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The post-transcriptional trans-acting regulator, *TbZFP3*, co-ordinates transmission-stage enriched mRNAs in *Trypanosoma brucei*

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**ABSTRACT**

Post-transcriptional gene regulation is essential to eukaryotic development. This is particularly emphasized in trypanosome parasites where genes are co-transcribed in polycistronic arrays but not necessarily co-regulated. The small CCCH protein, *TbZFP3*, has been identified as a trans-acting post-transcriptional regulator of Procyclin surface antigen expression in *Trypanosoma brucei*. To investigate the wider role of *TbZFP3* in parasite transmission, a global analysis of associating transcripts was carried out. Examination of a subset of the selected transcripts revealed their increased abundance through mRNA stabilization upon *TbZFP3* ectopic overexpression, dependent upon the integrity of the CCCH zinc finger domain. Reporter assays demonstrated that this regulation was mediated through 3'-UTR sequences for two target transcripts. Global developmental expression profiling of the cohort of *TbZFP3*-selected transcripts revealed their significant enrichment in transmissible stumpy forms of the parasite. This analysis of the specific mRNAs selected by the *TbZFP3*mRNP provides evidence for a developmental regulon with the potential to co-ordinate genes important in parasite transmission.

**INTRODUCTION**

The core machinery regulating mRNA stability and translation is well conserved throughout eukaryotic evolution, combining with transcriptional control factors to govern the overall expression of a gene (1,2). In addition to the core machinery, a myriad of organism- and tissue-specific trans-acting factors co-operate to finesse the genetic regulatory control of development. In recent years, the contribution of trans-acting factors that operate to control post-transcriptional, rather than transcriptional, processes have taken increasing prominence in our understanding of gene expression mechanisms. These exhibit regulation at the level of both individual genes and gene networks (3,4). Understanding the complexity of the underlying regulatory signals and machinery nonetheless remains a significant challenge.

One excellent model for the analysis of the post-transcriptional control of gene expression is the kinetoplastid parasites (5,6). These organisms are significant pathogens of the developing world and include *Trypanosoma brucei* (causing Human African Trypanosomiasis; HAT), *Trypanosoma cruzi* (causing South American Chagas’ disease) and *Leishmania* spp. that cause a variety of cutaneous and visceral maladies worldwide. Evolutionarily, the kinetoplastid parasites are among the earliest diverged eukaryotic organisms and exhibit a number of characteristics that distinguish them from the Opisthokont model organisms. In particular, their genome is organized into long polycistronic transcription units in which multiple genes are co-transcribed from dispersed unconventional transcriptional start sites (7,8). Despite their co-transcription, however, gene components of these post-transcriptional arrays often display differential expression, such as during the distinct developmental transitions that characterize the progression of kinetoplastid parasites through their complex life cycles (9–12). This differential expression is inevitably controlled at the post-transcriptional level, with regulatory signals being identified predominantly in the 3' untranslated region (UTR) (13,14) but also present in the 5'-UTR (14,15) and coding region (16) of several experimentally characterized genes.

Perhaps the best characterized models for gene expression control in kinetoplastids are the *procyclin* genes of *T. brucei*. These genes, comprised of *EP1*, *EP2*, *EP3* and *GPEET* isoforms, encode the major surface proteins on the parasite in the midgut of the tsetse fly (17,18),

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the vector for trypanosomiasis. These proteins differ slightly in their 3′-UTR sequences, which control their differential expression (19–22). Only recently have regulatory trans-acting proteins been identified that govern the differential expression of procyclin isoforms (22,23). The first of these, TbZFP3, is one of a family of small CCCH proteins (TbZFP1, TbZFP2, TbZFP3), which are conserved in kinetoplastids with each being implicit in trypanosome differentiation from mammalian bloodstream to tsetse midgut forms (24,25). Specifically, ectopic overexpression of TbZFP3 elevates the level of EP1 Procyclin protein expression at the expense of GPEET. Moreover, in RNA-immunoprecipitation experiments, TbZFP3 specifically selects the EP1 procyclin mRNA isoform, this being dependent upon both a negative regulatory element (Loop II) in the EP1 3′-UTR and the CCCH domain of TbZFP3, a predicted zinc finger involved in RNA binding in a range of eukaryotic proteins (26–29). Importantly, TbZFP3 promotes but is not necessary for the translation of the EP1 transcript, as deletion of the Loop II element suffices to both eliminate the TbZFP3 interaction as well as grossly upregulate the transcript and protein. This predicts that TbZFP3 competes with a negative regulator binding to the Loop II element and thereby acts as an anti-repressor to stabilize EP1 and promote its translation.

These analyses identified TbZFP3 as the first sequence-specific trans-regulator of surface coat regulation in trypanosomes. However, insight into the wider network of regulatory interactions involving this key regulator is lacking. Here, we have carried out a global analysis of the mRNAs that interact with the TbZFP3 mRNA, revealing a role for this regulator in the developmental events associated with parasite transmission from the mammalian bloodstream form to tsetse midgut form. Our findings generate a model whereby a cohort of developmentally regulated genes are co-stabilized in preparation for the signal to differentiate.

