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1 **Telomeric chromatin and TERRA**

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8

9 **Abstract:**

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

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**30 Introduction:**

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

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

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

57 chromatin features, like nucleosomal density. Historically, telomeric chromatin is viewed as  
58 generally heterochromatic, a type of chromatin that hinders DNA transactions. Yet the picture  
59 is even more complex because transcription also occurs at telomeric regions, an unusual  
60 feature in heterochromatin. This transcription leads to the production of long non-coding  
61 RNAs (lncRNAs) named TElomere Repeat-containing RNA (TERRA) [18-22]. It has been  
62 suggested that TERRA plays a role in telomerase inhibition [23-25], but also in telomerase  
63 recruitment [26, 27]. TERRA metabolism also might influence telomere maintenance by  
64 recombination [28-32]. This review will describe the current state of knowledge on telomeric  
65 chromatin regulation and the potential links with TERRA and its transcription. By analogy  
66 with the well-known function of non-coding RNAs (ncRNAs) at *Schizosaccharomyces pombe*  
67 centromeric regions, or the putative role of ncRNAs in recruiting chromatin regulators,  
68 TERRA molecules are viewed as important players in telomeric heterochromatin regulation  
69 [33-39]. Telomerase RNA component, another ncRNA, also is associated with telomeres and  
70 plays an essential role in telomere elongation by telomerase [40, 41]. Currently, this ncRNA  
71 is not considered to play a role in telomeric chromatin regulation, and therefore will not be  
72 discussed in this review. Another telomere-related small RNA was described in mouse (tel s-  
73 RNA), but its function in telomere biology awaits further characterization [42].

74

## 75 **Telomeric chromatin**

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

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

92

93 *S. cerevisiae* telomeres are 250bp-long and are essentially nucleosome-free [16].  
94 Subtelomeric regions, which are also made of distinct repeated DNA, are assembled into  
95 nucleosomes. However, the heterochromatin that forms and spreads along the subtelomeric  
96 regions depends on the binding of Rap1 (a shelterin protein) to its specific DNA motif  
97 essentially present on telomeres (**Figure 1A**) [49]. Rap1 then interacts with the Silence  
98 Information Regulation 4 (Sir4) protein that in turn recruits Sir3 and Sir2 to telomeres [49,  
99 50]. Sir2 catalyses the removal of acetyl groups from lysine 16 of histone H4 (H4K16Ac), and  
100 leads to a hypoacetylated and repressive chromatin conformation [51, 52]. Hypoacetylation  
101 creates a binding site for Sir3 and Sir4 [53, 54] that then recruit more Sir2, providing a simple  
102 molecular explanation for hypoacetylation spreading to sub-telomeric regions and TPE  
103 enforcement in this species. Loss of Sir4 disrupts heterochromatinization and at the same  
104 time promotes telomere shortening. This is because Sir4, in addition to its hypoacetylation  
105 role, facilitates telomerase recruitment at telomeres with the help of Ku proteins [55]. Thus,  
106 *S. cerevisiae* heterochromatin partly promotes telomerase recruitment, and is at least not  
107 inhibitory to lengthening.

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

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

114 In *S. pombe*, telomeric heterochromatinization also depends on a shelterin factor named  
115 Taz1 (**Figure 1B**). Taz1 tethers the SET domain-containing Clr4 to telomeres, and then Clr4  
116 catalyses H3K9me3 deposition to neighbouring nucleosomes [57]. This histone mark in turn  
117 creates a binding site for Swi6 (an orthologue of HP1) on telomeres [58, 59]. The histone  
118 deacetylase Snf2/Hdac-containing Repressor Complex (SHREC) also is recruited by Taz1  
119 and Swi6 to telomeres [60], and its Clr3-subunits deacetylates lysine 14 of histone H3. This  
120 contributes to the formation of repressive chromatin that spreads to sub-telomeric regions  
121 [60]. In *S. pombe*, the formation of sub-telomeric heterochromatin is promoted by the RNA  
122 interference (RNAi) machinery, as observed at centromeres [61]. Specifically, Dcr1, a subunit  
123 of the RNAi machinery, recruits Swi6 and the SHREC complex to telomeres [62]. Intriguingly,  
124 the direct involvement of the RNAi machinery in heterochromatin formation at telomeres  
125 seems to be specific to fission yeast, and is not observed at mammalian telomeres.  
126 Consistently, we never detected any RNAi pathway protein at heterochromatin purified from  
127 mammalian somatic cells [63-65].

