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Telomere and Subtelomere R-loops and Antigenic Variation in Trypanosomes

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Abstract

Trypanosoma brucei is a kinetoplastid parasite that causes African trypanosomiasis, which is fatal if left untreated. *T. brucei* regularly switches its major surface antigen, VSG, to evade the host immune responses. VSGs are exclusively expressed from subtelomeric expression sites (ESs) where *VSG* genes are flanked by upstream 70 bp repeats and downstream telomeric repeats. The telomere downstream of the active *VSG* is transcribed into a long-noncoding RNA (TERRA), which forms RNA:DNA hybrids (R-loops) with the telomeric DNA. At an elevated level, telomere R-loops cause more telomeric and subtelomeric Double-Strand Breaks (DSBs) and increase VSG switching rate. In addition, stabilized R-loops are observed at the 70 bp repeats and immediately downstream of ES-linked *VSGs* in RNase H-defective cells, which also have an increased amount of subtelomeric DSBs and more frequent VSG switching. Although subtelomere plasticity is expected to be beneficial to antigenic variation, severe defects in subtelomere integrity and stability increase cell lethality. Therefore, regulation of the telomere and 70 bp repeat R-loop levels is important for the balance between antigenic variation and cell fitness in *T. brucei*. Additionally, the high level of the active ES transcription favors accumulation of R-loops at the telomere and 70 bp repeats, providing an intrinsic mechanism for local DSB formation, which is a strong inducer of VSG switching.

Graphical Abstract

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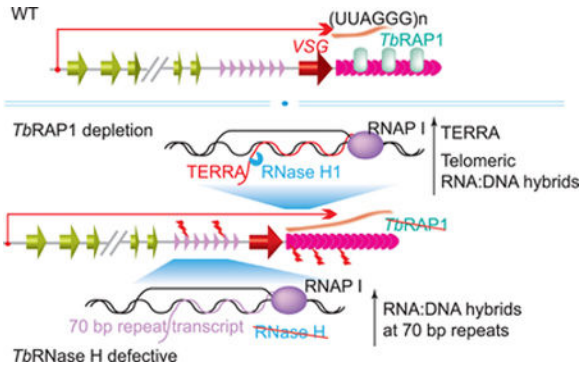

Keywords

R-loop; TERRA; telomere; 70 bp repeats; antigenic variation; *Trypanosomes*

Introduction

Transcription associated RNA:DNA hybrids or R-loops are a double-edged sword that plays important roles in certain cellular processes and causes genome instability [1]. Therefore, their localization and amount need to be tightly regulated. The telomeric tandem repeats in a number of organisms are transcribed into TERRA [2], which can form telomere R-loops that often affects genome stability [3] in humans, yeast, and *Trypanosoma brucei* cells [4]. Recent studies also showed that R-loops at the telomere and the subtelomere influence antigenic variation [5] in *T. brucei* [6–8]. The beneficial functions and adverse effects of R-loops have been reviewed extensively previously [1, 4, 9, 10]. Here we will focus on recent findings on effects of R-loops, particularly those at the telomere and the subtelomere, on antigenic variation in trypanosomes.

The R-loop structure

R-loops are three-stranded RNA-DNA structures with an RNA:DNA hybrid and a displaced single-stranded DNA [11]. The replication-associated RNA:DNA hybrids are usually 11 bp long (in Okazaki fragments) and transcription-associated RNA:DNA hybrids are generally 8 bp long (within the RNA polymerase active site) [12]. However, longer R-loops (> 1 kb) can also form [13]. Currently the thread back hypothesis is the best working model for R-loop formation: DNA behind a transcription bubble is negatively supercoiled, which has a tendency to unwind, allowing the nascent RNA to anneal with the template strand easily [1, 14]. In support of this, defective transcription elongation and termination, RNA splicing, and relaxation of supercoiled DNA all lead to elevated R-loop levels [15–18]. In addition, DNA nicks on the nontemplate DNA strand downstream of the promoter and G clusters facilitate initial R-loop formation, while subsequent expansion and stabilization of the RNA:DNA hybrid are enhanced by high G density and negative supercoiling [19].

R-loops play important roles and are necessary for several cellular processes [1]. First, in mammals, R-loops drive the programmed genomic rearrangement during immunoglobulin class switch in activated B-cells [20]. Second, R-loops formed at the promoter regions can

modulate gene expression [21–23]. Third, R-loops are associated with H3 S10 phosphorylation, a heterochromatic marker, suggesting that it has an important role in chromatin structure modulation [24]. Fourth, in human mitochondria, origin-specific DNA replication is initiated with a two step process: (1) transcription from an upstream promoter leads to accumulation of R- loops, and (2) processing the R-loops by RNase H1 generates 3′ ends for DNA replication by DNA polymerase γ [25]. However, R-loops are also well-known to be a genome instability factor [26]. The displaced single-stranded DNA can be easily mutated and contributes to transcription-associated mutagenesis [27], recombination, and DSBs [28, 29]. A stable R-loop structure can also block the progression of the replication fork [19,30], although the underlying mechanism is still not fully understood [3]. It is also possible that R-loops can trigger chromatin compaction [31–33], which in turn can block replication fork progression [3]. Additionally, nucleotide excision repair endonucleases can process R-loops into DSBs [34].

R-loops are quite stable, as RNA:DNA interactions are thermodynamically more stable than DNA:DNA pairing [35], and many enzymes are involved in dissolution of the R-loop. Several RNA helicases including Rho [36], DHX9 [37], and Senataxin [38] can unwind the RNA:DNA duplex. Ribonuclease H1 (RNase H1) can degrade the RNA strand of the RNA:DNA hybrid and requires a tract of at least four ribonucleotides in the substrate [39]. Eukaryotic Ribonuclease H2 (RNase H2), usually a trimer [40, 41], can resolve the R-loop [42, 43] and remove single ribonucleotides from genomic DNA [44–46]. These enzymes play important roles in maintaining an appropriate level of R-loops in cells [47].

Recent studies in a kinetoplastid parasite, *Trypanosoma brucei,* have shown that telomere and subtelomere R-loops can induce DNA damages, not only contributing to genome instability but also emerging as an important factor that influences antigenic variation [6–8]. These results further indicate that R-loops can have both beneficial and detrimental effects, and its level needs a tight regulation.

Antigenic variation in *T. brucei*

Trypanosomsa brucei causes human and animal African trypanosomiasis, which are usually fatal without treatment. *T. brucei* is transmitted by tsetse *(Glossina spp.)* and threatens millions of people in sub-Saharan Africa [48]. The bloodstream form (BF) *T. brucei* proliferates in the extracellular space of the mammalian host. Its major surface antigen, Variant Surface Glycoprotein (VSG), forms a dense protein layer [49], masks other invariant surface antigens, and can elicit strong immune responses [50]. However, *T. brucei* regularly switches its VSG coat, effectively evading its elimination by the host [5]. This antigenic variation is a critical pathogenesis mechanism and has two major aspects: VSG monoallelic expression and VSG switching.

