BRF1, a subunit of RNA polymerase III transcription factor TFIIIB, is essential for cell growth of *Trypanosoma brucei*

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SUMMARY

RNA polymerase III (Pol III) synthesizes small RNA molecules that are essential for cell viability. Accurate initiation of transcription by Pol III requires general transcription factor TFIIIB, which is composed of three subunits: TFIIB-related factor BRF1, TATA-binding protein and BDP1. Here we report the molecular characterization of BRF1 in *Trypanosoma brucei* (TbBRF1), a parasitic protozoa that shows distinctive transcription characteristics. *In silico* analysis allowed the detection in TbBRF1 of the three conserved domains located in the N-terminal region of all BRF1 orthologues, namely a zinc ribbon motif and two cyclin repeats. Homology modelling suggested that, similarly to other BRF1 and TFIIB proteins, the TbBRF1 cyclin repeats show the characteristic structure of five α-helices per repeat, connected by a short random-coiled linker. As expected for a transcription factor, TbBRF1 was localized in the nucleus. Knock-down of TbBRF1 by RNA interference (RNAi) showed that this protein is essential for the viability of procyclic forms of *T. brucei*, since ablation of TbBRF1 led to growth arrest of the parasites. Nuclear run-on and quantitative real-time PCR analyses demonstrated that transcription of all the Pol III-dependent genes analysed was reduced, at different levels, after RNAi induction.

Key words: BRF1, Pol III transcription, *Trypanosoma brucei*, gene expression.

INTRODUCTION

Trypanosoma brucei, a parasitic protozoa of the Trypanosomatidae family, is the etiologic agent of Human African Trypanosomiasis, also known as sleeping sickness. The parasite is transmitted through the bite of the tsetse fly (*Glossina* spp.) in the Sub-Saharan Africa, and without appropriate treatment it produces neurological disorders, including changes in the sleep–wake cycle, which may lead to a state of coma and death (Kennedy, 2013). *Trypanosoma brucei* is also important for presenting atypical characteristics of gene expression, such as RNA polymerase II (Pol II) polycistronic transcription, coupled with *trans*-splicing and polyadenylation to generate mature mRNAs (Martinez-Calvillo *et al*. 2010; Michaeli, 2011); and transcription of some protein-coding genes by the RNA polymerase I (Pol I) (Gunzl *et al*. 2003). Little is known in this parasite about RNA polymerase III (Pol III) transcription, despite its role in the synthesis of small essential RNA molecules, such as tRNAs, 5S rRNA, snRNAs and 7SL RNA (Willis, 1993; Dieci *et al*. 2007).

In higher eukaryotes and yeast, Pol III recognizes three main types of promoters that in most cases are located downstream of the transcription start site, within the gene itself. Type I promoters are present in 5S rRNA genes, and consist of three internal domains: box A, an intermediate element and box C. Type II promoters, characteristic of tRNA genes, consist of two conserved internal elements: boxes A and B. Type III promoters, found in U6 snRNA genes, contain elements that reside exclusively upstream of the coding sequence: a TATA box, a proximal sequence element and a distal sequence element (Schramm and Hernandez, 2002; White, 2011). Several transcription factors participate in Pol III transcription, including TFIIIA, TFIIIB, TFIIIC and SNAPc (Acker *et al*. 2013; Dieci *et al*. 2013). TFIIIB is a heterotrimeric factor composed of the TFIIB-related factor 1

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(BRF1), the TATA-binding protein (TBP) and BDP1. TFIIIB is involved in transcription of all the different types of Pol III genes, and it recruits and positions Pol III to the transcription start site and participates in promoter opening (Kassavetis and Geiduschek, 2006). Whereas in most organisms BRF1 participates in transcription of all Pol III genes, a human variant called BRF2 is responsible for transcription of genes with a type III promoter (Schramm *et al.* 2000). The BRF1 subunit of TFIIIB interacts directly with the Pol III subunits C160, C128, C34 and C17, as well as with the TFIIIC subunits Tfc1, Tfc4 and Tfc8 (Moir *et al.* 2002; Khoo *et al.* 2014). BRF1 is essential for the function of TFIIIB and consequently for the function of Pol III itself. The N-terminal half of BRF1 contains three domains with homology to the Pol II transcription factor TFIIB: a zinc ribbon motif and two cyclin or TFIIB-related repeats (Lopez-de-Leon *et al.* 1992). The C-terminal half of BRF1 does not show homology to TFIIB, and in several different yeast species it contains three conserved sequence domains, named BRF1 homology blocks I–III (Khoo *et al.* 2014).

In contrast to other eukaryotes, *T. brucei* Pol III not only transcribes the U6 snRNA gene, but also transcribes all the snRNA genes (Fantoni *et al.* 1994). Interestingly, snRNA genes in this parasite have a divergently oriented tRNA gene (or a tRNA-like) in their 5′-flanking region, and internal sequences from the neighbouring tRNA genes are required for the expression of the snRNAs (Nakaar *et al.* 1997). The only Pol III-related transcription factors identified and characterized in *T. brucei* are TBP (also known as TRF4, for TBP-related factor 4) and SNAPc (composed of three subunits in *T. brucei*) (Ruan *et al.* 2004; Das *et al.* 2005). These two transcription factors have been mainly studied in the context of transcription of the spliced-leader (SL) RNA genes, which are transcribed by Pol II (Gilinger and Bellofatto, 2001). Other Pol II transcription factors that are required for the expression of the SL RNA in *T. brucei* are TFIIA (Schimanski *et al.* 2005*a*), TFIIB (Palenchar *et al.* 2006; Schimanski *et al.* 2006), TFIIH (Lecordier *et al.* 2007; Lee *et al.* 2007) and the mediator complex (Lee *et al.* 2010).

Notably, neither TFIIIA nor TFIIIC has been identified in trypanosomatids. However, putative orthologues of BDP1 (Tb927.10.7840) and BRF1 (Tb927.11.470) have been identified in the *T. brucei* databases (Berriman *et al.* 2005; Gunzl *et al.* 2007). Tandem affinity purification experiments with a PTP-tagged version of TBP demonstrated that Tb927.11.470 co-purified with TBP; this confirmed the identity of Tb927.11.470 as the BRF1 orthologue in *T. brucei* (TbBRF1) (Schimanski *et al.* 2005*a*). Here we report that TbBRF1 contains the typical domains that are present in all the BRF1 orthologues: a zinc ribbon motif and two cyclin repeats. Additionally, TbBRF1 possesses a homology block I, conserved among yeast species and human. By generating TbBRF1 conditional knock-down cell lines, we show that TbBRF1 is needed for cell viability and that participates in Pol III transcription in *T. brucei* procyclic forms. Corresponding to its function, TbBRF1 was localized in the parasite nucleus.