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

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

140 site for HP1 $\alpha$  that recruits the Suppressor of Variegation 4-20 homologue 2 (SUV420H2)  
141 enzyme. SUV420H2 catalyses histone 4 lysine 20 trimethylation (H4K20me3), another mark  
142 of heterochromatin [71] with unclear functions. It was proposed that this pathway, which is  
143 very similar to what was described at pericentromeric regions, controls telomerase  
144 recruitment at telomeres, and controls the activation of telomerase-independent ALT [68, 71].  
145 In this classical model, heterochromatin thus negatively regulates telomere lengthening. DNA  
146 methyltransferases (DNMTs) also are involved in telomere length control in mammals [72].  
147 DNA methylation only occurs at subtelomeric regions because CpG sequences are not  
148 present in the telomere repeat motif [72-74]. As subtelomeric DNA is usually  
149 hypermethylated, it was suggested that in the mouse, this high level of DNA methylation  
150 somehow 'compacts' the telomeric fibre, rendering it less prone to damage and lengthening  
151 by recombination [72]. Intriguingly in human cells, loss of DNA methylation seems to have  
152 the opposite effect on telomere length compared with mouse cells [74-77]. In fact,  
153 subtelomeric chromatin is hypomethylated and telomeres are abnormally short in cells  
154 derived from patients with Immunodeficiency Centromeric instability and Facial anomalies  
155 (ICF) syndrome [75-77], who carry an inactivating mutation in *DNMT3B*. This shortening  
156 could be the consequence of defective telomere replication [76]. The different phenotypes  
157 observed in mouse and human cell lines might be due to the different average telomere  
158 length between the two models.

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

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

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

193

194



195 **TERRA biogenesis**

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

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

209 TERRA is a single-stranded lncRNA originating from the transcription of the C-rich telomeric  
210 DNA strand. Thus, TERRA is a G-rich RNA containing 5'-UUAGGG-3' repeats [18, 20-22,  
211 25]. TERRA length ranges from 100bp to more than 9kb in mammals [20, 91] and by  
212 consequence long TERRA transcripts are difficult to be detected by common Northern-Blot. In  
213 Yeast, TERRA range is about 400bp in yeast [22, 25]. TERRA levels are cell-cycle regulated  
214 [29, 69, 91]. Indeed, TERRA levels peak in early G1, and decrease in late G1, reaching the  
215 lowest level in late S phase, a time that roughly corresponds to telomere replication. After the  
216 G2/M phase, TERRA levels start to increase again. RNA polymerase II catalyses  
217 transcription at telomeres [18, 25, 73, 91, 92]. Subunits of the RNA polymerase I and III  
218 complexes also have been identified during the purification of telomeric chromatin [64];  
219 however, the biological significance of these associations is unclear and might not be linked  
220 to telomere transcription.

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

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

232 As TERRA is transcribed by RNA polymerase II, it contains a methyl-cap at the 5' end and a  
233 poly-A tail at the 3' end, like most mRNA species [18, 25, 91]. In yeast and human cells, all  
234 TERRA species are capped. In *S.cerevisiae*, almost all TERRA RNAs also have a poly-A tail  
235 (**Figure 2B**). In contrast, only 7% of human TERRA RNAs have a poly-A tail (**Figure 2C**) like  
236 TERRA in *S.Pombe* [27, 91], whereas this fraction has not been quantified in mouse cells  
237 [18]. In *S. cerevisiae*, poly-adenylation seems to require the action of the canonical poly-A  
238 polymerase (PAP1), but the precise molecular mechanisms of termination remain unclear  
239 because TERRA does not have the canonical poly-A signal (5'-AAUAAA-3') normally found  
240 at the 3' of most class II genes [25]. Indeed, in human cell lines, TERRA-poly-A(-) terminates  
241 preferentially with the 5'-UUAGG-3' sequence, whereas TERRA-poly-A(+) terminates with 5'-  
242 UUAGGG-3' [91], a finding which supports the idea of regulated 3' end TERRA processing.  
243 Moreover, as the TERRA DNA template strand mostly ends with the ATC-5' sequence [94],  
244 TERRA transcription might not process until the end of the telomere