There are more than 2,500 *VSG* genes and pseudogenes (all are located at subtelomeric regions) in the *T. brucei* genome [51]. Many of these are in large *VSG* gene arrays on megabase chromosomes (Fig. 1A, top) [52–54]. Individual *VSG* genes are also found at two-thirds of minichromosome subtelomeres (Fig. 1A, bottom) [51, 55, 56]. *VSG* genes located at these loci are normally not expressed. At the BF stage, VSGs are expressed exclusively from BF VSG Expression Sites (B-ESs, Fig. 1B, top), which are large polycistronic transcription units (PTUs) transcribed by RNA Pol I and are located immediately upstream of the

telomere on megabase or intermediate chromosomes (Fig. 1A, top and middle) [57, 58]. *VSG* is the last gene in any B-ES and is located within 2 kb from the telomeric TTAGGG repeats in all completely sequenced B-ESs, while the B-ES promoter is located 40 – 60 kb upstream (Fig. 1B, top) [52, 59, 60]. 70 bp repeats are found immediately upstream of the *VSG* gene in long arrays (3 – 20 kb) in B-ESs [52, 59]. *T. brucei* has multiple B-ESs (~ 15 in the Lister 427 strain), all with similar gene organization and ~ 90% sequence identity [59], but often having different *VSG* genes [59]. However, at any moment, only one B-ES is fully active, presenting a single type of VSG on the cell surface [49]. Monoallelic VSG expression ensures effectiveness of VSG switching, as the previously active VSG needs to be silenced for the parasite to avoid elimination by the host. Although detailed mechanisms are not fully understood, many factors have been shown to regulate VSG expression [61], such as chromatin structure [62–72], transcription elongation [73–75], inositol phosphate pathway [76, 77], nuclear lamina [78, 79], recruitment of sumoylated protein(s) to the active ES promoter [80], DNA replication initiation factors [81–84], a subtelomere and VSG-associated VEX complex [85, 86], and telomeric silencing [64, 87]. The VSG coat is lost when *T. brucei* is ingested by its insect vector, tsetse [88]. At the same time, *T. brucei* differentiates into the procyclic form (PF). After migrating to the salivary gland of tsetse, the infectious metacyclic form *T. brucei* expresses VSG again and is ready to be injected into a mammalian host [89]. At this stage, VSGs are expressed from metacyclic ES (M-ESs, Fig. 1B, bottom) that are also located at subtelomeric regions and are transcribed by RNA Pol I [90, 91], except that M-ESs are monocistronic transcription units with the promoter located < 5 kb from the telomere [92–96].

VSG switching only occurs in proliferative BF *T. brucei* cells [97]. It has two major pathways [98, 99]. *In-situ* switching occurs at the transcriptional level, where the originally active ES is silenced while a previously silent ES becomes fully transcribed, and no gene rearrangement is involved (Fig. 2A, bottom left) [9899]. The second and more frequent switching event is DNA recombination-mediated and includes two major types of process [100]. In crossover (CO, or telomere exchange, Fig. 2A, bottom right), a silent *VSG* (often ES-linked or at a minichromosome subtelomere) exchange places with the active *VSG* reciprocally, resulting in a new *VSG* being expressed from the originally active ES with no loss of genetic information [101, 102]. In gene conversion-mediated VSG switching (GC, Fig. 2A, top right), a previously silent *VSG* gene is copied into the active ES to replace the originally active VSG, which is subsequently lost [103, 104]. Theoretically, any functional *VSG* gene in the genome can act as a donor in GC-mediated VSG switching. However, sequences of different B-ESs are 90% identical [59], GC can often occur between B-ESs as they have long homologous sequences [103, 105]. Additionally, all ES-linked and minichromsome *VSGs* are expected to be good GC donors as they have long telomere repeats downstream and 70 bp repeats upstream, the same as the active *VSG* (Fig. 1). Indeed, telomere conversion-mediated VSG switching events have been observed [106]. When telomere-adjacent *VSGs* are used as donors, it is possible that VSG switching may occur through break-induced-replication (BIR) [107] instead of a classical GC (Fig. 2B). However, whether BIR occurs in VSG switching has not been investigated. Another type of GC has also been observed in VSG switching: several *VSG* genes can act as donors simultaneously, each donating a piece of the gene segment, resulting in a novel mosaic *VSG* gene [108–111]. In many published switching assays, *in situ* switching

events are less frequent than recombination-mediated ones, while gene conversion is the most prevalent [6,112–115].

Many proteins, especially those involved in DNA recombination, have been shown to play important roles in VSG switching. For example, RAD51 that mediates strand invasion in homologous recombination [116], RAD51–3 (a RAD51-related protein) [117], and BRCA2 [118], all facilitate VSG switching, while Topoisomerase 3 alpha [114], the RMI1 homologue [115], replication origin binding factor *Tb*ORC1 [81], and a RecQ helicase, RECQ2 [119], suppress VSG switching. Additionally, telomere proteins have been shown to suppress VSG switching [6, 112, 120, 121], while cells harboring an extremely short active *VSG*-adjacent telomere have a ~ ten-fold higher VSG switching rate than WT cells [122]. In addition, cells with defective RNase H enzymes appear to have a higher VSG switching frequency than WT cells [7, 8]. However, how VSG switching is initiated and regulated is less well-understood [123, 124].

T. brucei telomere proteins and antigenic variation

VSG is expressed exclusively from subtelomeric regions [60], and the telomere complex has been shown to play important roles in antigenic variation in *T. brucei* [6, 87, 112, 120, 121, 125]. Telomeres are nucleoprotein complexes that are essential for genome integrity and chromosome stability in eukaryotes [126, 127]. Proteins that directly bind the telomere DNA or associate with the telomere chromatin through protein-protein interaction play critical roles in all aspects of telomere functions, including proper maintenance of the telomere length [128], suppression of illegitimate DNA degradation, recombination, and repair at the chromosome ends [129], assembly of a telomere heterochromatin that represses subtelomeric gene expression [130], and regulation of the telomeric transcript level [131].

In *T. brucei,* besides the telomerase components that are required for telomere maintenance [132–134], several other telomere proteins have been identified [87, 121, 125, 135]. *Tb*TRF directly binds the duplex TTAGGG repeats through its C-terminal Myb domain [135], and the telomere DNA binding activity is essential for cell viability and telomere/subtelomere stability: cells transiently depleted of *Tb*TRF and the *Tb*TRF mutants with weakened telomere-binding activities have more VSG switching events that mostly involve the loss of the active ES [112]. A *Tb*TRF-interacting factor, *Tb*TIF2, is essential for normal cell growth and has a critical role in maintaining telomere/subtelomere integrity: depletion of *Tb*TIF2 results in increased amount of DSBs in ESs, and a transient depletion of *Tb*TIF2 leads to more frequent VSG switching, with most events involving the loss of the active ES [121]. TelAP1 is identified in the *Tb*TRF protein complex and in the complex that can interact with an oligonucleotide of telomeric sequence [125]. It is the only non-essential telomere protein identified so far, and TelAP1 null cells exhibit a faster VSG silencing kinetics when cells are differentiated from BF to PF *in vitro* [125].