MATERIALS AND METHODS

Bioinformatic analysis

Sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and tritrypDB (http://tritrypdb.org/tritrypdb/) (version 8.1) databases. Sequence alignments were generated using the ClustalΩ program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and shaded manually. Domain identification was performed using SMART 7 (http://smart.embl-heidelberg.de/), InterPro Scan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan/), Pfam (http://pfam.sanger.ac.uk/) and Superfamily 1.75 (http://supfam.org/SUPERFAMILY/hmm.html) pages. Secondary structure analysis was performed using PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/), NetSurfP 1.1 (http://www.cbs.dtu.dk/services/NetSurfP/), Jpred 3 (http://www.compbio.dundee.ac.uk/www-jpred/) and CFSSP (http://www.biogem.org/tool/chou-fasman/). Homology modelling was carried out using SWISS-MODEL (http://swissmodel.expasy.org/interactive) and Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The structure obtained was edited with the Swiss-PDBViewer program (http://www.expasy.org/spdbv/). The most suitable sequence of BRF1 for RNA interference (RNAi) experiments was selected using the trypanoFAN page (http://trypanofan.path.cam.ac.uk/trypanofan/main/).

Plasmid constructs

To generate plasmid p2T7-TbBRF1, a 445-bp fragment from TbBRF1-coding sequence was amplified with primers BRF1-RNAi-5′ (5′-AGGATCCAAGCTTGGATAGTATTGATAAG) and BRF1-RNAi-3′ (5′-ACTCGAGATTAGGTACAGGTGGTGCT) and inserted between the *Xho*I and *Bam*HI restriction sites of the RNAi vector p2T7-177 (Wickstead *et al.* 2002). For PTP-tagging, a 500-bp fragment from the C-terminal BRF1-coding sequence was amplified by PCR with primers BRF1-PTP-5′ (5′-GGGCCCTCAACCCTGATGATGTGGTGCC) and BRF1-PTP-3′ (5′-GCGGCCGCGCAGCGACCCATTCATC) and cloned into the *Apa*I and *Not*I restriction sites of the pC-PTP-BLA vector (Nguyen *et al.* 2007). To obtain plasmid pCold-TbBRF1, the entire

TbBRF1 gene was amplified with primers TbBRF1-SacI-F (5′-GAGCTCATGTCTAGTTG TTCGCATC) and TbBRF1-XbaI-R (5′-TCTAG AAGCGACCCATTCATCCTC) and cloned into the *Sac*I and *Xba*I restriction sites of the pCold1 expression vector (Takara Bio Inc.). For run-on analysis several DNA fragments from *T. brucei* were amplified by PCR and cloned into the pGEM-T Easy vector (Promega). The 18S rRNA fragment was amplified with primers 18S rRNA-5′ (5′-CGGCTTCCAGG AATGAAGG) and 18S rRNA-3′ (5′-CCCCTGAG ACTGTAACCTC); and procyclin with oligonucleotides procyclin-5′ (5′-ATGGCACCTCGTTCCC TTTA) and procyclin-3′ (5′-TTAGAATGCGGC AACGAGAG). The SL gene was amplified with primers SL-5′ (5′-TGTTTCCCATAAGTCT ACCG) and SL-3′ (5′-TATATATGAGTGAGT GAGTGTG); and α-tubulin with oligonucleotides α-tubulin-5′ (5′-AGAAGTCCAAGCTCGGCTAC AC) and α-tubulin-3′ (5′-GTAGTTGATGCCGC ACTTGAAG). Elp3b was amplified with oligonucleotides ELP3-5′ (5′-GGATCCAAGCTTTATT GAGGCGGAAATGAAGG) and ELP3-3′ (5′-CT CGAGATTTTCATGAACCCACGCTC); and 5S rRNA with primers 5S rRNA-5′ (5′-AAAGGT GCTTTTCTTCTTTTCT) and 5S rRNA-3′ (5′-GGAGAGAAGGGGAACTT). 7SL ncRNA was amplified with primers 7SL ncRNA-5′ (5′-GAATATCACTTGGCTTTGTCAAA) and 7SL ncRNA-3′ (5′-TCGGCAAAAGAAACCCA CTT); and U2 snRNA with oligonucleotides U2 snRNA-5′ (5′-ACTTTTGGATAAGGCGCTGC AT) and U2 snRNA-3′ (5′-GAGTGAACTTG AAGGACCAAAC). The tRNA-Arg fragment was amplified with oligonucleotides tRNA-Arg-5′ (5′-AAAAGGTTATTTCATATACGTTGGC) and tRNA-Arg-3′ (5′-TGCAAGAAGCGGTTCTT CCA); and tRNA-Phe with primers tRNA-Phe-5′ (5′-GAGTCACTTTCTGTTACGATAATAAAAA) and tRNA-Phe-3′ (5′-AGAGGAGCCGACCTTCA C). The *Leishmania major* tRNA-Tyr gene was amplified with oligonucleotides Lm36-TRNAT YR-5′ (5′-AGTGCCGAGAAGTTCGACG) and Lm36-TRNATYR-3′ (5′-TCGTCTCCGTTCCT GTTGC). All constructs were verified by sequencing.

Trypanosoma cell culture and transfection

Procyclic (tsetse midgut form) parasites of the *T. brucei* strain 29-13 (Wirtz *et al.* 1999) were cultured at 28 °C in SDM-79 medium supplemented with 10% fetal bovine serum, G418 (15 μg mL^{-1}) and hygromycin (50 μg mL^{-1}). Transfection by electroporation was performed as previously described (Foldynova-Trantirkova *et al.* 2005). Briefly, 1×10^8 cells in 0·5 mL electroporation buffer (25 mM HEPES, 120 mM KCl, 0·15 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM K_2HPO_4, 2 mM EDTA, pH 7·6) were transfected with 10 μg of linearized vector by electroporation at 1500 V, 50 μF and 500 Ω (BTX Electro Square Porator ECM 830). Transfectants containing an RNAi construct were selected with phleomycin (2·5 μg mL^{-1}), whereas transfectants containing a PTP-tagging construct were selected with blasticidin (10 μg mL^{-1}). For transfection, p2T7-TbBRF1 was linearized with *Not*I and pTbBRF1-PTP with *Bsm*I. Clonal cell lines were obtained by serial dilution in 96-well plates.

