al. Characterization and rescue of telomeric abnormalities in ICF syndrome type I fibroblasts. Front Oncol. 2013;3:35.
[76] Yehezkel S, Segev Y, Viegas-Pequignot E, Skorecki K, Selig S. Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions. Hum Mol Genet. 2008;17:2776-89.
[77] Sagie S, Toubiana S, Hartono SR, Katzir H, Tzur-Gilat A, Havazelet S, et al. Telomeres in ICF syndrome cells are vulnerable to DNA damage due to elevated DNA:RNA hybrids. Nat Commun. 2017;8:14015.
[78] Voigt P, LeRoy G, Drury WJ, 3rd, Zee BM, Son J, Beck DB, et al. Asymmetrically modified nucleosomes. Cell. 2012;151:181-93.
[79] Timms RT, Tchasovnikarova IA, Antrobus R, Dougan G, Lehner PJ. ATF7IP-Mediated Stabilization of the Histone Methyltransferase SETDB1 Is Essential for Heterochromatin Formation by the HUSH Complex. Cell Rep. 2016;17:653-9.
[80] Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, et al. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell. 2006;125:301-13.
[81] Karimi MM, Goyal P, Maksakova IA, Bilenky M, Leung D, Tang JX, et al. DNA methylation and SETDB1/H3K9me3 regulate predominantly distinct sets of genes, retroelements, and chimeric transcripts in mESCs. Cell Stem Cell. 2011;8:676-87.
[82] Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature. 2007;448:553-60.
[83] Becker JS, Nicetto D, Zaret KS. H3K9me3-Dependent Heterochromatin: Barrier to Cell Fate Changes. Trends Genet. 2016;32:29-41.
[84] Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature. 2006;441:349-53.
[85] Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol. 2007;27:3769-79.
[86] Wu K, Liu H, Wang Y, He J, Xu S, Chen Y, et al. SETDB1-Mediated Cell Fate Transition between 2C-Like and Pluripotent States. Cell Rep. 2020;30:25-36.e6.
[87] Kato M, Takemoto K, Shinkai Y. A somatic role for the histone methyltransferase Setdb1 in endogenous retrovirus silencing. Nat Commun. 2018;9:1683.
[88] Cooper S, Dienstbier M, Hassan R, Schermelleh L, Sharif J, Blackledge NP, et al. Targeting polycomb to pericentric heterochromatin in embryonic stem cells reveals a role for H2AK119u1 in PRC2 recruitment. Cell Rep. 2014;7:1456-70.
[89] Boros J, Arnoult N, Stroobant V, Collet JF, Decottignies A. Polycomb repressive complex 2 and H3K27me3 cooperate with H3K9 methylation to maintain heterochromatin protein 1alpha at chromatin. Mol Cell Biol. 2014;34:3662-74.
[90] Rudenko G, Van der Ploeg LH. Transcription of telomere repeats in protozoa. Embo j. 1989;8:2633-8.
[91] Porro A, Feuerhahn S, Reichenbach P, Lingner J. Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the presence of distinct and multiple regulatory pathways. Mol Cell Biol. 2010;30:4808-17.
[92] Deng Z, Wang Z, Stong N, Plasschaert R, Moczan A, Chen HS, et al. A role for CTCF and cohesin in subtelomere chromatin organization, TERRA transcription, and telomere end protection. Embo j. 2012;31:4165-78.