*Tb*RAP1 is a *Tb*TRF-interaction factor that associates with the telomere chromatin and is essential for cell viability [87]. Depletion of *Tb*RAP1 leads to derepression of all ES-linked silent *VSGs* (upto several thousand-fold), while the originally active *VSG* is expressed at ~ 50% of its WT level [64, 87]. Because transcription from silent B-ES promoters is detected but transcription elongation attenuates within a few kb [74, 75], it is hypothesized that depletion of

*Tb*RAP1 removes a blockage of transcription elongation, resulting in basal level VSG expression from all silent ESs [87]. This is consistent with the observation that promoter-less *VSGs* at minichromosome subelomeres are not derepressed after TbRAP1 depletion [6487]. Additionally, the *Tb*RAP1-mediated telomeric silencing is position-dependent, exerting strongest effects on telomere-adjacent *VSG* genes, weaker effects on *VSG pseudogenes* in the middle of B-ESs, and weakest effect on reporter genes inserted immediately downstream of the B-ES promoter [87]. The PF *T. brucei* cells proliferate in the midgut of tsetse and express procyclins as its major surface protein [136]. *VSGs* are normally silenced at this stage [88] but derepressed upon *Tb*RAP1 depletion [64]. Additionally, telomeric and subtelomeric chromatin structure is less compact when *Tb*RAP1 is removed in PF cells [64]. *Tb*RAP1 also plays an important role in maintaining telomere/subtelomere integrity and stability, which helps suppress VSG switching [6]. Interestingly, the underlying mechanism involves the telomeric transcript (TERRA) and telomere R-loops.

TERRA in *T. brucei*

The UUAGGG repeat-containing telomere transcript (TERRA) was first identified in *T. brucei* cells and several closely related kinetoplastid parasites three decades ago [137]. In recent years, TERRA has been detected in all eukaryotes tested [138–142] and has been shown to be the product of telomere repeat transcription in humans, mouse embryonic stem cells, both budding and fission yeasts, and plants [140, 141, 143–149]. Often, all telomeres are not transcribed [150, 151], and transcription from intrachromosomal telomeric sequences can be abundant [149]. TERRA has been shown to play important roles in telomere protection [138, 151153], length regulation [148, 154, 157], and recombination [158] in mammalian cells and yeasts. TERRA may also play a role in gene expression regulation in mouse embryonic stem cells [152]. In addition, an excessive amount of TERRA led to telomere and subtelomere instability in yeast [158], presumably due to its propensity to form telomere R-loops [159, 160], which is known to induce DSBs and cause genome instability [4,161].

The telomere transcript was detected in both BF and PF *T. brucei* cells and only a fraction of this RNA is polyadenylated [137]. Most strikingly, the TERRA level is resistant to 1 mg/ml alpha-amanitin, suggesting that TERRA is transcribed by RNA polymerase I [137]. Knowing that the active *VSG* is transcribed at a very high level from a subtelomeric ES by RNA Pol I that is resistant to alpha-amanitin, it was hypothesized that TERRA is transcribed by RNA Pol I as a product of read-through into the telomere repeats downstream of the active *VSG* gene [137]. This hypothesis was confirmed recently by Nanavaty *et al.* [6]. All B-ESs are PTUs [52, 59, 162], and nascent polycistronic transcripts are processed through trans-splicing, where a common spliced leader (SL) sequence is added to the 5′ end of individual mRNAs [163, 164]. After reverse transcription of total RNA using a CCCUAA primer, Nanavaty *et al.* were able to detect a PCR product using primers specific to the active *VSG* gene, indicating that the un-processed nascent RNA containing both the telomeric UUAGGG and the active *VSG* sequences exists, which confirms that TERRA indeed is transcribed from the telomere downstream of the active ES [6]. No TERRA product was detected from silent ES-adjacent telomeres [6]. However, *T. brucei* has more than 200 telomeres that do not host any ESs [52]. Whether TERRA can be transcribed from ES-free telomeres is still unknown. Our lab has now performed a TERRA FISH analysis in BF *T. brucei* cells using the TELC-PNA probe

(PNA Bio). We frequently observe only one or two TERRA foci in each *T. brucei* nucleus (Fig. 3A), suggesting that TERRA is transcribed from few telomeres if not only from the active ES-adjacent telomere. Additionally, we performed TERRA FISH and *Tb*TRF IF analysis simultaneously and found that TERRA is colocalized with *Tb*TRF at the telomere (Fig. 3A).

Interestingly, TERRA is detected in PF *T. brucei* cells where VSG is not transcribed [137, 165], suggesting that not all TERRA is transcribed as a read-through product. Our lab has also detected both UUAGGG and CCCUAA repeat-containing TERRA species in PF *T. brucei* cells (Fig. 3B). In addition to the TERRA species with various sizes (from 0.5 kb to 10 kb, shown in northern blotting as a smear), PF *T. brucei* also transcribes both G-rich and C-rich TERRAs with a more discrete size (~ 1 kb) (Fig. 3B). However, the origins of both UUAGGG and CCCUAA repeat containing TERRA species in PF *T. brucei* cells are currently unclear.

Although the function of *T. brucei* TERRA is not clear, TERRA has been shown to form telomere R-loops [6], and higher than WT levels of telomere R-loops result in an increased amount of subtelomeric and telomeric DSBs and an elevated VSG switching rate [6–8].

Telomere and subtelomere R-loops and antigenic variation

Telomere R-loops are detected in WT *T. brucei* cells [6] by the monoclonal antibody S9.6 that specifically recognizes the RNA:DNA hybrid [166]. Depletion of *Tb*RAP1 leads to not only a higher level of TERRA but also more telomere R-loops and an increased amount of telomeric and subtelomeric DSBs, which in turn cause more frequent VSG switching [6]. The increased amount of DSBs at telomeres and subtelomeres is mainly mediated by the increased amount of telomere R-loops, as expression of an ectopic allele of *Tb*RNase H1 in *Tb*RAP1-depleted cells brings telomere R-loops and telomeric/subtelomeric DSBs back to WT levels, which further reduces the VSG switching rate back to its WT level [6].

Although a higher level of TERRA is frequently associated with an elevated amount of telomere R-loops and *vice versa* [158, 160, 167, 168], this may not always be the case in *T. brucei.* It has been shown that the R-loop level is influenced by RNA processing [169, 170]. High transcription level and poor RNA processing can both lead to an increased level of R-loops. However, it is unknown whether *Tb*RAP1 plays any direct roles in dissolution of the telomere R-loop or whether *Tb*RAP1 affects premature RNA (containing both UUAGGG repeats and the upstream *VSG* sequences) processing. When an ectopic allele of *Tb*RNase H1 is expressed in the *Tb*RAP1-depleted cells, the TERRA level is still much higher than that in WT cells, even though the amount of telomere R-loop is reduced to the WT level [6], suggesting that TERRA and the telomere R-loop may be regulated independently. It would be interesting to further examine the relationship between telomere R-loop and TERRA in *T. brucei*.