245 As is the case for most mRNA, the poly-A tail stabilizes TERRA both in yeast and in human  
246 [25, 91]. It also correlates with TERRA sub-nuclear distribution in human cells: 60% of  
247 TERRA-poly-A(-) is in the nucleoplasm, while the remaining 40% is chromatin-associated.  
248 Conversely, TERRA-poly-A(+) molecules are mostly in the nucleoplasm [91]. This suggests  
249 that chromatin-associated TERRA is not poly-adenylated, and that TERRA-poly-A(+) and  
250 TERRA-poly-A(-) might underlie distinct functions.

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

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

263 In mammals, several groups identified TERRA production at many (if not all) telomeres [20,  
264 33, 73, 74, 92, 99], whereas others suggested that the bulk of TERRA is produced only from  
265 one or a very limited number of (sub)telomeric regions [100-102]. These two models have  
266 fundamentally different implication on the function of TERRA. The seminal study on TERRA  
267 showed transcription from several human telomeres [20], suggesting the presence of a  
268 transcription start site (TSS) on each of them. RNA fluorescent in situ hybridization indicates  
269 that most telomeres can be found associated with TERRA but this does not prove that  
270 TERRA is transcribed from each telomere, as TERRA could be made from a limited number  
271 of loci then addressed to other telomeres. Transcription from multiple telomeres was also  
272 demonstrated by the same group [74, 99]. Two subtelomeric promoter types, Type-I and  
273 Type-II, were identified at 1Kb and 5-10 Kb from the subtelomere-telomere boundary,  
274 respectively (**Figures 2C and 2D**) [33, 73, 92]. Both promoter types include CCCTC-binding  
275 factor (CTCF) binding sites and CpG island elements [73, 92]. At type I promoters, CpG  
276 islands are composed of three distinct repetitive tracts of 61bp, 29bp and 37bp in  
277 length. These elements are referred as "61-29-37 repeats" and are present at 13 distinct  
278 human chromosome ends [73]. These 61-29-37 repeats are bound by RNA polymerase II

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

289 The other model proposes that TERRA molecules are produced at a single or a limited  
290 number of telomeres [100-102]. Then, TERRA molecules can travel (via uncharacterized  
291 mechanisms) through the nucleus to other telomeres and also to a subset of genes to  
292 regulate the local expression and chromatin composition. One implication of this model is  
293 that telomere transcription, and therefore the inherent local chromatin remodelling, plays a  
294 limited role in telomere biology, while TERRA molecules are important. The other implication  
295 is that the status of one telomere has the potential to govern that of all the other telomeres. It  
296 was proposed that in U2OS human cancer cell lines, which maintain their telomeres with  
297 ALT, chromosomes 20 and X are the only TERRA producers [102]. U2OS ALT telomeres  
298 harbor heterochromatin features [65, 70] and non-physiological TERRA expression [24].  
299 Moreover these observations were not made in telomerase-positive cell lines [20]. Similarly,  
300 in mouse ES cells, the TERRA FISH signal forms two main dots that co-localize with the X  
301 chromosomes, and drive X chromosome pairing during X inactivation [101]. In Mouse  
302 Embryonic Fibroblasts (MEFs), the chromosome 18 telomere is the main source of TERRA  
303 [100]. In both cell types, TERRA signals are also found associated with other telomeres by  
304 FISH and with non-telomeric regions by CHIRT-seq [37, 103], suggesting a function in trans.  
305 Like the X telomere transcript in mouse ES cells, the chromosome 18 TERRA seems thus to  
306 travel to other genomic regions. It was proposed that in these cells, this interaction in trans

307 protects telomeres from the DNA damage response [100], therefore, presumably supporting  
308 an entirely different function from X chromosome pairing described in mouse ES cells [101].  
309 Live-cell imaging in a human cancer cell line, using MS2 knock-ins to track endogenous  
310 TERRA from one telomere, shows TERRA molecules diffusing into the nucleus [104],  
311 sometimes co-localizing as a cluster with one telomere. This result could reflect TERRA  
312 binding to another telomere in trans, or it could reflect the actual TERRA production from the  
313 modified telomere. Similar to the mouse situation, disrupting this single telomere TERRA  
314 transcript leads to the activation of the DNA damage response throughout the genome.  
315 In a model of a single telomere-producing TERRA [100-102], addressing TERRA function by  
316 disrupting the unique TERRA promoter should be straightforward. This was attempted, but  
317 TERRA RNA production was not entirely abrogated [100, 102], complicating the  
318 interpretation and clear conclusions about TERRA roles in trans.

