

6-3-2016

Trypanosoma brucei TIF2 and TRF Suppress VSG Switching Using Overlapping and Independent Mechanisms

Sanaa E. Jehi
Cleveland State University

Vishal Nanavaty
Cleveland State University

Bibo Li Ph.D.
Cleveland State University, B.LI37@csuohio.edu

Follow this and additional works at: https://engagedscholarship.csuohio.edu/scibges_facpub

 Part of the [Biology Commons](#), and the [Cell Biology Commons](#)

How does access to this work benefit you? Let us know!

Recommended Citation

Jehi SE, Nanavaty V, Li B. 2016. Trypanosoma brucei TIF2 and TRF suppress VSG switching using overlapping and independent mechanisms. PLoS One. 11(6):e0156746.

This Article is brought to you for free and open access by the Biological, Geological, and Environmental Sciences Department at EngagedScholarship@CSU. It has been accepted for inclusion in Biological, Geological, and Environmental Faculty Publications by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.

RESEARCH ARTICLE

Trypanosoma brucei TIF2 and TRF Suppress VSG Switching Using Overlapping and Independent Mechanisms

Sanaa E. Jehi^{1‡}, Vishal Nanavaty¹, Bibo Li^{1,2,3,4*}

1 Center for Gene Regulation in Health and Disease, Department of Biological, Geological, and Environmental Sciences, Cleveland State University, Cleveland, Ohio, United States of America, **2** The Rockefeller University, New York, New York, United States of America, **3** Department of Immunology, Cleveland Clinic Lerner Research Institute, Cleveland, Ohio, United States of America, **4** Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio, United States of America

‡ Current address: The Scripps Research Institute, Department of Molecular and Experimental Medicine, La Jolla, California, United States of America

* b.li37@csuohio.edu



OPEN ACCESS

Citation: Jehi SE, Nanavaty V, Li B (2016)

Trypanosoma brucei TIF2 and TRF Suppress VSG Switching Using Overlapping and Independent Mechanisms. PLoS ONE 11(6): e0156746. doi:10.1371/journal.pone.0156746

Editor: Ziyin Li, University of Texas Medical School at Houston, UNITED STATES

Received: March 6, 2016

Accepted: May 18, 2016

Published: June 3, 2016

Copyright: © 2016 Jehi et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by the National Institute of Allergy and Infectious Diseases, R01 grant (AI066095), <https://www.niaid.nih.gov/Pages/default.aspx>, PI: Li; Cleveland State University 2010 Faculty Research and Development, <http://www.csuohio.edu/>, PI: Li; The Center for Gene Regulation in Health and Disease at Cleveland State University research fund, <https://www.csuohio.edu/grhd/grhd>, PI: Li. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Trypanosoma brucei causes debilitating human African trypanosomiasis and evades the host's immune response by regularly switching its major surface antigen, VSG, which is expressed exclusively from subtelomeric loci. We previously showed that two interacting telomere proteins, *TbTRF* and *TbTIF2*, are essential for cell proliferation and suppress VSG switching by inhibiting DNA recombination events involving the whole active VSG expression site. We now find that *TbTIF2* stabilizes *TbTRF* protein levels by inhibiting their degradation by the 26S proteasome, indicating that decreased *TbTRF* protein levels in *TbTIF2*-depleted cells contribute to more frequent VSG switching and eventual cell growth arrest. Surprisingly, although *TbTIF2* depletion leads to more subtelomeric DNA double strand breaks (DSBs) that are both potent VSG switching inducers and detrimental to cell viability, *TbTRF* depletion does not increase the amount of DSBs inside subtelomeric VSG expression sites. Furthermore, expressing an ectopic allele of F2H-*TbTRF* in *TbTIF2* RNAi cells allowed cells to maintain normal *TbTRF* protein levels for a longer frame of time. This resulted in a mildly better cell growth and partially suppressed the phenotype of increased VSG switching frequency but did not suppress the phenotype of more subtelomeric DSBs in *TbTIF2*-depleted cells. Therefore, *TbTIF2* depletion has two parallel effects: decreased *TbTRF* protein levels and increased subtelomeric DSBs, both resulting in an acute increased VSG switching frequency and eventual cell growth arrest.

Introduction

Trypanosoma brucei is a protozoan parasite that causes fatal African trypanosomiasis in humans and nagana in cattle. While proliferating in the extracellular spaces of its mammalian

Competing Interests: The authors have declared that no competing interests exist.

host, bloodstream form (BF) *T. brucei* is directly exposed to the host immune surveillance. However, *T. brucei* regularly switches its major surface antigen, VSG, thereby evading the host's immune response [1].

There are more than 2,500 VSG genes and pseudogenes in the *T. brucei* genome [2,3]. Most are located within long VSG gene arrays at subtelomere regions of megabase chromosomes of *T. brucei* [3], and these VSGs are normally not expressed. In addition to eleven pairs of megabase chromosomes that contain all essential genes, *T. brucei* also has 4–5 intermediate chromosomes and ~100 copies of minichromosomes of only 50–150 kb [4,5]. Individual VSG genes are located at two thirds of minichromosome subtelomeres [2], which are not expressed but contribute to the large VSG gene pool for efficient VSG switching [5]. BF VSGs are expressed exclusively from subtelomeric VSG expression sites (ESs) [6,7], which are polycistronically transcribed by RNA polymerase I (RNAP I) [8] in a strictly monoallelic manner [9]. VSG is the last gene in any ES, located within 2 kb from the telomeric repeats and 40–60 kb downstream of the ES promoter [7]. There are 15 ESs in the *T. brucei* Lister 427 strain used in this study, but at any moment, only one ES is fully transcribed, resulting in a single type of VSG being expressed on the cell surface [9]. Most ESs are located on megabase chromosomes, but at least one ES is located on an intermediate chromosome [10].

VSG switching is an essential *T. brucei* pathogenesis mechanism enabling long-term *T. brucei* infections [1]. VSG switching occurs through two major pathways [11]. In an *in situ* switch, the originally active VSG ES becomes silent and a silent one becomes expressed, which does not involve gene rearrangements. Another major pathway for VSG switching is DNA recombination-based. In crossover (CO) or telomere exchange (TE), the active VSG gene and a silent subtelomeric VSG gene (in a silent ES or at a minichromosome subtelomere) exchange places, often together with their downstream telomere sequences [12]. No genetic information is lost in CO/TE. In gene conversion (GC), a silent VSG gene is duplicated into the active ES to replace the originally active VSG gene, which is subsequently lost [13]. When GC only encompasses the VSG vicinity, it is referred to as VSG GC. GC can also include most of the ES and even ES promoter regions, in which case it is referred to as ES GC. In many published studies, GC has been shown to be the most frequent event in VSG switching [14–19].

It has been shown that several proteins required for homologous recombination are important for VSG switching. At double strand break (DSB) sites, RAD51 binds the single stranded 3' overhang following 5' end resection and promotes strand invasion in DNA homologous recombination [20]. Deletion of *T. brucei* RAD51 and one of its paralogues, RAD51-3, significantly reduced the VSG switching frequency [21,22]. Deletion of BRCA2, a mediator facilitating the loading of RAD51 onto the single-stranded DNA [23], also decreased VSG switching frequencies [24]. On the other hand, deletion of Topoisomerase 3 alpha [15] and its interacting factor BMI1 [16] led to nearly 10 fold higher VSG switching frequencies, as the BLM-Topo3-BMI1 complex normally promotes resolution of double Holliday Junction and results in non-crossover events during homologous recombination [25,26].

How VSG switching is initiated and regulated is poorly understood. Recent studies have shown that inducing DSBs at 70 bp repeats located immediately upstream of the active VSG gene resulted in ~250 fold higher VSG switching frequencies [27]. However, although DSBs inside the active VSG ES are potent VSG switching inducers, they are also deleterious to cells and cause more than 85% of cell death (~85%, ~92%, and ~93% of cell death when DSBs are induced at ES promoter, between 70 bp repeats and the VSG gene, and downstream of the VSG gene, respectively) [28]. Therefore, maintaining subtelomere integrity is essential for *T. brucei* viability. Nevertheless, DSBs can be detected within the 70 bp repeats even in WT *T. brucei* cells, indicating that this is likely a key factor for VSG switching initiation [27]. Apparently,

balancing subtelomere stability and plasticity is important for parasite survival, and factors that influence the amount of subtelomere DSBs will influence VSG switching frequency.

So far, *T. brucei* TIF2 is the only protein that has been shown to influence the amount of subtelomeric DSBs [19]. *Tb*TIF2 is an intrinsic component of the *T. brucei* telomere protein complex [19]. It interacts tightly with the duplex telomere DNA binding factor, *Tb*TRF, which has been shown to play an essential role in maintaining the terminal telomere structure [29]. Telomere proteins are well-known for their roles in protecting the chromosome ends from illegitimate DNA processes including degradation, fusion, and recombination [30]. However, the functions of telomere proteins in maintaining subtelomere integrity are not clear. We found that depletion of *Tb*TIF2 resulted in a significant increase in the amount of DSBs at subtelomeres and subsequent elevated VSG switching frequency [19], demonstrating for the first time that telomere proteins also play important roles in maintaining subtelomere integrity. However, the underlying mechanism of *Tb*TIF2 function is not clear. Interestingly, we found that *Tb*TRF also suppresses VSG switching, and the telomeric DNA binding activity of *Tb*TRF is essential for this function [18]. Importantly, depletion of either *Tb*TIF2 or *Tb*TRF increases the amount of GC-mediated VSG switching events mostly involving the whole ES, suggesting that they may function in a same genetic pathway in influencing VSG switching [18,19]. In mammals, TIN2, the functional homologue of *Tb*TIF2 [19,31] interacts with both TRF1 and TRF2, which bind the duplex TTAGGG repeats [32–34] and are functional homologues of *Tb*TRF [18,29]. In addition, the interaction between TIN2 and TRF1 is essential for preventing TRF1 from being ADP-ribosylated by a TRF1-interacting factor, Tankyrase 1 [35,36], which releases TRF1 from the telomere DNA [37] and allows subsequent ubiquitination of TRF1 by SCF^{Fbx4} [38] followed by proteasome-mediated degradation [38,39]. Functions of TIN2, TRF1, and TRF2 are partly overlapping because TIN2 interacts with both TRF1 and TRF2 and is the central protein in the six-membered mammalian telomere complex called Shelterin [40].

