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Dynamic regulation of the *Trypanosoma brucei* transferrin receptor in response to iron starvation is mediated *via* the 3'UTR

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Abstract

The bloodstream form of the parasite *Trypanosoma brucei* obtains iron from its mammalian host by receptor-mediated endocytosis of host transferrin through its own unique transferrin receptor (*Tb*TfR). Expression of *Tb*TfR rapidly increases upon iron starvation by post-transcriptional regulation through a currently undefined mechanism that is distinct from the mammalian iron response system. We have created reporter cell lines by fusing the *Tb*TfR 3'UTR or a control Aldolase 3'UTR to reporter genes encoding GFP or firefly Luciferase, and inserted the fusions into a bloodstream form cell line at a tagged ribosomal RNA locus. Fusion of the *Tb*TfR 3'UTR is sufficient to significantly repress the expression of the reporter proteins under normal growth conditions. Under iron starvation conditions we observed upregulation of the mRNA and protein level of the *Tb*TfR 3'UTR fusions only, with a magnitude and timing consistent with that reported for upregulation of the *Tb*TfR. We conclude that the dynamic regulation of the *T. brucei* transferrin receptor in response to iron starvation is mediated *via* its 3'UTR, and that the effect is independent of genomic location.

Introduction

The obligate extracellular parasite *Trypanosoma brucei* has a complex digenetic lifecycle between a tsetse fly vector and a range of mammalian hosts. Mammals limit the availability of essential element iron to prevent growth of invasive pathogens by sequestering iron within the major serum glycoprotein transferrin (Tf). The bloodstream form of *T. brucei* has evolved a unique transferrin receptor (*Tb*TfR) that allows it to obtain iron through receptor mediated endocytosis of host transferrin (Tf) [1, 2]. After binding of host Tf to the *Tb*TfR in the flagella pocket, endocytosis occurs and subsequent acidification releases free iron for further processing and transportation, whilst the apo-Tf is degraded and the *Tb*TfR is recycled to the flagella pocket [3]. Different *Tb*TfR genes encode proteins with varying affinities for Tf from different mammals, and the occurrence of multiple *Tb*TfR genes has been suggested to allow the parasite to adapt to a wide host range [4, 5].

A subset of *T. brucei* genes are transcribed by RNA polymerase I (RNA pol I), including the essential Variant Surface Glycoprotein (VSG) which forms a dense surface coat that enables

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the parasite to evade the host's innate and adaptive immune responses, and which undergoes antigenic variation from a repertoire of ~1500 *VSG* genes [6]. Of the 15 subtelomeric VSG bloodstream expression sites (BES), only one is active at a time so that a single *VSG* is transcribed from a discrete location within the nucleus [7]. Antigenic variation requires replacement of the *VSG* within the active BES or a switch to a different BES, with the latter also causing a change in the identity of the expression site associated genes (*ESAGs*) that are expressed, as they are located on the *VSG* polycistronic transcriptional unit. The *VSG* promoter proximal genes *ESAG6* & *ESAG7* form the heterodimeric *Tb*TfR [1], which is evolutionarily distinct from mammalian TfR and structurally resembles a truncated VSG homodimer [8]. Both monomers of *Tb*TfR are extensively *N*-glycosylated and are membrane associated via the GPI anchor present on ESAG6 [9].

Under basal conditions only 3×10^3 *Tb*TfR heterodimers [1] are expressed despite *ESAG6 & 7* being located on the same polycistronic transcriptional unit as the highly abundant VSG (5×10^6 homodimers), demonstrating that transcript abundance and/or processing are differentially regulated. Under iron starvation conditions expression of the *Tb*TfR rapidly increases equally at the mRNA and protein level, with lack of increase in *VSG* mRNA suggesting that regulation occurs through a currently undefined post-transcriptional mechanism [10, 11]. Reducing the uptake of iron using the iron chelator deferoxamine, culturing with different mammalian serum, incubation with anti-TfR antibodies, or competition with apo-Tf all result in a rapid 2.5–5-fold upregulation of the *Tb*TfR and a corresponding increase in Tf uptake [5, 10–12]. Interestingly, *Tb*TfR upregulation occurs before intracellular iron stores are depleted and cells continue to divide for 48 h, suggesting that cells are responding to changes in iron flux. The mechanism is distinct from the post-transcriptional Iron Response Element (IRE) / Iron Response Protein (IRP) system found in mammals, as knockout of the *T. brucei* IRP-1 homologue aconitase has no effect on *Tb*TfR regulation [10]. As *Tb*TfR is a multi-gene family that occurs in an atypically regulated locus, and available antibodies cross-react with different ESAG6 & 7 glycoproteins, the direct study of *Tb*TfR regulation is challenging.