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

323

#### 324 **TERRA RNA functions:**

325 It has been proposed that lncRNAs regulate genome functions [105]. Specifically, they work  
326 by interacting with chromatin-modifying enzymes and nucleosome-remodelling factors to  
327 modulate chromatin structure. For this, they might recruit chromatin factors to specific  
328 genomic regions, or they might anchor chromatin factors away from target regions. lncRNAs  
329 could also act as scaffolds to build chromatin-modifying complexes, without necessarily  
330 targeting them to specific loci. As the lncRNA TERRA is associated with telomeres, it might  
331 play an important function in telomeric chromatin regulation. TERRA cannot be genetically  
332 inactivated easily, and consequently its physiological functions remain largely unknown.  
333 Several proteins that interact with this lncRNA were identified *in vitro* by affinity pull-downs  
334 [37, 38, 106, 107], including TRF1 and TRF2, HP1, the Origin Recognition Complex (ORC)

335 [38], several heterogeneous nuclear ribonucleoproteins (HnRNPs) [107], and also various  
336 interactors that need to be further characterized to determine whether TERRA acts as a local  
337 recruiter of biologically relevant activities. Since many relevant chromatin proteins  
338 promiscuously interact with long RNA *in vitro*, it will be important to determine whether *in*  
339 *vitro* interactors are recruited, and if the case, whether TERRA targets specific chromatin  
340 functions to telomeres. Nevertheless, some TERRA-related regulations are starting to  
341 emerge.

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

347 Telomeric transcription has the potential to generate local TERRA-telomere DNA hybrids that  
348 create R-loop structures and a displaced single-stranded G-rich telomeric DNA. As these  
349 structures generate replication stress, TERRA must be cleared from telomeres during S  
350 phase to ensure complete telomere replication [29, 110] (**Figure 3A**). R-loop clearance is  
351 ensured by an RNase H activity. In human ALT-positive cells, RNase H1 is highly enriched at  
352 telomeres and prevents RNA-DNA hybrid accumulation, which would otherwise promote  
353 increased homologous recombination (**Figure 2C**) [28]. In telomerase-negative yeast, RNase  
354 H1 and RNase H2 also act to limit RNA-DNA hybrid accumulation at telomeres (**Figure 2B**),  
355 thus controlling their elongation through homologous recombination (**Figure 3A**), [29, 30]. In  
356 yeast, RNA-DNA hybrid accumulation is regulated also by the THO complex, which is  
357 normally involved in mRNA export. Indeed, inactivation of Tho2p (a THO complex subunit)  
358 leads to RNA-DNA hybrid accumulation (**Figure 2C**) [30-32] and exonuclease 1-dependent  
359 telomere shortening [30]. These findings indicate that two different RNA processing  
360 pathways regulate telomere RNA-DNA hybrid levels [30, 32], with an apparently stronger  
361 action for RNaseH [32], but the connection between the two pathways are not known. In  
362 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 HP1 $\alpha$  [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

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

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

406 Finally, on the basis of TERRA interaction with Polycomb repressive complex 2 (PRC2)  
407 subunits *in vitro* [120], it was proposed that TERRA recruit Polycomb activities to telomeres  
408 [36]. It should be noted that in some cases the interaction between a chromatin enzyme and  
409 RNA inhibits the enzymatic activity. This is true for LSD1 [121], and EZH2 [125], suggesting  
410 that a simple model where TERRA mediates the recruitment of enzymes such as EZH2 to  
411 work at telomeres is probably incomplete.

