The zinc finger protein *Tc*ZFP2 binds target mRNAs enriched during *Trypanosoma cruzi* metacyclogenesis

Patricia Alves Mörking[1], Rita de Cássia Pontello Rampazzo[1], Pegine Walrad[2], Christian Macagnan Probst[1], Maurilio José Soares[1], Daniela Fiori Gradia[1], Daniela Parada Pavoni[1], Marco Aurélio Krieger[1], Keith Matthews[2], Samuel Goldenberg[1], Stenio Perdigão Fragoso[1]/+, Bruno Dallagiovanna[1]

[1]Instituto Carlos Chagas-Fiocruz, Curitiba, PR, Brasil [2]Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

Trypanosomes are parasitic protozoa in which gene expression is primarily controlled through the regulation of mRNA stability and translation. This post-transcriptional control is mediated by various families of RNA-binding proteins, including those with zinc finger CCCH motifs. CCCH zinc finger proteins have been shown to be essential to differentiation events in trypanosomatid parasites. Here, we functionally characterise Tc*ZFP2 as a predicted post-transcriptional regulator of differentiation in* Trypanosoma cruzi. *This protein was detected in cell culture-derived amastigotes and trypomastigotes, but it was present in smaller amounts in metacyclic trypomastigote forms of* T. cruzi. *We use an optimised recombinant RNA immunopreciptation followed by microarray analysis assay to identify* Tc*ZFP2 target mRNAs. We further demonstrate that* Tc*ZFP2 binds an A-rich sequence in which the adenosine residue repeats are essential for high-affinity recognition. An analysis of the expression profiles of the genes encoding the* Tc*ZFP2-associated mRNAs throughout the parasite life cycle by microarray hybridisation showed that most of the associated mRNAs were upregulated in the metacyclic trypomastigote forms, also suggesting a role for* Tc*ZFP2 in metacyclic trypomastigote differentiation. Knockdown of the orthologous* Trypanosoma brucei *protein levels showed ZFP2 to be a positive regulator of specific target mRNA abundance.*

Key words: *Trypanosoma cruzi* - CCCH zinc finger protein - RNA-binding protein - cell differentiation

In eukaryotes, post-transcriptional regulation involves *cis* control elements, which are generally present in the untranslated regions (UTRs) of mRNAs and *trans*-acting factors, such as non-coding RNAs and RNA-binding proteins (RBPs). RBPs are major regulators of mRNA processing, transport, stability and translation and are classified according to their RNA-binding domains (Lunde et al. 2007). Proteins with zinc finger domains may interact either with other proteins or with nucleic acids. Zinc finger domains with CCCH motifs bind to RNAs with high affinity and are present in proteins that are involved in the post-transcriptional regulation of mRNAs (Auweter et al. 2006). RBPs of this class have been identified in trypanosomatid protozoan parasites, which have been shown to contain approximately 50 proteins with at least one zinc finger domain (Hendricks & Matthews 2007, Kramer et al. 2010).

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PAM and RCPR contributed equally to this work.
+ Corresponding author: sfragoso@fiocruz.br
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Several species of trypanosomatids cause various diseases in humans and domestic animals, with major impacts on human health and on the economies of developing countries. The implicated species include *Trypanosoma cruzi*, *Trypanosoma brucei* spp and *Leishmania* spp. *T. cruzi* is the causal agent of Chagas disease, which affects millions of people in the Americas. This parasite has a complex life cycle, with two hosts and at least four well-defined forms (de Souza 2002). Within the insect vector, the non-infectious replicative forms (epimastigotes) are converted into non-replicating infectious forms (metacyclic trypomastigotes) in a process called metacyclogenesis. This differentiation process can be mimicked in vitro through the use of well-defined chemical media (Contreras et al. 1985, 1988) and this system constitutes an excellent model for studying differential gene expression throughout the life cycle of the parasite.

Gene expression is controlled almost entirely by post-transcriptional regulation in trypanosomes (reviewed by Clayton & Shapira 2007). This regulation typically involves polycistronic transcription, mRNA maturation by *trans*-splicing and the editing of mitochondrial transcripts. No canonical RNA pol II promoters have been described and a clear case in which transcription initiation is regulated for this polymerase has yet to be reported. Moreover, genes that are present in the same polycistronic unit and transcribed as a single pre-mRNA display different patterns of expression, suggesting that regulation occurs during the later stages of mRNA metabolism. Post-transcriptional regulation in trypano-

somes has been reported to primarily involve the control of mRNA stability and translation. The exosome and deadenylation complexes have been identified as the primary regulators of transcript stability via several different degradation pathways (reviewed by Clayton & Shapira 2007, Haile & Papadopoulou 2008). In *T. cruzi,* RBPs of the RRM type are involved in the stage-specific regulation of mucin surface proteins, which bind to the ARE-like elements in the 3' UTRs of mRNAs (D'Orso et al. 2003). Translational regulation has also been described in trypanosomes and may involve a number of mechanisms, including the control of elongation or termination (Nardelli et al. 2006) and the differential mobilisation of transcripts to polysomes (Ávila et al. 2003, Walrad et al. 2009). RBPs associate together in macromolecular structures called ribonucleoprotein particles (RNPs), which may also form complex structures, such as various types of RNA granules. The processing bodies and stress granules that are generated in response to stress conditions have been described for *T. cruzi* (Cassola et al. 2007) with different dynamics at different stages of the parasite life cycle (Holetz et al. 2007).

A subfamily of small RBPs with a single CCCH zinc finger motif has been described in trypanosomes. In *T. brucei,* three different proteins from this subfamily (*Tb*ZFP1, *Tb*ZFP2 and *Tb*ZFP3) have been described and are involved in cell differentiation. *Tb*ZFP1 displays stage-specific expression, whereas *Tb*ZFP2 and *Tb*ZFP3 are constitutively expressed (Hendricks et al. 2001, Hendricks & Matthews 2005, Paterou et al. 2006). Knockout studies and RNAi assays have shown that these proteins are important for the progression of the parasite life cycle (Hendricks et al. 2001, Hendricks & Matthews 2005, Paterou et al. 2006). Immunoprecipitation assays have shown that *Tb*ZFP3 associates with procyclin transcripts, modulating their translation (Walrad et al. 2009). We previously identified protein *Tc*ZFP1 in *T. cruzi* and isolated two orthologues of the *T. brucei Tb*ZFP2 and *Tb*ZFP3 proteins (Mörking et al. 2004). The biochemical properties of these proteins were subsequently analysed in vitro (Caro et al. 2005). Interestingly, it has been shown that in both *T. brucei* and *T. cruzi,* these small ZFP proteins interact both in vivo and in vitro (Caro et al. 2005, Paterou et al. 2006), which suggests that the proteins may regulate similar biological processes in a coordinated manner. Supporting this theory, the *Tb*ZFP1-interacting domain of *Tb*ZFP3 is essential for protein function and polysomal association (Paterou et al. 2006).

Here, we examine the function of *Tc*ZFP2 in *T. cruzi* and its role as a post-transcriptional regulator that promotes *T. cruzi* differentiation to the human infectious metacyclic trypomastigote forms. We used a variation of RNA immunoprecipitation followed by microarray analysis (RIP-CHIP) that uses the recombinant RBP to identify mRNA targets (rRIP-CHIP) (Townley-Tilson et al. 2006). We further demonstrate that *Tc*ZFP2 binds an A-rich sequence for which the adenosine residue repeats are essential for high-affinity recognition. Knockdown of the orthologous *T. brucei* protein levels shows ZFP2 to be a positive regulator of specific target mRNA abundance.

MATERIALS AND METHODS

Parasite culture and transfection - T. cruzi culture - T. cruzi clone Dm28c (Contreras et al. 1985) was used throughout this study. Epimastigotes of *T. cruzi* Dm28c were cultured at 28ºC in liver infusion tryptose (LIT) medium supplemented with 10% bovine foetal serum. The culture was initiated by adding 5 x 10^5 1 x 10^6 cells mL^{-1} and the parasites were harvested when the culture reached a cell density of 1-2 x 10^7 cells mL^{-1} (log-phase parasites).

To obtain metacyclic trypomastigotes, *T. cruzi* epimastigotes were allowed to differentiate under chemically defined conditions (TAU3AAG medium) as previously described (Bonaldo et al. 1988, Contreras et al. 1988). Briefly, epimastigotes in the late exponential growth phase were harvested from LIT medium by centrifugation and were subjected to nutritional stress for 2 h in triatomine artificial urine (TAU) (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 8 mM sodium phosphate buffer, pH 6.0) at a density of 5 x 10^8 cells mL^{-1}. The epimastigotes were subsequently used to inoculate cell culture flasks containing TAU3AAG (TAU supplemented with 50 mM sodium glutamate, 10 mM L-proline, 2 mM sodium aspartate and 10 mM glucose) at a density of 5 x 10^6 cells mL^{-1} at 28ºC. Metacyclic trypomastigotes were purified by DEAE-51 chromatography from the TAU3AAG culture supernatant after 72 h of incubation.