The BES that is usually active in *T. brucei* bloodstream form culture-adapted cell lines is BES1, which expresses *ESAG6 & 7* genes with nanomolar affinity for bovine Tf, but that bind to canine Tf only poorly [4, 5]. Prolonged iron starvation (>7 days) induced by changing from growth in media supplemented with bovine serum to canine serum selects for cells that have altered the identity of the expressed *ESAG6 & 7* and *VSG*, either by switching to another BES or replacing the genes in the active BES1 [5, 12]. Switching events can be prevented by supplementing the canine serum with bovine Tf, demonstrating that the adaption is driven by iron starvation [4]. Under normal physiological conditions the concentration of available host Tf is unlikely to limit trypanosome growth, but uptake may become limiting in later stages of an infection when competition with anti-*Tb*TfR antibodies and/or host anaemia come into consideration [12, 13].

There is mounting evidence of the importance of the 3'UTR in the post-transcriptional regulation of developmentally regulated genes in *T. brucei*. The stage-specific regulation of both RNA pol I transcribed VSG [14] and procyclin [15], and the RNA pol II transcribed COX genes [16] has been demonstrated to occur, at least in part, due to recognition of motifs within their respective 3'UTRs. The developmental regulation of *ESAG9* depends on a 34-nucleotide bifunctional element in the 3'UTR that confers both positive and negative regulation [17], and an RNA binding protein that negatively regulates *ESAG9* has recently been identified through a genome-wide RNA interference screen [18]. Here, we investigate the importance of the *Tb*TfR 3'UTR in the dynamic regulation of *Tb*TfR in response to iron starvation using a simplified reporter system. By fusing the *ESAG6*-3'UTR to reporter genes encoding GFP or firefly Luciferase (fLUC), we demonstrate that the 3'UTR alone is sufficient to confer dynamic regulation of gene expression in response to iron starvation.

Material and methods

Cell lines

The culture adapted monomorphic *T. brucei brucei* Lister 427 bloodstream form 2T1 cell line [19], containing a tagged RRNA locus, were cultured in HMI-11T [20] containing 0.2 μg/mL Puromycin and 0.5 μg/mL Phleomycin at 37 ^{0}C in a 5% CO_2 incubator. Transfected 2T1 cell lines were selected and maintained with 2.5 μg/mL Hygromycin in place of Puromycin.

Cloning of ESAG6 3'UTR

RNA was extracted from ~1 × 10^7 logarithmic phase cells using the RNeasy plus kit (Qiagen) according to the manufacturer's instructions. A two-step RT-PCR reaction was performed by first transcribing 0.25 μg of RNA using an Omniscript Reverse Transcriptase (Promega) with a Oligo-dT adapter primer (5′-CGCGTCGACTAGTACTTTTTTTTTTTTTTT-3′) and then using 5 μl of the resulting cDNA as a template for a PCR amplification using Hot-start RED-Taq (Sigma) with primers specific for BES1 *ESAG6* ORF [7] (5′-GCAGTACATTTGAGTCTTT-3′) and the adapter sequence (5'-CGCGTCGACTAGTAC-3'). The resulting amplicon was ligated into pGEM-T-easy (Promega) prior to DNA sequencing.