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



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

425

#### 426 **Conclusions:**

427 Telomeric chromatin features and telomeric transcripts actively participate in telomere  
428 stability that is required for ensuring genome stability. From yeast to human cells, telomeric  
429 chromatin has been defined as silent heterochromatin, due to TPE. Typical constitutive  
430 heterochromatin marks are enriched at chromosome ends: Sir4-Sir3-Sir2 in *S. cerevisiae*  
431 [49, 51, 52, 54], and H3K9me3 and Swi6/HP1 in *S. pombe* and mammals [57, 58, 65].  
432 However, in most cells, telomeres are nucleosome-poor or have poorly positioned  
433 nucleosomes, a situation which generally disfavours constitutive heterochromatin formation.  
434 The organization of telomeric heterochromatin remains unclear and controversial, which is  
435 why its function is difficult to define. Mechanisms of TERRA mediated functions is  
436 challenging to address due to the telomeric RNA repeated sequence and the different  
437 producing genomic loci, and therefore we are facing major technical challenges. Moreover, in  
438 order to fully understand the interplay between TERRA and chromatin regulation, measuring  
439 TERRA steady-state levels will not be sufficient. We will need to detail the TERRA  
440 transcription mechanisms with more dedicated methods such as nuclear run-on, studying the  
441 RNA polymerase II phosphorylation state and common mechanisms linked to transcriptional  
442 elongation and termination, which are determined by chromatin. Discovering a significant  
443 function for TERRA in regulating telomeric chromatin will thus require robust and quantitative  
444 tools that await further development.

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778 **Figures:**

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



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

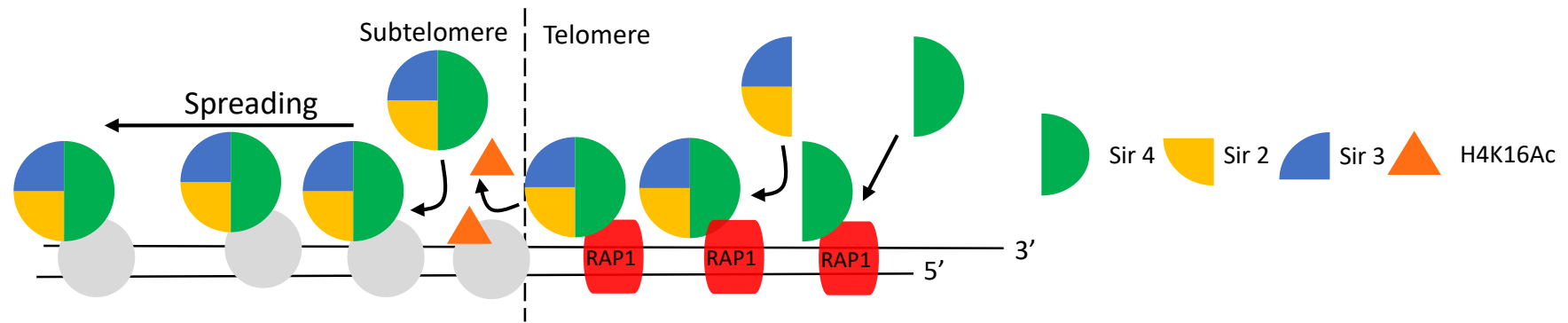
791 **Figure 2: Telomere transcription.** (A) The telomere transcriptome of *S. pombe*. G-rich  
792 RNAs include telomeric TERRA RNAs and sub-telomeric ARRET RNAs. C-rich RNAs  
793 include telomeric ARIA RNAs.  $\alpha$ ARRET are sub-telomeric RNAs complementary to the  
794 ARRET RNAs. (B) *S. cerevisiae*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