Because *Tb*TIF2 interacts with *Tb*TRF, and because depletion of either protein leads to an acute increase in VSG switching frequencies with similar switching mechanisms and eventual cell death [18,19], we decided to examine whether these two proteins function in the same pathway, which will help reveal the underlying mechanism of *Tb*TIF2's function in maintaining subtelomere stability. In this work, we found that depletion of *Tb*TIF2 decreased *Tb*TRF protein levels but did not affect its mRNA levels. We further found that *Tb*TRF was degraded by the proteasome upon depletion of *Tb*TIF2. However, depletion of *Tb*TRF did not increase the amount of DSBs inside the subtelomeric VSG ESs. Furthermore, expression of an ectopic *Tb*TRF WT allele delayed *Tb*TRF degradation, mildly improved cell growth, and partially suppressed the phenotype of elevated VSG switching frequency in *Tb*TIF2 RNAi cells. However, this did not suppress the phenotype of increased amount of subtelomeric DSBs in *Tb*TIF2 RNAi cells. Therefore, our observations indicate that increased amounts of subtelomeric DSBs and decreased *Tb*TRF protein levels are two independent and parallel consequences of *Tb*TIF2 depletion, both contributing to increased VSG switching frequencies and eventual cell growth arrest.

Materials and Methods

Chromatin IP (ChIP)

ChIP was performed exactly the same way as described in [18]. Briefly, Cells were fixed with 1% Formaldehyde for 30 min at room temperature and cell lysate was sonicated in a Bioruptor (Diagenode Corp.) at medium level for 6 cycles (30 sec on/30sec off per cycle) at 4°C. IP was carried out using protein G Dynabeads (Life Technologies) coupled with a *Tb*TRF antibody

[29] followed by wash, elution, reverse crosslinking, and DNA isolation. ChIP products were hybridized with a TTAGGG repeat or a 50 bp repeat probe in slot blot Southern analysis.

VSG Switching Assay

Switching assays were performed according to [18,41]. Detailed switching assay protocol is included in the supplemental materials.

Ligation mediated PCR (LMPCR)

LMPCR were performed the same way as described previously [19,27]. Briefly, in each ligation reaction, 2 μ g of genomic DNA was either treated or not treated with 2 μ l of T4 DNA Polymerase (3000 U/ml, New England BioLabs) in the presence of 200 μ M dNTP and then ligated with 10 μ l annealed adaptor. Three 1:3 serial dilutions of the ligated products were prepared and used in subsequent PCR using Hotstart Platinum[®] Taq DNA Polymerase (Life Technologies) and a touchdown PCR program.

MG-132 treatment

T. brucei cells were incubated in medium with 25 μ M of MG-132 for 6 hours before proteins were extracted for western analysis. For RNAi induced samples, MG-132 was added to the medium 6 hours prior to the time point of harvesting cells.

Results

TbTIF2 protects *TbTRF* from being degraded by the 26S proteasome

We observed a similar growth arrest phenotype upon RNAi induction in *TbTIF2* and *TbTRF* RNAi cells [19,29]. In addition, a transient *TbTIF2* or *TbTRF* RNAi induction resulted in a significant increase in VSG switching frequency with most VSG switchers arising from subtelomeric gene rearrangements that resulted in the loss of the whole active ES [18,19]. Therefore, we hypothesized that *TbTIF2* and *TbTRF* function in the same genetic pathway in maintaining subtelomere stability.

We first tested whether *TbTRF* protein levels were affected by *TbTIF2*. Indeed, induction of *TbTIF2* RNAi led to depletion of not only the endogenous FLAG-HA-HA (F2H) tagged *TbTIF2* (Fig 1A) but also the endogenous *TbTRF* protein (Fig 1A). In contrast, protein levels of *TbRAP1*, another intrinsic component of the *T. brucei* telomere complex [42], were not affected (Fig 1A). Northern blotting analysis showed that only the *TbTIF2* mRNA was knocked-down by *TbTIF2* RNAi, while *TbTRF* mRNA levels were not affected (Fig 1B), indicating that *TbTIF2* is required for maintaining *TbTRF* protein levels.

To investigate whether *TbTRF* is degraded by the proteasome upon *TbTIF2*-depletion, we treated *TbTIF2* RNAi cells with and without MG-132, which is a specific proteasome inhibitor and has previously been successfully used in *T. brucei* [45]. We found that in cells not treated with MG-132, *TbTIF2* was depleted upon induction of RNAi (the relative levels of *TbTIF2*-F2H are 100%, 81%, and 79% at 0, 24, and 40 hrs after induction, respectively), which led to a quick *TbTRF* depletion (Fig 1C). In contrast, in cells treated with MG-132, although *TbTIF2* was again depleted by RNAi at a very similar rate (the relative levels of *TbTIF2*-F2H are 100%, 82%, and 72% at 0, 24, and 40 hrs, respectively), *TbTRF* protein levels were not decreased (Fig 1C), indicating that *TbTRF* is degraded by the proteasome when *TbTIF2* is absent. As a control, we also examined the protein levels of *TbPSA6* and *TbAUK1*. *TbPSA6* is the A6 subunit of the 20S proteasome and is not degraded by the proteasome [46], while *TbAUK1*, the *T. brucei* Aurora like kinase, is degraded by the proteasome [47]. As expected, adding MG-132 blocks

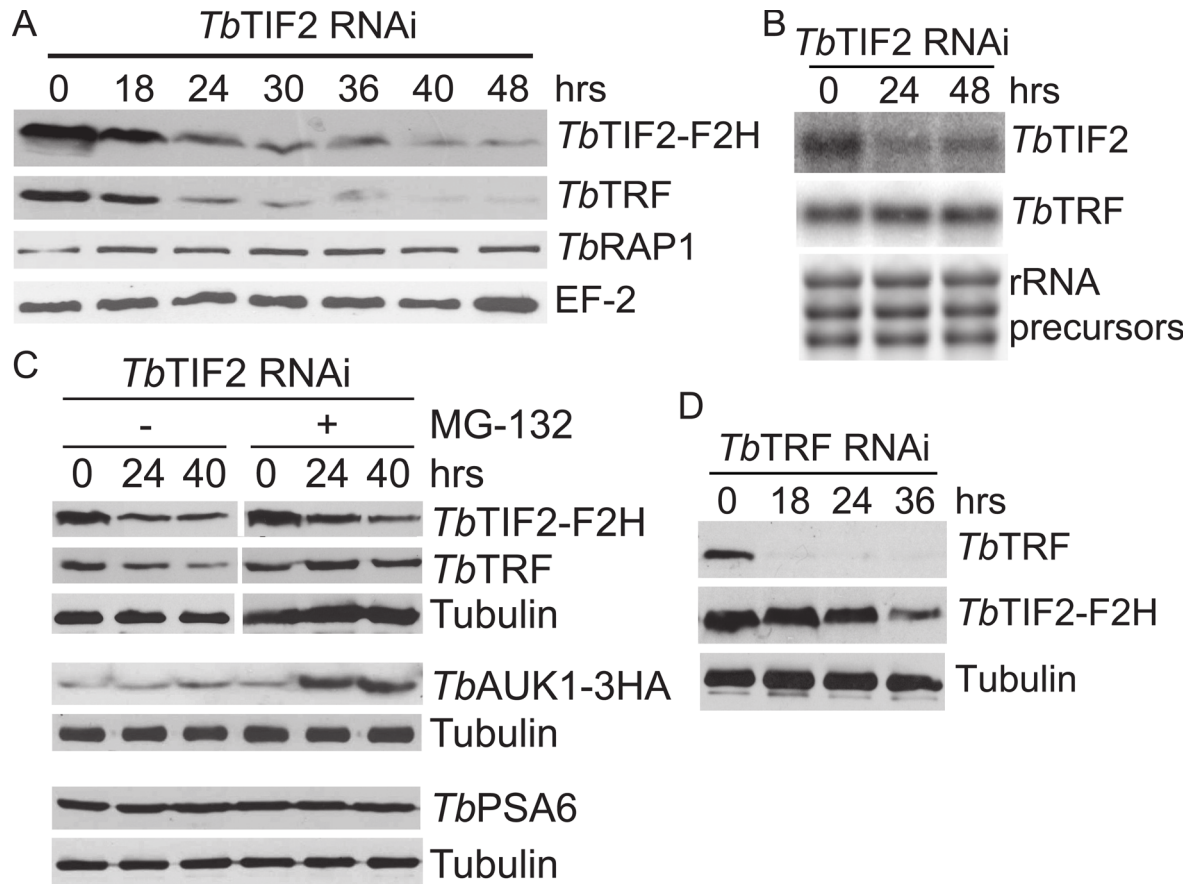


Fig 1. *TbTIF2* is essential for maintaining normal *TbTRF* protein levels. (A) Western blotting was performed with whole cell extracts prepared at various time points after induction of *TbTIF2* RNAi (shown on top). (B) Northern blotting shows that *TbTRF* mRNA levels were not affected in *TbTIF2*-depleted cells. rRNA precursors were shown as a loading control. (C) Western blotting was performed using whole cell extracts prepared from *TbTIF2* RNAi cells treated with and without MG-132 (Sigma), an inhibitor of the 26S proteasome. *TbAUK1* is normally degraded by the 26S proteasome while *TbPSA6* is not. (D) Western analysis showed that *TbTIF2* protein levels are not sensitive to *TbTRF* depletion. In western analyses, *TbTIF2*-F2H and *TbAUK1*-3HA were detected using an HA monoclonal antibody (F-7, SantaCruz Biotechnology). *TbTRF* [29], *TbRAP1* [42], *TbPSA6* [43], and tubulin [44] were detected by their respective antibodies. EF-2 was detected using a goat polyclonal antibody against human EF-2 (Santa Cruz Biotechnology). All primary antibodies were diluted 1,000 fold. In this and other figures, proteins from 15 million cells were loaded each lane for western blotting, except when detecting tubulin, proteins from only 0.5 million cells were loaded each lane.