Generation of reporter cell lines

The pRPaΔ^{GFP} X vector, a version of the pRPa^{GFP} X vector [19] with tetracycline operator removed, was a kind gift from Sam Alsford, LSTHM. The firefly Luciferase (fLUC) ORF was PCR amplified from the pCRm-LUC-HYG vector [21] (a kind gift from Phillip Yates, Oregon Health & Science University, USA) using a mutagenic forward primer incorporating a *Hind*III site (underlined) to removes an internal *ApaI* site (mismatch in bold) (5′-ATTATAAGCTTATGGAAGATGCCAAAAACATTAAGAA**A**GGCCCAGGG-3′) and a reverse primer incorporating a *Bam*HI site (underlined) (5′-TTCGCGGATCCTCACACGGCGATCTTGC-3′). The PCR product was ligated into pGEM-T-easy (Promega) to allow DNA sequencing prior to subsequent subcloning into pRPaΔ^{GFP} X using the *Hind*III and *Bam*HI sites to replace the GFP-stuffer protein fusion, resulting in pRPaΔ^{fLUC} that contains the *fLUC* gene fused to the aldolase 3'UTR.

The 335bp *ESAG6* 3'UTR was PCR amplified from *T. brucei* gDNA using a forward primer incorporating *Xba*I (italics) and *Bam*HI (underlined) sites (5′-AATGA*TCTAGA*TAGGGATCCGGGAAGGATGCGAC-3′) and reverse primer incorporating an *Apa*I (underlined) site (5′-AATAGGGCCCAGTAGAATTAGTCTAGTTT-3′). Digestion with *Xba*I and *Apa*I allowed subcloning into pRPaΔ^{GFP} X, replacing the stuffer protein X and creating pRPaΔ^{GFP}-*ESAG6-3'UTR* where the *GFP* gene is fused to *ESAG6-3'UTR*. Digestion of the same PCR product with *Bam*HI and *Apa*I allowed subcloning into pRPaΔ-*fLUC* creating pRPaΔ^{fLUC}-*ESAG6-3'UTR* where the *fLUC* gene is fused to *ESAG6-3'UTR*. Finally, the *ESAG6-3'UTR* of pRPaΔ^{GFP}-*ESAG6-3'UTR* was replaced with the aldolase 3'UTR of pRPaΔ^{GFP} X using the *Bam*HI and *Apa*I sites creating pRPaΔ-*GFP* that contains the *GFP* gene fused to the aldolase 3'UTR.

The reporter constructs were digested with *Asc*I, ethanol precipitated and resuspended in water prior to transfection. 3 μg DNA was mixed with 1 × 10^7 2T1 cells suspended in transfection buffer [22] in a 2 mm cuvette (BioRad) and electroporated in an Amaxa Nucleofector (Lonza Biosciences) using program X-001. Cells were allowed to recover for 6h at 37 ^{0}C, 5% CO_2 in HMI-11T antibiotics before selection with 2.5 μg/mL Hygromycin and 0.5 μg/mL Phleomycin.

Induction of iron starvation

The iron chelator deferoxamine (Sigma Aldrich) was added to *T. brucei* cell cultures at 25 μM to induce iron starvation. For serum switching starvation experiments, cells were harvested at

800 × g, washed once in HMI-11T without serum, and resuspended in HMI-11T containing either 10% fetal calf serum (Labtech) or 10% donor dog serum (Labtech) with and without the addition of 200 μg/ml holo bovine transferrin (Sigma).

Western blots

Cells were counted, harvested at 800 × g, the pellet resuspended at 1×10^6 cells/μl in 1 × SDS loading buffer (Melford), and the cells lysed for 5 min at 95 ^{0}C. The lysate was separated on a 10% Tris-acetate SDS-PAGE gel, transferred to a PVDF membrane using a Trans blot Turbo semi-dry blotter (Biorad) and blocked overnight in 5% w/w ECL blocking agent (GE Healthcare) in TTBS (0.05% Tween 20 in Tris buffered saline). The expression of GFP was detected with a rabbit anti-GFP primary (1:1000, Roche) and an anti-rabbit-HRP conjugated secondary (1:20,000, GE Healthcare) with expression level quantified with Clarity ECL chemiluminescent detection (Biorad) in a ChemiDoc XPS+ system (Biorad). The detection of tubulin using mouse anti-tubulin KMX-1 primary (1:100; Gift from Keith Gull, Oxford) and anti-mouse-HRP secondary (1:20,000, GE Healthcare) was used to verify equal sample loading, and to normalise the GFP signal.