[93] Vrbsky J, Akimcheva S, Watson JM, Turner TL, Daxinger L, Vyskot B, et al. siRNA-mediated methylation of Arabidopsis telomeres. PLoS Genet. 2010;6:e1000986.
[94] Sfeir AJ, Chai W, Shay JW, Wright WE. Telomere-end processing the terminal nucleotides of human chromosomes. Mol Cell. 2005;18:131-8.
[95] Negishi Y, Kawaji H, Minoda A, Usui K. Identification of chromatin marks at TERRA promoter and encoding region. Biochem Biophys Res Commun. 2015;467:1052-7.
[96] Beishline K, Vladimirova O, Tutton S, Wang Z, Deng Z, Lieberman PM. CTCF driven TERRA transcription facilitates completion of telomere DNA replication. Nat Commun. 2017;8:2114.
[97] Caslini C, Connelly JA, Serna A, Broccoli D, Hess JL. MLL associates with telomeres and regulates telomeric repeat-containing RNA transcription. Mol Cell Biol. 2009;29:4519-26.
[98] Zhu X, Gustafsson CM. Distinct differences in chromatin structure at subtelomeric X and Y' elements in budding yeast. PLoS One. 2009;4:e6363.
[99] Feretzaki M, Lingner J. A practical qPCR approach to detect TERRA, the elusive telomeric repeat-containing RNA. Methods. 2017;114:39-45.
[100] Lopez de Silanes I, Grana O, De Bonis ML, Dominguez O, Pisano DG, Blasco MA. Identification of TERRA locus unveils a telomere protection role through association to nearly all chromosomes. Nat Commun. 2014;5:4723.
[101] Chu HP, Froberg JE, Kesner B, Oh HJ, Ji F, Sadreyev R, et al. PAR-TERRA directs homologous sex chromosome pairing. Nat Struct Mol Biol. 2017;24:620-31.
[102] Montero JJ, Lopez de Silanes I, Grana O, Blasco MA. Telomeric RNAs are essential to maintain telomeres. Nat Commun. 2016;7:12534.
[103] Marion RM, Montero JJ, Lopez de Silanes I, Grana-Castro O, Martinez P, Schoeftner S, et al. TERRA regulate the transcriptional landscape of pluripotent cells through TRF1-dependent recruitment of PRC2. Elife. 2019;8.
[104] Avogaro L, Querido E, Dalachi M, Jantsch MF, Chartrand P, Cusanelli E. Live-cell imaging reveals the dynamics and function of single-telomere TERRA molecules in cancer cells. RNA Biol. 2018;15:787-96.
[105] Yao RW, Wang Y, Chen LL. Cellular functions of long noncoding RNAs. Nat Cell Biol. 2019;21:542-51.
[106] Scheibe M, Arnoult N, Kappei D, Buchholz F, Decottignies A, Butter F, et al. Quantitative interaction screen of telomeric repeat-containing RNA reveals novel TERRA regulators. Genome Res. 2013;23:2149-57.
[107] Lopez de Silanes I, Stagno d'Alcontres M, Blasco MA. TERRA transcripts are bound by a complex array of RNA-binding proteins. Nat Commun. 2010;1:33.
[108] Pfeiffer V, Lingner J. TERRA promotes telomere shortening through exonuclease 1-mediated resection of chromosome ends. PLoS Genet. 2012;8:e1002747.
[109] Balk B, Dees M, Bender K, Luke B. The differential processing of telomeres in response to increased telomeric transcription and RNA-DNA hybrid accumulation. RNA Biol. 2014;11:95-100.
[110] Maicher A, Kastner L, Dees M, Luke B. Deregulated telomere transcription causes replication-dependent telomere shortening and promotes cellular senescence. Nucleic Acids Res. 2012;40:6649-59.
[111] Silva B, Pentz R, Figueira AM, Arora R, Lee YW, Hodson C, et al. FANCM limits ALT activity by restricting telomeric replication stress induced by deregulated BLM and R-loops. Nat Commun. 2019;10:2253.
[112] Pan X, Chen Y, Biju B, Ahmed N, Kong J, Goldenberg M, et al. FANCM suppresses DNA replication stress at ALT telomeres by disrupting TERRA R-loops. Sci Rep. 2019;9:19110.