MATERIALS AND METHODS

Trypanosomes
SDM-79 medium (30) was used to culture procyclic form T. brucei. Transfected cells lines expressing inducible TbZFP3-TY, TbZFP3CCAHY-TY or TbZFP3 (NoTag) were described previously (22). Cells were harvested in logarithmic phase growth at 2–6 × 10⁶ cells/ml. Logarithmic procyclic s427–449 stage cells were induced for ectopic expression using 1 μg/ml tetracycline. RNA was harvested at 0 h, 30, 60, 90, 120, 240, 360 and 480 min and prepared via RNasea Qiagen columns (isolating ≥200 nt) and DNAse treated as per the manufacturer’s instructions, then pooled for Illumina digital-tag expression analysis.

Illumina digital-tag expression analysis
Transcripts isolated from either anti-TbZFP3 RNA IPs or whole procyclic cell lysate were reverse transcribed and subject to Illumina digital-tag sequencing by the ‘Gene Pool’ facility at Edinburgh University (genepool.bio.ed.ac.uk) and by MWG Eurofins. Sequence identities of ~5 × 10⁵ (RNA IP) and 1 × 10⁶ (total mRNA) quality reads were determined using the T. brucei 927 ORF and UTR sequences available from the TriTrypDB database website [tritrypdb.org; (32)].

Transcript stability analysis
Parental and transfected logarithmic procyclie stage cells were induced or uninduced for ectopic ‘TbZFP3-TY’ or ‘TbZFP3-NoTag’ expression with tetracycline for 72 h then treated with 5 μg/ml actinomycinD. RNA was harvested at 0, 5, 30, 60, 90, 120, 240, 360 and 480 min and prepared via RNasea Qiagen columns (isolating ≥200 nt) as per the manufacturer’s instructions.

Quantitative RT-PCR
Quantitative RT-PCR was conducted as described previously (22) using the following primers to amplify regions of candidate transcripts: TbSmB (Tb927.2.4540); CTTCACAACATACCCGCAC, CTAACCTCCCG AGTTGCC; TbGrpE (Tb927.6.2170); CTCGTGTTGCT CAGTCTCCC, TCCAAACCTCTCAAGCG; TbBP23 (Tb927.10.11270); ATGGTGTTCTACAGGTG CGCC, ACCGACGTTCATCAAGTTG; TbCyc7 (Tb927.6.5020); TCCCATTTGATGAGGACACATG, GGGAG ACTGCAAGAACATAACCTTG; TbMCP (Tb11.03.0870); ATACGCTGCTACCAGGCTTAC, AGCGCA GCTTAAAACACAGACG; TbKrepa5 (Tb927.8.680); CGAATGGAGAGGAGGTTGAG, CACTCCAACGT AGCGACTG; TbSmf (Tb09.211.1695); GTGGAAG AGCAACAGAGAGGC; GTGCAAGAATGAGGA GG; TbRpb7 (Tb11.01.6090); GAATTCTCCGCGGGT CT, TAAACCCTACCTGGC; TbRpb8 (Tb11.02.5790); ATTAGGAAGACACCTTCACCG, GCATGCG ATAAACTCAAACTG; Tb927.5.720: TGTCAATGATCG TCGTGTCG; TTGCCTGACATCTCTCACA, CCT GTGCAGGTTATACGCTGC; TbNmd3 (Tb927.7.970); AGTTGCAAGAGTTGTC, CATTCTCAAGGGTGC; TbSm15K (Tb927.6.4340); GTTCATTCTCTGCT TGGGC, TTCCTGATGCTACCCCTACG.

Primers for actin, epl and zfp1 were as described previously (22).

Western and northern blot analyses
Western and northern blots were as described with quantifications derived by G:Box Chemi analyses (Syngene). Probes to the ORFs of target transcript candidates and controls were created using Roche DIG RNA Labelling Kit (Sp6/T7) according to the manufacturer’s instructions. Primers were as above.

CAT ELISA assay

3'-UTR regulatory regions of candidate target transcripts were PCR-amplified and cloned into the BamHI/XhoI sites of the pH617 CAT reporter vector from which the Tet-operator sequences had been removed (a gift of P. Macgregor, University of Edinburgh). Primers to amplify and insert the intergenic regions of target candidates were as follows: T7RBp23: GGATCCCAAGCTAG TAATTAAGTGAGTCG, CTCGAGACCACCTTTCTCAACAGGCTC, 255 bp 3' of ORF; T7SmR: GGATCCGCGATCCTTTTACCATCCTGAGAAATTCCGTAGGTAAATGTGCT, 1.2 kb 3' of ORF.

CAT-expression vectors were transfected into procyclic form s427-parasites containing the pH449 plasmid for tetracycline regulated expression (33), with and without the pH451 vector (33) for tetracycline inducible expression of 'ZFP3 No Tag', 'ZFP3-TY' and 'ZFP3cAh-TY' (22). Transfectant cell lines were selected for growth in 1 μg/ml Puromycin and retested for inducibility of ectopic TbZFP3 expression. Cell lines were induced for ectopic expression in 1 μg/ml Tetracycline for 72 h and protein, RNA and CAT samples were harvested simultaneously. CAT protein expression was examined using the Roche CAT ELISA kit according to the manufacturer’s instructions. Trypanosome culture (1 ml) was concentrated by microfuge centrifugation. After removal of the supernatant, the cell pellet was washed three times with cold PBS, before being lysed with 1 ml of Roche lysis buffer for 25 min at room temperature. After removal of cell debris by centrifugation, 500 μl aliquots were snap frozen and stored at -80°C. Samples were measured at three dilutions in duplicate at 1, 3, 5, 7, 10, 12.5, 15 and 20 min within standard parameters, generated using a CAT calibration curve (r² = 0.995 or above). Results were consistent between all dilutions and time points.