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

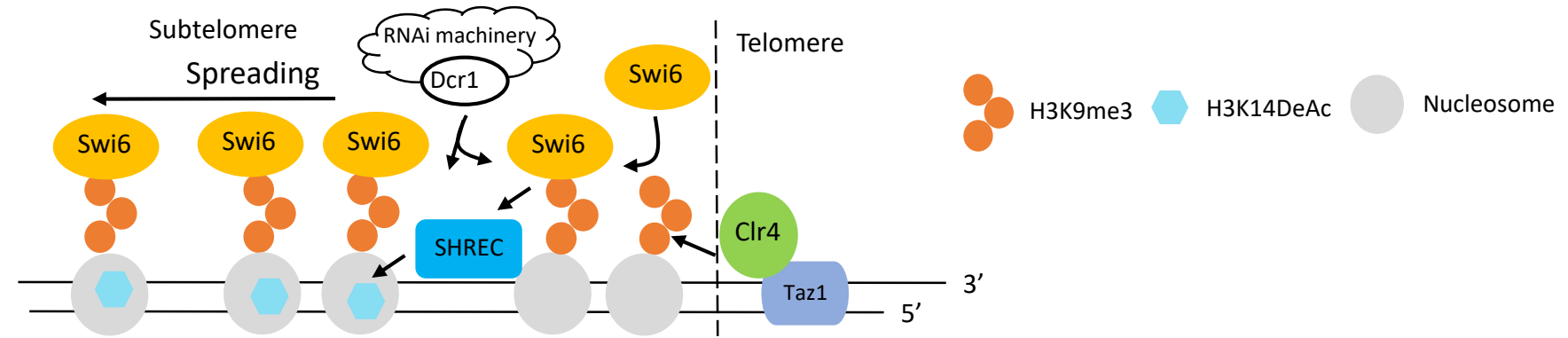
808 DNA damage repair pathway activation and telomere degradation. (D) TERRA regulates  
809 heterochromatin formation at telomeres. TERRA interacts with different heterochromatin  
810 factors and tethers them to telomeres.



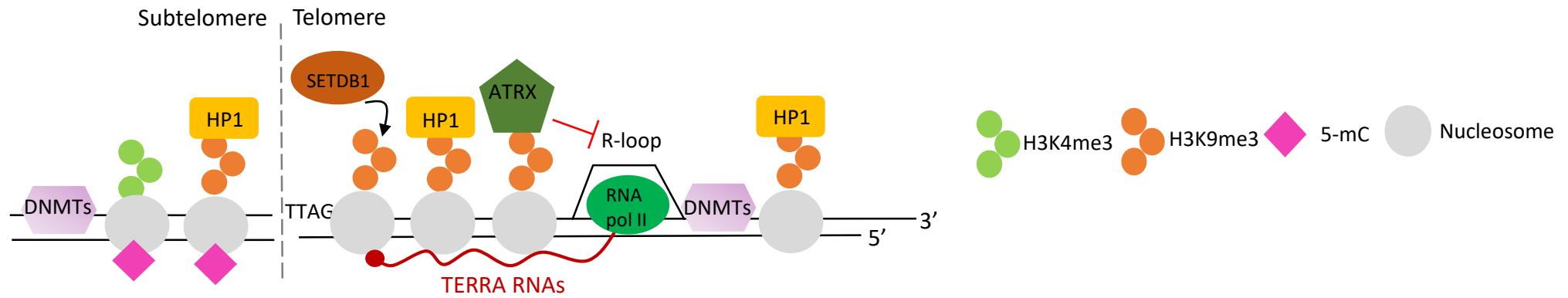
A *S.Cerevisiae* :