To obtain cell-derived trypomastigotes, metacyclic trypomastigote forms were collected as described above and were used to infect Vero cells. The Vero cells were grown in RPMI medium supplemented with 5% bovine foetal serum (Invitrogen, Carlsbad, CA, USA), 100 UI/mL penicillin, 10 µg/mL streptomycin and 2 mM glutamine at 37ºC in an atmosphere of 5% CO_2 until the cells reached 50-70% confluence. The cell monolayer was subsequently infected with metacyclic trypomastigotes (150 parasites for 1 host cell). After 24 h, the medium was discarded to remove the parasites in the supernatant. The cells were then washed once with RPMI and new medium was added to the culture flask. Cell-derived trypomastigotes were released into the supernatant four days after infection and were harvested by centrifugation at 5,000 *g* for 10 min.

Culture amastigotes were obtained by disrupting the Vero cells 10 days after the culture was infected with metacyclic trypomastigotes as described above. The amastigotes were harvested by centrifugation at 1,000 *g* for 5 min.

T. brucei culture - Procyclic form *T. brucei* Lister 427 trypanosomes engineered for *Tb*ZFP2-TY ectopic expression and *Tb*ZFP2 RNAi have been described previously (Hendricks et al. 2001). These trypanosomes were regenerated by Amaxa nucleofection and were selected and cultured in SDM-79 media as previously described (Paterou et al. 2006).

The cloning and production of the recombinant protein and antibodies - The *Tczfp2* gene (GenBank accession ABW69369) was amplified by polymerase chain reaction (PCR) using the primers *Tc*ZFP2f (GGGGGGATCCATGTCCTACCCGAATCGTTA) and *Tc*ZFP2r

(GGGGAAGCTTTCACTGGGTCTGTGCGGGCA) and was subsequently inserted into a pQE30 plasmid (Qiagen, Germany) digested with BamHI and HindIII restriction enzymes. The restriction enzyme sites are underlined, whereas the start and stop codons are in bold font. The resulting construct was used to transfect *Escherichia coli* M15. The recombinant protein was purified under native conditions using Ni-NTA resin (Qiagen, Germany) according to the manufacturer's protocol. A polyclonal antiserum (anti-*Tc*ZFP2) was raised in BALB/c mice through immunisation with the *Tc*ZFP2 recombinant protein.

Gel shift assays - Binding reactions and an electrophoretic mobility shift assay (EMSA) were performed as previously described (Mörking et al. 2004) with 0.5 ng of the appropriate radioactively labelled oligonucleotides (10,000 cpm). The binding reactions were performed by adding 500 ng of the recombinant *Tc*ZFP2 protein to the reaction mixture under the same conditions.

Oligoribonucleotides were obtained from Qiagen Operon (Supplementary data). The oligonucleotides were all end-labelled with T4 polynucleotide kinase (Roche, Switzerland) and [γ-^{32}P] ATP (Amersham Biosciences, England) as previously described (Mörking et al. 2004).

The apparent dissociation constants (K_d) of the ribonucleoprotein complexes of the recombinant *Tc*ZFP2 protein with different probes were determined by EMSA as previously described (Mörking et al. 2004).

RNA pull-down assay - For the recombinant protein pull-down assays, 200 pmol of recombinant His-tagged *Tc*ZFP2 protein were incubated with 90 µg of total RNA from epimastigotes in 500 µL of EMSA buffer (1 mM Tris-HCl pH 8.0, 1 mM KCl, 1 mM $MgCl_2$ and 1 mM DTT) at 4ºC for 1 h in the presence of heparin and spermidine as competitors. After the incubation, reaction mixtures were incubated with 200 µL of Ni-NTA resin (Qiagen, Germany) overnight at 4ºC. Bound and unbound RNA samples were recovered. The bound sample was washed with the same buffer three times. After washing, the RNA present in both of the fractions was purified.

RNA purification - RNA from the pull-down assays was purified with an RNeasy mini kit (Qiagen, Germany). Linearly amplified RNA (aRNA) was generated with the MessageAmp aRNA kit (Ambion, USA) according to the manufacturer's instructions. cDNA was synthesised from 1 µg of total or affinity-purified RNAs with random primers (USB, USA) and reverse transcriptase IMPROM II (Promega, USA) as recommended by the manufacturer.

Microarray analysis - The microarray was constructed with 70-mer oligonucleotides. Due to the hybrid and repetitive nature of the sequenced *T. cruzi* strain, all of the coding regions (CDS) identified in the genome (version 3) were retrieved and clustered by the BLASTClust program using the criteria of 40% coverage and 75% identity. For probe design, we used ArrayOligoSelector software (v. 3.8.1) with a parameter of 50% G+C content. We obtained 10,359 probes for the longest *T. cruzi* CDS of each cluster, 393 probes corresponded to the genes of an external group (*Cryptosporidium hominis*) and 64 spots contained only spotting solution (SSC 3X), resulting in a total of 10,816 spots. These oligonucleotides were spotted from a 50 µM solution onto poly-L-lysine-coated slides and were cross-linked with 600 mJ of ultraviolet irradiation. Each probe corresponding to a *T. cruzi* gene was identified according to the *T. cruzi* Genome Consortium annotation (genedb.org). We compared bound and unbound RNA, which were extracted from three independent pull-down assays in a dye-swap design that included four slides. Microarray images were analysed by Spot software (hca-vision.com/product_spot.html). The Limma package (Smyth 2004) was used for background correction using the normexp method, intra-slide normalisation by the printtiploess method and inter-slide normalisation by the quantile method. The results for the two intra-slide probe replicates were subsequently averaged. The pull-down results were averaged and probes displaying more than a two-fold difference between the bound and unbound fractions were selected.

Quantitative real time PCR (qRT-PCR) analysis - Total RNA from parental and transgenic procyclic cell lines was harvested and purified using Qiagen RNAeasy columns using on-column DNAse digestion. cDNA was generated using an oligo(dT_{18}) primer for reverse transcription and subsequent qRT-PCR reactions were performed as previously described (Walrad et al. 2009). The actin cDNA was amplified as previously described (Walrad et al. 2009). The primers used to amplify the *T. brucei* orthologs of *Tc*ZFP2-associated transcripts are shown in Supplementary data.

Ethics - The animal experiments were approved by the Ethical Committee on Animal Experimentation of the Oswaldo Cruz Foundation (protocol P-0434/07).

RESULTS

*Tc*ZFP2 is a cytoplasmic protein displaying reduced expression in the metacyclic stage.

His-tagged *Tc*ZFP2 recombinant protein (Fig. 1) was used to raise a polyclonal antiserum in mice. On Western blots of cell extracts from epimastigotes, we detected a band approximately 20 kDa in size that corresponded to the expected molecular mass of *Tc*ZFP2. This protein was also detected in cell culture-derived amastigotes and trypomastigotes, but it was present in lesser amounts in metacyclic trypomastigote forms (Fig. 2A). A detailed analysis of the metacyclogenesis process revealed that *Tc*ZFP2 was present at similar levels in different parasites and that it was present in lesser amounts only in metacyclic forms (Fig. 2B).

The identification of putative TcZFP2-associated mRNAs - We used an optimised version of the RIP-CHIP to identify the transcripts bound by *Tc*ZFP2 (Townley-Tilson et al. 2006). The recombinant protein was bound to Ni-NTA-agarose resin and was then incubated with total RNA purified from epimastigote cells. RNA-protein complexes were allowed to form in vitro and RNA was purified from the bound and flow-through fractions and then amplified. The resulting cRNAs were labelled and hybridised to a *T. cruzi* oligonucleotide microarray. We conducted three independent pull-down assays, each of which was followed by microarray hybridisation. As

a negative control, we conducted a mock assay in which no recombinant protein was added and an independent assay using a recombinant GST protein. No RNA was detectable in the bound fractions of both assays, even after amplification; thus, no microarray hybridisations were performed (not shown). A *T. cruzi* oligonucleotide microarray was analysed by competitive hybridisation using the non-bound fraction as the reference population in dye-swap assays.

Enrichment was observed for 223 genes among the three independent assays (Supplementary data). It has been suggested that RBPs also regulate the function of the associated mRNAs in trypanosomes (Noé et al. 2008, Kabani et al. 2009, Ouellette & Papadopoulou 2009). Therefore, we looked for functional relationships between the identified mRNAs. An analysis of the enriched genes showed that most genes (178) were annotated as "hypothetical proteins" in the *T. cruzi* GeneDB. We found mRNAs encoding proteins involved in RNA processing and transport, cytochrome B complex proteins and dynein-associated proteins (Fig. 3, Supplementary data). Interestingly, the mRNA encoding the *Tc*ZFP2 protein was present in the pull-down fraction, suggesting that this protein may be regulating the expression of its own transcripts.