Immunofluorescence microscopy

Procyclic 427 cells with eYFP-labelled Scd6 were washed three times in PBS, then divided equally between SDM-79 media at 27°C, SDM-79 media at 41°C (heat shock) and fresh PBS at 27°C (2 h glucose starvation). Parasites were then fixed in 4% paraformaldehyde for 20 min, washed three times in PBS, quenched in PBT (Triton X-100):glycine (10 mg/ml), washed three times in PBS:BSA and settled onto polylysine-labelled slides. Slides were then incubated with α-3ZFP3 [1:500] or fresh PBS:BSA 1 h. Slides were washed three times in PBS, incubated in anti-rabbit Alexa-633 [1:5000] (Invitrogen) in PBS:BSA 1 h, washed two times in PBT, stained with 4',6-diamidino-2-phenylindole, washed two times in PBS and mounted in Mowiol:Phenylene diamine for confocal imaging. Confocal images were captured on a LEICA SP5 confocal microscope and analysed via VOCILITY (Perkin Elmer).

In situ hybridization

Stumpy form AnTat 1.1 cells were harvested from mice 6-day post-inoculation, examined to confirm morphology, then fixed fresh from blood purification 20 min in 4% paraformaldehyde (pH 7.5), centrifuged at 700 g 10 min, washed three times in PBS, quenched in PBT (2% Triton X-100):10 mg/ml glycine and allowed to settle onto polyK-labelled slides 20 min in a humidity chamber. Slides were then washed three times in PBS:10% blocking reagent (Roche), blocked 2 h in hybridization buffer (5 × SSC, 50% formamide, 2% block, 0.02% SDS) and hybridized overnight in either the sense or anti-sense DIG-labelled oligo probes or hybridization buffer alone overnight in a humidity chamber. The slides were then washed once in 4 × SSC:10% formamide, twice in 4 × SSC, once in 2 × SSC, once in PBS, blocked for 1 h in PBS (0.1% Triton X-100):10% block, incubated 1 h with sheep α-DIG [1:6000] and/or α-TbZFP3 [1:500], washed four times in PBT (0.1% Triton X-100), twice in PBS, incubated 1 h with α-sheep Alexa 633 (1:2500), then washed twice in PBT (0.1% Triton X-100), three times in PBS, DAPI stained, washed four times in PBS and mounted in Mowiol:PDA [10:1].

Transcript expression analysis

To highlight stage-regulated concentration distinctions of genes that associate with TbZFP3, a figure was generated using the EdgeR Bioconductor package for R (34). Comparative transcript concentrations between stumpy and slender form T. brucei was plotted with the ‘plotSmean’ function of EdgeR using log² fold change against the log² mean count for each mRNA. Additionally, the top 100 transcripts in the TbZFP3mRNP were highlighted in black.

RESULTS

Selection of transcripts that co-associate with the TbZFP3mRNP

We used a previously optimized RNA immunoprecipitation (RIP) approach to select mRNAs that associate with the TbZFP3mRNP (22). As a control, extracts were incubated alternatively in the presence of a peptide matching the epitope recognized by the TbZFP3-specific antibody (Figure 1A). Analysis of the immunoprecipitated material from four replicate extractions confirmed that there was effective selection of TbZFP3, this being efficiently blocked in the presence of peptide. Associating RNA was then isolated from the combined extracts derived in the absence of blocking peptide, and the extracted polyA+ mRNAs subjected to Digital-Tag (Solexa) gene expression analysis on an Illumina platform. The resulting reads were then aligned to T. brucei TREU927/4 open reading frames and to a data set of untranslated regions generated by RNaseq analysis of T. brucei s427 procyclic form trypanosomes (8,10). This allowed the identification and quantitation of transcript tags from cDNAs either in their coding region or
untranslated region. In total, 5790 individual genes could be identified by ORF analysis, with a further 2478 genes being incorporated through 3'-UTR inclusion in the analysis.

To identify the transcripts within the TbZFP3mRNP, the ratio of each transcript’s abundance in the TbZFP3-selected pool versus the unselected mRNA population was analysed (Supplementary Figure S1). Validating the specificity of the approach used, EP1 and GPEET procyclin transcripts were both enriched in the selected mRNA pools (4.94- and 5.06-fold, respectively; Supplementary Table S1) whereas EP2 procyclin was specifically underrepresented (0.593-fold enrichment) and EP3 was absent (Supplementary Figure S1). This result matched our previous qRT-PCR and regulatory analysis and provided important positive and negative controls that validate this strategy (22). In total, 179 mRNAs were identified as being enriched in the TbZFP3mRNP pool at least 5-fold, the 48 transcripts showing at least 10-fold enrichment with respect to peptide-blocked material for 10 target transcripts (Supplementary Figure S2).

Transcripts selected by the TbZFP3mRNP are also regulated by ectopic over expression of TbZFP3. Having identified that TbZFP3 RIP co-selected a cohort of diverse transcripts, we tested whether it could regulate these transcripts to validate the relevance of their selection. Hence, we examined the levels of a subset of the selected transcripts in cell lines capable of the ectopic overexpression of TbZFP3, or a mutant of this protein with the CCCH domain disrupted by point mutation. Further to this, reporter gene assays were used to evaluate the contribution of the 3'-UTR sequences to their regulation, and to determine control at the protein level.