Indirect immunofluorescence

Mid-log cells were harvested, washed twice with 1 × PBS and resuspended to obtain a 300 000 cells μL cell density. From this cell suspension 5 μL were spread onto a poly-L-lysine-coated glass slide. Cells were fixed with 4% paraformaldehyde for 30 min at 4 °C and permeabilized with 0·05% Triton X-100, for 2 min at room temperature. Then, cells were blocked with 1% cold fish skin gelatin and 2% BSA for 1 h at room temperature. After that, cells were incubated with rabbit anti-protein C polyclonal antibody (Delta Biolabs) diluted at 1:25 with blocking solution for 1 h at room temperature, and washed with 1× PBS and 0·05% Tween 20. Next, cells were incubated with a secondary goat anti-rabbit antibody conjugated with Alexa 488 diluted at 1:400 with blocking solution at room temperature, and washed with 1× PBS and 0·05% Tween. Finally, cells were mounted with Vectashield – DAPI solution (Vector Laboratories Inc.). Images were obtained using a Leica microscope (SP5, DM 16000, Mo) and analysed with the LAS AF software.

Analysis of RNAi knock-down cell line

In the knock-down cell line, silencing of BRF1 was induced by adding doxycycline (2 μg mL^{-1}) to the medium (Dox+ or induced culture). As a control, the same cell line was grown without doxycycline (Dox− or non-induced culture) in parallel with the induced culture. Both cultures were counted daily and diluted to 2×10^6 cells mL^{-1}. For growth curves, cell number was calculated as the product of the cumulative cell density and the dilution factor. Total RNA was extracted with TRI reagent (Sigma) at different time points and fractionated by formaldehyde-MOPS agarose gels. After electrophoresis, RNA was transferred to Hybond N+ membranes (Amersham) by capillary action. TbBRF1 mRNA was detected with a 300-bp probe that corresponds to the 3′-UTR, which was labelled with [α-^{32}P]dCTP using the High Prime labelling system (Amersham). Whole-cell protein was extracted at different time points, fractionated by SDS–PAGE and transferred to PVDF membranes. TbBRF1 protein was detected with a specific polyclonal antibody (see below) and a horseradish peroxidase-conjugated secondary antibody, and developed using an ECL kit (GE Healthcare).

Production of TbBRF1 polyclonal antibody

Escherichia coli BL21 (DE3) competent cells were transformed with the pCold-TbBRF1 construct. Expression of TbBRF1 recombinant protein (TbBRF1r) was induced with 1 mM IPTG at 37 °C for 16 h. The TbBRF1r protein was purified by Ni-Sepharose 6 Fast Flow chromatography (GE Healthcare) following the manufacturer's instructions. The purity of the recombinant protein was examined by SDS–PAGE using 15% polyacrylamide gels. The anti-TbBRF1r polyclonal antibody was produced by inoculating a 4-week-old male New Zealand rabbit intramuscularly two times at 3-week intervals with purified TbBRF1r protein (100 μg) plus TiterMax Gold adjuvant (Sigma) at a 1:1 ratio. The rabbit was bled weekly to check for antibody production by Western blot analysis. Serum was collected 15 days after the last immunization. Preimmune normal rabbit serum was collected before immunization and used as a negative control in all experiments with rabbit antibodies. The specificity of the anti-TbBRF1 polyclonal antibody was confirmed by Western blot analysis against TbBRF1r and parasite extracts.

Nuclear run-on experiments

These experiments were performed as described elsewhere (Martinez-Calvillo *et al*. 2003; Padilla-Mejia *et al*. 2015), with nuclei isolated from 2×10^8 mid-log cells incubated for 48 h in the presence of doxycycline. Labelled nascent RNA was hybridized to Hybond filters (Amersham) containing dots of 2 μg of plasmid DNA. Hybridization was performed for 48 h at 50 °C in a solution containing 50% formamide, 5× SSC (1× SSC is 0·15 M NaCl and 0·015 M sodium citrate), 0·2% SDS, 4× Denhardt's reagent and 100 μg mL^{-1} salmon sperm DNA. Post-hybridization washes were carried out in 0·1× SSC and 0·1% SDS at 65 °C. RNA signals were quantified by densitometry using the MultiGauge software.

Quantitative real-time PCR

Briefly, 1 μg of total RNA from the induced (for 24 and 48 h) and non-induced TbBRF1 RNAi cultures was used as template for the first strand cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen) and 50 ng of random hexamers (Invitrogen). The cDNA was analysed by quantitative real-time PCR (qPCR) assays using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) in a Rotor-Gene 3000 cycler (Corbett Research) according to the manufacturer's recommendations. All qPCR reactions were performed at least in triplicate, using primers and conditions that were optimized to produce a single amplicon of the correct size. Each amplification product was analysed for specificity by both agarose gel electrophoresis and melt curve analysis. Standard curves for primer pairs were derived from genomic DNA and cDNA dilution series and ranged in their r^2 value from 0·98 to 1·0. PCR efficiencies were near to 100% for all the genes, so the data were analysed by the $2^{-\Delta\Delta C_q}$ method. For normalization of the data we used 18S rRNA as a reference gene, and all values were represented relative to non-induced treatments. The 18S rRNA (*Tb927.2.1452*) was amplified with primers 18sqFw (5′-GGGATACTCAAACCCATCCA) and 18sqRv (5′-CCCTTTAACAGCAACAGCATTA); and the tRNA^{ARG} (*Tb927.8.2859*) with ArgqFw (5′-GGTCTCGTGGCGCAATG) and ArgqRv (5′-CGATCCCGGCAGGACTC). The tRNA^{ALA} (*Tb927.7.6821*) was amplified with oligonucleotides AlaqFw (5′-GGGGATGTAGCTCAGATGG) and AlaqRv (5′-TGGAGAAGTTGGGTATCGATC); and procyclin (*Tb927.6.510*) with Procyclin-5 (5′-ATGGCACCTCGTTCCCTTTA) and ProcqRv (5′-CTTTGCCTCCCTTCACGATAAC). TFIIB (*Tb927.9.5710*) was amplified with primers Tf2bqFw (5′-GAACAGGGAACGCACATTAG) and Tf2bqRv (5′-TTGTTGACTTTGGTCACTTCC); and α-tubulin (*Tb927.1.2340*) with TubqFw (5′-GGGCTTCCTCGTGTATCA) and TubqRv (5′-GCTTGGACTTCTTGCCATAG). Elp3b (*Tb927.8.3310*) was amplified with oligonucleotides Elp3qFw (5′-TAAGGGTATCCGGTGCAAAG) and Elp3qRv (5′-CTGGCGCGAAACTCATTAAC); and *Tb927.9.2780* (a hypothetical protein) with primers TbZ5qFw (5′-GCTGGGAGTCTACATGGATAAC) and TbZ5qRv (5′-AGTACGGACAGCGCATAATC).