Several RBPs regulate their own transcripts through positive or negative feedback regulatory loops, which suggests that such autoregulatory mechanisms are characteristic of RBPs that are involved in controlling gene expression (Pullmann et al. 2007). Indeed, our results suggest that these mechanisms may be a conserved feature of RBPs in eukaryotes.

TcZFP2 binds an A-rich, short sequence in the 3'UTR of a target transcript - To confirm the RNA pull-down assay results, we conducted an EMSA using an RNA oligo

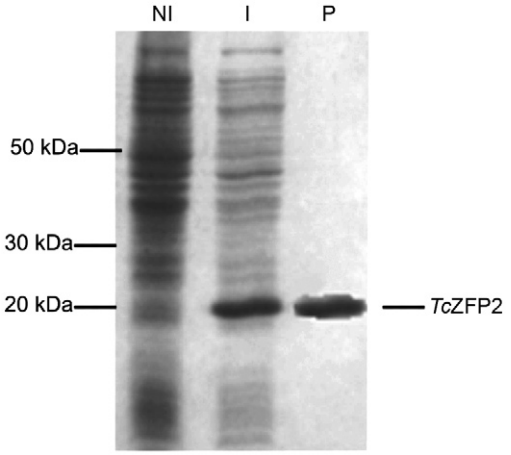

Fig. 1: *Tc*ZFP2 was expressed in *Escherichia coli* and purified by Ni-NTA affinity chromatography. Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Coomassie Blue. Molecular weight markers (kDa) are indicated. I: IPTG-induced cultures; NI: cellular extracts from non-induced; P: purified protein.

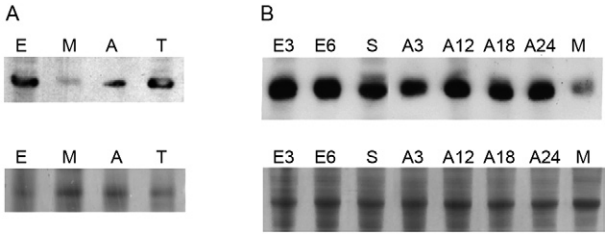

Fig. 2: *Tc*ZFP2 production is downregulated in metacyclic trypomastigotes. A: we analyzed the profile of *Tc*ZFP2 production in *Trypanosoma cruzi* epimastigotes (E), metacyclic trypomastigotes (M), amastigotes (A) and trypomastigote forms (T); B: *Tc*ZFP2 levels during *T. cruzi* metacyclogenesis in vitro; E3: exponential phase; E6: stationary phase epimastigotes; M: metacyclic trypomastigotes; S: stressed parasites, parasites adhering to the plate for 3 h (A3), 12 h (A12), 18 h (A18) and 24 h (A24). Coomassie Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis gel containing the same protein extracts as used in A and B (lower panels).

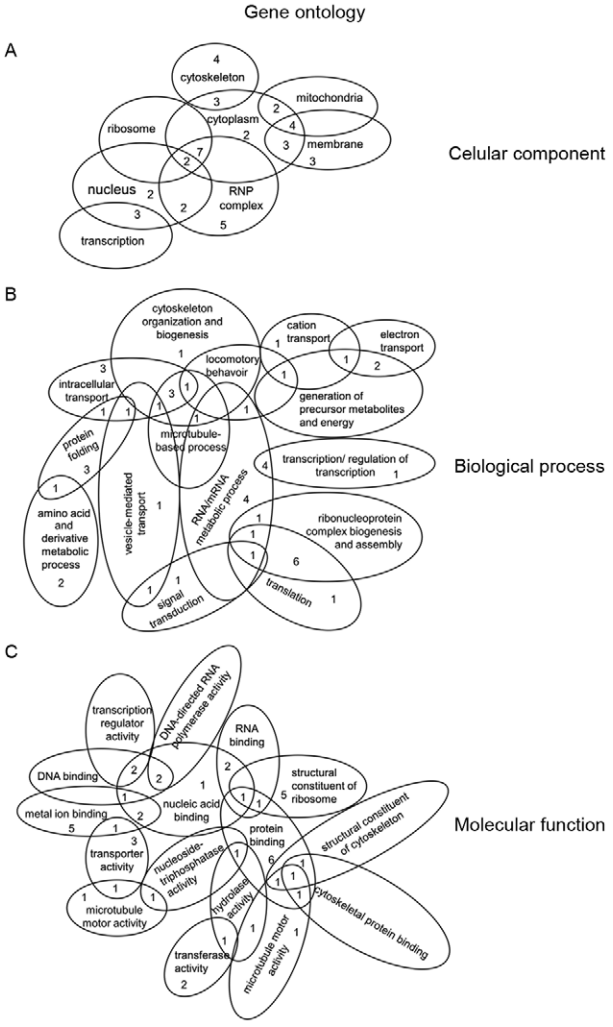

Fig. 3: gene ontology (GO) terms for the putative *Tc*ZFP2-regulated mRNAs. Venn diagram of overlaps between GO terms for the cellular components (A), molecular functions (B) and biological processes (C) of the annotated mRNAs bound by *Tc*ZFP2 in RNA immunopreciptation followed by microarray analysis assays. RNP: ribonucleoprotein particle.

(TUTR) containing the first 64 nt from the 3' UTR of the *Tc*ZFP2 mRNA as a probe (EST with GenBank accession CF889184.1) because this transcript was present in the pull-down fraction (Fig. 4A). The *Tc*ZFP2 recombinant protein formed a stable complex with the probe on EMSA (Fig. 4B, Lane 2). We subsequently conducted a more detailed analysis to identify the putative binding sequence of *Tc*ZFP2. Overlapping oligoribonucleotides spanning the entire putative UTR were synthesised and tested by EMSA (Fig. 4A). *Tc*ZFP2 formed a stable complex with the UTR4 probe (Fig. 4B, Lane 10). Competition assays using the TUTR as a probe and the five partial sequences

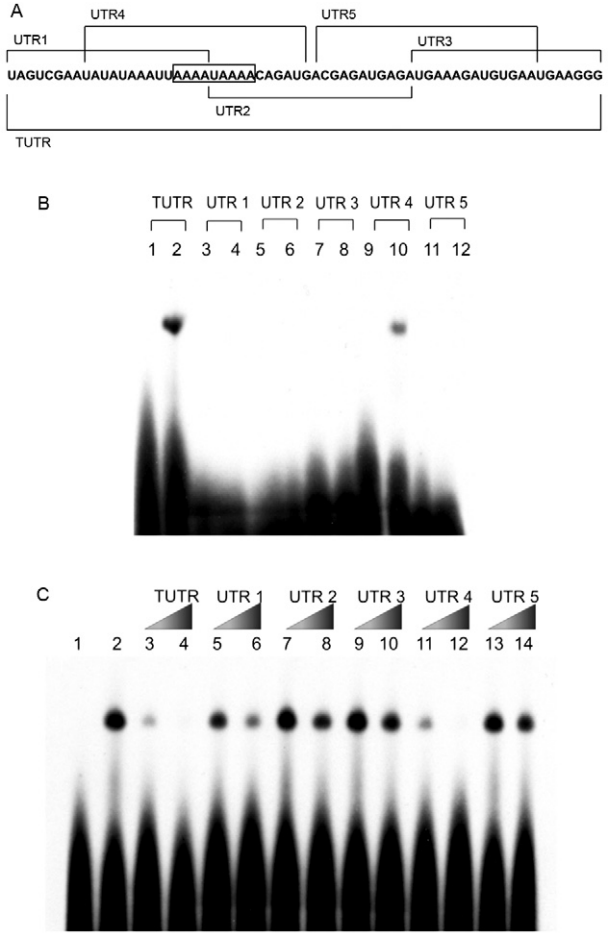

Fig. 4: the binding of *Tc*ZFP2 to the 3' untranslated region (UTR) of the *Tc*ZFP2 mRNA is highly sequence-specific. A: schematic diagram of the putative 3'UTR of the Tc*ZFP2* mRNA. This region was used as a template for the synthesis of oligoribonucleotides for electrophoretic mobility shift assay, as shown. Boxed motif shows the putative binding site; B: gel mobility shift assay to assess binding capacity. Free radiolabelled probes are shown in Lanes 1, 3, 5, 7, 9 and 11. Binding reactions with 500 ng of the recombinant *Tc*ZFP2 and 0.5 ng of the corresponding labelled probe (approx. 10.000 cpm) are shown in Lanes 2, 4, 6, 8, 10 and 12; C: specificity assays for the TUTR oligoribonucleotide. Effects on formation of the *Tc*ZFP2 + TUTR probe complex of a 1 (Lanes 3, 5, 7, 9, 11, 13) and 100-fold molar excess of unlabeled competitors (Lanes 4, 6, 8, 10, 12, 14). Free-labelled TUTR probe (Lane 1); Binding reaction for *Tc*ZFP2 + TUTR labelled probe (Lane 2).

as unlabelled competitors showed that only the UTR4 probe abolished the formation of *Tc*ZFP2-RNA complexes (Fig. 4C, Lanes 11, 12). A certain degree of competition was observed when high concentrations of the unlabelled UTR1 competitor were tested (Fig. 4C, Lane 6).