Initially the abundance of three of the TbZFP3-selected transcripts (Tb927.2.4540, ‘SmB’, 143-fold enriched; Tb927.6.4340, ‘TbSm15K’, 0.1-fold enriched; Tb927.7.970, ‘actin’, 0.26-fold enriched), was barely detectable in the selected sample, despite being readily detectable in unselected total mRNA (Figure 1B, ‘Negative control’). Independent quantitative analysis by qRT-PCR further validated the selection, demonstrating enrichment of between 5- and 1000-fold with respect to peptide-blocked material for 10 target transcripts (Supplementary Figure S2).
by a point mutation (ZFP3 CCAH-TY) (36). In each cell line the tetracycline-inducible expression of the ectopic proteins was confirmed by western blotting using the TY1-specific antibody, BB2 (37) (Figure 2A). Significantly, overexpression of TbZFP3-TY generated an increased abundance of each of the associating transcripts as detected by northern blotting (Figure 2B; compare lanes 3 and 4). This upregulation averaged ~10-fold when these and eight additional selected transcripts were quantitated by qRT-PCR (Supplementary Figures S3 and S4). In contrast, wild-type procyclic forms showed no tet-induced changes in target transcript abundance (Figure 2B, lanes 1 and 2) and mutation within the CCCH domain of TbZFP3 prevented regulation, although in this case the level of expressed protein was less (Figure 2A and B; lanes 5 and 6). Importantly, the upregulation of associating transcripts by TbZFP3 levels was specific as none of the negative control transcripts were elevated (Figure 2C). These results indicate that increased levels of TbZFP3 upregulate levels of associating transcripts, this requiring an intact CCCH domain.

Table 1. Transcripts exhibiting 10-fold or greater enrichment after selection by TbZFP3 RNA-immunoprecipitation with respect to their tag frequency in unselected RNA

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</tbody>
</table>

The relative expression of each transcript in pleomorphic slender and stumpy forms is also shown. Two very highly expressed ‘Hypothetical conserved’ transcripts (Tb09.160.0465, Tb927.4.1300) are present in the table, but no function is evident for these.
We have previously demonstrated that \textit{Th}ZFP3 regulates \textit{EP1 procyclin} gene expression through regulatory elements within the 3'-UTR (22). In order to assess whether the 3'-UTR's of other target transcripts were implicit in \textit{Th}ZFP3-dependent regulation, the intergenic region spanning from the stop codon of either \textit{Rbp23}, \textit{SmB} or \textit{GrpE} to the borders of the next gene were inserted into constitutive CAT reporter constructs (Figure 3A). These CAT-targetUTR reporter constructs were then stably transfected into multiple cell lines (Figure 3A) including the parental control and lines that ectopically express either native \textit{Th}ZFP3 ('ZFP3 No Tag'), \textit{Th}ZFP3-TY or the mutant protein \textit{Th}ZFP3 CCAH-TY. The resulting lines (summarized in Figure 3A) were then tested for tet-inducible ectopic \textit{Th}ZFP3 expression using antibodies detecting either \textit{Th}ZFP3 or the TY tag (Figure 3B). This revealed that all reporter cell lines exhibited inducible \textit{Th}ZFP3 expression, except for the 'CAT-SmB' reporter in the 'ZFP3 No Tag' cell line, where ectopic expression was considerably leaky, such that equivalent ectopic \textit{Th}ZFP3 expression was observed whether induced or not.

Figure 4A shows the effect of \textit{Th}ZFP3 ectopic overexpression upon these \textit{CAT} reporters. For both \textit{Rbp23} and \textit{SmB}, the 3'-UTR sequence was sufficient to generate an inducible increase in \textit{CAT} mRNA in response to ectopic \textit{Th}ZFP3-TY expression (Figure 4A, left panel; lanes 2 and 3 for \textit{Rbp23}, and lanes 4 and 5...
for SmB), whereas the TbZFP3-CCAH-TY mutant generated no effect (Figure 4A, left panel, lanes 6–9). Similarly, untagged ectopic TbZFP3 elevated the Rbp23 reporter transcript levels upon induction (Figure 4A, right panel, lanes 7 and 8). For the SmB reporter, the leakiness of ectopic TbZFP3 protein in this cell line (Figure 3B) required comparison with the parental wild-type procyclic form cells and those capable of the inducible ectopic expression of untagged TbZFP3 (ZFP3-No Tag), TbZFP3-TY or ZFP3 CCAH-TY. The respective cell lines generated in each case are summarized in the chart. ND = not done. (B) Western blots of inducible ectopic TbZFP3 expression in lines transfected with the respective CAT reporter constructs. Note that for the CAT-SmB/ZFP3-No tag cell line, the level of ectopic TbZFP3 expression was equivalent in uninduced and induced cells. Hence, for this cell line expression comparisons of the CAT reporter were made with the parental CAT-SmB transfected line. Relative loading is indicated by the Coomassie stained gel images in each case. ND = Not done.

Figure 3. Cell lines created to investigate the regulation by TbZFP3 of target mRNA 3’-UTRs. (A) Chloramphenicol acetyltransferase (CAT) Reporter strategy. Intergenic sequences downstream of Rbp23, SmB and GrpE coding regions were inserted individually into a reporter construct adjacent to the CAT coding region. In each case, the inserted sequence length is indicated in the Table in Panel A, as is the predicted endogenous 3’-UTR length for each transcript. Each reporter was transfected into parental wild-type procyclic form cells and those capable of the inducible ectopic expression of untagged TbZFP3 (ZFP3-No Tag), TbZFP3-TY or ZFP3 CCAH-TY. The respective cell lines generated in each case are summarized in the chart. ND = not done. (B) Western blots of inducible ectopic TbZFP3 expression in lines transfected with the respective CAT reporter constructs. Note that for the CAT-SmB/ZFP3-No tag cell line, the level of ectopic TbZFP3 expression was equivalent in uninduced and induced cells. Hence, for this cell line expression comparisons of the CAT reporter were made with the parental CAT-SmB transfected line. Relative loading is indicated by the Coomassie stained gel images in each case. ND = Not done.
included in the reporter construct, i.e. the GrpE 3'-UTR or ORF.