Two recent studies on *T. brucei* ribonuclease H enzymes also showed that R-loops at the telomere and the subtelomere influence VSG switching frequencies [7, 8]. *T. brucei* has two RNase H enzymes, a non-essential TbRNase H1 [171] and an essential *Tb*RNase H2 [8]. DRIP-seq experiments (R-loop immunoprecipitation followed by high-throughput sequencing

analysis) were done to map which genomic loci have R-loops in WT and RNase H defective cells [7, 8, 172]. In WT cells, R-loops are detected at the region immediately downstream of the active *VSG* gene, and a much lower level of R-loops is observed immediately downstream of a silent *VSG* gene [7, 8]. More R-loops are clearly detected at both of these regions in *Tb*RNase H1 null cells [7]. Depletion of *Tb*RNase H2A (the catalytic subunit of TbRNase H2) has a similar phenotype [8], indicating that both RNase H enzymes influence R-loop levels at the telomere and subtelomere junction.

Interestingly, in both *Tb*RNase H1 null and *Tb*RNase H2A-depleted cells, increased amounts of R-loops are detected across the active and silent ESs, with the most prominent increase at the 70 bp repeats [7, 8]. The heterogeneous ~ 70 bp repeats are found upstream of most *VSG* genes (Fig. 1 & 2) [51]. The repeats often contain varying numbers of tandem TAA triplets and their sizes range from 66 to 81 bp [173, 174]. Two highly conserved motifs (AGTGTTGTGAGTGTG and TATAATAAGAGCAGTAAT) have been identified and 83% of the 70 bp repeats being studied contain one or both of these motifs [175]. In *VSG* gene arrays, usually few copies of 70 bp repeats are upstream of a *VSG* gene [52, 162]. While in B-ESs, 3 – 20 kb of 70 bp repeats are upstream of the *VSG* gene [59]. In WT BF cells, no stable transcripts from the 70 bp repeats have been detected, even though the active ES is highly transcribed by RNA Pol I, suggesting that RNA processing is very efficient. However, in *Tb*RNase H1 null and *Tb*RNase H2A-depleted cells, a significant amount of R-loops are detected at the 70 bp repeat region in both active and silent ESs [7, 8]. Consistent with the notion that R-loops often induce DNA damages [3], more γH2A (DNA damage associated histone H2A with phosphorylated T130 [176]) associates with the active ES chromatin, particularly at the telomere-proximal region, in *Tb*RNase H1 null cells [7], suggesting telomere-proximal R-loops are more stable. When *Tb*RNase H2A is depleted, much more γH2A associates with both active and silent ES chromatin throughout the whole ES [8]. Furthermore, more switchers are observed to have shed the originally active VSG on the cell surface in the RNase H defective cells, suggesting that these cells have an increased VSG switching rate than WT cells [7, 8]. Therefore, increased amounts of telomere/subtelomere R-loops in RNase H defective cells are linked with elevated DNA damage levels at ESs and more frequent VSG switching [7, 8]

In *Tb*RNase H defective cells, an increased amount of R-loops is detected at the telomere/subtelomere junction [7, 8]. It is likely that telomere R-loops are also stabilized in these cells, although this has not been tested directly. It is also unknown whether TERRA levels are increased when the RNase H enzymes are deleted or depleted. In *Tb*RNase H1 null and TbRNase H2A-depleted cells, the mRNA levels of a number of silent *VSGs* are increased (upto several ten-fold) [7, 8], suggesting that *Tb*RNase H1 and H2 may be important for VSG silencing. R-loops have been shown to affect gene expression in other organisms [21–23]. Therefore, it is possible that VSG expression is affected by nearby R-loops and that TERRA level is also increased in *Tb*RNase H defective cells. On the other hand, frequent VSG switching in the RNase H defective cells [7, 8] could also lead to mildly increased VSG mRNA levels when a cell population is examined. Cells expressing both the originally active VSG and an originally silent VSG simultaneously are observed by IF in the *Tb*RNase H defective cells [7, 8]. However, these cells might be in the middle of a VSG switching process. Whether the TERRA level is affected by the RNase H enzymes needs further investigation.

It is important to note that functions of *Tb*RNase H1 and H2 are not limited at ES regions [7, 8, 172]. A recent DRIP-seq analysis detected R-loops in multiple *T. brucei* genome loci in WT cells [172]. In *Tb*RNase H1 null cells, increased levels of R-loops are also observed at transcription start sites of RNA Pol II transcribed PTUs, while no significant increase of DNA damage at these sites is seen [172]. It is also unknown whether *Tb*RNase H1 affects mRNA levels of genes located outside of ESs. On the other hand, depletion of *Tb*RNase H2A leads to a dramatic increase in the amount of genomic DNA damage, particularly at the transcription initiation sites [8]. Additionally, several tens of genes other than *VSG,* ESAG*,* or procyclin genes exhibit increased mRNA levels when *Tb*RNase H2A is depleted [8].

Subtelomeric regions are often composed of various repeats and gene families [177–179]. High polymorphism in the subtelomere is frequently observed among different chromosome ends and individuals in humans [180, 181], yeast [177, 182], fly [183], plant [184], and fungal pathogens [185, 186]. *T. brucei* subtelomeres also exhibit dynamic variations: the *T. brucei* homologous megabase chromosome pairs often differ greatly in size (Fig. 1A) [187]. Several factors contribute to this size polymorphism: subtelomeric ESs and *VSG* gene arrays have different sizes, telomere lengths vary at different chromosome ends, and repetitive chromosomal regions vary in size [188]. Importantly, two-thirds of the size polymorphisms are due to variations in subtelomeric regions, while chromosomal core regions, containing all essential genes, are relatively stable [53].

Telomere dysfunctions are well-known to induce genome instabilities. At the telomere vicinity, unprotected telomeres lead to chromosome end-to-end fusions [189, 190], anaphase bridges [191, 192], and telomere recombination [193, 194]. Telomere fusions in human cells can further induce a persistent mitotic arrest that leads to greatly increased cell lethality [195]. At a global level, telomere crisis results in dicentric chromosome formation and subsequent chromothripsis and kataegis [191]. Dysfunctional telomeres can also lead to subtelomere instability in yeast [196, 197]. In telomerase null cells, Type I and Type II survivors use DNA recombination-dependent mechanisms to maintain their telomere length [196], where Type I survivors amplify their subtelomeric Y’ elements in a Rad51-dependent pathway [197, 198]. Studies in *T. brucei* have shown that depletion of telomere proteins, *Tb*TRF, *Tb*RAP1, and *Tb*TIF2, all result in unstable subtelomeres [6, 112, 120, 121].