Based on the competition pattern, an analysis of the probe sequence suggested that the putative recognition element might be an A-rich sequence ($A_{1-4}UA_{1-4}$). We tested this hypothesis by synthesising oligoribonucleotide probes in which the central uridine base was replaced by a guanine (MUT1) (UAUAAAUUAAAAGAAAACAGAUG) or in which the central adenine residues of the quadruplet were replaced by cytosines (MUT2) (UAUAAUUACCAUACCACAGAUG). Binding assays revealed that *Tc*ZFP2 formed complexes with the UTR4 and MUT1 probes, yielding bands of similar intensities, whereas recognition of the MUT2 probe was less efficient (Fig. 5). We analysed the interaction between the recombinant *Tc*ZFP2 protein and the UTR4 and mutant probes in detail by studying the K_d of the ribonucleoprotein complexes observed. The recombinant *Tc*ZFP2 recognised the UTR4 probe with an apparent K_d of approximately 800 nM (Fig. 5A). Although *Tc*ZFP2 complexes were clearly formed at low protein concentrations, with both the UTR4 and the MUT1 probes (50 ng), quantitative binding affinity analysis revealed a two-fold difference in the affinities of the two probes. The K_d values obtained for the MUT1 probe were similar to those obtained for a poly(A) probe (2.300 and 2.000 nM, respectively) (Fig. 5B, 5D), suggesting that *Tc*ZFP2 recognises A-rich sequences, albeit with a lower affinity. In contrast, the affinity obtained for the MUT2 probe was almost 10 times lower than that of the specific probe (K_d 8.300 nM) (Fig. 5C).

Competition assays in which the complete UTR was used as a probe and the UTR4 and mutant oligomers were used as unlabelled competitors showed that the UTR4 and MUT1 probes competed efficiently (Fig. 6). We cannot exclude the possibility that *Tc*ZFP2 also recognises an RNA secondary structure, but our results strongly suggest that the target element is defined by the linear sequence. Competition assays using Mut2 showed that the A residues are essential for high-affinity binding. However, *Tc*ZFP2 also bound the A-rich Mut1 sequence with slightly lower affinity. These data provide valuable insight, but further comparative analysis of these sequences to the UTRs of other transcript targets is required to define the exact *Tc*ZFP2 recognition motif.

TbZFP2 genetic assays in T. Brucei - RNA interference was used to analyse changes in the levels of the *Tc*ZFP2-associated mRNAs and these assays were conducted using the *T. brucei* system because *T. cruzi* lacks the machinery required for RNA interference. Hence, the orthologous gene *Tb*ZFP2 was targeted by RNAi, with the assumption that there was conservation between the putative *Tc*ZFP2 targets of the two species.

When the *Tb*ZFP2 levels were analysed after RNAi, we noted considerable depletion of the *Tb*ZFP2 mRNA, although the leakiness of the tetracycline-inducible system was such that the uninduced cell lines exhibited comparable levels of ablation. Consistent with this, there were large decreases in *Tb*ZFP2 protein levels in both

induced and uninduced samples (Fig. 7A). Therefore, comparisons within this experiment (Fig. 7) were conducted using the uninduced *Tb*ZFP2 cell line and the parental PTT procyclic cell line. When the abundances of the predicted *Tb*ZFP2 target transcripts were assayed by qPCR (with *actin* and *TbPTP1* used as negative control transcripts), most abundances were considerably lower in response to reduced levels of *Tb*ZFP2 compared to the parental line (Fig. 7B).

In addition, the *Tb*ZFP2 protein was ectopically overexpressed with a C-terminal Ty1 tag. As before, levels of the putative *Tb*ZFP2-regulated transcripts were analysed by qRT-PCR, with *actin* and *TbPTP1* again used as negative control transcripts. The levels of mRNA for the putative target genes displayed no significant variations upon *Tb*ZFP2 ectopic overexpression (Fig. 8).

We looked for the presence of the $A_{1-4}UA_{1-4}$ element in the putative 3'UTRs of the *T. brucei* transcripts. All of the transcripts analysed contained the putative binding motif. These results suggest that *Tb*ZFP2 targets similar transcript pools to *Tc*ZFP2 and that *Tb*ZFP2 acts as a positive regulator, such that when it is depleted, the abundance of target mRNAs decreases.

TcZFP2 target mRNAs are upregulated in infectious metacyclic forms - We analysed the expression profiles of the genes encoding the *Tc*ZFP2-associated mRNAs throughout the parasite life cycle by microarray hybridisation. Most of the associated mRNAs were upregulated in the metacyclic trypomastigote forms (Fig. 9), which suggests that *Tc*ZFP2 plays a role in metacyclic trypomastigote differentiation.

DISCUSSION

Over the last 10 years, major efforts have been devoted to the identification of the transcripts bound and regulated by RBPs and the characterisation of the gene regulatory networks controlled by them (Sanchez-Diaz & Penalva 2006). The ribonomic approach is based on the genome-wide identification of mRNAs associated with RBPs (Tenenbaum et al. 2002). This approach has been used to identify the putative mRNA targets of the *T. brucei* PUF family (Luu et al. 2006) and an RRM-type protein (Estevez 2008). In *T. cruzi,* we have identified mRNAs associated with TcPUF6, which is an RBP of the PUF family, by TAP-TAG purification followed by microarray hybridisation (Dallagiovanna et al. 2008). Here, we used a modified version of the RIP-CHIP assay to identify the transcripts bound by *Tc*ZFP2. We simplified the assay by binding the recombinant protein directly to a Ni-NTA-agarose resin, overcoming the need for an immunoprecipitation step. This modification made it possible to identify the putative target transcripts forming complexes with *Tc*ZFP2.

It has been suggested that RBPs also regulate the function of the associated mRNAs in trypanosomes (Noé et

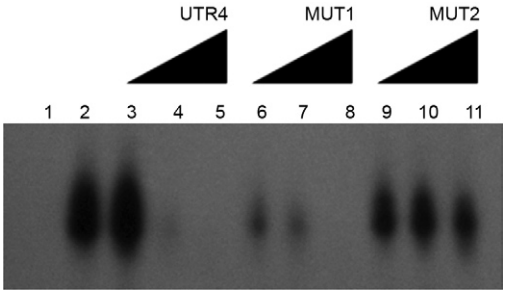

Fig. 6: competition assays with the mutated ribonucleotides. Lane 1: TUTR free radiolabelled probe (approx. 10.000 cpm); 2: binding reaction with 500 ng of the recombinant *Tc*ZFP2; 3-5: untranslated region (UTR)4-ribonucleotide was added as an unlabelled competitor in a 1, 10 or 100-fold molar excess, respectively, and incubated at room temperature for 10 min prior to the addition of the labelled probe. Same with the MUT1 (Lanes 6-8) and MUT2-ribonucleotide (Lanes 9-11). Gels were pre-run at 200V at 4ºC and then run with the samples for 3-4 h at 250 V at 4°C.