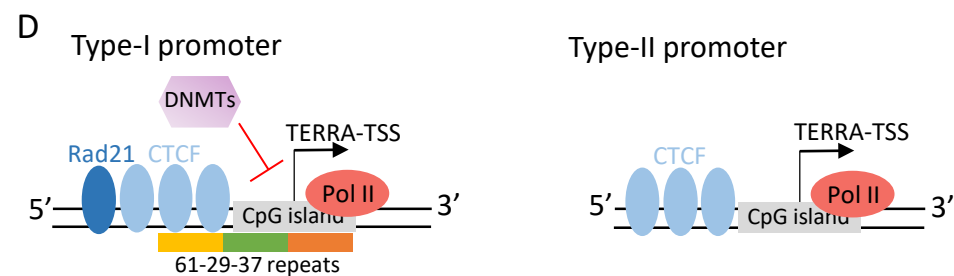
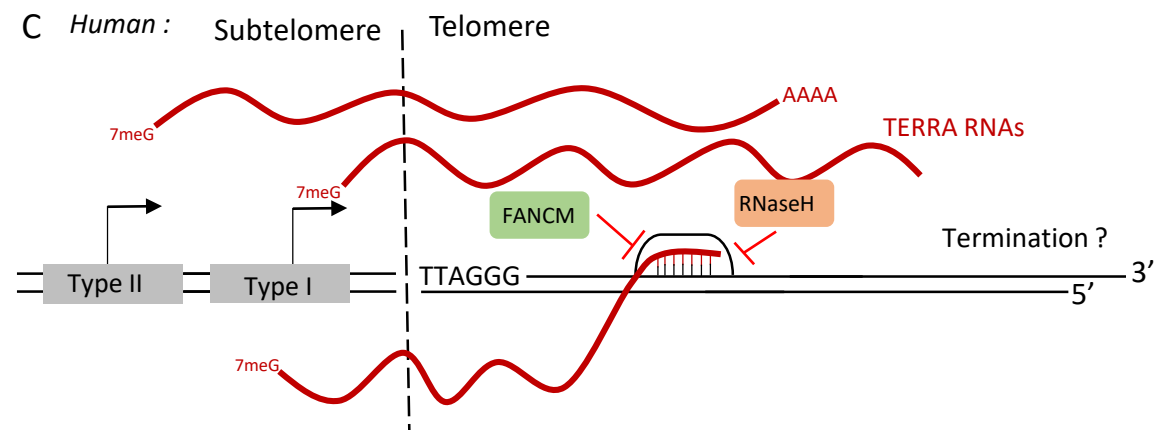
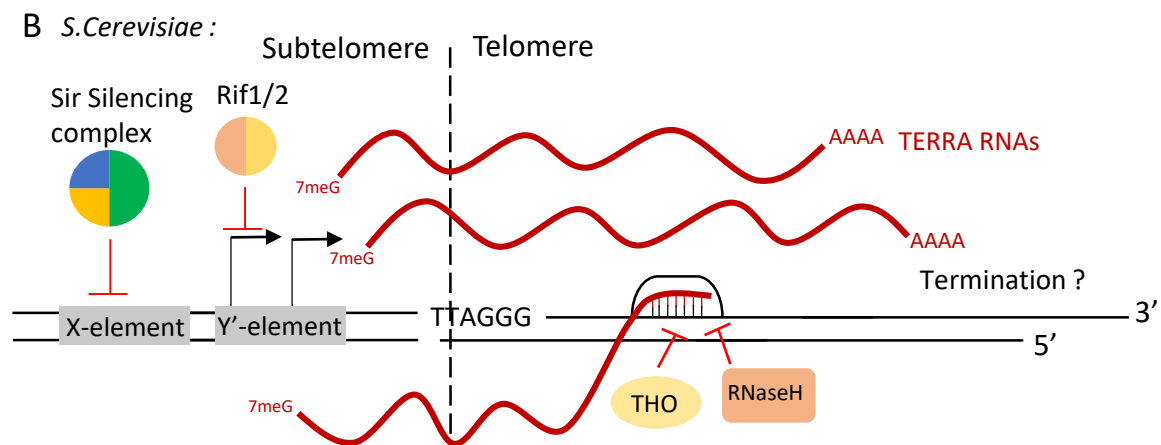
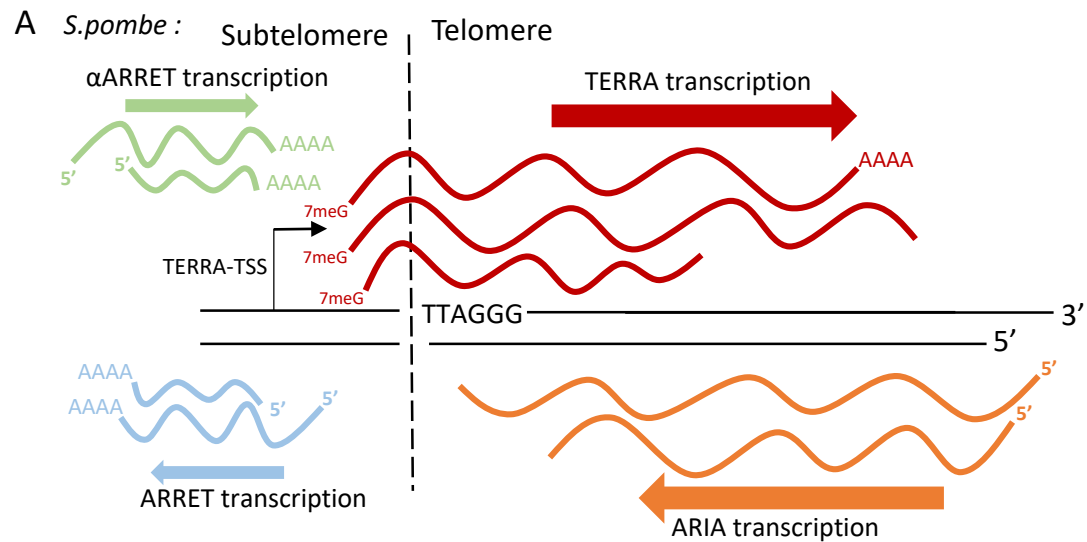