doi:10.1371/journal.pone.0156746.g001

the activity of the proteasome and the *TbAUK1* protein level was higher in these cells than in cells not treated with MG-132 (Fig 1C). In contrast, the *TbPSA6* protein level was not affected by MG-132 (Fig 1C). In this particular induction, the *TbAUK1* protein level appears to be slightly increased upon *TbTIF2* depletion in the absence of MG-132 (Fig 1C). To better examine the effect of *TbTIF2* depletion on *TbAUK1* protein levels, we repeated the induction three more times and quantified the *TbAUK1*-3HA protein levels in Adobe Photoshop using tubulin or EF-2 as a loading control. On average (calculated from four independent inductions), *TbAUK1*-3HA protein levels changed from 100% at 0 hr to 106.8% at 24 hrs and 103.8% at 40 hrs after induction, indicating that *TbTIF2* does not affect *TbAUK1* protein levels significantly. Therefore, *TbTIF2* regulates *TbTRF* protein levels by inhibiting its degradation by the proteasome.

We also examined both *TbTRF* and *TbTIF2* protein levels in *TbTRF* RNAi cells. Upon RNAi induction, we only observed depletion of *TbTRF*, while the *TbTIF2* protein level was

stable for at least 24 hrs. The mild decrease in *TbTIF2* protein levels after 36 hrs of *TbTRF* RNAi induction is likely due to the severe growth defect in *TbTRF*-depleted cells [29].

TbTRF does not affect the amount of subtelomere DNA double strand breaks (DSBs)

The fact that depletion of *TbTIF2* leads to depletion of *TbTRF* further suggests that *TbTIF2* and *TbTRF* function in the same pathway to influence VSG switching and allow normal cell proliferation. To further investigate this possibility, we tested whether depletion of *TbTRF* results in more subtelomeric DSBs as we observed in *TbTIF2* RNAi cells. Due to extremely limited availability of specific antibodies against γ H2A, which has been shown to be deposited at the chromatin with DSBs [48], we used Ligation-mediated PCR (LMPCR) analysis (Fig 2A) to detect and estimate the amount of DSBs in active and silent VSG ESs as we did previously [19]. LMPCR has a higher resolution than γ H2A Immunofluorescence analysis (IF) so that we can determine whether DSBs are in subtelomeric VSG ESs, while IF can only reveal whether DSBs are in telomere vicinity in general (can be either telomeric or subtelomeric or both).

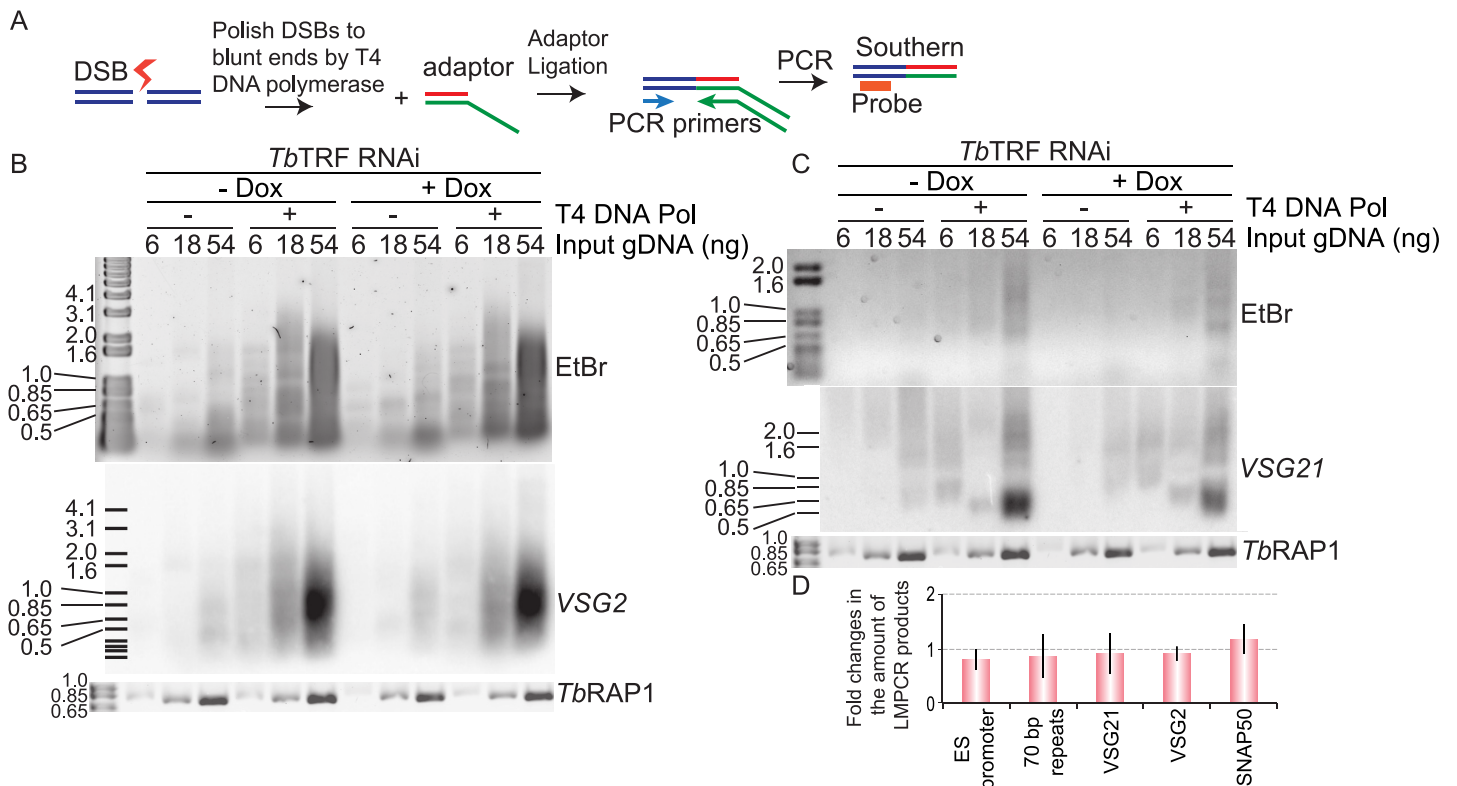


Fig 2. *TbTRF* depletion does not affect the amount of DSBs inside VSG ESs. (A) Principle of LMPCR assay. After DSBs (represented by a bolt) form, an adaptor is ligated with the genomic DNA at the break sites if they have blunt ends. Treating genomic DNA with T4 DNA polymerase converts staggered broken ends into blunt ends. The ligated products are then amplified by PCR using a locus-specific forward primer and the adaptor-specific reverse primer. The PCR amplified products are subsequently detected by locus-specific probes in Southern analysis. (B & C) LMPCR analyses were performed in *TbTRF* RNAi cells. The LMPCR products were hybridized with *VSG2* (B) and *VSG21* (C). In panels B & C, the Ethidium Bromide (EtBr)-stained LMPCR products are shown at the top, the Southern blot result is shown in the middle, and the PCR products using primers specific to the *TbRAP1* gene (as a loading control) are shown at the bottom. The amounts of input genomic DNA, either treated (+) or not treated (-) with T4 DNA polymerase, were marked on top of each lane. (D) Quantification of the change in the amounts of LMPCR products (with T4 DNA pol treatment using 54 ng input gDNA) from three independent experiments. Average values are shown. Error bars represent standard deviations.

doi:10.1371/journal.pone.0156746.g002

Surprisingly, although DSBs were still detected, the amount of DSBs within VSG ESs was not increased when *Tb*TRF was depleted (Fig 2). We examined the single-copy active *VSG2* (Fig 2B) and silent *VSG21* (Fig 2C) and multi-copy ES promoter (S1A Fig) and 70 bp repeat regions (S1B Fig), and the results are reproducible. Quantification of LMPCR product levels indicated that there is no significant change observed in the DSB levels inside VSG ESs before and after depletion of *Tb*TRF (Fig 2D). In addition, depletion of *Tb*TRF did not affect the DSB levels at a chromosome internal *SNAP50* gene locus (S1C Fig). Therefore, depletion of *Tb*TIF2 appears to have two independent effects: decreasing *Tb*TRF protein levels and increasing subtelomeric DSB levels.

Induced expression of an ectopic *Tb*TRF allele slightly improves cell growth in *Tb*TIF2 RNAi cells

To further investigate the functional relationship between *Tb*TRF and *Tb*TIF2 in maintaining subtelomere stability and in cell survival, we introduced an ectopic F2H-tagged WT allele of *Tb*TRF into the *Tb*TIF2 RNAi strain used previously for examining *Tb*TIF2's effect on VSG switching, S/TIF2i [19]. We have shown that F2H-*Tb*TRF has essential *Tb*TRF functions and can rescue mutant phenotypes in *Tb*TRF RNAi cells [18,29]. *Tb*TIF2 RNAi and expression of F2H-*Tb*TRF in S/TRFi+F2H-*Tb*TRF cells can be induced simultaneously by adding doxycycline [49]. Upon adding doxycycline, we observed induced F2H-*Tb*TRF expression within the first 48 hours (Fig 3A). However, expression of F2H-*Tb*TRF was not stably maintained 72 hrs after the induction, because *Tb*TIF2 was depleted soon after induction of *Tb*TIF2 RNAi, as shown in northern blotting analysis (Fig 3B). In addition, we also observed that the endogenous *Tb*TRF protein level decreased significantly 48 hrs after inducing *Tb*TIF2 RNAi (Fig 3A). Even so, at 24–48 hrs after induction, the *Tb*TRF protein level in S/TIFi+F2H-*Tb*TRF cells has not significantly decreased as in S/TIF2i cells, suggesting that expression of the ectopic F2H-*Tb*TRF allele resulted in more *Tb*TRF proteins in the cell that presumably require longer time to be completely degraded after removal of *Tb*TIF2.