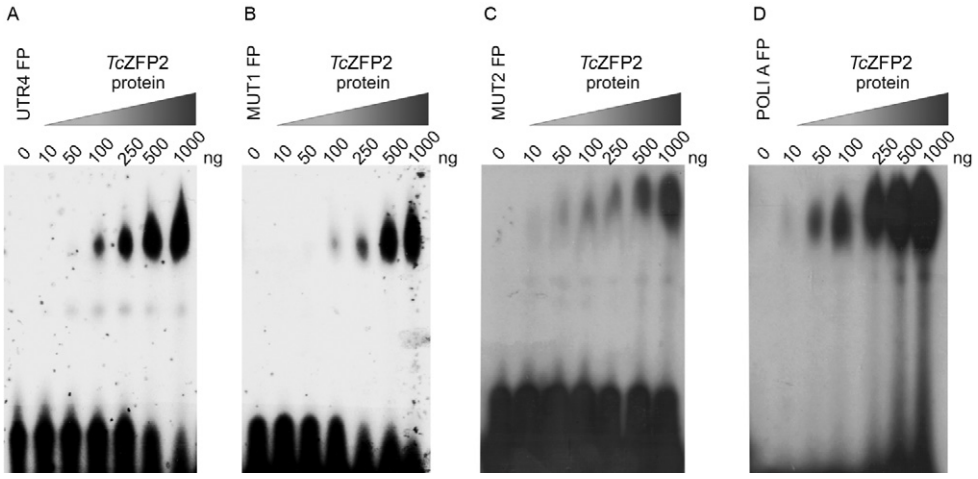

Fig. 5: affinity analysis of the complexes formed by the recombinant *Tc*ZFP2 and the oligoribonucleotides. Binding reactions with the untranslated region (UTR)4 (A), MUT1 (B), MUT2 (C) and POLI A (D) probes were carried out with various amounts of the recombinant *Tc*ZFP2 protein and 0.5 ng of the labelled probe (approx. 10.000 cpm). Lanes: binding reactions with 10, 50, 100, 250, 500 and 1.000 ng of protein, respectively. FP: free probe.

al. 2008, Kabani et al. 2009, Ouellette & Papadopoulou 2009). Therefore, we looked for functional relationships between the identified mRNAs. Most of the transcripts encoded hypothetical proteins, but some of these proteins were nuclear-encoded mitochondrial proteins, motor proteins or proteins involved in RNA transport. Interestingly, *Tc*ZFP2 bound its own transcript. Several RBPs regulate their own transcripts through positive or negative feedback regulatory loops. Pullmann et al. (2007) suggested that such autoregulatory mechanisms are characteristic of RBPs that are involved in controlling gene expression. Indeed, our results suggest that these mechanisms may be conserved features of RBPs in eukaryotes.

We defined the putative 3'UTR of the *Tc*ZFP2 transcript by searching the EST database. We subsequently used this sequence to confirm binding on EMSA. *Tc*ZFP2 was found to bind with high specificity to an A-rich region in the 3'UTR sequence. We cannot exclude

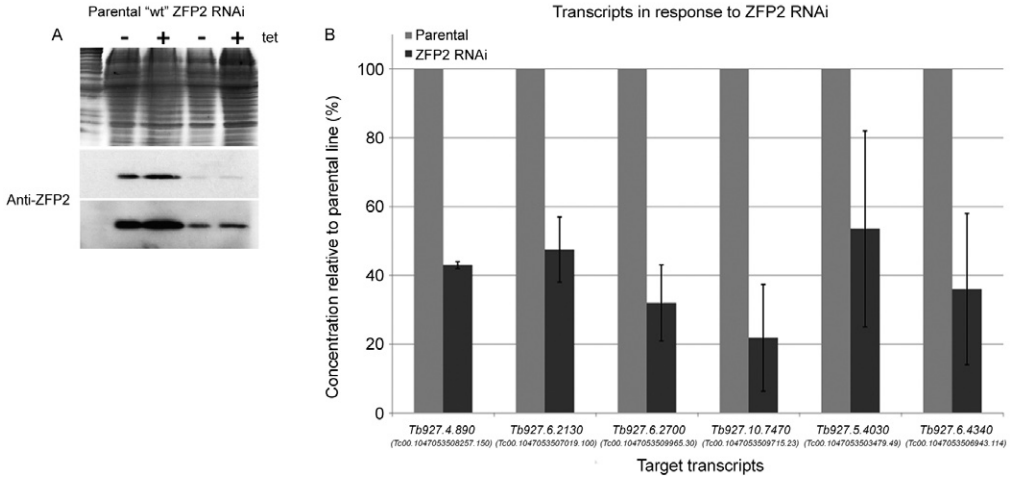

Fig. 7: *Tb*ZFP2 knockdown specifically reduces levels of putative targets. A: *Tb*ZFP2 protein levels in parental vs. tetracycline-induced or uninduced p2T7i-*TbZFP2* PTT procyclic *Trypanosoma brucei* cells. The Coomassie-stained gel indicates relative loading, the middle image shows *Tb*ZFP2 protein levels and the lower image represents a longer exposure of the same blot. Since no dramatic change was observed in the levels of *Tb*ZFP2 in either cell line in response to tetracycline, total RNA was harvested from parental *T. brucei* PTT cells and p2T7i-*TbZFP2* PTT procyclic cells, both in the absence of tetracycline; B: identities and relative concentrations of the syntenic *T. brucei* orthologs of the *Tc*ZFP2-associating transcripts in response to reduced levels of *Tb*ZFP2. The concentrations represent the relative percentages of each transcript normalized to actin transcripts in each sample. Bars indicate standard deviation.

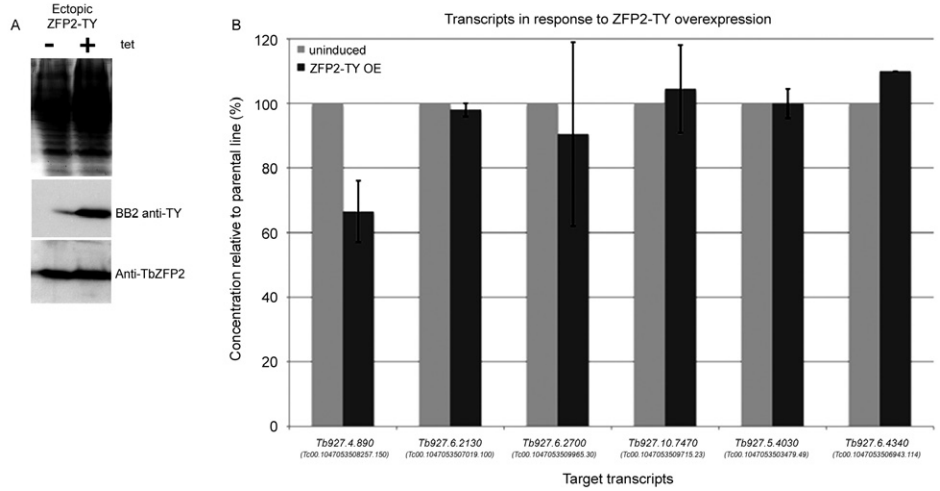

Fig. 8: *Trypanosoma brucei* transcript levels in response to *Tb*ZFP2 overexpression. A: *Tb*ZFP2 protein in uninduced vs. Tetracycline-induced pHD451-ZFP2-TY procyclic *T. brucei* cells. Coomassie-stained gel indicates relative loading. Upper film indicates relative ectopic *Tb*ZFP2-TY expression. BB2 monoclonal antibody recognizes the Ty1 motif [provided by Prof Keith Gull (Oxford)]. This antibody therefore recognizes only the ectopic *Tb*ZFP2-TY and allowed us to confirm the inducible overexpression of *Tb*ZFP2. Lower film indicates relative levels of endogenous *Tb*ZFP2. As demonstrated previously, anti-*Tb*ZFP2 antibody does not recognize *Tb*ZFP2-TY (Hendriks et al. 2001); B: identities and relative concentrations of *T. brucei* homologs of *Tc*ZFP2-associating transcripts in response to ectopic *Tb*ZFP2-TY induction. Concentrations represent relative percentages normalized to actin. Bars indicate standard deviation.

the possibility that *Tc*ZFP2 also recognises an RNA secondary structure, but our results strongly suggest that the target element is defined by the linear sequence. Competition assays showed that the U residue in the central position was essential for high-affinity binding. However, *Tc*ZFP2 also bound A-rich sequences with slightly lower affinity, thereby increasing the number of putative targets. Further analysis is required to define the exact recognition motif.