B *S.pombe* :

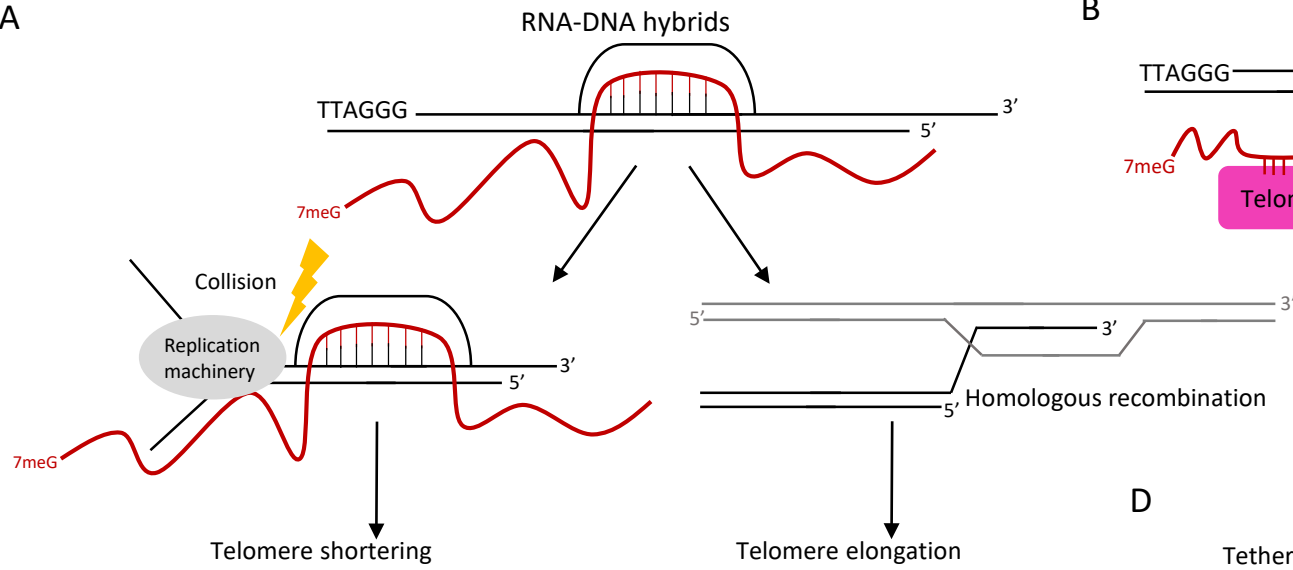


C *Mouse ES cells* :

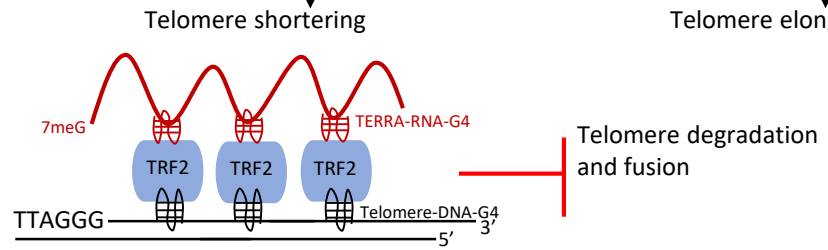




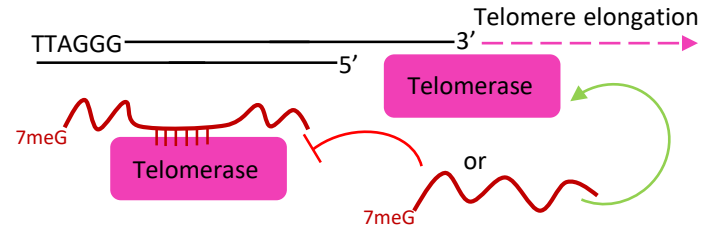
A



C



B



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