Luciferase assays

The expression of the fLUC reporter gene was quantified by incubating 100 μl of a 5×10^5 cells/ml *T. brucei* culture in white 96 well plates with an equal volume the OneGlo luciferase reagent (Promega) for 5 min, with the resulting luminescence detected using a Fluoroskan ascent FL plate reader (Thermo Scientific) with a 10 sec acquisition time per well.

qRT-PCR

Cells were lysed and total RNA extracted using the EZNA total RNA kit (Omega Bio-TEK), treated with DNase I (Thermo Scientific), and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to manufacturer's instructions. qRT-PCR amplification was carried out using FastStart Universal SYBR Green master mix (Roche) on a LightCycler 96 realtime PCR instrument (Roche) using primers for the *fLUC* (fLUC-F `5′-GACACCGCTATCC TCAGCGT-3′` and fLUC-R `5′- CATGAGCACGACCCGAAAGC -3′`) and an Actin control (Actin-F `5′- GTACCACTGGCATTGTTCTCG -3′` and Actin-R `5′-CTTCATGAGATATTCC GTCAGGTC-3′`). The cycling parameters were as follows: initial denaturation at 95 ^{0}C for 600 secs; 40 cycles at 95 ^{0}C for 10 secs and at 60 ^{0}C for 30 secs, followed by a melt curve step. Quantification cycle for the mRNA's of interest were first normalised to an Actin reference mRNA, and then converted to fold change relative to the untreated *fLUC-3'ALD* signal.

Immunofluorescence microscopy

Approximately 1×10^6 cells were harvested by centrifugation at 800 × g, washed once with 1 × PBS and allowed to adhere to glass slides for 5 min. Slides were incubated with 4% PFA for 5 min at room temperature, washed twice in 1 × PBS for 5 minute. A drop of Fluoroshield with DAPI (Sigma) was added to the slide, a coverslip was applied and the slide examined on a Leica DMRXA2 fluorescent microscope.

Results

Identification of the ESAG6-3'UTR

In order to investigate whether the 3'UTR of the *Tb*TfR is involved in its dynamic regulation it was necessary to first identify the size of the 3'UTR, which due to its telomeric location is not present in the previous polyadenylation site mappings [23, 24]. As expression of ESAG7 may

not be essential [7], we focussed our efforts on ESAG6. To identify the size of the *ESAG6 3'UTR* expressed in the active BES1, a two-step RT-PCR was performed with an oligo-dT adapter primer to transcribe cDNA and subsequent amplification of the *ESAG6-3'UTR* with primers specific for BES1 *ESAG6* open reading frame and the adapter sequence. Sequencing of the resulting amplicons identified that polyadenylation occurred at closely spaced sites 317, 330 or 335 bases downstream of the *ESAG6* stop codon. The observed heterogeneity and 3'UTR length are consistent with previous observations in *T. brucei* [23, 24]. Sequence alignment revealed a high level of sequence conservation between the 13 copies of the *ESAG6-3'UTR* found in the 15 different BES [7], with pairwise comparison of the nucleotide sequences revealing 91.0–99.7% identity [25] (Fig 1 & Figure A in S1 File). Interestingly the 3'UTR of the putative non-telomeric copy of ESAG6 (Tb927.9.15680) was less conserved, with only 38.9–42.2% sequence identity with the telomeric sequences [25] (Figure B in S1 File).

Expression of reporters with the ESAG6-3'UTR under normal growth conditions

To investigate the potential involvement of the *ESAG6*-3'UTR in regulation of the *Tb*TfR, constructs were created with the reporter genes *GFP* or firefly Luciferase (*fLUC*) ORF followed by either the aldolase (*ALD*) 3'UTR or the 335 bp *ESAG6*-3'UTR (Fig 2). The reporter genes were located downstream of a RRNA promoter and integrated into the tagged *RRNA* locus in the 2T1 cell line to ensure high levels of constitutive transcription by RNA pol I whilst avoiding positional effects [19].