Consistent with our observation of delayed *Tb*TRF depletion in S/TIF2i+F2H-*Tb*TRF cells, we found that these cells showed mildly better growth than S/TIF2i cells (Fig 3C). In S/TIF2i cells, cell growth slowed down significantly by 24 hrs and was completely arrested by 72 hrs after induction of *Tb*TIF2 RNAi, while in S/TIF2i+F2H-*Tb*TRF cells, cell growth slowed down by 48 hrs and was completely arrested by 96 hrs after induction. Therefore, maintaining *Tb*TRF protein levels for a longer time slightly delayed the cell growth arrest phenotype in *Tb*TIF2 RNAi cells. This further supports the idea that depletion of *Tb*TIF2 caused two parallel effects that both lead to cell growth arrest.

We previously found that when the DNA binding activity of *Tb*TRF is significantly impaired [less than 20% of *Tb*TRF is associated with the telomeric chromatin in Chromatin IP (ChIP) experiment], the VSG switching frequency is increased [18]. To examine whether *Tb*TRF's telomere binding is affected in S/TIF2i and S/TIF2i+F2H-*Tb*TRF cells, we performed ChIP analysis using a *Tb*TRF antibody [29]. In S/TIF2i cells, the association between *Tb*TRF and the telomere chromatin decreased significantly upon *Tb*TIF2 depletion (Fig 3D, top), as *Tb*TRF is degraded. In S/TIF2i+F2H-*Tb*TRF cells, within 30 hrs after induction of *Tb*TIF2 RNAi, the association of *Tb*TRF with the telomeric DNA was decreased mildly, but ~75% of *Tb*TRF still remained at the telomere (Fig 3D, bottom), indicating that expression of the ectopic F2H-*Tb*TRF helped to keep sufficient *Tb*TRF at the telomere within a short frame of time after *Tb*TIF2 was depleted. This mild decrease of *Tb*TRF binding at the telomere is not expected to affect VSG switching frequencies based on our previous observations [18].

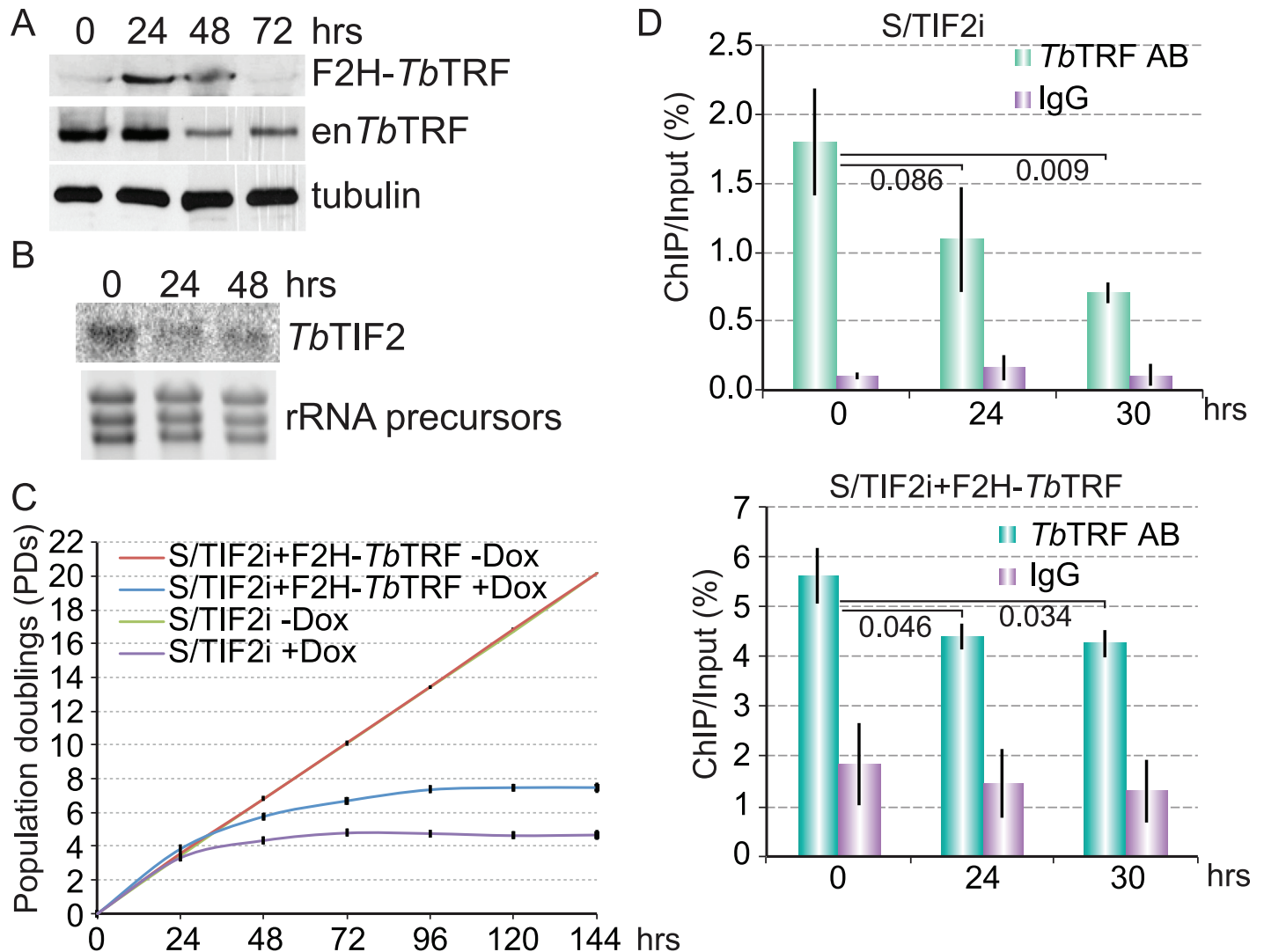


Fig 3. Inducing the expression of an ectopic F2H-*TbTRF* allele in *TbTIF2* RNAi cells. (A) Western analysis showing the expression of the ectopic F2H-*TbTRF* and eventual depletion of both endogenous and ectopic *TbTRF* proteins upon depletion of *TbTIF2*. (B) Northern analysis showing the depletion of *TbTIF2* mRNA upon induction of RNAi. rRNA precursors were shown as a loading control. (C) Growth curves show that expression of an ectopic F2H-*TbTRF* allele slightly improved the cell growth in *TbTIF2* RNAi cells. (D) Quantification of ChIP analysis using *TbTRF* antibody and IgG (as a control) performed in S/TIF2i cells (top) and in S/TIF2i+F2H-*TbTRF* cells (bottom). ChIP products were hybridized with a TTAGGG repeat probe. As a control, ChIP products were also hybridized with a 50 bp repeat (located upstream of ES promoters) probe, and the quantification results are shown in S2 Fig. Average values were calculated from three independent experiments. Error bars represent standard deviations. Numbers represent P values (unpaired t-test) between groups of values as indicated.

doi:10.1371/journal.pone.0156746.g003

Expression of ectopic *TbTRF* does not suppress the phenotype of more subtelomeric DSBs in *TbTIF2* RNAi cells

We found that depletion of *TbTRF* does not increase the subtelomeric amount of DSBs (Fig 2), suggesting that more subtelomeric DSBs in *TbTIF2*-depleted cells are independent of decreased *TbTRF* protein levels. If this is the case, expression of the ectopic F2H-*TbTRF* in *TbTIF2* RNAi cells will not suppress the phenotype of increased DSB levels. To confirm this, we performed the LMPCR analysis (Fig 2A) in S/TIF2i+F2H-*TbTRF* cells to detect DSBs at subtelomeres. Indeed, 24 hrs after induction of *TbTIF2*, when *TbTRF* level had not decreased significantly and more than 75% of *TbTRF* were still associated with the telomere, we observed a significant

increase in the amount of subtelomeric DSBs (Fig 4) the same as in S/TIF2i cells [19]. We tested the active *VSG2* (S3A Fig) and ψ_{ESI} pseudogene (Fig 4A), the silent *VSG21* (Fig 4B) and ψ_{ESI1} pseudogene (data not shown), and 70 bp repeats (S3B Fig) and observed similar results at all tested loci. To compare the fold change in DSB levels in S/TIF2i+F2H-*TbTRF* cells with that in S/TIF2i cells [19], we also quantified the fold change in DSB levels at multiple gene loci including the active *VSG2* and ψ_{ESI} loci, the silent *VSG21* and ψ_{ESI1} loci, 70 bp repeats, and the chromosome internal *SNAP50* locus (Fig 4C). In S/TIF2i+F2H-*TbTRF* cells, depletion of *TbTIF2* led to ~ 1.5–3.5 fold increase in subtelomeric DSB levels, which is the same as in S/TIF2i cells [19]. This observation also confirmed that induction of RNAi in S/TIF2i+F2H-*TbTRF* cells efficiently depleted *TbTIF2* as we did previously in S/TIF2i cells.