[113] Xu Y, Kaminaga K, Komiyama M. G-quadruplex formation by human telomeric repeats-containing RNA in Na+ solution. J Am Chem Soc. 2008;130:11179-84.
[114] Martadinata H, Heddi B, Lim KW, Phan AT. Structure of long human telomeric RNA (TERRA): G-quadruplexes formed by four and eight UUAGGG repeats are stable building blocks. Biochemistry. 2011;50:6455-61.
[115] Biffi G, Tannahill D, Balasubramanian S. An intramolecular G-quadruplex structure is required for binding of telomeric repeat-containing RNA to the telomeric protein TRF2. J Am Chem Soc. 2012;134:11974-6.
[116] Redon S, Zemp I, Lingner J. A three-state model for the regulation of telomerase by TERRA and hnRNPA1. Nucleic Acids Res. 2013;41:9117-28.
[117] Le PN, Maranon DG, Altina NH, Battaglia CL, Bailey SM. TERRA, hnRNP A1, and DNA-PKcs Interactions at Human Telomeres. Front Oncol. 2013;3:91.
[118] Roach RJ, Garavis M, Gonzalez C, Jameson GB, Filichev VV, Hale TK. Heterochromatin protein 1alpha interacts with parallel RNA and DNA G-quadruplexes. Nucleic Acids Res. 2020;48:682-93.
[119] Takahama K, Miyawaki A, Shitara T, Mitsuya K, Morikawa M, Hagihara M, et al. G-Quadruplex DNA- and RNA-Specific-Binding Proteins Engineered from the RGG Domain of TLS/FUS. ACS Chem Biol. 2015;10:2564-9.
[120] Wang X, Goodrich KJ, Gooding AR, Naeem H, Archer S, Paucek RD, et al. Targeting of Polycomb Repressive Complex 2 to RNA by Short Repeats of Consecutive Guanines. Mol Cell. 2017;65:1056-67.e5.
[121] Hirschi A, Martin WJ, Luka Z, Loukachevitch LV, Reiter NJ. G-quadruplex RNA binding and recognition by the lysine-specific histone demethylase-1 enzyme. Rna. 2016;22:1250-60.
[122] Shirai A, Kawaguchi T, Shimojo H, Muramatsu D, Ishida-Yonetani M, Nishimura Y, et al. Impact of nucleic acid and methylated H3K9 binding activities of Suv39h1 on its heterochromatin assembly. Elife. 2017;6.
[123] Johnson WL, Yewdell WT, Bell JC, McNulty SM, Duda Z, O'Neill RJ, et al. RNA-dependent stabilization of SUV39H1 at constitutive heterochromatin. Elife. 2017;6.
[124] Kondo K, Mashima T, Oyoshi T, Yagi R, Kurokawa R, Kobayashi N, et al. Plastic roles of phenylalanine and tyrosine residues of TLS/FUS in complex formation with the G-quadruplexes of telomeric DNA and TERRA. Sci Rep. 2018;8:2864.
[125] Zhang Q, McKenzie NJ, Warneford-Thomson R, Gail EH, Flanigan SF, Owen BM, et al. RNA exploits an exposed regulatory site to inhibit the enzymatic activity of PRC2. Nat Struct Mol Biol. 2019;26:237-47.
[126] Levy MA, Kernohan KD, Jiang Y, Berube NG. ATRX promotes gene expression by facilitating transcriptional elongation through guanine-rich coding regions. Hum Mol Genet. 2015;24:1824-35.
[127] Nguyen DT, Voon HPJ, Xella B, Scott C, Clynes D, Babbs C, et al. The chromatin remodelling factor ATRX suppresses R-loops in transcribed telomeric repeats. EMBO Rep. 2017;18:914-28.
[128] Dyer MA, Qadeer ZA, Valle-Garcia D, Bernstein E. ATRX and DAXX: Mechanisms and Mutations. Cold Spring Harb Perspect Med. 2017;7.
[129] Sadic D, Schmidt K, Groh S, Kondofersky I, Ellwart J, Fuchs C, et al. Atrx promotes heterochromatin formation at retrotransposons. EMBO Rep. 2015;16:836-50.

Figures:

Figure 1: Telomeric heterochromatin establishment and players involved. (A) In *S.cerevisiae,* the shelterin component Rap1 recruits Sir4, Sir3, and Sir2. Sir2