Most telomere dysfunctions result in severe genome instability and cause cell growth defects [129]. In *T. brucei,* VSG is essential [199], and damages to the active *VSG* gene are generally poorly tolerated: Introducing an artificial DSB (an I-SceI cut) within or near the active *VSG* gene leads to cell death in more than 80% of the cell population [200], which also leads to a 250-fold higher VSG switching rate [201]. It is possible that the I-SceI cut is not repaired efficiently due to continued I-SceI expression. However, the location of the damage site appears to be a critical factor, as inducing the same I-SceI cut in a silent ES is much better tolerated [200]. *Tb*TIF2 and *Tb*RAP1 are essential proteins that associate with the telomere chromatin [87, 121], and depletion of these proteins induces DNA damages mainly in the active and silent ESs [6, 121]. These observations are consistent with the idea that DNA damages in the active ES are poorly tolerated. Depletion of *Tb*RNase H2A results in global increased amounts of DNA damages at transcription initiation sites [8], suggesting that *Tb*RNase H2A may be important for genome integrity at non-subtelomeric regions. Therefore, it is hard to

interpret whether the increased amounts of DNA damages in ESs contribute significantly to the growth defect in *Tb*RNase H2A-depleted cells. On the other hand, some telomeric dysfunction and subtelomeric damages are better tolerated, contributing to subtelomeric plasticity. Single damages within silent ESs do not severely affect cell growth [200]. Telomeres downstream of the silent ESs can be as short as < 100 bp without inducing any cell growth defect [202]. Additionally, cells do not experience cell cycle arrest when a single telomere (without any adjacent ES) is deleted [203]. Therefore, damages that do not directly disrupt the active *VSG* gene seem less detrimental to cell growth than the ones that do. However, *Tb*RNase H1 null cells represent an exception, where an increased amount of DNA damages is detected in the active ES, but the cells are viable [7]. Although it is not strictly comparable among different studies, loss of *Tb*RNase H1 does not seem to cause an as high level of DNA damage in ESs as depletion of *Tb*TIF2 or *Tb*RAP1 [6, 7, 121]. In the latter two cases, DNA damages are observed in both active and silent ESs, while loss of *Tb*RNase H1 only causes a moderate increase in the amount of DNA damages in the active ES [6, 7, 121]. Therefore, at *T. brucei* subtelomeres, the amount of damages probably also contributes to its effect on cell fitness. In consistence with this, some DNA breaks are detected at 70 bp repeat region in the active ES in WT cells [201], suggesting that cells can tolerate a low level of subtelomeric damages, although the exact amount of telomere and subtelomere damages in WT cells is unknown.

These observations suggest that the balance between plasticity and integrity at the *T. brucei* subtelomere is a key factor of keeping a balance between antigenic variation and cell fitness, both important for parasite survival. A similar balancing act is necessary for proper telomere maintenance in human ALT cells [168], where a telomerase-independent and DNA recombination-dependent telomere maintenance mechanism is critical for cell survival [204]. In ALT cells, overexpression of TERRA leads to an increased level of telomere R-loops and many more telomere recombination events [168]. Importantly, depletion of RNase H1 in these cells leads to an accumulation of telomere R-loops and C-circle excision-mediated rapid telomere shortening [168].

Overexpression of RNase H1 in ALT cells reduces the amount of telomere R-loops, which hinders telomere recombination potential and results in gradual telomere shortening [168]. Therefore, perturbing telomere R-loop levels in ALT cells disrupts the delicate balance between recombination-mediated telomere attrition and maintenance.

Achieving a good balance between telomere/subtelomere stability and subtelomere variation may be feasible through an introduction of the right amount of telomere/subtelomere damages [205]. In *Kluyveromyces lactis,* variation in a subtelomeric gene family encoding β-galactosidase allows yeast to better cope with different nutrition [206], a scenario not too different from VSG switching in *T. brucei.* Mild telomere dysfunction that does not induce global genome instability leads to increased variation of the subtelomere β-galactosidase-coding genes, while severe telomere dysfunction causes complete deletion of these genes [206]. Therefore, mild telomere dysfunction can serve as a beneficial drive for subtelomere variations that allow cells to better adapt to environmental stresses [205]. The *Tb*RNase H1 null cells [7] may be a good example of having a mild telomere/subtelomere dysfunction in *T. brucei,* as the amount of R-loop-induced subtelomeric DNA damages appears not severe

enough to trigger cell growth defect but sufficient to stimulate VSG switching, which is presumably beneficial for a long-term parasite survival inside the mammalian host. Since *Tb*RNase H1 null cells are viable, it would be interesting to examine whether these cells have increased virulence when infecting an animal host.

Telomere/subtelomere R-loops and initiation of VSG switching

Homologous recombination (HR) is a major pathway for VSG switching [100]. For ES-linked and minichromosome *VSGs,* the telomere sequence is found downstream of *VSG*[51, 52, 59]. Additionally, most *VSG* genes have a common 14 nt sequence in their 3′UTR region [51]. It has been proposed that the common 14 nt VSG 3′UTR sequence and sometimes the telomere sequence can serve as the downstream homologous arm, while the 70 bp repeats can serve as the upstream homologous arm for efficient homologous recombination that mediates a VSG switch [207]. However, an earlier study showed that the 70 bp repeats upstream of the *VSG* gene in an ES is not required for VSG switching [103]. In a recent study that introduces an I-SceI cut in the active ES to initiate VSG switching, it is found that 70 bp repeats in the active ES promote selection of *VSG* donors from the genomic archive rather than only from silent ESs [207].

Although a DSB is not absolutely required for HR, it is a good inducer for HR and frequently repaired by HR [208]. Therefore, it has been hypothesized that DSBs are an important trigger of VSG switching [123, 201]. In support of this, introducing an artificial DSB immediately upstream of the active *VSG* leads to a more than 250-fold higher VSG switching frequency [201], and DSBs introduced near the active *VSG* (both upstream and downstream) result in efficient VSG switching [200]. DSBs can also be detected in 70 bp repeats by Ligation mediated PCR analysis in WT *T. brucei* cells [201], although the amount of telomeric and subtelomeric DNA damage in WT cells has not been carefully quantified. Interestingly, the active ES-adjacent telomere experiences frequent large fragment deletions [209], which may be a consequence of TERRA transcription by RNA Pol I [6, 137] that depletes most nucleosomes in the active ES [67, 68] and may also interfere with the binding of *Tb*TRF at the telomere. Therefore, DNA breaks in both the telomere and 70 bp repeats are possible to serve as an inducer for VSG switching. Several mechanisms are possible for DSB formation in these repetitive sequences.