RESULTS

TbBRF1 possesses the three conserved domains

Tb927.11.470 was previously identified as the BRF1 orthologue in *T. brucei* (TbBRF1) (Schimanski *et al*. 2005*a*, 2006). A multiple sequence alignment of TbBRF1 and homologues in other eukaryotes showed that TbBRF1 contains the three conserved domains located in the N-terminal half of the protein: a zinc ribbon motif and two imperfect cyclin repeats (also known as TFIIB-related repeats) (Fig. 1A). The presence of these domains was confirmed using the SMART, InterPro Scan, Pfam and Superfamily servers. The three domains are also present in the BRF1 orthologues of the related trypanosomatids *L. major* (LmjF.25.0440) and *Trypanosoma cruzi* (TcCLB.507093.180)

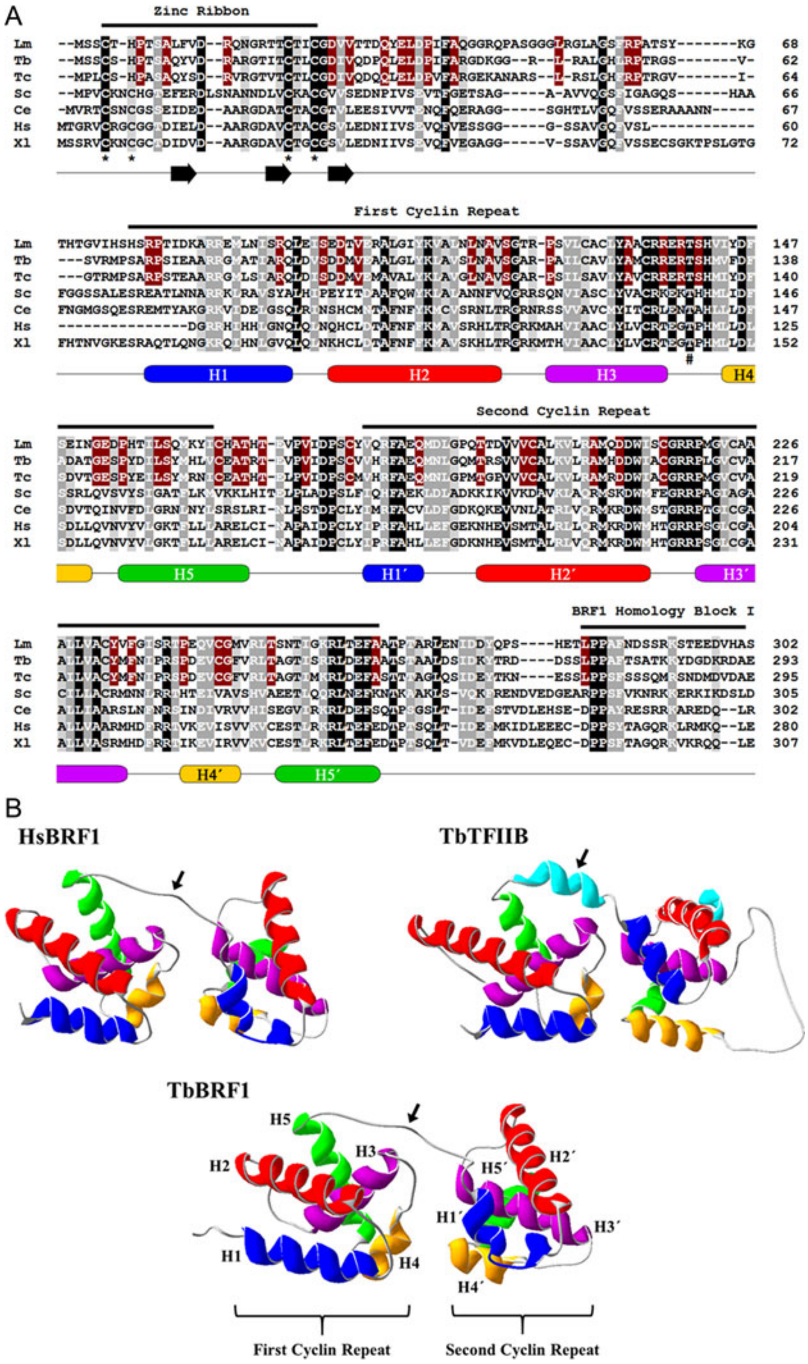

Fig. 1. Sequence and predicted structure analyses of TbBRF1. (A) Multiple sequence alignment of the N-terminal region of BRF1 from *Leishmania major* (Lm, LmjF.25.0440), *Trypanosoma brucei* (Tb, Tb927.11.470), *Trypanosoma cruzi* (Tc, TcCLB.507093.180), *Saccharomyces cerevisiae* (Sc, CAA68968.1), *Caenorhabditis elegans* (Ce, NP_495526.1), *Homo sapiens* (Hs, AAH86856.1) and *Xenopus laevis* (Xl, NP_001088063.1). Complete conservation is denoted by black shading, conserved substitutions are indicated by dark-grey shading with white lettering and semi-conserved substitutions are denoted by light-grey shading with black lettering, according to the ClustalΩ program. Trypanosomatid-specific conserved residues are shaded in red with white letters. The zinc ribbon domain, both cyclin or TFIIB-related repeats and the BRF1 homology block I, are indicated. Asterisks (*) indicate the four zinc-binding residues in the zinc ribbon. The hash character (#) indicates a Thr residue that is phosphorylated in *S. cerevisiae* and that is conserved in all the species analysed. Predicted secondary structure elements are shown below the TbBRF1 sequence. The β-strands are denoted by black arrows, whereas α-helices by rounded rectangles. The five α-helices of the first cyclin repeat (H1–H5) and the second cyclin repeat (H1′–H5′) are shown in colour. (B) Predicted three-dimensional structure of the N-terminal region of TbBRF1. Homology-modelling was performed for TbBRF1 (bottom image), BRF1 from *H. sapiens* (HsBRF1) and TFIIB from *T. brucei* (TbTFIIB), using the crystal structure of human TFIIB as a template. The five α-helices of the first cyclin repeats (H1–H5) and the second cyclin repeats (H1′–H5′) are shown in the same colours, which correspond to the colours shown in panel (A). For simplicity, cyclin repeats and α-helices are only labelled in the TbBRF1 model. The arrows indicate the random-coiled region that separates the first and second cyclin repeats in TbBRF1 and HsBRF1, which is substituted by an α-helix in TbTFIIB. The quality of the homology models was evaluated with the Mod Eval program, showing a score of 0·95.