Expression of ectopic *TbTRF* in *TbTIF2* RNAi cells partially suppresses the phenotype of increased VSG switching frequency

Our observations suggest that the two effects of *TbTIF2* depletion, increased subtelomeric DSB levels and decreased *TbTRF* protein levels, are independent of each other. Still, both are expected to contribute to subsequent increased VSG switching frequency. To test this, we estimated the VSG switching frequencies in S/TIF2+F2H-*TbTRF* cells the same way we did previously [18]. Both S/TIF2i and S/TIF2+F2H-*TbTRF* strains were derived from the HSTB261 strain, in which the active *VSG2*-containing ES are marked with a *Blasticidin resistance* (*BSD*) marker immediately downstream of the ES promoter and a *Puromycin resistance* (*PUR*)–*Thymidine Kinase* (*TK*) fusion gene between the active *VSG2* and the 70 bp repeats [15].

To be able to compare current results with previous observations, we performed the switching assay in S/TIF2i, S/TIF2+F2H-*TbTRF*, and S/*ev* control cells that carry an empty RNAi construct, using exactly the same switching protocol as before [18]. Cells were induced for 30 hrs. Then doxycycline was removed by extensive washing [19]. This allows cells to recover from the growth arrest so that VSG switchers can subsequently be obtained. To enrich for VSG switchers, cells were first mixed with MACS beads conjugated with a *VSG2* monoclonal antibody. Non-switchers that still express *VSG2* are trapped with MACS beads, while switchers were in the flow through fraction, which is further selected by ganciclovir [15]. VSG switchers should no longer express the *TK* gene targeted immediately upstream of the active *VSG2*, and cells not expressing *TK* are resistant to ganciclovir, a nucleoside analog [50].

We found that VSG switching frequencies in S/TIF2i+F2H-*TbTRF* cells are lower than that in S/TIF2i cells but are higher than that in S/*ev* cells (Fig 5A), supporting the idea that expression of an ectopic F2H-*TbTRF* allele partially suppressed the phenotype of increased VSG switching frequency in *TbTIF2* depleted cells.

By determining the expression status and genotypes of the markers in the obtained VSG switchers, we can reveal the underlying mechanisms of switching events [15,18,19]. Previously, we found that although VSG switchers that lost the original active ES are already the most frequent switching events (70%), depletion of *TbTIF2* further increased the percentage of such VSG switchers (80–90%) [19]. Here we estimated the VSG switching frequency in S/TIF2i cells using the MACS-based VSG switching assay and observed the same phenotype, where VSG switchers that lost the original active ES consist of 90% of all switchers (Fig 5B). In addition, expression of the ectopic F2H-*TbTRF* reduced the percentage of this type of switchers to 80% (Fig 5B), which is between the levels in S/TIF2i cells and S/*ev* cells [19]. Therefore, examination of VSG switching in S/TIF2i+F2H-*TbTRF* cells further indicates that expressing F2H-*TbTRF* and maintaining cellular *TbTRF* protein levels at the time of performing switching assays partially suppresses phenotypes in *TbTIF2* RNAi cells.

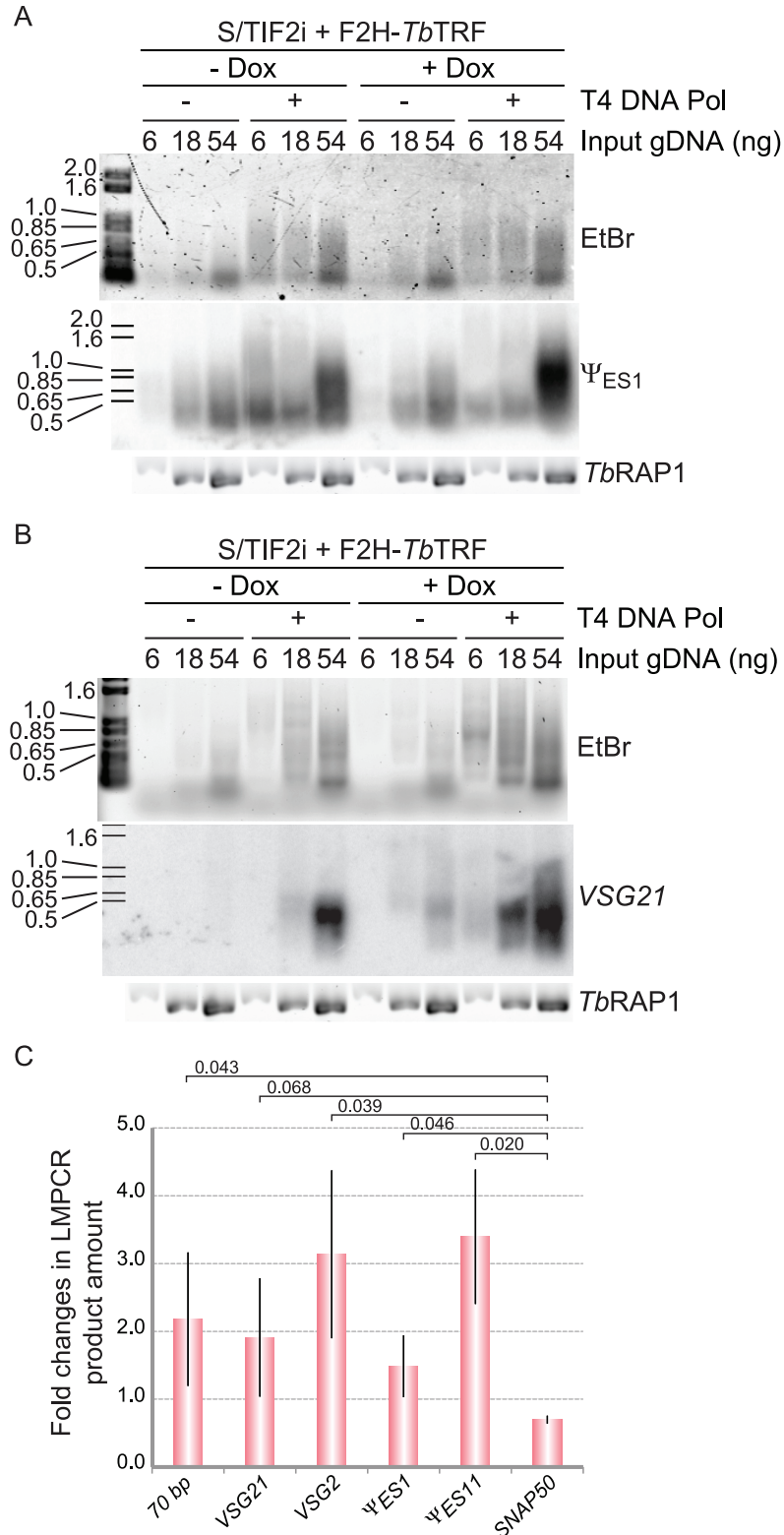


Fig 4. Expression of ectopic F2H-*TbTRF* does not suppress the phenotype of increased subtelomeric DSB numbers in *TbTIF2* RNAi cells. LMPCR analyses were performed in S/TIF2i+F2H-*TbTRF* cells. LMPCR products were hybridized with *VSG pseudogene* Ψ_{ES1} (A) and *VSG21* (B). Ethidium Bromide (EtBr)-stained LMPCR products are shown at the top, the Southern blot result is shown in the middle, and the PCR

products using primers specific to the *TbRAP1* gene (as a loading control) are shown at the bottom. (C) Quantification of the change in the amounts of LMPCR products (with T4 DNA pol treatment using 54 ng input gDNA) from three independent experiments. Average values are shown. Error bars represent standard deviations. Numbers represent P values of unpaired t-tests between pairs of data groups as indicated.

doi:10.1371/journal.pone.0156746.g004

Discussion

Human African trypanosomiasis is fatal without treatment. However, only few drugs are available for treating *T. brucei* infections, all with severe side effects. We previously showed that both *Tb*TIF2 and *Tb*TRF are essential for normal cell proliferation and have weak sequence homology with their mammalian homologues only within functional domains [19,29]. Therefore, *Tb*TIF2 and *Tb*TRF could serve as anti-parasite drug targets. However, the underlying mechanisms of *Tb*TIF2 and *Tb*TRF functions are unclear, and whether *Tb*TIF2 and *Tb*TRF function in the same genetic pathway is unknown. Our current investigation reveals that the functions of these two telomere proteins are not identical but are partially overlapping.

The mechanisms of *Tb*TIF2 and *Tb*TRF functions are at least partially overlapping. *Tb*TRF interacts with *Tb*TIF2, and both suppress subtelomeric gene conversion events involving the whole active VSG ES [18,19]. More importantly, depletion of *Tb*TIF2 also leads to depletion of *Tb*TRF (Fig 1). Clearly, decreased *Tb*TRF protein levels in *Tb*TIF2-depleted cells is an important contributing factor for the growth defect and increased VSG switching frequency, as *Tb*TRF is essential for normal cell growth and also suppresses VSG switching [18,29].