First, as repetitive sequences, both telomeres and 70 bp repeats can be difficult to be replicated due to strand slippage during DNA replication [210] and secondary structure formation [211] (such as the G-quadruplex structure formed by the telomere G-rich strand DNA [212] and the non-H bonded structure formed by TTA/TAA in the 70 bp repeats [213]). In mammalian and yeast cells, several telomere proteins have been shown to play important roles in ensuring proper telomere replication [214–216]. Whether *T. brucei* telomere proteins have similar functions still requires further investigation. Nevertheless, obstacles in DNA replication often lead to stalled replication fork and eventual DSBs [217, 218]. Second, it is shown that the active B-ES is replicated in early S phase [119]. Hence, the transcription machinery may collide with the DNA replication machinery in the active B-ES and its adjacent telomere, which often causes fork stalling and collapsing followed by DSB formation [219]. Third, the active ES and its adjacent telomere are transcribed at high levels by

RNA Pol I [6, 220], which can induce more DSB formation with or without the R-loop formation. RNA Pol I transcription depletes nucleosomes from the active ES [67, 68] and can, presumably, also remove some *Tb*TRF from the telomere. The exposed DNA in the active ES and the adjacent telomere is likely more vulnerable to nuclease attack, which can result in more DSBs. Additionally, the traverse of RNA polymerase along repetitive DNA allows formation of DNA secondary structure, which helps DSB formation [221]. Furthermore, the high levels of transcription can induce transcription-associated recombination (TAR) [222], and RNA Pol I transcription in BF *T. brucei* cells stimulates homologous recombination > 3 fold [223]. Finally, the telomere and subtelomere R-loops may further increase the chance of DSB formation in this area. We now know that TERRA forms R-loops with the telomeric DNA [6], and an increased level of telomere R-loops leads to an increased amount of DSBs in ESs and more frequent VSG switching [6]. Additionally, R-loops are stabilized at the 70 bp repeats in *Tb*RNase H1 null and *Tb*RNase H2A-depleted cells, which is also linked with increased amounts of DNA damages in ESs and more frequent VSG switching [7, 8]. Transcribing the active *VSG* with the flanking repetitive sequences provides a couple of intrinsic mechanisms to induce DSBs in the local region, either with or without the formation of local R-loops, which ensures a good chance of VSG switching through homologous recombination.

R-loops at the telomere and 70 bp repeats clearly contribute to more efficient antigenic variation [6–8]. However, the underlying mechanisms of the formation and dissolution of these R-loops are not well-understood. Functions of *T. brucei* TERRA are not clear, and whether high levels of TERRA always lead to high levels of telomere R-loops is not known. Similarly, it is unclear whether the RNA transcribed from the 70 bp repeats can only exist as part of the R-loop in *Tb*RNase H defective cells. Finally, *Tb*RNase H1 null cells appear to have achieved a good balance between improved subtelomeric plasticity and sufficient genome stability [7]. A better understanding about the detailed functions of *Tb*RNase H1, particularly at the subtelomere, would be revealing for the positive and negative effects of telomere/subtelomere R-loops on antigenic variation and genome stability.

Telomere biology in other microbial pathogens and the potential link with pathogen virulence

Two kinetoplastid parasites that are closely related to *T. brucei* also cause debilitating human diseases: *Trypanosoma cruzi* causes Chagas disease, which can lead to serious heart and digestive problems [224]. Leishmaniasis is caused by over 20 species of *Leishmania.* The disease severely decreases the life quality and causes heavy economic burdens [225]. Telomeres in all three kinetoplastid parasites have the TTAGGG repetitive sequence [162, 226, 227]. Homologues of all known *T. brucei* telomere proteins can be easily identified in *T. cruzi* and *Leishmania* genomes [228, 229]. Additionally, TERRA has been detected in *Leishmania* [165]. However, it is unknown whether *T. cruzi* transcribes its telomeric sequence. It is also unclear whether R-loops are formed in *T. cruzi* and *Leishmania.* Still, the essential telomere function in maintaining genome stability is expected to be conserved in both *T. cruzi* and *Leishmania.* In addition, subtelomere stability may be an important factor for *T. cruzi* virulence. *T. cruzi* is able to infect any host cell but mainly macrophages, fibroblasts and epithelial cells [230]. Infective forms of *T. cruzi* express surface transsialidase proteins

[231, 232], and their conserved peptide motifs are important for interacting with cytokeratin and mediate host-parasite interaction [233]. Decreasing trans-sialidase expression also contributes to the loss of *T. cruzi* virulence [234]. Interestingly, the gp85 gene family that encodes trans-sialidase is located at subtelomeric regions [235], and maintaining telomere and subtelomere stability is expected to help maintain stable gp85 gene copy numbers. Therefore, it will be interesting to investigate whether gp85 expression is affected by telomeric silencing, whether any R-loop is formed at telomere/subtelomere, and whether these R-loops affect gp85 gene family stability or their expression.

Several other microbial pathogens that undergo antigenic variation also host their variable surface antigens at subtelomeres [179], including *Pneumocystis jirovecii* that causes pneumonia in immunodeficient patients, in which DNA recombination appears to be the major pathway of antigenic variation [185]. A better understanding of telomere functions and how telomere R-loops influence telomere and subtelomere stability will help us better understand the mechanisms of antigenic variations in these human pathogens.

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Research Highlights

- In *T. brucei,* a protozoan parasite that causes African trypanosomiasis and undergoes antigenic variation, the major surface antigen-coding *VSG* gene is transcribed from telomere-adjacent expression sites, where it is flanked by the telomere and 70 bp repeats
- In *Tb*RAP1-depleted cells, an increased amount of telomere R-loops leads to an elevated amount of telomere/subtelomere DSBs and more frequent VSG switching
- Removal of *Tb*RNase H enzymes results in accumulation of R-loops at the 70 bp repeats and at the telomere-subtelomere junctions, more DSBs in VSG expression sites, and more frequent VSG switching
- R-loop levels at the telomere and 70 bp repeats influence the balance between antigenic variation and cell fitness
- The high level of transcription of the active ES by RNA Pol I promotes accumulation of R-loops at the telomere and 70 bp repeats. This provides an intrinsic mechanism for DSB formation in the active ES, which is a strong inducer of VSG switching.