There is strong evidence to suggest that post-transcriptionally regulated operons exist in trypanosomatid parasites. Metabolically related genes display tight co-regulation in trypanosomes (Mayho et al. 2006, Noé et al. 2008, Queiroz et al. 2009). Developmentally regulated RNA regulons have been identified in both *T. brucei* and *T. cruzi* (Dallagiovanna et al. 2008, Queiroz et al. 2009). Intriguingly, *Tc*ZFP2 levels are lower in metacyclic cells. This finding appears to be incompatible with

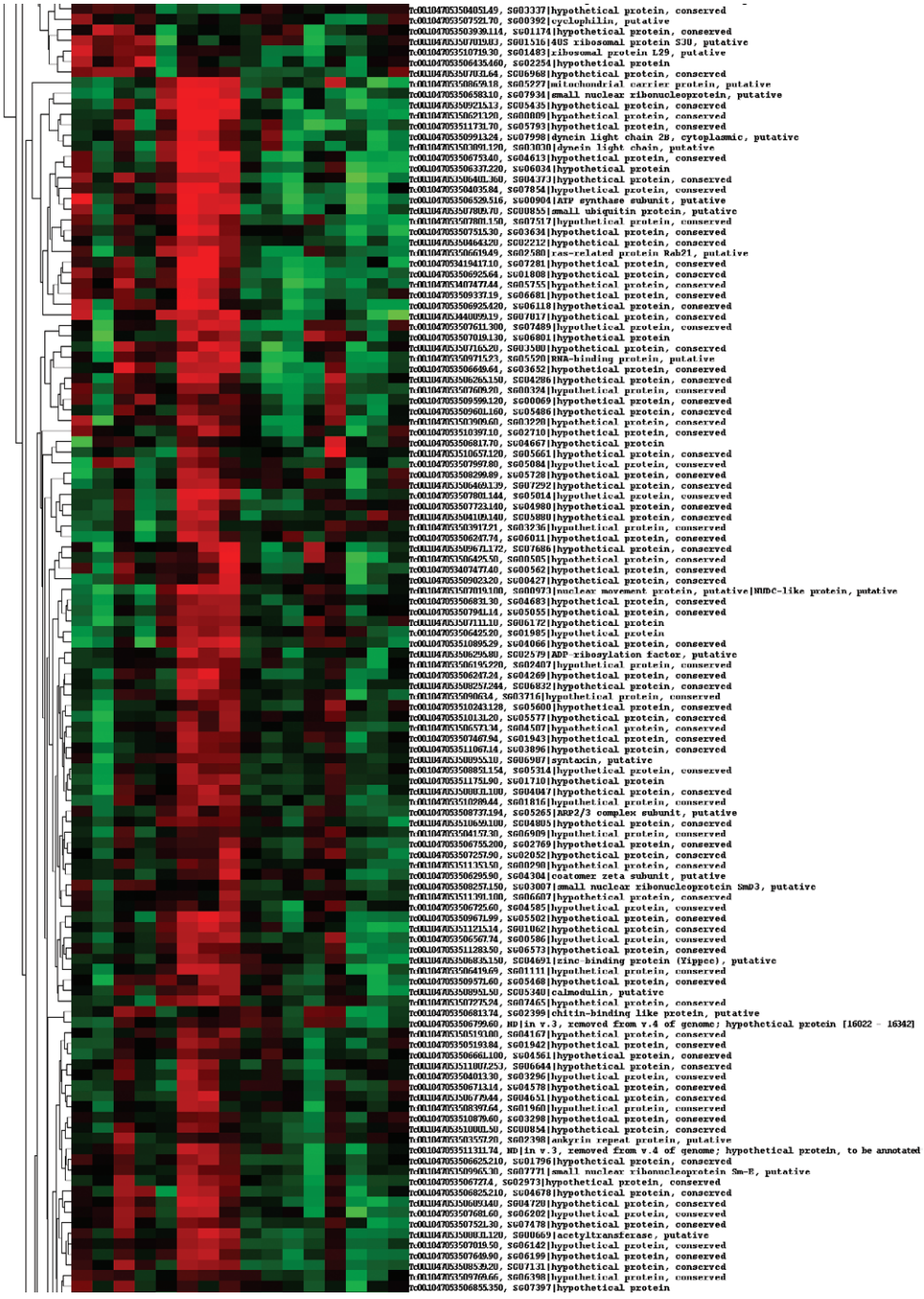

Fig. 9: *Tc*ZFP2-associated mRNAs are upregulated in metacyclic forms. Microarray analysis of the polysomal RNA fraction during differentiation showed that the mRNAs bound by *Tc*ZFP2 were produced preferentially in metacyclic forms. Each column represents an independent experiment. Ama: amastigotes forms; Epi: epimastigotes; Met: metacyclic trypomastigotes; Trp: cell-derived trypomastigotes.

the mRNA-stabilising function proposed. RNA PolII activity has been observed to be much weaker in infectious forms (at least an order of magnitude lower than in non-infectious forms), which results in lower levels of total mRNA in the cell (Queiroz et al. 2009). Therefore, we can also assume that smaller amounts of the regulator protein are required to obtain a positive effect. Our data for *T. brucei* support this assumption because the *Tb*ZFP2 knockdown reduces the abundance of associating transcripts. The fact that overexpression does not significantly affect the same targets could simply indicate that the levels in the cell are sufficient to ensure mRNA stability. Hence, depletion decreases stability, but overexpression does not push stability beyond already maximal levels.

It is possible that *Tc*ZFP2 is in a competitive equilibrium with a putative negative regulator in epimastigotes. This negative regulator may be absent in metacyclic forms, resulting in the observed transcript stabilisation and upregulation. Another hypothesis is that *Tc*ZFP2 association with other protein complexes involving compartmentalisation or sequestration may be occurring during the metacyclic stage of *T. cruzi*. One clear example is *T. cruzi* PUF-6 (TcPUF6). In epimastigotes, TcPUF6 co-localises with the decapping activator TcDhh1, which supports the notion that TcPUF6 might target mRNAs for degradation. In contrast, TcPUF6 and TcDhh1 do not co-localise in metacyclic trypomastigote forms, which suggests the absence of interaction and, consequently, the upregulation of associated mRNAs in the metacyclic forms (Dallagiovanna et al. 2008).

Recently, a novel CCCH zinc finger protein (*Tb*ZC3H20) was shown to be enriched in insect procyclic forms of *T. brucei* and also to positively modulate the stability of its mRNA targets (Ling et al. 2011). The binding of the *Tb*ZC3H20 to poly(A) or the recruitment of distinct components of the translational machinery are among the mechanisms suggested to avoid the degradation of target mRNA.

In *T. brucei,* the knockdown of the orthologous *Tb*ZFP2 abolishes differentiation into the circulating forms found in the bloodstream (Hendricks et al. 2001). In addition, the related *Tb*ZFP1 protein is also involved in controlling kinetoplast segregation; this protein has been shown to interact with other members of this protein family (Hendricks & Matthews 2005, Paterou et al. 2006). Thus, one conserved function of these proteins in trypanosomes may be the regulation of kinetoplast segregation during differentiation. This phenotypic effect was dose-dependent, which reveals the need for a delicate protein balance to regulate the differentiation process. More recently, Walrad et al. (2011) performed a global survey of the mRNAs that co-associate with the *Tb*ZFP3 mRNP. The results showed that the selected mRNAs were stabilised by *Tb*ZFP3 and the associated transcripts were predominantly more abundant in the transmission stage of *T. brucei* for stumpy forms. This result implicates *Tb*ZFP3 mRNP as a trans-acting factor defining a regulon in these parasites, controlling the changes in gene expression that accompany the life-cycle development of the parasite. Together, these results demonstrate that ZFP2 and ZFP3 proteins have important roles in regulating trypanosome differentiation.

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Oligoribonucleotides used for electrophoretic mobility shift assay

Primer designation	Nucleotide sequence (5'-.....-3')
TUTR	UAGUCGAAUAUAUAAAUUAAAAUA AAACAGAUGACGAGAUGAGAUGAA AGAUGUGAAUGAAGGG
UTR1	UAGUCGAAUAUAUAAAUUAAAA
UTR2	UAAAACAGAUGACGAGAUGAGA
UTR3	UGAAAGAUGUGAAUGAAGGG
UTR4	UAUAAAUUAAAAUAAAACAGAUG
UTR5	ACGAGAUGAGAUGAAAGAUGUG
MUT1	UAUAAAUUAAAAGAAAACAGAUG
MUT2	UAUAAAUUACCAUACCACAGAUG

UTR: untranslated region.

Oligonucleotides used for quantitative real time-polymerase chain reaction

Trypanosoma brucei gene ID	Nucleotide sequence (5'-.....-3')
*Tb*927.4.890	ATGGGCGTGTTACTGACCTC TTCTTTGGCTTCTTACGGGA
*Tb*927.10.15150	TGCAATCGTTTCTCATGCTC CACTGGAAGCCACACTTCAAAC
*Tb*927.6.2130	AGGCATGAAGGGTCAATCAC CGGAGATATGTTTGCTCGGTG
*Tb*927.6.2700	TTAAGCCGACTGTTGTCGTG3 TGCCAATAGGGTGAATGACAC
*Tb*927.10.7470	GCGAATTCTCCTTGTAACGG CTAGCGCCCTACGATCTCTG
*Tb*927.5.4030	TGGATCGTCGTGTTGTTGTTTC GGGTTCTGTTGAAGGATTGC
*Tb*927.6.4340	GTTTCCTTTCCTTCTTGGGT TGGACACAACTGTCTGCCTC

*Tc*ZFP2 associated transcripts identified in the recombinant t RNA immunopreciptation followed by microarray analysis assay