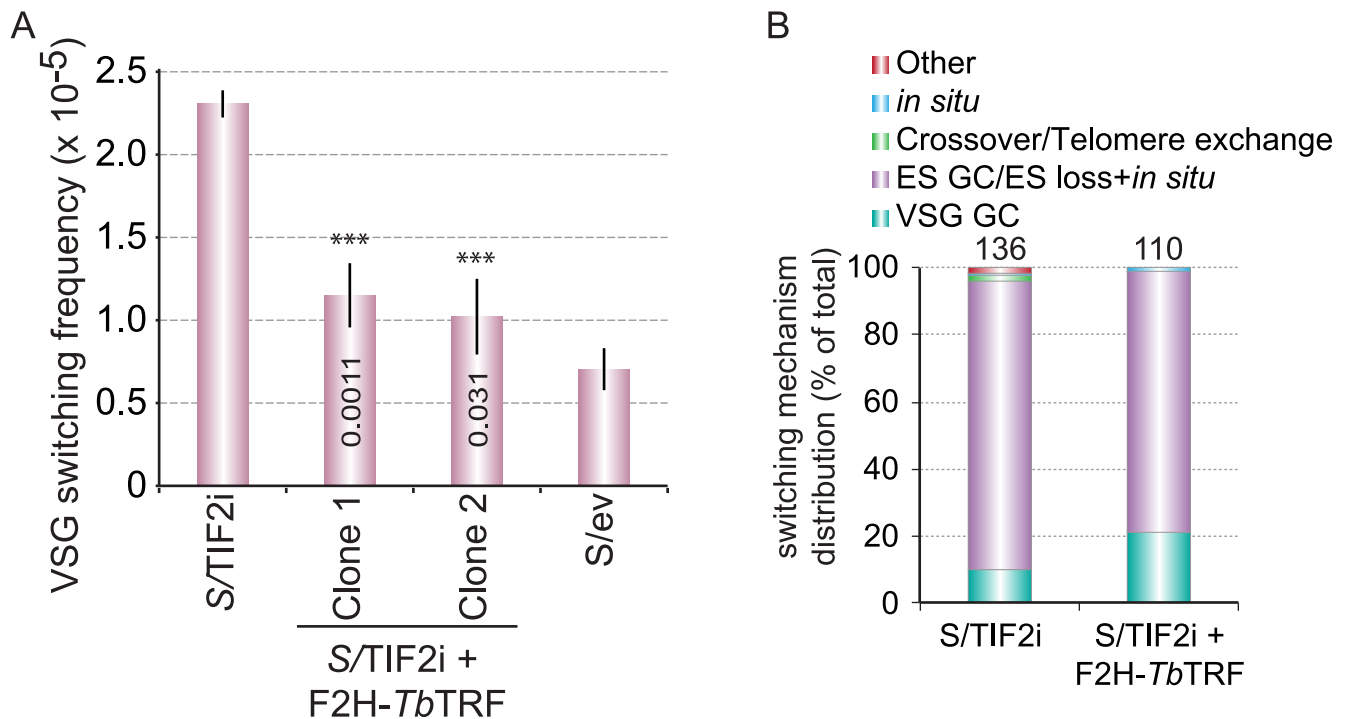


Fig 5. Expressing an ectopic F2H-*Tb*TRF allele partially suppresses the phenotype of increased VSG switching frequency in *Tb*TIF2 RNAi cells. (A) VSG switching frequencies in several strains. Averages were calculated from three independent experiments. Error bars represent standard deviations. Numbers labeled on middle columns are P values (unpaired t-test) calculated between switching frequencies in S/TIF2i+F2H-*Tb*TRF cells and that in S/ev cells. Asterisks represent P values (unpaired t-test) calculated between values in S/TIF2i+F2H-*Tb*TRF cells and that in S/TIF2i cells. ***, P<0.001. (B) VSG switching mechanisms in different strains. The total number of analyzed switchers in each strain was indicated on top of the corresponding column.

doi:10.1371/journal.pone.0156746.g005

*Tb*TIF2 has an additional important function in maintaining subtelomere integrity [19], which we now show is independent of *Tb*TRF. Depletion of *Tb*TRF does not affect the amount of DSBs inside VSG ESs (Fig 2); expressing an ectopic WT allele of *Tb*TRF in S/TIF2i cells does not suppress the phenotype of more subtelomere DSBs but mildly improved the cell growth and partially suppressed the phenotype of increased VSG switching frequency. Therefore, functions of *Tb*TIF2 and *Tb*TRF are not identical.

Based on our observations, we conclude that the functional interaction pathways between *Tb*TIF2 and *Tb*TRF are as follows: *Tb*TIF2 depletion has two independent effects: increased amount of subtelomere DSBs and decreased *Tb*TRF protein levels. Both effects are known to contribute to growth defect and increased VSG switching frequencies [18,19,29]. First, DSBs induced in the active VSG ES are detrimental to *T. brucei* cells and cause >85% of cell death [28]. At the same time, DSBs induced near the 70 bp repeats upstream of the active VSG gene are also a potent inducer for VSG switching [27,28]. Therefore, the increased amount of subtelomere DSBs in *Tb*TIF2 RNAi cells is an important contributor for cell growth arrest and increased VSG switching frequency. Second, *Tb*TRF is known to have an essential role in maintaining the terminal telomere structure, which is likely the reason why it is essential for normal cell proliferation [29]. *Tb*TRF also suppresses subtelomere gene conversion events that lead to VSG switching [18]. Therefore, decreased *Tb*TRF protein levels induced by *Tb*TIF2 depletion are also an important factor contributing to an acute elevated VSG switching frequency and eventual growth arrest.

It is interesting that *Tb*TIF2 is essential for maintaining subtelomere integrity, while its interacting factor *Tb*TRF is not. Since *Tb*TRF binds the duplex telomere DNA directly [29], it is possible that *Tb*TRF is more important for maintaining telomere than subtelomere integrity. Our previous observation that *Tb*TRF is important for maintaining the terminal telomere structure is consistent with this view [29]. Mammalian TRF2, the functional homologue of *Tb*TRF [29], is also essential for maintaining the telomere terminal G-overhang structure and for preventing chromosome end-to-end fusions through the non-homologous end-joining (NHEJ) pathway [51–53]. We speculate that loss of *Tb*TRF may also result in chromosome end fusions and subsequent breakage–fusion–bridge cycle, which often result in large terminal chromosome deletions [54]. Because VSG is essential, if the chromosome end containing the active VSG ES is lost, then only VSG switchers can survive. As a consequence, *Tb*TRF depletion would result in increased VSG switching [18]. In this case, the DNA break sites can locate well upstream of the active VSG ES, hence were not detected in the LMPCR analysis where ES-specific probes were used. Although the essential player in NHEJ, DNA ligase IV, has not been identified in *T. brucei* [55], micro-homology mediated end-joining has been observed in *T. brucei* [55,56], which can also mediate telomere end fusions. Due to the fact that *T. brucei* chromosomes do not condense during mitosis and the limited sensitivity of our current molecular tools, telomere end-to-end fusions have not been detected in *T. brucei* yet. Developing more sensitive molecular tools would help their detection in the future.

Previous studies indicated that DSBs downstream of the active VSG gene mostly lead to switchers that lost the whole active ES [28]. Therefore, it is also possible that *Tb*TRF depletion may cause DSBs only in the telomere repeats that lead to the loss of the active VSG ES and subsequent VSG switching. Although these cells have telomerase activity that can elongate telomere sequences after telomere breaks [57,58], it has been shown that the active telomere are more prone to large telomere truncations than silent telomeres [59], presumably because the active telomere is expressed [60], which make it more fragile [61]. DSBs inside the telomere repeats may not be easily detected by the LMPCR analysis, as the most telomere-proximal locus-specific probe is located at the 5' end of the VSG gene, and DSBs located a few kilobases downstream may not be efficiently detected by LMPCR due to limited PCR efficiency.

Although we have identified *Tb*RAP1 as another *Tb*TRF interacting telomere protein [42], *Tb*RAP1, but not *Tb*TRF or *Tb*TIF2, is essential for subtelomeric VSG silencing [19,42,62]. In addition, we have not detected any direct interaction between *Tb*RAP1 and *Tb*TIF2. Furthermore, in yeast 2-hybrid analysis, *Tb*RAP1 interacts with *Tb*TRF weakly [42], while *Tb*TIF2 interacts with *Tb*TRF very strongly [19]. Therefore, we anticipate that *Tb*TIF2 and *Tb*TRF function independently from *Tb*RAP1.

Depletion of *Tb*TRF does not increase the amount of DSBs in subtelomeric ESs, while depletion of *Tb*TIF2 does, indicating that *Tb*TIF2 has essential functions independent of *Tb*TRF. Therefore, it would be interesting to investigate whether recruiting *Tb*TIF2 to telomeres is completely dependent on *Tb*TRF, as *Tb*TRF has a duplex telomere DNA binding activity [29] while *Tb*TIF2 does not have any functional domain suggesting DNA binding activity [19]. Although we have not identified any telomere proteins other than *Tb*TRF [29], *Tb*TIF2 [19], and *Tb*RAP1 [42] in *T. brucei*, it is very possible that the *T. brucei* telomere complex has more than just three protein members. For example, *T. brucei* has a telomere G-rich 3' overhang at the very end [63]. Although this overhang is short, we expect it to be bound and protected by proteins with single stranded DNA binding activity. It has been shown that Leishmania RPA1 can bind telomeric single stranded DNA [64]. Since *T. brucei* and Leishmania are closely related, it is possible that *T. brucei* RPA1 can also bind telomeric G-overhang. If *Tb*TIF2 interacts with single-stranded telomere DNA binding factors, it can also be recruited to the telomere independently of *Tb*TRF.

Our studies further validate that both *Tb*TIF2 and *Tb*TRF are essential for normal *T. brucei* proliferation. Since these proteins are only weakly homologous to their mammalian counterparts [19,29], they are good potential targets for anti-parasite agents. In addition, our results indicate that targeting *Tb*TIF2 would be more efficient than targeting *Tb*TRF, as *Tb*TIF2 depletion causes simultaneous depletion of *Tb*TRF in addition to increased subtelomeric DSB numbers. In human cells, TIN2 stabilizes TRF1 proteins by preventing Tankyrase 1 from modifying TRF1 [35,36], which in turn prevents TRF1 from being ubiquitinated by SCF^{FBX4} [38] and subsequent degradation by the 26S proteasome [38,39]. However, tankyrase 1 and SCF^{FBX4} homologs have not been identified in *T. brucei*, and whether *Tb*TRF is ubiquitinated before it is degraded by the proteasome is unknown. Further studies on *Tb*TRF modifications will help to reveal the detailed mechanisms of how *Tb*TIF2 regulates *Tb*TRF protein levels.