Having demonstrated that ectopic TbZFP3 overexpression increases CAT reporter mRNA levels controlled by both Rbp23 or SmB 3'-UTR’s, we investigated whether this upregulation translates to the CAT protein level via quantitative CAT-ELISA assay (Figure 4B). However, in neither case was inducible elevation of the reporter protein observed upon overexpression of either TbZFP3-TY (Figure 4B, left hand panel) or untagged TbZFP3 (Figure 4B, right hand panel). This revealed that, unlike EP1 procyclin (22), ectopic TbZFP3 expression specifically increased target mRNA abundance but generated no significant change in protein levels for the examined transcript targets. This suggests that translation is restricted despite the elevated mRNA abundance for these reporter mRNAs.

To determine the basis of the increased abundance of the selected mRNAs, the decay of SmB (TbZFP3-selected) and TbSm15K (non-selected) transcripts were assayed after actinomycinD treatment of cells induced, or not, to overexpress TbZFP3. Initially, we confirmed the inducible overexpression of TbZFP3 in the cells (Supplementary Figure S5) and examined the relative levels of SmB, Rbp23 and GrpE versus the non-target negative controls TbSM15k and SmB and actin. Semi-quantitative northern blot data confirmed the specific upregulation for the selected transcripts (data not shown). Thereafter, the decay of TbSm15K and SmB mRNA was analysed in induced and uninduced cells by qRT-PCR (Figure 5A and B). This revealed that the decay of TbSm15K was unaffected by the overexpression of TbZFP3 (F1 = 0.10, P = 0.759; Figure 5A) whereas SmB exhibited reduced decay rates in the TbZFP3-induced line compared to the uninduced cells (F1 = 5.62, P = 0.050, Figure 5B), doubling the SmB transcript half-life from 40 to 80 min. Semi-quantitative northern blot data confirmed this response for the GrpE and Rbp23 transcripts (data not shown). These assays indicated that TbZFP3-selected transcripts are stabilized by TbZFP3 overexpression, providing an explanation for the elevated abundance of target transcripts in steady state mRNA.

TbZFP3/mRNP transcripts are enriched in the parasite transmission stage. Having confirmed the specificity and regulation of TbZFP3-associated transcripts, we

Figure 4. The 3'-UTR’s of SmB and Rbp23 are sufficient for regulation by TbZFP3. (A) Ectopic overexpression of TbZFP3-TY (left panel) or untagged TbZFP3 (right panel) increases levels of CAT-Rbp23 and CAT-SmB, matching the effect on the endogenous mRNAs. This upregulation is dependent upon the CCCH predicted RNA-binding domain of TbZFP3. In contrast, the CAT-GrpE transcript was not significantly changed in response to ectopic TbZFP3 expression, unlike endogenous GrpE mRNA (Supplementary Figure S3). In each case, a northern blot is shown detecting CAT mRNA. Relative loading is indicated by EtBr stained rRNA. The relative fold increases for each reporter mRNA, based on their chemifluorescent signal, are highlighted above the lane numbers used for comparison, these being derived from an independent experiment. For the CAT-SmB reporter in the ‘TbZFP3 No Tag’ line, the leakiness of ectopic protein expression necessitated comparison with the PCF control line (lanes 3 and 4). (B) Corresponding CAT protein levels are not significantly upregulated in response to ectopic TbZFP3-TY (left-hand panel) or TbZFP3 (right-hand panel) overexpression. In each case CAT protein levels were determined by CAT-ELISA assay and normalized to the uninduced ZFP3-TY line (left-hand panel) or PCF (without tetracycline) containing the reporter constructs, but no ectopic TbZFP3.
investigated whether they exhibited evidence of functional co-ordination or co-expression during the parasite’s lifecycle. To achieve this, the relative abundance of each TbZFP3-RIP selected transcript was compared between different developmental forms of the parasite, namely bloodstream ‘slender’ forms, ‘intermediate’ forms and ‘stumpy’ forms, and cultured tsetse fly midgut procyclic forms (Supplementary Figure S6). The bloodstream forms were derived from Day 3 (slender), Day 5 (intermediate) and Day 7 (stumpy) of a mouse infection, and replicate samples were generated from two independently isolated T. brucei strains (AnTAT1.1 or EATRO), thereby ensuring that any identified developmental profile was consistent between strains. These RNAs were then subject to Illumina Digital Tag gene expression analysis to derive the abundance of individual transcripts at each developmental stage and thereafter those transcripts selected by TbZFP3 analysed for their relative expression profile. Figure 6 and Supplementary Figure S6 show the relative expression of the top 100 (>7-fold enriched; Figure 6A and B), 200 (>4.75-fold enriched), 300 (>3.5-fold enriched), 400 (>3-fold enriched) and 500 (>2.5-fold enriched) TbZFP3-selected transcripts compared to the total pool of all transcripts in slender or stumpy forms. Strikingly, the selected transcripts were significantly overrepresented in the stumpy developmental form in both strains of T. brucei tested. Furthermore, the extent of representation directly correlated with the relative enrichment of the transcripts in the TbZFP3-selected material (Figure 6B). Hence, the top 100 enriched transcripts in the TbZFP3 selected pool showed significantly elevated expression in the stumpy derived mRNA pool ($\chi^2 = 10.8, df = 2, P < 0.005$), with progressively less evidence for stumpy-enriched expression when the top 200, top 300 and top 400 TbZFP3-selected transcripts were considered (Figure 6B). Correspondingly, the TbZFP3-RIP selected transcripts were enriched in ‘intermediate forms’, albeit less dramatically than in stumpy forms, and were underrepresented in the mRNA pool enriched in slender forms (Supplementary Figure S6 and Figure 6).