Observation of the GFP reporter cell lines by immunofluorescence microscopy confirmed cytosolic expression of GFP, with the *ESAG6*-3'UTR reporter cells producing noticeably diminished GFP signal compared to the *ALD*-3'UTR reporter cells (Fig 3A). The steady state expression level of GFP during exponential growth was quantified by western blotting with anti-GFP antibodies for three independent clones, revealing that expression of GFP in the *ESAG6*-3'UTR reporter cells was repressed by ~80% compared to the *ALD*-3'UTR reporter cells (Fig 3B). Similarly, measurement of luciferase activity in the fLUC expressing cell lines revealed a significant difference between the *ESAG6*-3'UTR and *ALD*-3'UTR for each of three independent clones, with the fusion of the *ESAG6*-3'UTR repressing the expression level by ~90% (Fig 3C). The level of expression of *Tb*TfR has previously been reported to increase up to 5-fold at high cell density [27], and we observed similar cell density dependent changes in luciferase activity in the *ESAG6*-3'UTR fusion (Fig 3D).

The ESAG6 3'UTR mediates an iron starvation response

The addition of the iron chelator deferoxamine to *T. brucei* cells reduces the availability of Tf-bound iron, and results in a 2.4–3.7-fold increase in *Tb*TfR protein and mRNA levels after 5

```
BES1.ESAG6-3'UTR   GGGAAGGATGCGACCGAAACTGCGCTGCTTAGCGTGAAAGATTATGGTAATGGAGGGTTGGGAAAG-ATTGGGGAAACAA
Conservation        ************* *** ******** ** * ***** **** ** ********** * ****  ** ** * *****

BES1.ESAG6-3'UTR   ATACCTATTTCTTTTATTTGGGGGAACAAATGGGCAAAAGTAACGTAAGTTTCCAGTGGGAGTGGTATGTGTGTGTGTAT
Conservation       * ****************** ************* *   ******* ****** ** ** ******   *  ***** **

BES1.ESAG6-3'UTR   GGGGCTGGCTAAGGAAAGATGTGAGTTCGGCATGTGGTATGTACAAGCTACGAAAACGTGTGAAACAAAACGAGATGTAA
Conservation       *** * *  *************  ****** ********* ********* *******  ***** *******  * ***

BES1.ESAG6-3'UTR   GGGGAAAATGTAACAACCAACTATGTTAAATT-TCAG--GAGACTATTTTTCAAATTTAGTTACAACAAAGTAAATGTCA
Conservation       **** ************ * ** ** ****** * *   ** ************************** ********* *
                      ▼              ▼    ▼
BES1.ESAG6-3'UTR   AATAATGCCAACTATAAAGGAAAA
Conservation       ***** ************ *****
```

Fig 1. Identification of the *ESAG6*-3'UTR. The observed polyadenylation sites are indicated with triangles above the sequence, and sequence identity with the other 13 BES *ESAG6*-3'UTR sequences indicated by stars below the sequence. Sequences immediately downstream of the stop codon were aligned with T-Coffee [26].