Supporting Information

S1 Fig. LMPCR analysis of subtelomeric DSBs in *Tb*TRF RNAi cells.

(EPS)

S2 Fig. Control ChIP experiments to examine association of *Tb*TRF and the 50 bp repeats.

(EPS)

S3 Fig. LMPCR analysis of subtelomeric DSBs in S/TIFi+F2H-*Tb*TRF cells.

(EPS)

S1 Table. S/TIF2i Switcher phenotype and genotype characterization.

(DOCX)

S2 Table. S/TIF2i+F2H-*Tb*TRF Switcher phenotype and genotype characterization.

(DOCX)

S3 Table. List of primers used in this study.

(DOCX)

S1 Procedure. Supplemental Experimental Procedure. (PDF)

Acknowledgments

We thank Dr. Hee-Sook Kim and Dr. George A. M. Cross for sending us the HSTB261 strain. We thank Dr. Ziyin Li for the *Tb*AUK1-3HA tagging construct and the *Tb*PSA6 antibody. Dr. Keith Gull is thanked for providing us with tubulin antibodies. Raina Liu is thanked for her comments on the manuscript.

Author Contributions

Conceived and designed the experiments: BL SJ. Performed the experiments: SJ VN. Analyzed the data: SJ BL. Contributed reagents/materials/analysis tools: BL SJ. Wrote the paper: BL SJ VN.

References

1. Barry JD, McCulloch R (2001) Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. *Adv Parasitol* 49: 1–70. PMID: [11461029](#)
2. Cross GAM, Kim HS, Wickstead B (2014) Capturing the variant surface glycoprotein repertoire (the VSGnome) of *Trypanosoma brucei* Lister 427. *Mol Biochem Parasitol* 195: 59–73. doi: [10.1016/j.molbiopara.2014.06.004](#) PMID: [24992042](#)
3. Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC et al. (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science* 309: 416–422. PMID: [16020726](#)
4. Melville SE, Leech V, Navarro M, Cross GAM (2000) The molecular karyotype of the megabase chromosomes of *Trypanosoma brucei* stock 427. *Mol Biochem Parasitol* 111: 261–273. PMID: [11163435](#)
5. Alsford S, Wickstead B, Ersfeld K, Gull K (2001) Diversity and dynamics of the minichromosomal karyotype in *Trypanosoma brucei*. *Mol Biochem Parasitol* 113: 79–88. PMID: [11254956](#)
6. de Lange T, Borst P (1982) Genomic environment of the expression-linked extra copies of genes for surface antigens of *Trypanosoma brucei* resembles the end of a chromosome. *Nature* 299: 451–453. PMID: [7121582](#)
7. Hertz-Fowler C, Figueiredo LM, Quail MA, Becker M, Jackson A, Bason N et al. (2008) Telomeric expression sites are highly conserved in *Trypanosoma brucei*. *PLoS ONE* 3: e3527. doi: [10.1371/journal.pone.0003527](#) PMID: [18953401](#)
8. Gunzl A, Bruderer T, Laufer G, Schimanski B, Tu LC, Chung HM et al. (2003) RNA polymerase I transcribes procyclin genes and variant surface glycoprotein gene expression sites in *Trypanosoma brucei*. *Eukaryot Cell* 2: 542–551. PMID: [12796299](#)
9. Cross GAM (1975) Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology* 71: 393–417. PMID: [645](#)
10. Berriman M, Hall N, Sheader K, Bringaud F, Tiwari B, Isobe T et al. (2002) The architecture of variant surface glycoprotein gene expression sites in *Trypanosoma brucei*. *Mol Biochem Parasitol* 122: 131–140. PMID: [12106867](#)
11. Myler PJ, Allison J, Agabian N, Stuart K (1984) Antigenic variation in African trypanosomes by gene replacement or activation of alternative telomeres. *Cell* 39: 203–211. PMID: [6091912](#)
12. Rudenko G, McCulloch R, Dirksmulder A, Borst P (1996) Telomere exchange can be an important mechanism of variant surface glycoprotein gene switching in *Trypanosoma brucei*. *Mol Biochem Parasitol* 80: 65–75. PMID: [8885223](#)
13. Scholler JK, Myler PJ, Stuart KD (1989) A novel telomeric gene conversion in *Trypanosoma brucei*. *Mol Biochem Parasitol* 35: 11–19. PMID: [2761571](#)
14. Robinson NP, Burman N, Melville SE, Barry JD (1999) Predominance of duplicative VSG gene conversion in antigenic variation in African trypanosomes. *Mol Cell Biol* 19: 5839–5846. PMID: [10454531](#)
15. Kim HS, Cross GAM (2010) TOPO3alpha influences antigenic variation by monitoring expression-site-associated VSG switching in *Trypanosoma brucei*. *PLoS Pathog* 6: e1000992. doi: [10.1371/journal.ppat.1000992](#) PMID: [20628569](#)
16. Kim HS, Cross GAM (2011) Identification of *Trypanosoma brucei* RMI1/BLAP75 homologue and its roles in antigenic variation. *PLoS One* 6: e25313. doi: [10.1371/journal.pone.0025313](#) PMID: [21980422](#)

17. Benmerzouga I, Concepcion-Acevedo J, Kim HS, Vandoros AV, Cross GAM, Klingbeil MM et al. (2013) *Trypanosoma brucei* Orc1 is essential for nuclear DNA replication and affects both VSG silencing and VSG switching. *Mol Microbiol* 87: 196–210. doi: [10.1111/mmi.12093](https://doi.org/10.1111/mmi.12093) PMID: [23216794](https://pubmed.ncbi.nlm.nih.gov/23216794/)
18. Jehi SE, Li X, Sandhu R, Ye F, Benmerzouga I, Zhang M et al. (2014) Suppression of subtelomeric VSG switching by *Trypanosoma brucei* TRF requires its TTAGGG repeat-binding activity. *Nucleic Acids Res* 42: 12899–12911. doi: [10.1093/nar/gku942](https://doi.org/10.1093/nar/gku942) PMID: [25313155](https://pubmed.ncbi.nlm.nih.gov/25313155/)
19. Jehi SE, Wu F, Li B (2014) *Trypanosoma brucei* TIF2 suppresses VSG switching by maintaining subtelomere integrity. *Cell Res* 24: 870–885. doi: [10.1038/cr.2014.60](https://doi.org/10.1038/cr.2014.60) PMID: [24810301](https://pubmed.ncbi.nlm.nih.gov/24810301/)
20. Holthausen JT, Wyman C, Kanaar R (2010) Regulation of DNA strand exchange in homologous recombination. *DNA Repair (Amst)* 9: 1264–1272.
21. McCulloch R, Barry JD (1999) A role for RAD51 and homologous recombination in *Trypanosoma brucei* antigenic variation. *Genes Dev* 13: 2875–2888. PMID: [10557214](https://pubmed.ncbi.nlm.nih.gov/10557214/)
22. Proudfoot C, McCulloch R (2005) Distinct roles for two RAD51-related genes in *Trypanosoma brucei* antigenic variation. *Nucleic Acids Res* 33: 6906–6919. PMID: [16326865](https://pubmed.ncbi.nlm.nih.gov/16326865/)
23. Zhao W, Vaithiyalingam S, San Filippo J, Maranon DG, Jimenez-Sainz J, Fontenay GV et al. (2015) Promotion of BRCA2-Dependent Homologous Recombination by DSS1 via RPA Targeting and DNA Mimicry. *Mol Cell* 59: 176–187. doi: [10.1016/j.molcel.2015.05.032](https://doi.org/10.1016/j.molcel.2015.05.032) PMID: [26145171](https://pubmed.ncbi.nlm.nih.gov/26145171/)
24. Hartley CL, McCulloch R (2008) *Trypanosoma brucei* BRCA2 acts in antigenic variation and has undergone a recent expansion in BRC repeat number that is important during homologous recombination. *Mol Microbiol* 68: 1237–1251. doi: [10.1111/j.1365-2958.2008.06230.x](https://doi.org/10.1111/j.1365-2958.2008.06230.x) PMID: [18430140](https://pubmed.ncbi.nlm.nih.gov/18430140/)
25. Li B (2015) DNA double-strand breaks and telomeres play important roles in *Trypanosoma brucei* antigenic variation. *Eukaryot Cell* 14(3): 196–205. doi: [10.1128/EC.00207-14](https://doi.org/10.1128/EC.00207-14) PMID: [25576484](https://pubmed.ncbi.nlm.nih.gov/25576484/)
26. Sarbajna S, West SC (2014) Holliday junction processing enzymes as guardians of genome stability. *Trends Biochem Sci* 39: 409–419. doi: [10.1016/j.tibs.2014.07.003](https://doi.org/10.1016/j.tibs.2014.07.003) PMID: [25131815](https://pubmed.ncbi.nlm.nih.gov/25131815/)
27. Boothroyd CE, Dreesen O, Leonova T, Ly KI, Figueiredo LM, Cross GAM et al. (2009) A yeast-endonuclease-generated DNA break induces antigenic switching in *Trypanosoma brucei*. *Nature* 459: 278–281. doi: [10.1038/nature07982](https://doi.org/10.1038/nature07982) PMID: [19369939](https://pubmed.ncbi.nlm.nih.gov/19369939/)
28. Glover L, Alsford S, Horn D (2013) DNA break site at fragile subtelomeres determines probability and mechanism of antigenic variation in African trypanosomes. *PLoS Pathog* 9: e1003260. doi: [10.1371/journal.ppat.1003260](https://doi.org/10.1371/journal.ppat.1003260) PMID: [23555264](https://pubmed.ncbi.nlm.nih.gov/23555264/)
29. Li B, Espinal A, Cross GAM (2005) Trypanosome telomeres are protected by a homologue of mammalian TRF2. *Mol Cell Biol* 25: 5011–5021. PMID: [15923618](https://pubmed.ncbi.nlm.nih.gov/15923618/)
30. Stewart JA, Chaiken MF, Wang F, Price CM (2012) Maintaining the end: Roles of telomere proteins in end-protection, telomere replication and length regulation. *Mutat Res* 730: 12–19. doi: [10.1016/j.mrfmmm.2011.08.011](https://doi.org/10.1016/j.mrfmmm.2011.08.011) PMID: [21945241](https://pubmed.ncbi.nlm.nih.gov/21945241/)
31. Kim SH, Kaminker P, Campisi J (1999) TIN2, a new regulator of telomere length in human cells. *Nat Genet* 23: 405–412. PMID: [10581025](https://pubmed.ncbi.nlm.nih.gov/10581025/)
32. Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Tempst P et al. (1995) A human telomeric protein. *Science* 270: 1663–1667. PMID: [7502076](https://pubmed.ncbi.nlm.nih.gov/7502076/)
33. Broccoli D, Smogorzewska A, Chong L, de Lange T (1997) Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat Genet* 17: 231–235. PMID: [9326950](https://pubmed.ncbi.nlm.nih.gov/9326950/)
34. Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gilson E (1997) Telomeric localization of TRF2, a novel human telobox protein. *Nat Genet* 17: 236–239. PMID: [9326951](https://pubmed.ncbi.nlm.nih.gov/9326951/)
35. Ye JZ, de Lange T (2004) TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. *Nat Genet* 36: 618–623. PMID: [15133513](https://pubmed.ncbi.nlm.nih.gov/15133513/)
36. Smith S, Giriati I, Schmitt A, de Lange T (1998) Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* 282: 1484–1487. PMID: [9822378](https://pubmed.ncbi.nlm.nih.gov/9822378/)
37. Smith S, de Lange T (2000) Tankyrase promotes telomere elongation in human cells. *Curr Biol* 10: 1299–1302. PMID: [11069113](https://pubmed.ncbi.nlm.nih.gov/11069113/)
38. Lee TH, Perrem K, Harper JW, Lu KP, Zhou XZ (2006) The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance. *J Biol Chem* 281: 759–768. PMID: [16275645](https://pubmed.ncbi.nlm.nih.gov/16275645/)
39. Chang W, Dynek JN, Smith S (2003) TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres. *Genes Dev* 17: 1328–1333. PMID: [12782650](https://pubmed.ncbi.nlm.nih.gov/12782650/)
40. de Lange T (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 19: 2100–2110. PMID: [16166375](https://pubmed.ncbi.nlm.nih.gov/16166375/)