Gene	Description	FC
Tc00.1047053505807.29	Hypothetical protein, conserved	4,515
Tc00.1047053506825.210	Hypothetical protein, conserved	4,360
Tc00.1047053507957.140	Trans-sialidase (pseudogene), putative	4,282
Tc00.1047053507165.20	Hypothetical protein, conserved	4,239
Tc00.1047053504069.110	Hypothetical protein, conserved	3,729
Tc00.1047053509055.54	Hypothetical protein, conserved	3,670
Tc00.1047053505193.80	Hypothetical protein, conserved	3,610
Tc00.1047053504625.74	Hypothetical protein, conserved	3,568
Tc00.1047053506213.20	Hypothetical protein, conserved	3,512
Tc00.1047053506529.516	ATP synthase subunit, putative	3,464
Tc00.1047053506893.40	Hypothetical protein, conserved	3,429
Tc00.1047053506691.64	Cytochrome C oxidase copper chaperone, putative	3,425
Tc00.1047053507163.90	Hypothetical protein, conserved	3,371
Tc00.1047053503925.90	Hypothetical protein, conserved	3,353
Tc00.1047053504001.20	Hypothetical protein, conserved	3,287
Tc00.1047053508831.120	Acetyltransferase, putative	3,264
Tc00.1047053509599.120	Hypothetical protein, conserved	3,260
Tc00.1047053511807.253	Hypothetical protein, conserved	3,246
Tc00.1047053506753.40	Hypothetical protein, conserved	3,226
Tc00.1047053506925.120	Eukaryotic initiation factor 5a, putative	3,201
Tc00.1047053506265.150	Hypothetical protein, conserved	3,195
Tc00.1047053508299.89	Hypothetical protein, conserved	3,175
Tc00.1047053506943.114	U6 snrna-associated Sm-like protein LSm5p	3,165
Tc00.1047053508231.64	Hypothetical protein, conserved	3,082
Tc00.1047053401469.10	Zinc finger protein 2, putative	3,080
Tc00.1047053509671.172	Hypothetical protein, conserved	3,078
Tc00.1047053510397.10	Hypothetical protein, conserved	3,076
Tc00.1047053506883.69	Hypothetical protein, conserved	3,067
Tc00.1047053503891.54	Nucleolar RNA-binding protein, putative	3,064
Tc00.1047053507019.100	Nuclear movement protein, putative	3,045
Tc00.1047053507801.144	Hypothetical protein, conserved	3,031
Tc00.1047053507521.44	Hypothetical protein, conserved	2,992
Tc00.1047053507611.300	Hypothetical protein, conserved	2,989
Tc00.1047053506779.44	Hypothetical protein, conserved	2,959
Tc00.1047053506583.10	Small nuclear ribonucleoprotein, putative	2,959
Tc00.1047053503479.49	U6 snRNA-associated Sm-like protein LSm7p	2,953
Tc00.1047053508153.994	Cytochrome C oxidase copper chaperone, putative	2,950
Tc00.1047053506945.200	Mago nashi-like protein, putative	2,925
Tc00.1047053507019.130	Hypothetical protein	2,922
Tc00.1047053506831.30	Hypothetical protein, conserved	2,896
Tc00.1047053506337.220	Hypothetical protein	2,887
Tc00.1047053509715.23	RNA-binding protein, putative	2,877
Tc00.1047053507723.140	Hypothetical protein, conserved	2,844
Tc00.1047053419417.10	Hypothetical protein, conserved	2,842
Tc00.1047053506401.360	Hypothetical protein, conserved	2,842
Tc00.1047053508737.194	ARP2/3 complex subunit, putative	2,832
Tc00.1047053508851.154	Hypothetical protein, conserved	2,809
Tc00.1047053506925.64	Hypothetical protein, conserved	2,795
Tc00.1047053508817.160	Trichohyalin, putative	2,776
Tc00.1047053503425.20	Glutaredoxin-like protein, putative	2,771
Tc00.1047053507625.186	Hypothetical protein, conserved	2,769
Tc00.1047053506247.24	Hypothetical protein, conserved	2,764

↳

Gene	Description	FC
Tc00.1047053504109.174	Hypothetical protein, conserved	2,764
Tc00.1047053506363.40	Hypothetical protein, conserved	2,751
Tc00.1047053506713.14	Hypothetical protein, conserved	2,746
Tc00.1047053506813.74	Chitin-binding like protein, putative	2,742
Tc00.1047053509215.13	Hypothetical protein, conserved	2,742
Tc00.1047053505193.84	Hypothetical protein, conserved	2,740
Tc00.1047053506567.74	Hypothetical protein, conserved	2,723
Tc00.1047053407477.40	Hypothetical protein, conserved	2,709
Tc00.1047053504157.30	Hypothetical protein, conserved	2,698
Tc00.1047053507801.150	Hypothetical protein, conserved	2,676
Tc00.1047053506817.70	Hypothetical protein	2,675
Tc00.1047053506755.200	Hypothetical protein, conserved	2,669
Tc00.1047053507991.129	Cyclophilin, putative	2,656
Tc00.1047053506649.64	Hypothetical protein, conserved	2,654
Tc00.1047053506425.50	Hypothetical protein, conserved	2,648
Tc00.1047053504035.84	Hypothetical protein, conserved	2,624
Tc00.1047053506295.80	ADP-ribosylation factor, putative	2,622
Tc00.1047053509693.194	Hypothetical protein, conserved	2,607
Tc00.1047053507949.69	Hypothetical protein, conserved	2,606
Tc00.1047053508897.100	Dynein light chain, putative	2,602
Tc00.1047053509671.99	Hypothetical protein, conserved	2,592
Tc00.1047053507521.30	Hypothetical protein, conserved	2,583
Tc00.1047053507941.14	Hypothetical protein, conserved	2,579
Tc00.1047053509011.54	Ubiquinol-cytochrome C reductase, putative	2,576
Tc00.1047053508659.18	Mitochondrial carrier protein, putative	2,573
Tc00.1047053506573.34	Hypothetical protein, conserved	2,567
Tc00.1047053511865.54	Protein transport protein Sec61 gamma subunit, putative	2,566
Tc00.1047053506625.210	Hypothetical protein, conserved	2,561
Tc00.1047053510879.60	Hypothetical protein, conserved	2,561
Tc00.1047053508257.150	Small nuclear ribonucleoprotein SmD3, putative	2,552
Tc00.1047053506925.460	Hypothetical protein, conserved	2,550
Tc00.1047053510731.104	Hypothetical protein, conserved	2,536
Tc00.1047053508503.30	Hypothetical protein	2,528
Tc00.1047053507019.50	Hypothetical protein, conserved	2,528
Tc00.1047053510243.128	Hypothetical protein, conserved	2,522
Tc00.1047053510609.34	Hypothetical protein, conserved	2,521
Tc00.1047053506363.10	Hypothetical protein	2,519
Tc00.1047053510657.120	Hypothetical protein, conserved	2,516
Tc00.1047053506469.139	Hypothetical protein, conserved	2,513
Tc00.1047053511291.120	RNA polymerase III C11 subunit, putative	2,505
Tc00.1047053509913.24	Dynein light chain 2B, cytoplasmic, putative	2,484
Tc00.1047053503917.21	Hypothetical protein, conserved	2,482
Tc00.1047053503891.120	Dynein light chain, putative	2,477
Tc00.1047053510131.20	Hypothetical protein, conserved	2,473
Tc00.1047053511211.104	Hypothetical protein, conserved	2,472
Tc00.1047053509055.60	Hypothetical protein, conserved	2,467
Tc00.1047053509769.66	Hypothetical protein, conserved	2,464
Tc00.1047053508257.244	Hypothetical protein, conserved	2,460
Tc00.1047053511283.194	Hypothetical protein, conserved	2,458
Tc00.1047053511003.180	Hypothetical protein, conserved	2,457
Tc00.1047053508257.204	Hypothetical protein, conserved	2,438
Tc00.1047053508307.150	Hypothetical protein, conserved	2,435
Tc00.1047053509337.19	Hypothetical protein, conserved	2,435
Tc00.1047053504013.30	Hypothetical protein, conserved	2,431