(Fig. 1A). Sequence identity of TbBRF1 ranges from 52 to 67% for the *L. major* and *T. cruzi* orthologues, respectively; and 22–26% for other eukaryotes. Sequence conservation is higher in the N-terminal half of BRF1 than in the C-terminal half (Fig. 1A and data not shown). The predicted mass of TbBRF1 (67·6 kDa) is similar to the predicted masses for the orthologues in *Saccharomyces cerevisiae* (66·9 kDa), *T. cruzi* (67·7 kDa), *Homo sapiens* (71·1 kDa) and *Xenopus laevis* (73·7 kDa). Interestingly, the predicted size of the BRF1 orthologue in *L. major* (77·2 kDa) is almost 10 kDa larger than TbBRF1, due to insertions in the C-terminal region of the former (data not shown).

In contrast to the N-terminal half, the C-terminal half of BRF1 shows a low degree of sequence conservation. However, in *S. cerevisiae* this region contains three domains (homology blocks I–III) that are conserved in yeast species and human (Khoo *et al.* 2014). TbBRF1 contains a relatively conserved homology block I, but lacks the other two conserved domains (Fig. 1A and data not shown).

TbBRF1 predicted structure

Zinc ribbon domains usually fold into β-sheet structures (Chen *et al.* 2000). Accordingly, the zinc-binding motif in TbBRF1 is predicted to form a β-sheet, while the rest of the protein is composed of α-helices (Fig. 1A). The TbBRF1 cyclin repeats show the characteristic structure of five α-helices per repeat, connected by a short random-coiled linker (Fig. 1A) (Noble *et al.* 1997). Thus, TbBRF1 presents the typical BRF1 secondary structure. To further examine the structure of TbBRF1, the hypothetical three-dimensional structure of the cyclin repeats was obtained by homology modelling, using the crystal structure of human TFIIB as a template. As controls, we generated the structures of human BRF1 and TbTFIIB (Fig. 1B), obtaining models that were practically identical to those previously reported (Ibrahim *et al.* 2009; Khoo *et al.* 2014). When comparing the predicted structures for TbBRF1 and *H. sapiens* BRF1 we found a very similar architecture, which consists of the two defined and characteristic cyclin motifs, each of them folded into five α-helices (Fig. 1B). On the other hand, while the structure of the first cyclin repeat of TbBRF1 and first module of TbTFIIB is conserved, the structure of the second cyclin repeat of TbBRF1 and the second module of TbTFIIB is very different; moreover, the random-coil linker that connects both cyclin repeats in TbBRF1 is replaced by an α-helix structure in TbTFIIB (Fig. 1B) (Ibrahim *et al.* 2009). Altogether, the data presented suggest that TbBRF1 possesses all the attributes that are present in the BRF1 orthologues from other species.

TbBRF1 localizes to the nucleus

The TbBRF1 sequence was compared to nuclear localization signals (NLS) previously identified in trypanosomatids (Marchetti *et al.* 2000), and a putative NLS was identified (554-RKRRR-558) in the C-terminal half of the protein, in accordance with the expected nuclear localization of TbBRF1. To determine whether TbBRF1 is actually a nuclear protein, we produced a cell line where TbBRF1 was labelled with a C-terminal PTP tag to perform indirect immunofluorescence experiments. The PTP tag consists of protein A and protein C epitopes separated by a tobacco etch virus protease cleavage site (Schimanski *et al.* 2005*b*). For these experiments, a specific polyclonal antibody recognizing the protein C epitope was used on fixed and permeabilized parasites. As expected for a transcription factor, TbBRF1 was localized in the nucleus of transfected parasites (Fig. 2). The observed TbBRF1 punctate signal resembles the nuclear distribution reported for TFIIIB and TFIIIC in human and fission yeast (Haeusler and Engelke, 2006).

TbBRF1 is indispensable for cell survival

To evaluate whether TbBRF1 is essential for cell growth, we knock-down TbBRF1 *in vivo* by RNAi. Thus, a 445-bp fragment from the TbBRF1-coding sequence, encompassing nucleotides 780–1224, was amplified and cloned into the p2T7-177 vector, which contains two opposite tetracycline-inducible T7 RNA polymerase promoters to generate double-stranded RNA (Wickstead *et al.* 2002). The resultant vector was transfected into procyclic *T. brucei* cell line 29–13 that constitutively expresses the tetracycline repressor and T7 RNA polymerase (Wirtz *et al.* 1999). The transfected population was cloned by limiting dilution, and a clonal cell line was selected for further analysis. Synthesis of TbBRF1 double-stranded RNA was induced by the addition of the tetracycline analogue doxycycline. In the absence of doxycycline, cells grew normally; in contrast, cells stop growing 2 days after RNAi induction, leading to cell death 2 or 3 days later (Fig. 3A). A Northern blot analysis was performed to confirm the TbBRF1 mRNA depletion after induction of RNAi, observing that the TbBRF1 mRNA level was decreased by around 95% on day 3 post-induction (Fig. 3B).

In order to analyse the levels of the TbBRF1 protein in the knock-down culture, we obtained TbBRF1r and produced antibodies against it (anti-TbBRF1r) (data not shown). When analysing the TbBRF1 protein in the knock-down culture, we observed that its levels were reduced by around 70% on day 3 post-induction, as verified by Western blot analysis using the specific anti-TbBRF1r antibody (Fig. 3C). Altogether, these results demonstrate that TbBRF1 is essential for