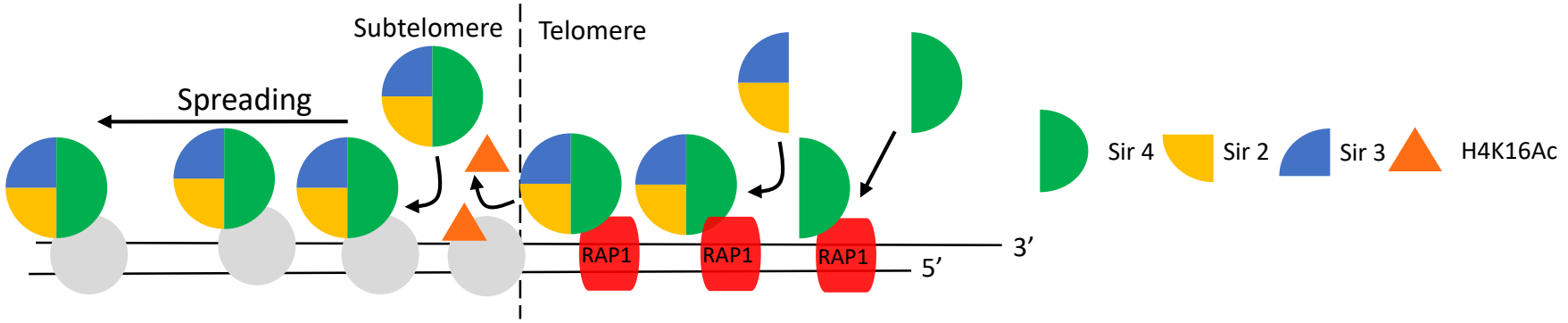
deacetylates H4K16, promoting more Sir4, Sir3, and Sir2 binding and spreading of repressive chromatin to the subtelomeric region. (B) In *S. pombe,* the shelterin factor Taz1 interacts with the histone methyltransferase Clr4 that trimethylates H3K9 and creates binding sites for Swi6. Swi6 recruits SHREC that deacetylates H3K14. Moreover, the RNAi machinery also contributes to Swi6 and SHREC recruitment, contributing to heterochromatin formation. (C) In mouse embryonic stem (ES) cells, SETDB1 trimethylates H3K9. This creates a binding site for HP1 and promotes recruitment of other heterochromatin factors, such as ATRX and DNA methyltransferases (DNMTs). Telomeric heterochromatin is also present at subtelomeres, where DNMTs methylate CpG motifs.

Figure 2: Telomere transcription. (A) The telomere transcriptome of *S. pombe*. G-rich RNAs include telomeric TERRA RNAs and sub-telomeric ARRET RNAs. C-rich RNAs include telomeric ARIA RNAs. αARRET are sub-telomeric RNAs complementary to the ARRET RNAs. (B) *S. cerevisiae* telomeres are transcribed into TERRA RNAs. Transcription starts from the Y'-element in the sub-telomeric region. RNase H and the THO complex destabilize RNA-DNA hybrids. (C) In human cells, telomere DNA is transcribed into TERRA RNAs. Transcription starts from Type-I or Type-II promoters. RNase H and FANCM prevents aberrant accumulation of RNA-DNA hybrids on telomeres. (D) Type-I and Type-II promoters include CTCF binding sites and CpG island promoter elements. CpG island of Type-I promoter are composed of repetitive "61-29-37 repeats" and repressed by DNMTs.

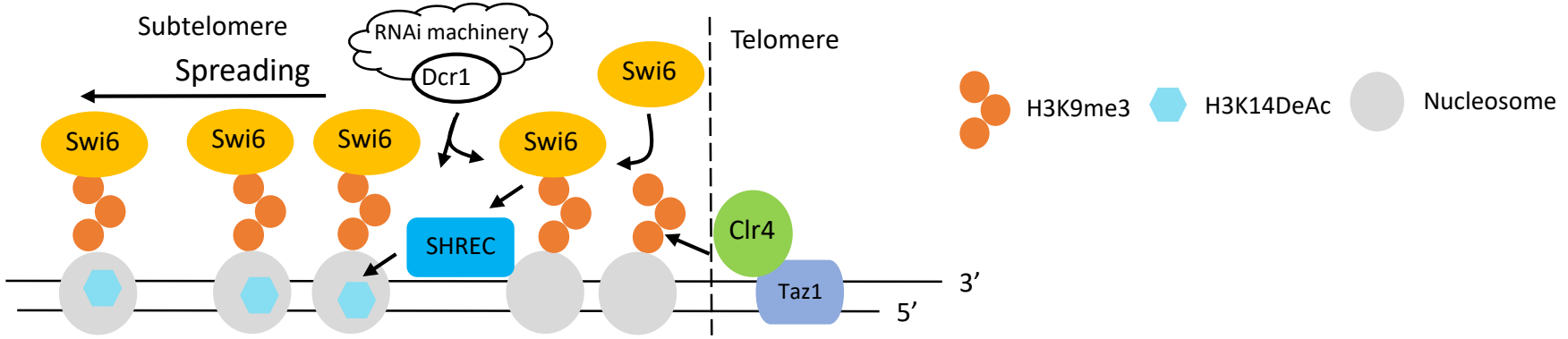
Figure 3: TERRA hypothetic functions (A) TERRA regulates telomere length. TERRA forms co-transcriptional RNA-DNA hybrids that can lead to telomere shortening by interfering with the replication machinery, or to telomere elongation, through homologous recombination. (B) TERRA controls telomerase activity either by inhibiting or by stimulating its recruitment to telomeres. (C) TERRA RNAs promote telomere integrity. TERRA is associated with telomeres, through TRF2 binding, and prevents the