To investigate whether the enriched genes showed any functional co-ordination, the Gene Ontology (GO) of the top 100 enriched mRNAs was investigated using the GOstat software package (39). This identified GO groups that were over-represented in the selected list compared to the overall list of T. brucei GO annotated genes (www.geneDb.org; 8 December 2011 update). Using a stringent P-value cutoff of 0.01, this analysis revealed five GO groups that showed significant over-abundance in the top 100 TbZFP3mRNP transcripts compared to a randomized GO annotated T. brucei gene set (Figure 7). These groups were ‘Ribonucleoprotein Complex’ ($P = 0.002$), ‘Macromolecule Biosynthetic Complex’ ($P = 0.003$), ‘Translation’ ($P = 0.009$), ‘Lipase Activity’ ($P = 0.01$) and ‘Cytosolic Large Ribosomal Subunit’ ($P = 0.01$). These GO identities make up the majority of the TbZFP3mRNP pool and are also enriched in stumpy enriched mRNA pool, contrasting with the total transcriptome population (Figure 7).

Combined, these analyses demonstrated that TbZFP3 preferentially co-associated with transmission stage enriched mRNAs, invoking the presence of a novel developmental regulon. Moreover, the selected mRNAs were enriched for molecules likely to be necessary as parasites prepare for the extensive changes in gene expression and protein synthesis upon vector uptake.

TbZFP3 associates into Procyolic form cytoplasmic granules upon serum starvation but not in stumpy forms. Having determined that TbZFP3 preferentially associates with transcripts enriched in stumpy forms, we investigated whether this was co-ordinated through any higher order mRNP structure. In eukaryotic cells, transcripts can be stabilized through their association with cytoplasmic granules, which can provide storage sites under conditions of stress or nutritional starvation. Several predicted RNA binding proteins in kinetoplastids localize into cytoplasmic foci upon serum starvation, or in

Figure 5. TbZFP3 stabilizes associating transcripts. (A) mRNA quantitation derived from triplicate qRT-PCR assays of TbSm15K mRNA (not selected by TbZFP3) at time points after treatment with actinomycin D. Samples were derived from cells induced, or not, to ectopically express TbZFP3, with values expressed as a percentage of the starting abundance. Using a GLM with % of starting transcript as the response variable, the presence/absence of TET was not a significant factor ($F_1 = 0.10, P = 0.759$). (B) As in A, but assayed for the abundance of the SmB (selected by TbZFP3). The steady state abundance of SmB is enhanced upon TbZFP3 ectopic expression, hence comparisons between induced and uninduced samples are expressed as a percentage of the abundance at time = 0 h in each case. In this case, the presence/absence of TET was a significant factor ($F_1 = 5.62, P = 0.050$).
response to heat stress (38,39). Although several classes of mRNP granule have been observed in trypanosomatids, the molecule SCD6 is believed to be diagnostic for cytoplasmic P body granules (38). To determine whether TbZFP3 could associate with these mRNP granules, the cellular location of this molecule was established by expressing a SCD6-eYFP fusion in procyclic forms [a kind gift of Dr Mark Carrington; (38)]. In SDM79 procyclic cell medium, both TbZFP3 and SCD6-YFP were dispersed throughout the cell cytoplasm (Figure 8A). However, when incubated in serum-free phosphate buffered saline for 2 h, both proteins coalesced into discrete cytoplasmic foci that colocalized (74% correspondence) (Figure 8B). Unlike Scd6, however, this redistribution of TbZFP3 was not observed in response to heat shock (42°C; data not shown), indicative of a starvation-specific rather than broader stress-induced relocalization. This indicates that TbZFP3 associates with P body granules specifically upon serum starvation in procyclic forms.

To probe the functional significance of this P-body association, we examined TbZFP3 localization in stumpy forms, when cells might be predicted to be under stresses analogous to PBS starvation in procyclic forms. Although reagents are not available to visualize SCD6 in pleomorphic bloodstream forms, stumpy cells harvested from a mouse infection and purified from host blood by DEAE chromatography were stained for TbZFP3 to identify granule-like structures similar to those seen in procyclic forms. The same cells were also analysed by in situ hybridization, to visualize EP procyclin transcript localization. This EP-specific mRNA probe detects all three EP procyclin mRNA isoforms, including EP2 and EP3, which do not associate with TbZFP3. However, we anticipated that EP1 procyclin mRNA and TbZFP3 protein might colocalize into detectable P body-like structures. Figure 8C shows that in stumpy forms, TbZFP3 exhibited a diffuse but punctate cytoplasmic staining, with some local concentration in areas of the cytoplasm. Similarly, procyclin transcripts visualized with an anti-sense probe were diffusely located, whereas a sense probe generated no detectable signal. However, large, discrete foci as induced by serum starvation in procyclic cells were not evident in stumpy cells, despite some local concentration of both signals (arrowed in Figure 7C). Hence, TbZFP3 can associate with P bodies upon serum starvation in procyclic forms, but analogous structures are not obvious in transmissible stumpy forms.
DISCUSSION