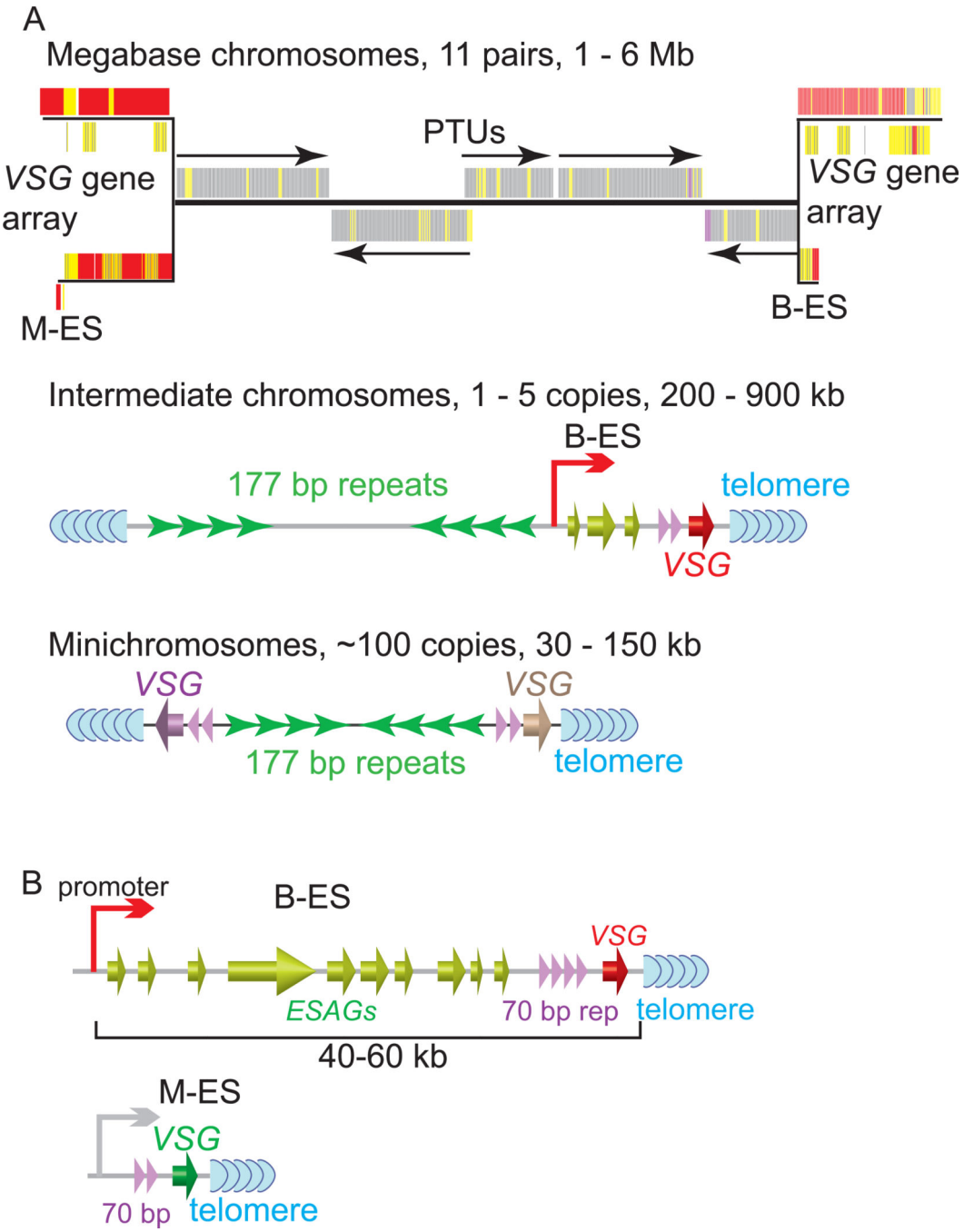

Figure 1.

(A) Schematic diagram of a megabase chromosome (top), an intermediate chromosome (middle), and a minichromosome (bottom) in *T. brucei,* with their type, number, and size indicated. The central region of the two homologous megabase chromosomes are the same and is shown once, while their subtelomeres are different and shown separately (top). Individual genes are represented as short colored bars. Grey, functional genes; red, *VSG* genes; yellow, pseudogenes. PTUs and their transcription directions are marked with arrows. *VSG* gene arrays, a B-ES, and an M-ES are shown. A B-ES is shown at one subtelomere of

the intermediate chromosome (middle). Individual *VSG* genes are shown at subtelomeres of the minichromosome (bottom) (B) A representative B-ES (top) and an M-ES (bottom) is shown. *ESAGs: Expression Site Associated Genes.*

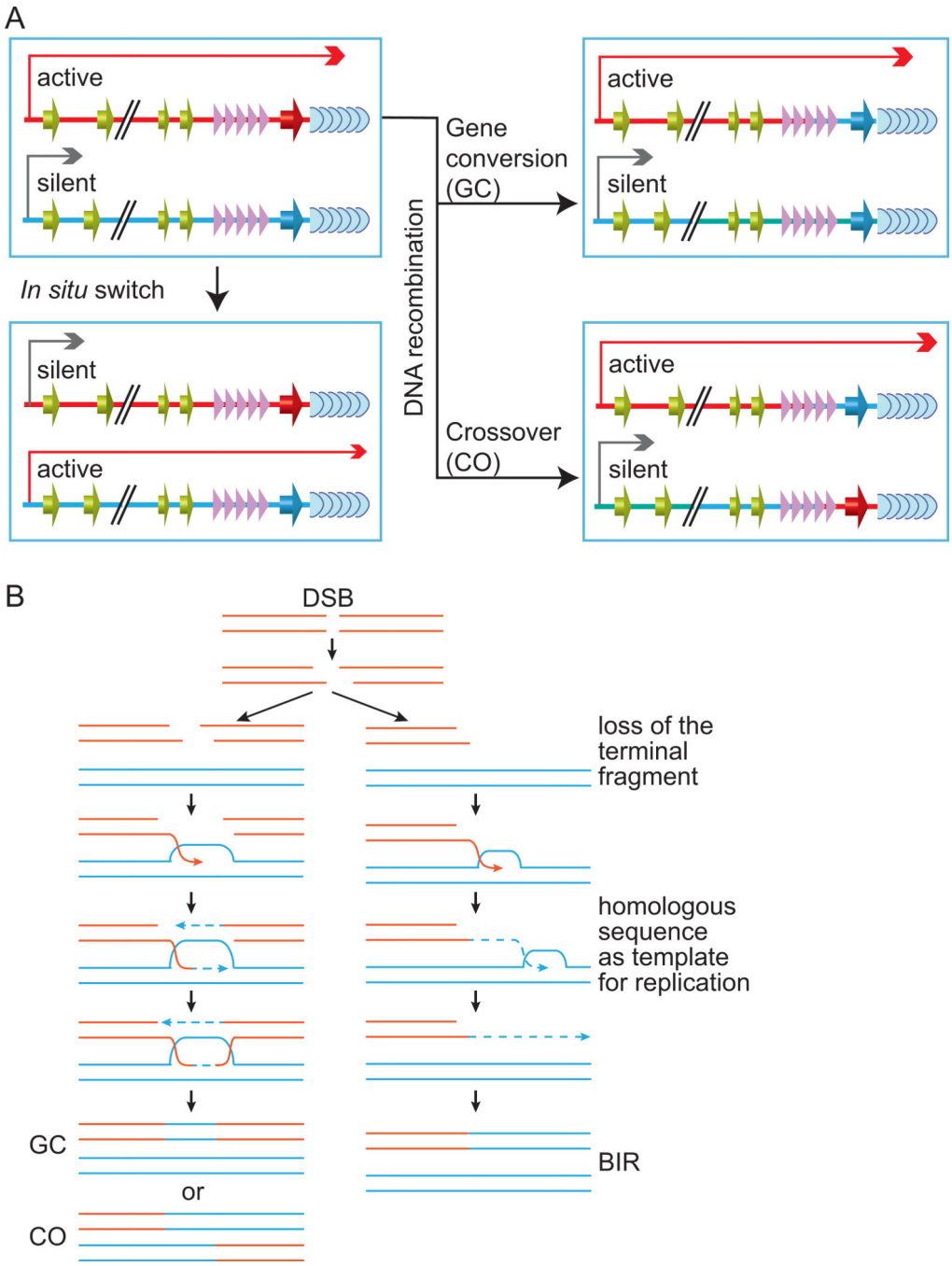

Figure 2.