DNA damage repair pathway activation and telomere degradation. (D) TERRA regulates heterochromatin formation at telomeres. TERRA interacts with different heterochromatin factors and tethers them to telomeres.

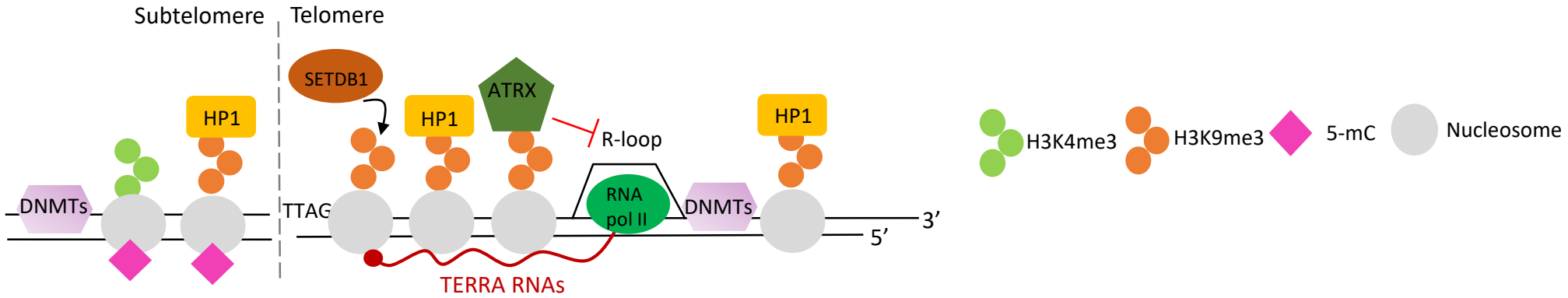
A *S.Cerevisiae :*

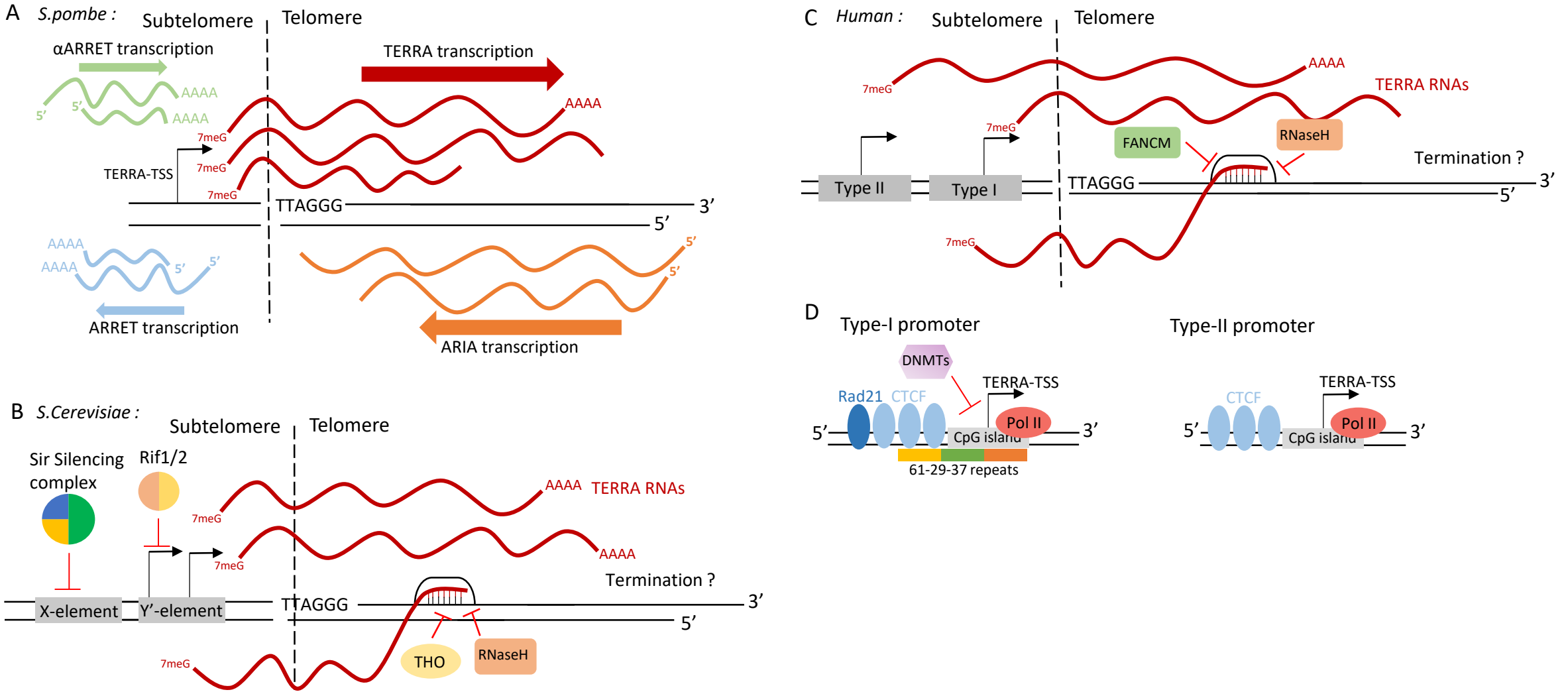


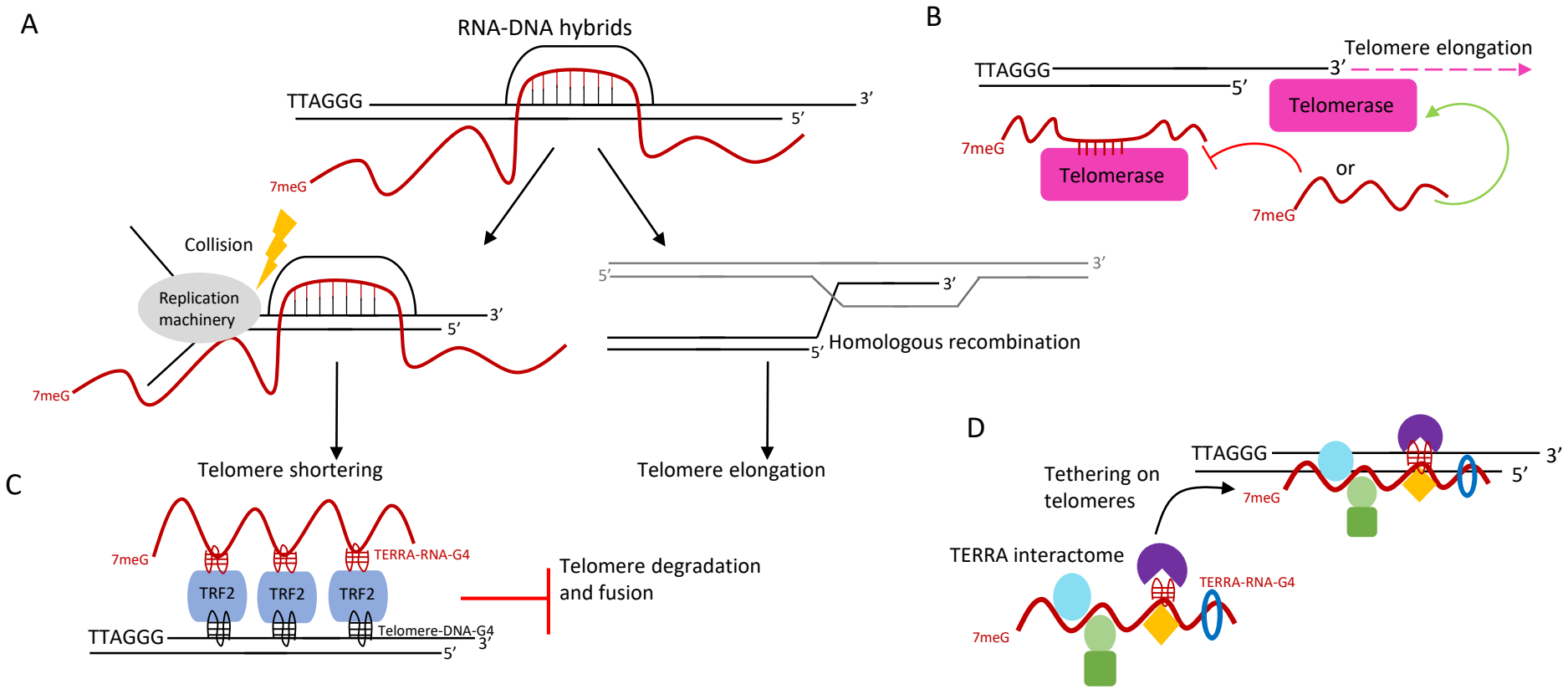
B *S.pombe :*

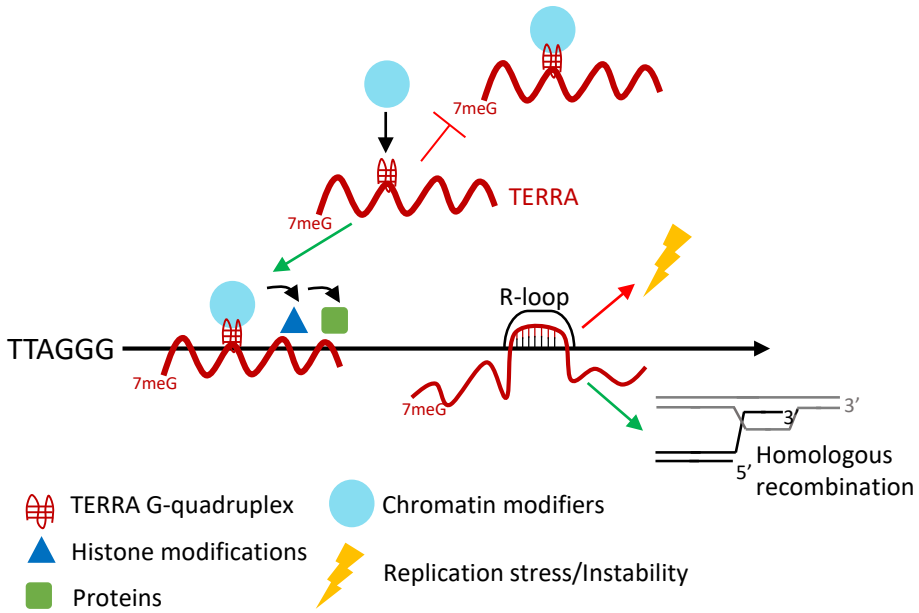


C *Mouse ES cells :*




A
S.pombe :
Subtelomere