Although there are large numbers of predicted RNA binding proteins encoded in the genomes of kinetoplastid parasites (36, 41), only in a few instances have target transcripts been identified. Of these, the best characterized are (i) the cell cycle box binding proteins, CSBPA and B, which recognize a conserved octamer sequence in the UTR’s of cell cycle-regulated transcripts (15), (ii) Puf 9 (42), which also has a putative role with cell cycle-regulated mRNAs, (iii) DRBD3, an RGG domain protein that appears to associate with the mRNAs of membrane proteins (43) and (iv) the small CCCH protein family comprising \(TbZFP1\), \(TbZFP2\) and \(TbZFP3\). Of the latter, each is less than 140 amino acids and co-associate in procyclic forms, aided by complementary protein interaction domains (31). The \(TbZFP\) proteins have each been implicated in regulating developmental processes, namely kinetoplast repositioning \([TbZFP1; (26)]\) or the efficiency of differentiation as monitored by the expression of the Procyclin surface proteins and morphology \([TbZFP2, TbZFP3 (24, 31)]\). In the case of the \(TbZFP3mRNP\), the interaction with \(procyclin\) mRNA was found to be direct. Specifically, the \(TbZFP3mRNP\) associated with the Loop II element of the \(EP1\) procyclin protein at the cell surface (22), this being dependent on the integrity of the \(TbZFP3\) CCCH domain.

Here, we have carried out a global survey of the mRNAs that co-associate with the \(TbZFP3mRNP\). This has revealed, firstly, that the selected mRNAs were stabilized by \(TbZFP3\) and, secondly, that the selected transcripts were predominantly more abundant in the transmission stage of trypanosomes, stumpy forms. This implicates \(TbZFP3\) mRNP as a trans-acting factor defining a developmental regulon in these parasites.

The approach used to identify mRNAs that associate with the \(TbZFP3\) mRNP involved co-immunoprecipitation of mRNAs by an anti-peptide antibody specific for \(TbZFP3\) (22). The selected transcripts were then identified by their relative enrichment with respect to unselected mRNA, this being determined quantitatively at a global level by use of Illumina Digital-tag expression analysis. This approach offers a number of benefits. Firstly, by use of an anti-peptide antibody, a blocking peptide control could be incorporated into the selection regime, ensuring that interactions were specific for the target protein, \(TbZFP3\). Secondly, by use of an antibody to the endogenous protein we could ensure that the physiological stoichiometry of mRNA–mRNP interactions in the cell was preserved and avoid the need for affinity tags to be incorporated into the protein ligand. The latter is an important consideration since we have observed that incorporation of a 10 amino acids TY tag into the C-terminus of \(TbZFP3\) alters selection of procyclin isoform mRNAs (our unpublished data).

Finally, by use of high-throughput Digital-tag transcriptome analysis, we could accurately identify and quantify selected transcripts, exploiting the available ORF and the 3’-UTR data generated by RNA-seq analysis of trypanosome life cycle stages (10).

Analysis of the developmental expression profile of the transcripts co-associated with \(TbZFP3\) revealed an enrichment of mRNAs whose expression is elevated in stumpy forms, which are poised for development when taken up in a tsetse bloodmeal (44, 45). Indeed, there was a strong correlation between the extent of enrichment after \(TbZFP3\) RIP and stumpy-enriched expression, this trend being observed in two independently isolated parasite lines capable of transmission. This observation is consistent with the established roles of \(TbZFP\) proteins in bloodstream to procyclic form differentiation (22, 36, 25, 26) and the enrichment of CCCH proteins in differentiation events recently observed by high-throughput RNAi

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**Figure 7.** GO analysis of the \(TbZFP3mRNP\) associating transcripts. Representation of the GO terms enriched in the \(TbZFP3-RIP\) selected transcripts (A) versus the relative abundance of the same GO classes in the total (B) or stumpy enriched (C) gene set. \(P\)-values were calculated using the GOStat package (40).
Coupled with the regulation by \( TbZFP3 \) of \( EP1 \) procyclin, the earliest marker of development to procyclic forms, we propose that the small CCCH proteins are implicit in controlling the changes in gene expression that accompany life-cycle development upon entry to the tsetse fly. Supporting this, \( TbZFP3 \) co-associated with mRNAs functionally linked to gene regulation and new protein synthesis, a profile expected for control of the early events during developmental progression upon entry into the tsetse fly midgut.

Analysis of a subset of transcripts selected by \( TbZFP3 \)- RIP revealed that each showed increased abundance after \( TbZFP3 \) levels were elevated by ectopic expression, leading to enhanced mRNA stability. This differs from our earlier observations with \( procyclin \) mRNAs where \( TbZFP3 \) overexpression did not alter levels of the specifically selected \( EP1 \) and \( GPEET \) mRNAs (22). Also contrasting with \( EP1 \) procyclin regulation by \( TbZFP3 \), we did not find evidence for enhanced protein expression of the target mRNAs linked to elevated \( TbZFP3 \) expression. While this evidence may superficially appear to conflict and indicate distinct regulatory functions, in all cases examined \( TbZFP3 \) acts to positively regulate associating targets. Hence, through stabilization it appears \( TbZFP3 \) potentiates but does not ensure the translation of associating transcripts. This model is consistent with global analysis of mRNA and protein levels observed in the related kinetoplastid, \textit{Leishmania donovani}, where mRNA were predicted to be stabilized and directly targeted for translation upon a differentiation signal (47,48).