(A) VSG switching mechanisms. Light blue soft arrow heads, telomere repeats; purple arrow heads, 70 bp repeats; red and dark blue 3D arrows, *VSG* genes; green 3D arrows, *ESAGs;* long red line with an arrow head, active transcription from the B-ES promoter; grey short line with an arrow head, short-distance transcription from the silent B-ES promoter. VSG switching pathways are explained in the text. (B) The classical double Holliday Junction pathway of homologous recombination to generate either GC or CO products is shown on the left. In comparison, the BIR pathway that usually occurs at the telomere is shown on the

right. The BIR and GC products are indistinguishable because the telomeres downstream of the *VSG* genes have the same tandem repeat sequence.

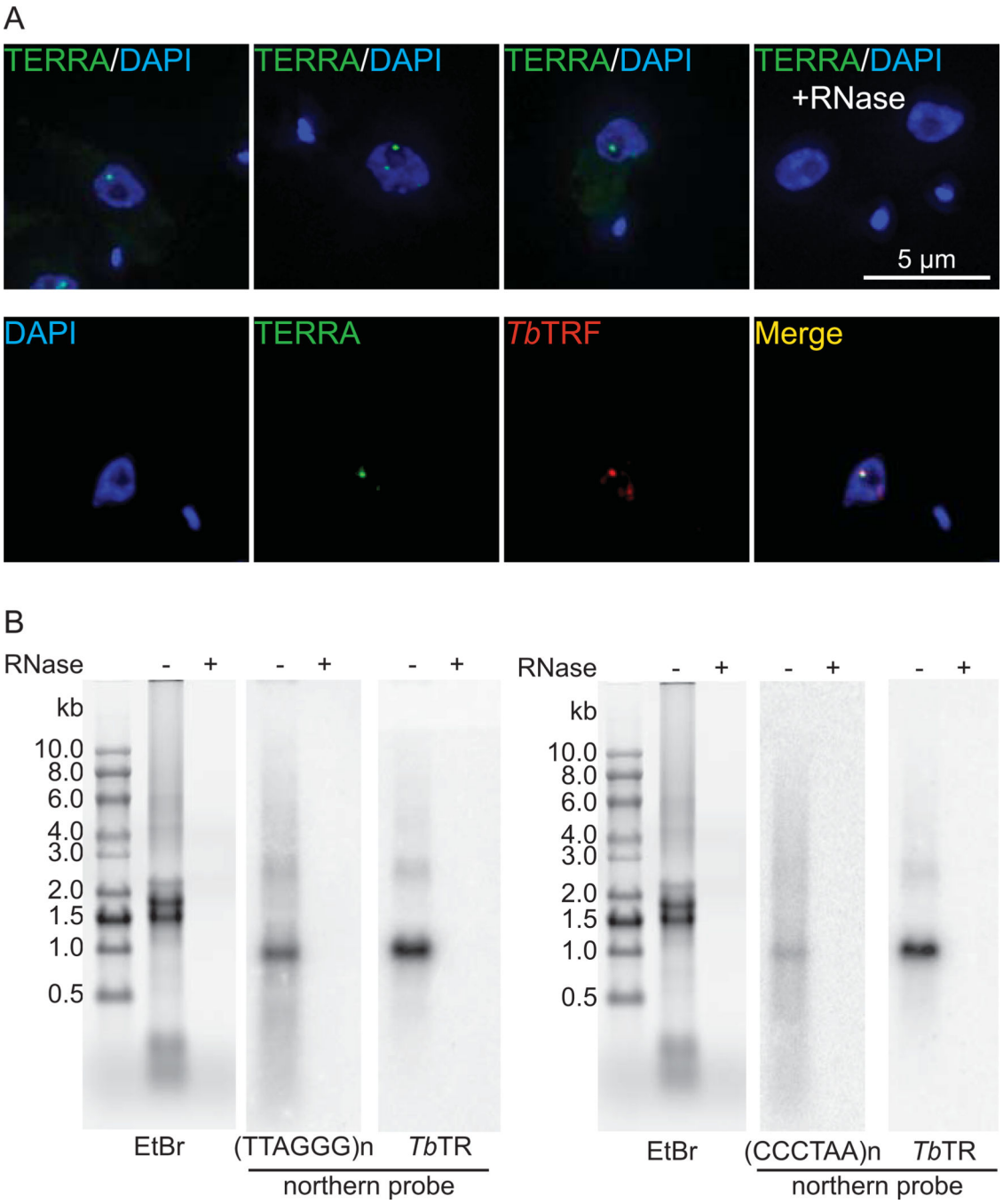

Figure 3.

(A) Subnuclear localization of TERRA. Top, TERRA FISH was performed in BF WT *T. brucei* cells with an Alexa488-conjugated PNA probe containing $(CCCTAA)_3$ (TELC probe, PNA Bio). Hybridization was done at 37°C without denaturation. Treating cells with RNase A eliminated all nuclear punctate signals (right image in the top row), confirming that the observed signals represent RNA molecules. Bottom, *Tb*TRF IF was performed using a rabbit antibody recognizing *Tb*TRF [135] and an Alexa594-conjugated donkey anti-rabbit 2nd antibody. TERRA FISH was performed the same way as in (A). DNA was stained by DAPI.

The large DAPI-positive circle is the *T. brucei* nucleus, while the smaller DAPI-positive dot is the kinetoplast. All images are of the same scale, with the scale bar shown in the top right panel. (B) Northern blot of PF *T. brucei* total RNA. In both left and right panels, the EtBr-stained agarose gel images are shown on the left. Hybridization using the *Tb*TR probe was done as a loading control (shown on the right). An 800 bp $(TTAGGG)_n$-containing DNA fragment was labeled with radioactive dGTP in the absence of dCTP to generate a probe that was used to detect the $(CCCUAA)_n$-containing TERRA (left). The same probe was labeled with radioactive dCTP in the absence of dGTP to generate a probe that was used to detect $(UUAGGG)_n$-containing TERRA (right). Hybridizations were done at 50°C. 10 units of RNase A and 20 units of RNase One were added in the RNase treatment control samples.