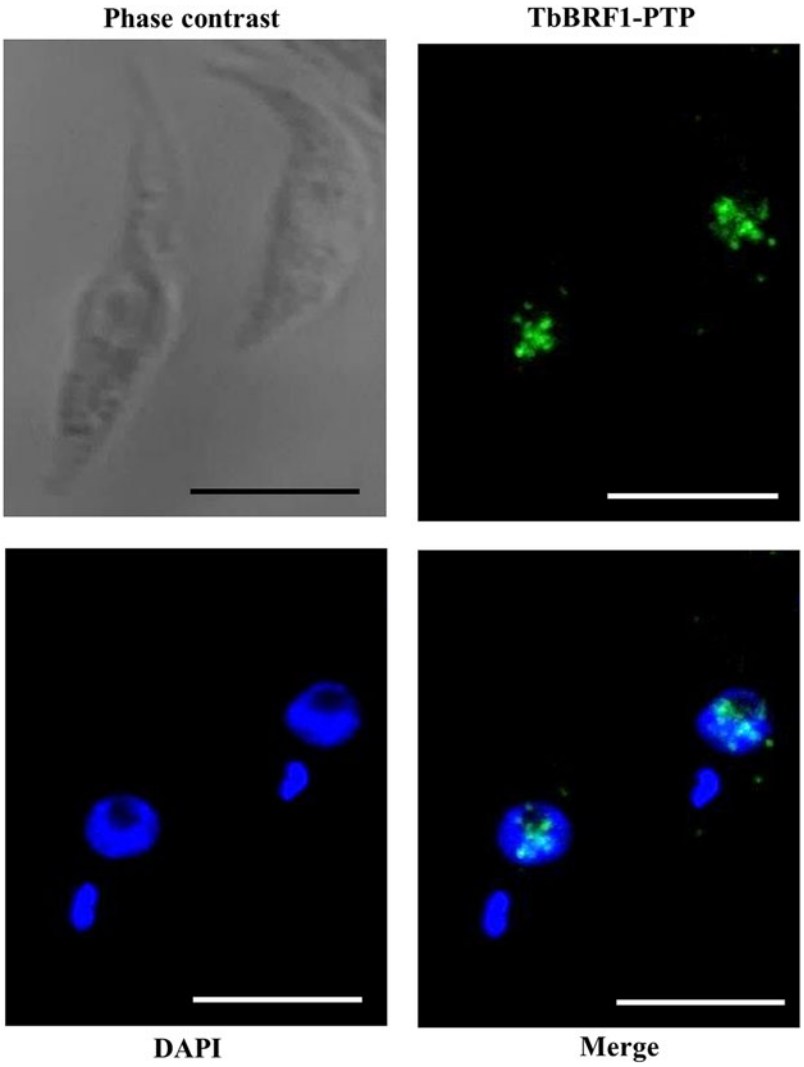

Fig. 2. TbBRF1 is localized in the nucleus. PTP-tagged TbBRF1 was detected by immunofluorescence with a rabbit anti-protein C polyclonal antibody and an Alexa 488-conjugated anti-rabbit secondary antibody. Nuclei and kinetoplast were stained with DAPI. Scale bar indicates 10 μm.

cell survival in procyclic forms of *T. brucei*, as has been reported in *S. cerevisiae* (Kassavetis *et al.* 1991; Colbert and Hahn, 1992).

Ablation of TbBRF1 affects Pol III transcription

To confirm the participation of TbBRF1 in Pol III-mediated transcription, radiolabelled nascent transcripts were obtained by run-on assays with isolated nuclei from TbBRF1 RNAi cultures that were induced for 48 h (Dox+) or non-induced (Dox−) (Fig. 4). The Pol III genes analysed were: 5S rRNA, U2 snRNA, 7SL RNA, tRNA-Arg, tRNA-Phe and tRNA-Tyr. As controls, three Pol II-transcribed genes were included (*α*-tubulin, Elp3b and SL RNA), as well as 18S rRNA and procyclin, which are transcribed by Pol I. The autoradiograph presented in Fig. 4A is a representative result of three independent experiments obtained 2 days after Dox induction. Figure 4B shows the quantification of the dot blot signal intensities from the three independent experiments, where transcription signal obtained with the non-induced cells was set to 100%. As expected, transcription signal of tRNA-Arg, tRNA-Phe, tRNA-Tyr, U2 snRNA and 7SL RNA was clearly reduced to ~24–36% of the control value. The 5S rRNA was also reduced, but to a lesser extent (~65%). Thus, these results confirm the participation of TbBRF1 in transcription of all types of Pol III genes. This is the expected result, taking into consideration that a BRF2 orthologue has not been found in *T. brucei* (Berriman *et al.* 2005). Regarding Pol I transcription, signal of the 18S rRNA was not affected, while transcription signal of the protein-coding gene procyclin was slightly reduced to ~87%. Intriguingly, Pol II transcription of *α*-tubulin was reduced to ~29% after RNAi induction with doxycycline, while signal of Elp3b was also reduced to ~55%. By contrast, transcription signal for SL RNA, which is also transcribed by Pol II, was slightly reduced to 78% of the control value.

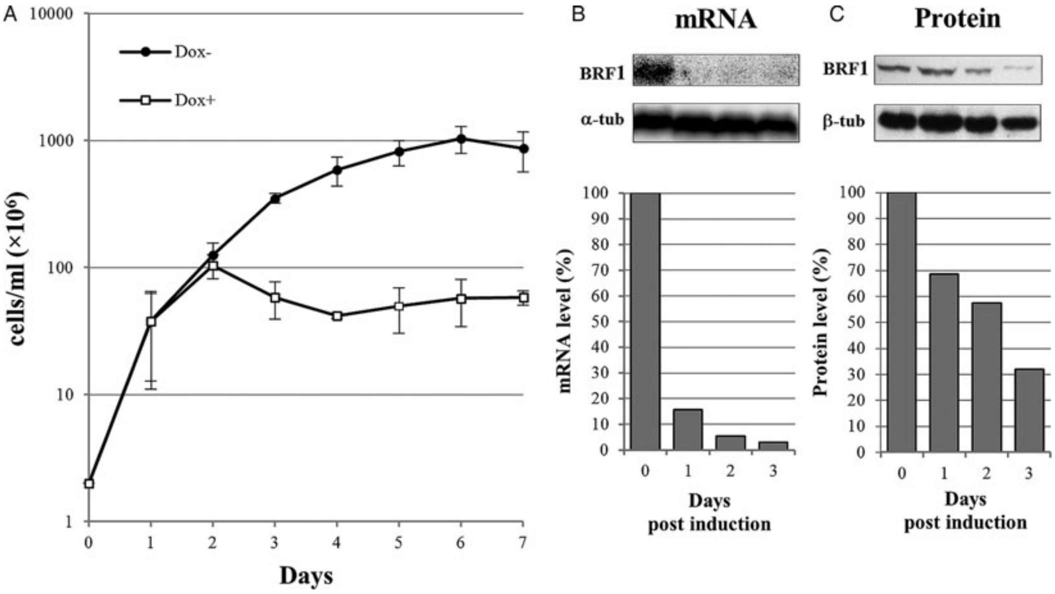

Fig. 3. TbBRF1 is essential for cell growth of procyclic forms of *Trypanosoma brucei*. (A) Growth curve of a clonal cell line under non-induced (Dox−) and doxycycline-induced (Dox+) conditions. Cells were counted and diluted daily to a density of 2×10^6 cells mL^{-1}. The values represent the cumulative cell density multiplied by the dilution factor. Data points reflect the means of triplicate experiments. Standard deviation bars are shown. (B) Northern blot analysis of TbBRF1 mRNA in non-induced cells (0 days), and cells induced for 1, 2 or 3 days. The bands shown here and from an independent experiment were quantified and plotted, considering as 100% the RNA level obtained in the non-induced culture. Values represent means of the two experiments. TbBRF1 mRNA levels were normalized to the level of the α-tubulin mRNA (loading control). (C) Western blot analysis of TbBRF1 protein in non-induced cells (0 days), and cells induced for 1, 2 or 3 days using the specific anti-TbBRF1r polyclonal antibody at 1:1000 dilution. The bands shown here and from an independent experiment were quantified and plotted, considering as 100% the protein level obtained in the non-induced culture. Values represent means of the two experiments. TbBRF1 protein levels were normalized to the level of the β-tubulin protein (loading control).