The regulatory distinctions for different \( TbZFP3 \) mRNP target mRNAs may result from several processes. Firstly, different transcript classes may have different rate-limiting steps in their regulatory control. \textit{Procyclin} represents an mRNA that must be exquisitely regulated to prevent the premature appearance of protein on the surface of bloodstream form parasites, where it could provoke a strong immune response against the parasite. Consequently, this transcript must be stringently limited by transcript-specific translational repression and transcript destabilization (21), a restriction counteracted by the

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**Figure 8.** \( TbZFP3 \) association in cytoplasmic P granules. Panels (A) and (B) show Procyclic form cells engineered to express \( T8SCD6 \)-YFP were incubated in SDM-79 (A) or PBS for 2h, (B) and then the location of \( T8SCD6 \)-YFP (green) or \( TbZFP3 \) (red) determined by immunofluorescence. The third panel in each case represents the image derived from DAPI staining to visualize the parasite nucleus and kinetoplast, whereas the fourth panel shows a merge of the \( T8SCD6 \)-YFP, \( TbZFP3 \) and DAPI panels. After PBS starvation the \( T8SCD6 \)-YFP and \( TbZFP3 \) signals colocalize in discrete cytoplasmic granules or P Bodies (arrowed). Panels (C) and (D) show the signal generated when slender (SL) and stumpy (ST) cells are probed for the location of \textit{ep procyclin} mRNA (panel C, anti-sense probe, panel D, sense probe) and \( TbZFP3 \), with DAPI staining of the cells being shown in the third panel and a merge of all staining in the fourth panel. The \( TbZFP3 \) signals do not localize into enlarged P bodies, unlike serum starved procyclic forms. However, some concentration, in particular regions is observed (arrowed).
contribute to secondary structural features in the target mRNAs may shared the same sequence motifs. This is not surprising as the relationship was not simple, and not all enriched transcripts are likely to be associated with the Loop II regulatory region of the mRNA 3′-UTR (49). In contrast, for other target transcripts, target mRNAs are stabilized by TbZFP3, whereas protein expression might be restricted by a more general translational control mechanism or other regulatory factors. Secondly, variation in the precise protein composition of TbZFP3mRNP(s) may exist for different transcripts or transcript classes. In this scenario, TbZFP3 could associate with distinct protein factors altering mRNP specificities that regulate different target mRNA classes. Finally, we cannot rule out limitations imposed by the reporter system used. Here, two 3′-UTR sequences (for SmB and RBP23) were found to recapitulate the elevated target mRNA observed when TbZFP3 was ectopically expressed. However, if other sequences in the 5′-UTR or coding region contribute to gene regulation, matching the scenario for GrpE and at least one other transcript (16), then our assays may not fully represent the regulatory consequences of increased TbZFP3 levels. In all cases, however, other factors in addition to TbZFP3 must operate to differentially regulate gene expression because unlike our perturbation experiments, the endogenous levels of TbZFP3 do not dramatically differ between bloodstream and procyclic forms (31). One such factor is likely to be TbZFP1, which is induced during differentiation and interacts with TbZFP3 (31). Another is the differential polysome association of TbZFP3 between life cycle stages (40).

How is the specificity of TbZFP3 regulation achieved? A bioinformatic analysis of the 3′-UTR sequences of the selected transcripts revealed an enrichment of sequence motifs that distinguish EP1 and GPEET from EP2 and EP3 3′-UTR sequences, supportive of a sequence specific interaction (Supplementary Figure S7). However, this relationship was not simple, and not all enriched transcripts shared the same sequence motifs. This is not surprising as secondary structural features in the target mRNAs may contribute to TbZFP3 recognition, such structural motifs being difficult to predict and identify by computational means alone. Also, as discussed earlier, TbZFP3 may associate with different mRNP complexes with different specificities, complicating the identification of conserved motifs among the global cohort of transcripts co-selected with TbZFP3. Although further analysis of the RNA–protein and protein–protein interactions of this trans-acting regulatory factor are necessary, its association into cytoplasmic granules containing TbSCD6 in procyclic forms demonstrate its involvement in the higher order mRNA regulatory complexes within the cell (Figure 8).

To conclude, we have exploited a proven and physiologically relevant strategy to identify the cellular population of mRNAs specifically associated with the small trans-acting post-transcriptional regulator, TbZFP3. In each case, TbZFP3 was found to act as a positive regulator of gene expression, generating increased stability for associated mRNAs, matching another CCCH class protein in T. brucei (49). Strikingly, the selected mRNAs were enriched in transmission stages of the parasite, suggesting the possibility of a developmental post-transcriptional operon directed by TbZFP3, rather than a more limited functional operon. Moreover, the broad range of enrichment for different transcripts suggests that there will be considerable complexity and nuance in the regulation of specific transcript and transcript groups by CCCH family RNA regulators. By identification of the full complement of TbZFP3 mRNA targets, these interactions and regulatory events can now be analysed in detail, greatly extending earlier ‘one transcript-one regulator’ models for gene regulation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–7.

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