↳

Gene	Description	FC
Tc00.1047053509063.4	Hypothetical protein, conserved	2,420
Tc00.1047053506855.350	Hypothetical protein	2,408
Tc00.1047053507515.30	Hypothetical protein, conserved	2,395
Tc00.1047053508397.64	Hypothetical protein, conserved	2,387
Tc00.1047053511353.50	Hypothetical protein, conserved	2,383
Tc00.1047053510761.73	Dynein-associated protein, putative	2,380
Tc00.1047053509571.60	Hypothetical protein, conserved	2,375
Tc00.1047053506885.300	Hypothetical protein, conserved	2,367
Tc00.1047053506661.100	Hypothetical protein, conserved	2,366
Tc00.1047053506223.130	Hypothetical protein, conserved	2,366
Tc00.1047053506425.20	Hypothetical protein	2,363
Tc00.1047053503865.30	Hypothetical protein, conserved	2,359
Tc00.1047053510719.30	Ribosomal protein L29, putative	2,356
Tc00.1047053507775.40	Hypothetical protein, conserved	2,354
Tc00.1047053507873.34	Hypothetical protein, conserved	2,351
Tc00.1047053505555.20	Hypothetical protein, conserved	2,350
Tc00.1047053511731.70	Hypothetical protein, conserved	2,347
Tc00.1047053508851.150	Hypothetical protein, conserved	2,346
Tc00.1047053511003.170	Hypothetical protein, conserved	2,345
Tc00.1047053507809.70	Small ubiquitin protein, putative	2,330
Tc00.1047053511311.74	In v.3, removed from v.4 of genome; hypothetical protein, to be annotated (newly added gene) (36855-36601)	2,329
Tc00.1047053509063.20	Hypothetical protein, conserved	2,322
Tc00.1047053509023.20	Hypothetical protein, conserved	2,311
Tc00.1047053508153.634	Hypothetical protein, conserved	2,309
Tc00.1047053506529.30	Hypothetical protein, conserved	2,308
Tc00.1047053504109.140	Hypothetical protein, conserved	2,306
Tc00.1047053506223.90	Hypothetical protein, conserved	2,304
Tc00.1047053508539.20	Hypothetical protein, conserved	2,304
Tc00.1047053510001.50	Hypothetical protein, conserved	2,297
Tc00.1047053503557.20	Ankyrin repeat protein, putative	2,293
Tc00.1047053507257.30	Hypothetical protein, conserved	2,289
Tc00.1047053506977.49	Ribosomal proteins L36, putative	2,283
Tc00.1047053507467.94	Hypothetical protein, conserved	2,281
Tc00.1047053506727.150	Hypothetical protein, conserved	2,281
Tc00.1047053507611.280	Cytochrome C oxidase subunit IX, putative	2,270
Tc00.1047053506195.220	Hypothetical protein, conserved	2,258
Tc00.1047053506247.350	Hypothetical protein, conserved	2,248
Tc00.1047053504109.24	Dynein light chain, putative	2,246
Tc00.1047053506863.4	Dephospho-CoA kinase, putative	2,245
Tc00.1047053503939.114	Hypothetical protein, conserved	2,241
Tc00.1047053506625.140	Hypothetical protein, conserved	2,236
Tc00.1047053506419.69	Hypothetical protein, conserved	2,234
Tc00.1047053508323.84	Peptidyl-prolyl cis-trans isomerase, putative	2,223
Tc00.1047053511575.90	Hypothetical protein	2,222
Tc00.1047053506943.90	Hypothetical protein, conserved	2,220
Tc00.1047053503525.10	Hypothetical protein, conserved	2,213
Tc00.1047053507111.10	Hypothetical protein	2,213
Tc00.1047053511283.50	Hypothetical protein, conserved	2,209
Tc00.1047053508209.159	Hypothetical protein, conserved	2,201
Tc00.1047053503527.50	Hypothetical protein, conserved	2,200
Tc00.1047053506799.60	In v.3, removed from v.4 of genome; hypothetical protein (16022-16342)	2,198

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Gene	Description	FC
Tc00.1047053508461.380	Calmodulin, putative	2,196
Tc00.1047053504073.20	Hypothetical protein, conserved	2,196
Tc00.1047053510659.100	Hypothetical protein, conserved	2,190
Tc00.1047053505171.44	Hypothetical protein, conserved	2,187
Tc00.1047053506473.6	Hypothetical protein, conserved	2,186
Tc00.1047053511751.90	Hypothetical protein	2,183
Tc00.1047053407477.44	Hypothetical protein, conserved	2,181
Tc00.1047053507257.90	Hypothetical protein, conserved	2,179
Tc00.1047053503999.70	Ras-family member, GTP-binding protein, putative	2,175
Tc00.1047053472777.9	Hypothetical protein, conserved	2,173
Tc00.1047053511067.14	Hypothetical protein, conserved	2,171
Tc00.1047053507993.89	Hypothetical protein, conserved	2,166
Tc00.1047053504109.180	Hypothetical protein, conserved	2,160
Tc00.1047053508461.480	60S ribosomal protein L23, putative	2,155
Tc00.1047053504741.10	Hypothetical protein, conserved	2,155
Tc00.1047053511391.100	Hypothetical protein, conserved	2,152
Tc00.1047053507023.270	Hypothetical protein, conserved	2,150
Tc00.1047053510895.29	Hypothetical protein, conserved	2,147
Tc00.1047053509599.100	Hypothetical protein	2,145
Tc00.1047053503929.20	Ribosomal protein L13, putative	2,142
Tc00.1047053509965.30	Small nuclear ribonucleoprotein Sm-E, putative	2,141
Tc00.1047053507681.60	Hypothetical protein, conserved	2,137
Tc00.1047053508153.620	Hypothetical protein, conserved	2,137
Tc00.1047053509733.134	Hypothetical protein, conserved	2,136
Tc00.1047053504643.20	Hypothetical protein, conserved	2,133
Tc00.1047053440099.19	Hypothetical protein, conserved	2,132
Tc00.1047053507019.83	40S ribosomal protein S30, putative	2,128
Tc00.1047053508831.100	Hypothetical protein, conserved	2,128
Tc00.1047053508257.190	Hypothetical protein, conserved	2,123
Tc00.1047053504741.124	Hypothetical protein, conserved	2,110
Tc00.1047053511215.14	Hypothetical protein, conserved	2,108
Tc00.1047053511837.40	Hypothetical protein, conserved	2,105
Tc00.1047053503909.60	Hypothetical protein, conserved	2,103
Tc00.1047053506295.90	Coatomer zeta subunit, putative	2,098
Tc00.1047053506943.110	Hypothetical protein	2,089
Tc00.1047053507009.80	Hypothetical protein, conserved	2,088
Tc00.1047053506619.49	Ras-related protein Rab21, putative	2,088
Tc00.1047053507993.36	Hypothetical protein, conserved	2,087
Tc00.1047053506835.150	Zinc-binding protein (Yippee), putative	2,084
Tc00.1047053506435.460	Hypothetical protein	2,078
Tc00.1047053510257.24	Hypothetical protein, conserved	2,078
Tc00.1047053507649.90	Hypothetical protein, conserved	2,076
Tc00.1047053508955.10	Syntaxin, putative	2,075
Tc00.1047053508479.170	Hypothetical protein, conserved	2,072
Tc00.1047053507641.180	Endochitinase, putative	2,072
Tc00.1047053508277.300	Hypothetical protein, conserved	2,065
Tc00.1047053507275.24	Hypothetical protein, conserved	2,059
Tc00.1047053507669.200	Hypothetical protein, conserved	2,055
Tc00.1047053504051.49	Hypothetical protein, conserved	2,055
Tc00.1047053506727.4	Hypothetical protein, conserved	2,054
Tc00.1047053510289.44	Hypothetical protein, conserved	2,053
Tc00.1047053507609.20	Hypothetical protein, conserved	2,045
Tc00.1047053507031.64	Hypothetical protein, conserved	2,043
Tc00.1047053506247.74	Hypothetical protein, conserved	2,039

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Gene	Description	FC
Tc00.1047053507979.20	Hypothetical protein, conserved	2,038
Tc00.1047053509601.160	Hypothetical protein, conserved	2,037
Tc00.1047053506743.190	Hypothetical protein, conserved	2,036
Tc00.1047053507997.80	Hypothetical protein, conserved	2,035
Tc00.1047053508177.20	Carbohydrate kinase, thermoresistant glucokinase, putative	2,035
Tc00.1047053506575.54	Hypothetical protein, conserved	2,027
Tc00.1047053507521.70	Cyclophilin, putative	2,023
Tc00.1047053506725.60	Hypothetical protein, conserved	2,023
Tc00.1047053508951.50	Calmodulin, putative	2,021
Tc00.1047053503575.34	Ribosomal protein L38, putative	2,018
Tc00.1047053508409.250	Hypothetical protein, conserved	2,016
Tc00.1047053508013.90	Hypothetical protein	2,010
Tc00.1047053504171.50	Hypothetical protein, conserved	2,009
Tc00.1047053504105.60	Hypothetical protein, conserved	2,009
Tc00.1047053507771.114	Hypothetical protein, conserved	2,007
Tc00.1047053506925.420	Hypothetical protein, conserved	2,004
Tc00.1047053507949.29	Hypothetical protein, conserved	2,003

223 enriched genes observed in the three independent assays using *Trypanosom cruzi* oligonucleotide microarray analyzed by competitive hybridization using the non bound fraction as the reference population in dye-swap assays. FC: fold change.