To further analyse the abundance of different transcripts in TbBRF1 RNAi cultures, qPCR experiments were performed with total RNA from cultures induced for 24 and 48 h (Fig. 5). As expected, a strong reduction in the abundance of tRNA-Arg and tRNA-Ala was observed 48 h post-induction. By contrast, the abundance of the mRNAs from TFIIB, α-tubulin, Elp3b and Tb927.9.2780 was not affected in the knock-down cultures. Procyclin's mRNA abundance was not affected either. Thus, it is likely that the observed signal reduction for α-tubulin and Elp3b in the nuclear run-on assay represents an indirect effect of the ablation of TbBRF1, resulting from the reduced synthesis of Pol III transcripts needed for translation and trans-splicing.

DISCUSSION

The results presented here indicate that TbBRF1 possesses all the sequence and structural attributes that are present in the BRF1 orthologues from other organisms. For instance, the N-terminal region of TbBRF1 contains a zinc ribbon domain and two cyclin repeats, as reported for other BRF1 orthologues and for TFIIB. Zinc ribbon motifs in the BRF1-TFIIB family contain the sequence $C–X_2–C/H–X_{15–17}–C–X_2–C$ (Hahn and Roberts, 2000). In trypanosomatids, the BRF1 orthologues possess the sequence $C–X–H–X_{15}–C–X_2–C$, which is a well-conserved domain, except for the fact that C and H are separated by only one amino acid in the first part of the motif (Fig. 1A). In contrast, the sequence of the zinc-binding region in TFIIB from *T. brucei* (TbTFIIB) is identical to the consensus ($C–X_2–C–X_{16}–C–X_2–C$) (Palenchar *et al.* 2006; Schimanski *et al.* 2006). It has been shown that the TFIIB zinc ribbon is necessary for Pol II recruitment into the preinitiation complex (Buratowski and Zhou, 1993). Nevertheless, the BRF1 zinc-binding domain is not needed for Pol III recruitment, but instead is required for promoter opening (Hahn and Roberts, 2000). Thus, although zinc ribbons in TFIIB and BRF1 have similar sequence and structure, they are involved in different functions.

It is noteworthy that TbBRF1 possesses two canonical copies of the cyclin repeat, considering that in TbTFIIB the second repeat is atypical (Palenchar *et al.* 2006; Schimanski *et al.* 2006; Ibrahim *et al.* 2009). It is plausible that TbTFIIB second module and linker region, which folds into an α-helix structure instead of a random-coil structure, have evolved to set specific characteristics

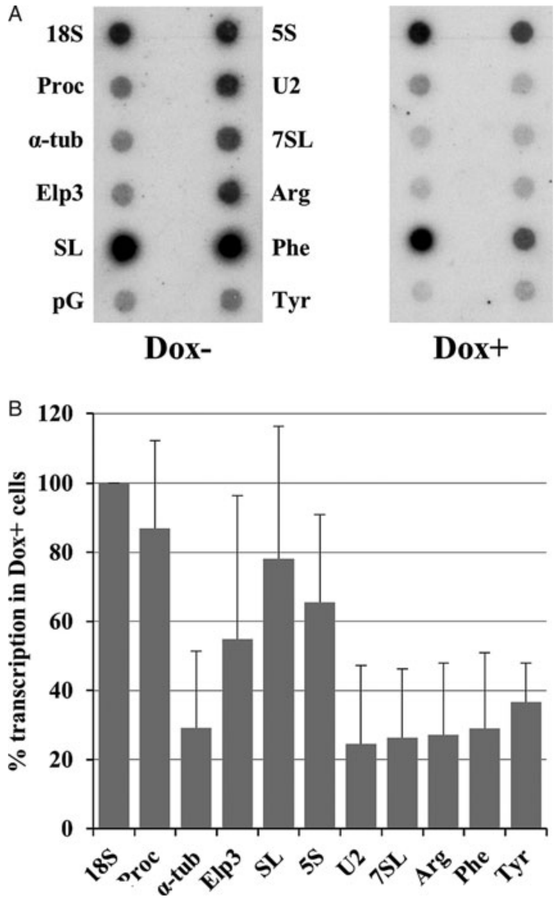

Fig. 4. Effect of TbBRF1 depletion on Pol III transcription. (A) Nuclear run-on assays carried out with nuclei isolated from *Trypanosoma brucei* cultures that were either induced with doxycycline for 2 days (Dox+) or non-induced (Dox−). Labelled nascent RNA was hybridized to dot blots of double-stranded DNAs (2 μg) cloned into pGEM-T Easy. The Pol III genes analysed were: 5S rRNA (5S), U2 snRNA (U2), 7SL RNA (7SL), tRNA-Arg (Arg), tRNA-Phe (Phe) and tRNA-Tyr (Tyr). 18S rRNA (18S) and procyclin (Proc), transcribed by Pol I, were also analysed. Also, three genes transcribed by Pol II were included: α-tubulin (α-tub), Elp3b (Elp3) and spliced-leader RNA gene (SL). As control, an empty vector was also analysed (pG). (B) Signals obtained for each gene in panel (A), and from two more independent experiments, were quantified and plotted, considering as 100% the signal obtained in the Dox− experiment. Values represent means of the three experiments. Standard deviation bars are shown. All RNA levels were normalized to the level of the 18S rRNA.

required to participate in Pol II transcription in trypanosomatids, which is unusual in different aspects (Gunzl *et al*. 2007; Das *et al*. 2008). The two cyclin repeats present in TbBRF1 are 21·5% identical (Fig. 1A). This identity is higher than the one observed between both BRF1 repeats from *H. sapiens* (15%) and *S. cerevisiae* (14%). Homology modelling suggested that the TbBRF1 cyclin repeats show the characteristic structure of five α-helices per repeat, connected by a short random-coiled linker. Interestingly, TbBRF1 also contains a BRF1 homology block I, which is conserved among yeast species and human (Fig 1A).

Since the function of BRF1 is regulated by phosphorylation of specific amino acids (Felton-Edkins *et al*. 2003), we performed an *in silico* search for potential phosphorylation sites in TbBRF1. We found several potential amino acids, including a threonine located in the first cyclin repeat (T-131) that is conserved in all the species analysed (Fig. 1A, and data not shown), and that in *S. cerevisiae* is phosphorylated by the mitogen-activated protein kinase ERK (Felton-Edkins *et al*. 2003). The presence of this putative phosphorylation site in TbBRF1 suggests that, similarly to other organisms, in *T. brucei* the function of TFIIIB is regulated by phosphorylation.