41. Hovel-Miner GA, Boothroyd CE, Mugnier M, Dreesen O, Cross GAM, Papavasiliou FN (2012) Telomere length affects the frequency and mechanism of antigenic variation in *Trypanosoma brucei*. *PLoS Pathog* 8: e1002900. doi: [10.1371/journal.ppat.1002900](https://doi.org/10.1371/journal.ppat.1002900) PMID: [22952449](https://pubmed.ncbi.nlm.nih.gov/22952449/)
42. Yang X, Figueiredo LM, Espinal A, Okubo E, Li B (2009) RAP1 is essential for silencing telomeric variant surface glycoprotein genes in *Trypanosoma brucei*. *Cell* 137: 99–109. doi: [10.1016/j.cell.2009.01.037](https://doi.org/10.1016/j.cell.2009.01.037) PMID: [19345190](https://pubmed.ncbi.nlm.nih.gov/19345190/)
43. Li Z, Zou CB, Yao Y, Hoyt MA, McDonough S, Mackey ZB et al. (2002) An easily dissociated 26 S proteasome catalyzes an essential ubiquitin-mediated protein degradation pathway in *Trypanosoma brucei*. *J Biol Chem* 277: 15486–15498. PMID: [11854272](https://pubmed.ncbi.nlm.nih.gov/11854272/)
44. Woods A, Sherwin T, Sasse R, MacRae TH, Baines AJ, Gull K (1989) Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J Cell Sci* 93: 491–500. PMID: [2606940](https://pubmed.ncbi.nlm.nih.gov/2606940/)
45. Wei Y, Hu H, Lun ZR, Li Z (2013) The cooperative roles of two kinetoplastid-specific kinesins in cytokinesis and in maintaining cell morphology in bloodstream trypanosomes. *PLoS One* 8: e73869. doi: [10.1371/journal.pone.0073869](https://doi.org/10.1371/journal.pone.0073869) PMID: [24069240](https://pubmed.ncbi.nlm.nih.gov/24069240/)
46. Huang L, Shen M, Chernushevich I, Burlingame AL, Wang CC, Robertson CD (1999) Identification and isolation of three proteasome subunits and their encoding genes from *Trypanosoma brucei*. *Molecular & Biochemical Parasitology* 102: 211–223.
47. Li Z, Gourguechon S, Wang CC (2007) Tousled-like kinase in a microbial eukaryote regulates spindle assembly and S-phase progression by interacting with Aurora kinase and chromatin assembly factors. *J Cell Sci* 120: 3883–3894. PMID: [17940067](https://pubmed.ncbi.nlm.nih.gov/17940067/)
48. Glover L, Horn D (2012) Trypanosomal histone gammaH2A and the DNA damage response. *Mol Biochem Parasitol* 183: 78–83. doi: [10.1016/j.molbiopara.2012.01.008](https://doi.org/10.1016/j.molbiopara.2012.01.008) PMID: [22353557](https://pubmed.ncbi.nlm.nih.gov/22353557/)
49. Wirtz E, Leal S, Ochatt C, Cross GAM (1999) A tightly regulated inducible expression system for dominant negative approaches in *Trypanosoma brucei*. *Mol Biochem Parasitol* 99: 89–101. PMID: [10215027](https://pubmed.ncbi.nlm.nih.gov/10215027/)
50. Valdes J, Taylor MC, Cross MA, Ligtenberg MJL, Rudenko G, Borst P (1996) The viral thymidine kinase gene as a tool for the study of mutagenesis in *Trypanosoma brucei*. *Nucleic Acids Research* 24: 1809–1815. PMID: [8657559](https://pubmed.ncbi.nlm.nih.gov/8657559/)
51. van Steensel B, Smogorzewska A, de Lange T (1998) TRF2 protects human telomeres from end-to-end fusions. *Cell* 92: 401–413. PMID: [9476899](https://pubmed.ncbi.nlm.nih.gov/9476899/)
52. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T (1999) p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 283: 1321–1325. PMID: [10037601](https://pubmed.ncbi.nlm.nih.gov/10037601/)
53. Celli GB, de Lange T (2005) DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion. *Nat Cell Biol* 7: 712–718. PMID: [15968270](https://pubmed.ncbi.nlm.nih.gov/15968270/)
54. Murnane JP (2006) Telomeres and chromosome instability. *DNA Repair (Amst)* 5: 1082–1092.
55. Burton P, McBride DJ, Wilkes JM, Barry JD, McCulloch R (2007) Ku heterodimer-independent end joining in *Trypanosoma brucei* cell extracts relies upon sequence microhomology. *Eukaryot Cell* 6: 1773–1781. PMID: [17693593](https://pubmed.ncbi.nlm.nih.gov/17693593/)
56. Conway C, Proudfoot C, Burton P, Barry JD, McCulloch R (2002) Two pathways of homologous recombination in *Trypanosoma brucei*. *Mol Microbiol* 45: 1687–1700. PMID: [12354234](https://pubmed.ncbi.nlm.nih.gov/12354234/)
57. Dreesen O, Cross GAM (2006) Consequences of telomere shortening at an active VSG expression site in telomerase-deficient *Trypanosoma brucei*. *Eukaryot Cell* 5: 2114–2119. PMID: [17071826](https://pubmed.ncbi.nlm.nih.gov/17071826/)
58. Sandhu R, Sanford S, Basu S, Park M, Pandya UM, Li B et al. (2013) A trans-spliced telomerase RNA dictates telomere synthesis in *Trypanosoma brucei*. *Cell Res* 23: 537–551. doi: [10.1038/cr.2013.35](https://doi.org/10.1038/cr.2013.35) PMID: [23478302](https://pubmed.ncbi.nlm.nih.gov/23478302/)
59. Bernards A, Michels PAM, Lincke CR, Borst P (1983) Growth of chromosome ends in multiplying trypanosomes. *Nature* 303: 592–597. PMID: [6304531](https://pubmed.ncbi.nlm.nih.gov/6304531/)
60. Rudenko G, Van der Ploeg LH (1989) Transcription of telomere repeats in protozoa. *EMBO J* 8: 2633–2638. PMID: [2511008](https://pubmed.ncbi.nlm.nih.gov/2511008/)
61. Dreesen O, Li B, Cross GAM (2007) Telomere structure and function in trypanosomes: a proposal. *Nat Rev Microbiol* 5: 70–75. PMID: [17160000](https://pubmed.ncbi.nlm.nih.gov/17160000/)
62. Pandya UM, Sandhu R, Li B (2013) Silencing subtelomeric VSGs by *Trypanosoma brucei* RAP1 at the insect stage involves chromatin structure changes. *Nucleic Acids Res* 41: 7673–7682. doi: [10.1093/nar/gkt562](https://doi.org/10.1093/nar/gkt562) PMID: [23804762](https://pubmed.ncbi.nlm.nih.gov/23804762/)
63. Sandhu R, Li B (2011) Examination of the telomere G-overhang structure in *Trypanosoma brucei*. *J Vis Exp*
64. Neto JL, Lira CB, Giardini MA, Khater L, Perez AM, Peroni LA et al. (2007) *Leishmania* replication protein A-1 binds *in vivo* single-stranded telomeric DNA. *Biochem Biophys Res Commun* 358: 417–423. PMID: [17498665](https://pubmed.ncbi.nlm.nih.gov/17498665/)

Copyright of PLoS ONE is the property of Public Library of Science and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.