Telomere
αARRET transcription
TERRA transcription
AAAA
5'
7meG
TERRA-TSS
TTAGGG
3'
ARRET transcription
ARIA transcription
B
S.Cerevisiae :
Sir Silencing complex
Rif1/2
TERRA RNAs
Termination ?
X-element
Y'-element
THO
RNaseH
C
Human :
FANCM
Type II
Type I
D
Type-I promoter
Type-II promoter
DNMTs
Rad21
CTCF
Pol II
CpG island
61-29-37 repeats


A
RNA-DNA hybrids
TTAGGG
3′
5′
7meG
Collision
Replication machinery
3′
5′
7meG
5′
3′
3′
5′
Homologous recombination
Telomere shortering
Telomere elongation
B
Telomere elongation
TTAGGG
3′
5′
Telomerase
7meG
Telomerase
or
7meG
C
7meG
TERRA-RNA-G4
TRF2
TRF2
TRF2
TTAGGG
Telomere-DNA-G4
3′
5′
Telomere degradation and fusion
D
Tethering on telomeres
TTAGGG
3′
5′
7meG
TERRA interactome
TERRA-RNA-G4
7meG


7meG
TERRA
7meG
TTAGGG
R-loop
7meG
7meG
3′
3′
5′
Homologous
recombination
TERRA G-quadruplex
Chromatin modifiers
Histone modifications
Replication stress/Instability
Proteins