Ablation of TbBRF1 by RNAi showed that this protein is essential for the viability of procyclic forms of *T. brucei* (Fig. 3). And nuclear run-on analysis demonstrated that transcription of tRNAs, U2 snRNA and 7SL RNA was strongly reduced (to ~24–36% of the control value) after RNAi induction. Intriguingly, 5S rRNA transcription was reduced to a lesser extent (to ~65% of the control value) (Fig. 4). The reduction in the abundance of tRNAs in the knock-down cultures was also shown by qPCR assays (Fig. 5). These experiments also demonstrated that the TFIIB mRNA remains unaffected in the BRF1 knockdown, in spite of the sequence similarity between BRF1 and TFIIB. Therefore, the observed effects on cell viability and Pol III transcription are due to TbBRF1 ablation.

Depletion of TbBRF1 seemed to reduce Pol II transcription of α-tubulin and Elp3b (Fig. 4), although the steady-state abundance of these and other mRNAs was not affected after RNAi induction (Fig. 5). Thus, it is possible that a reduced synthesis of Pol III transcripts needed for translation (rRNA 5S and tRNAs) and trans-splicing (snRNAs), resulted in an overall decrease in Pol II transcription, considering that changes in Pol III transcription drive alterations in mRNA translation and cell growth (Goodfellow and White, 2007; White, 2011; Moir and Willis, 2013). Indeed, a genome-wide study in *S. cerevisiae* showed that more than 4% of the Pol II-transcribed genes exhibited significant changes in expression levels in a slow-growing thermosensitive strain defective in Pol III transcription due to the presence of mutations in the BRF1 subunit (Conesa *et al*. 2005). Similar results were obtained with strains bearing mutations in the C160 subunit of Pol III and in two different subunits of TFIIIC, showing that a major remodelling of genome expression is evoked to allow yeast cells to adapt to defects in Pol III transcription (Conesa *et al*. 2005). Therefore, the observed reduction in transcription of α-tubulin and Elp3b is most likely a secondary effect caused by ablation of Pol III products.

In conclusion, our results show that Tb927.11.470 is indeed the BRF1 orthologue in *T. brucei*, since: (1)

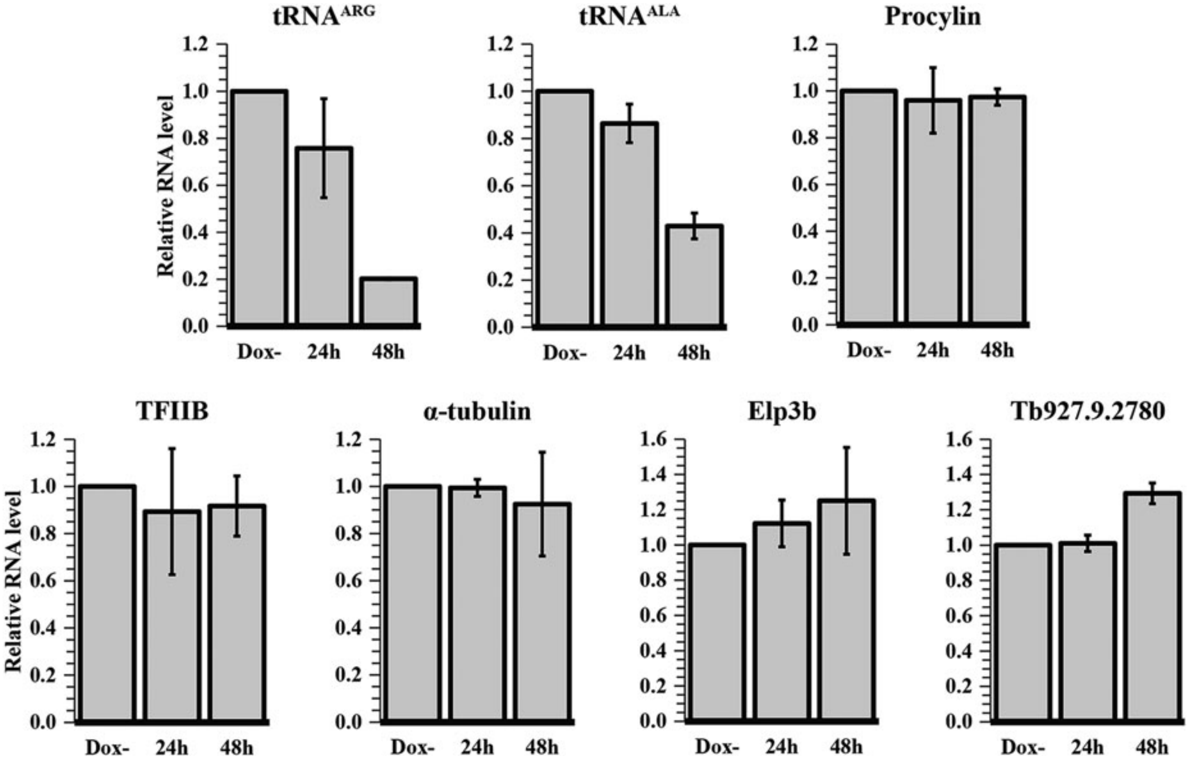

Fig. 5. Silencing of TbBRF1 affects abundance of Pol III transcripts. Quantitative real-time PCR analysis of total RNA from the induced (for 24 and 48 h) and non-induced (Dox−) TbBRF1 RNAi cultures. The analysis included cDNAs derived from two Pol III transcripts (tRNA-Arg and tRNA-Ala), one Pol I transcript (procyclin) and four Pol II transcripts (TFIIB, α-tubulin, Elp3b and Tb927.9.2780). All qPCR reactions were performed at least in triplicate, using primers and conditions that were optimized to produce a single amplicon of the correct size. Error bars indicate standard deviations.

it possesses the three typical BRF1 conserved sequences in the N-terminal half (a zinc ribbon motif and two imperfect cyclin repeats); (2) homology modelling indicates that the predicted structures of the N-terminal region for TbBRF1 and *H. sapiens* BRF1 show very similar architectures, with each cyclin repeat folded into five α-helices; (3) the C-terminal region of TbBRF1 contains the BRF1 homology block I, conserved in yeast species and human; (4) TbBRF1 localizes to the nucleus, as expected for a transcription factor; (5) similarly to BRF1 in yeast, TbBRF1 is essential for cell survival; (6) reduced transcriptional signals were observed for all Pol III-transcribed genes in the TbBRF1 knock-down cell line. Thus, the results presented here substantially increase our understanding of Pol III transcription in *T. brucei*. Future studies will help determine whether highly divergent orthologues for transcription factors TFIIIA and TFIIIC, which have not been identified in trypanosomatids, are present in this group of early branched eukaryotes.

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