https://doi.org/10.1371/journal.pone.0206332.g001

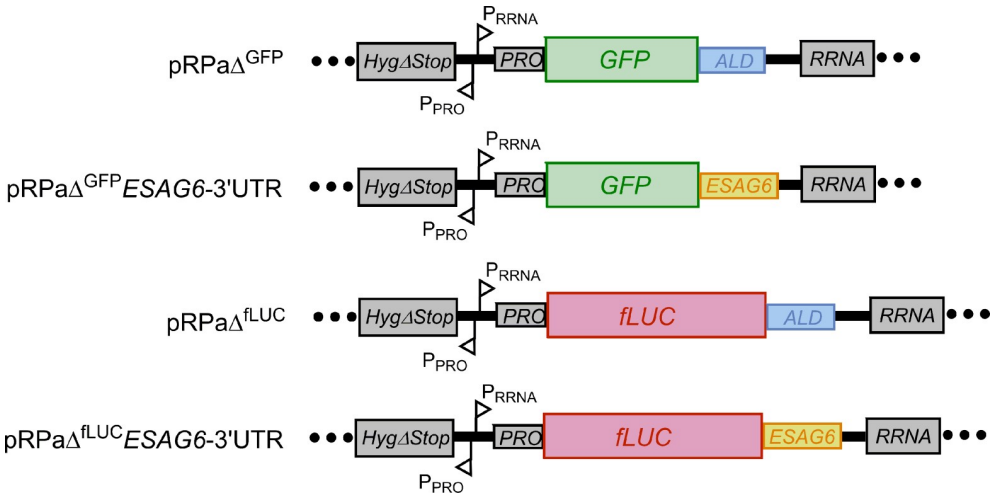

Fig 2. Reporter constructs used in this study. RNA processing regions and promoters: *HygΔStop*—portion of the hygromycin resistance targets the Hyg-tagged RRNA locus; P_{RRNA}—RRNA promoter; P_{PRO}—procyclin promoter; *PRO*—procyclin 5'UTR; *ALD*—Aldolase 3'UTR; *ESAG6*—*ESAG6*-3'UTR; GFP–green fluorescent protein; fLUC–firefly luciferase.

https://doi.org/10.1371/journal.pone.0206332.g002

hours [10]. Treatment of the two fLUC reporter cell lines with deferoxamine for 5 hours reduced the luciferase activity of the *ALD*-3'UTR reporter cells by 0.8-fold, but significantly increased the luciferase activity of the *ESAG6*-3'UTR reporter cells by ~10-fold, so that the

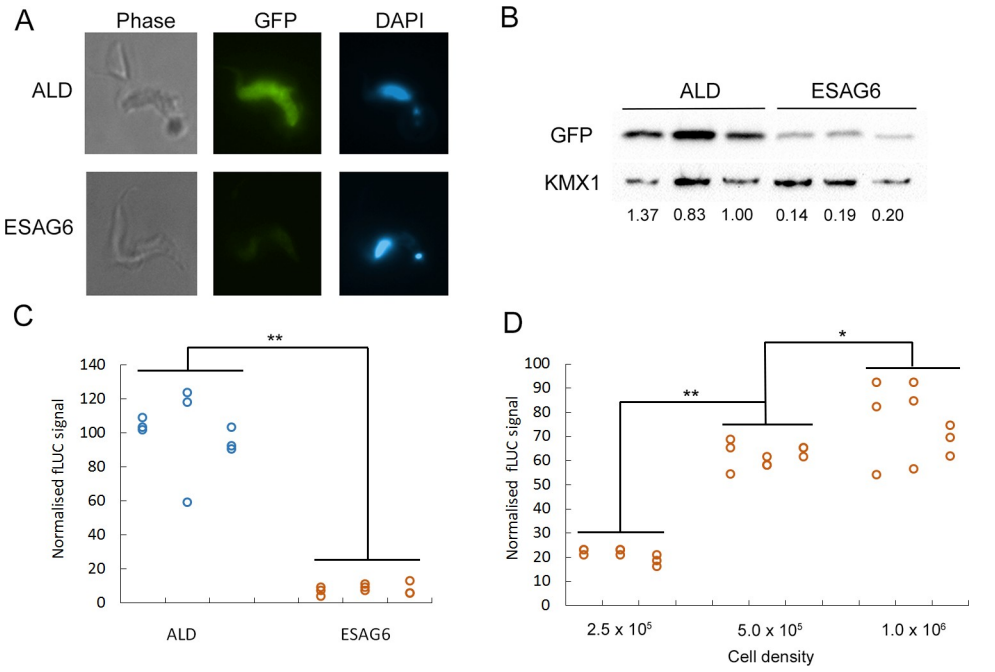

Fig 3. Reporter protein expression under normal culture conditions. A. Immunofluorescence of GFP reporter constructs. B. Quantification of GFP expression by western blotting, using anti-GFP and KMX1 as a loading control. C. Luciferase activity assay, D. Luciferase activity of the *ESAG6*-3'UTR cell line varies with cell density. Data in panel B-D represents three independent clones of each cell line. Luciferase signal is normalised to the aldolase-3'UTR signal; data point for three biological replicates shown. ALD–aldolase-3'UTR, ESAG6 –*ESAG6*-3'UTR. * $p < 0.05$, ** $p < 0.001$.

https://doi.org/10.1371/journal.pone.0206332.g003

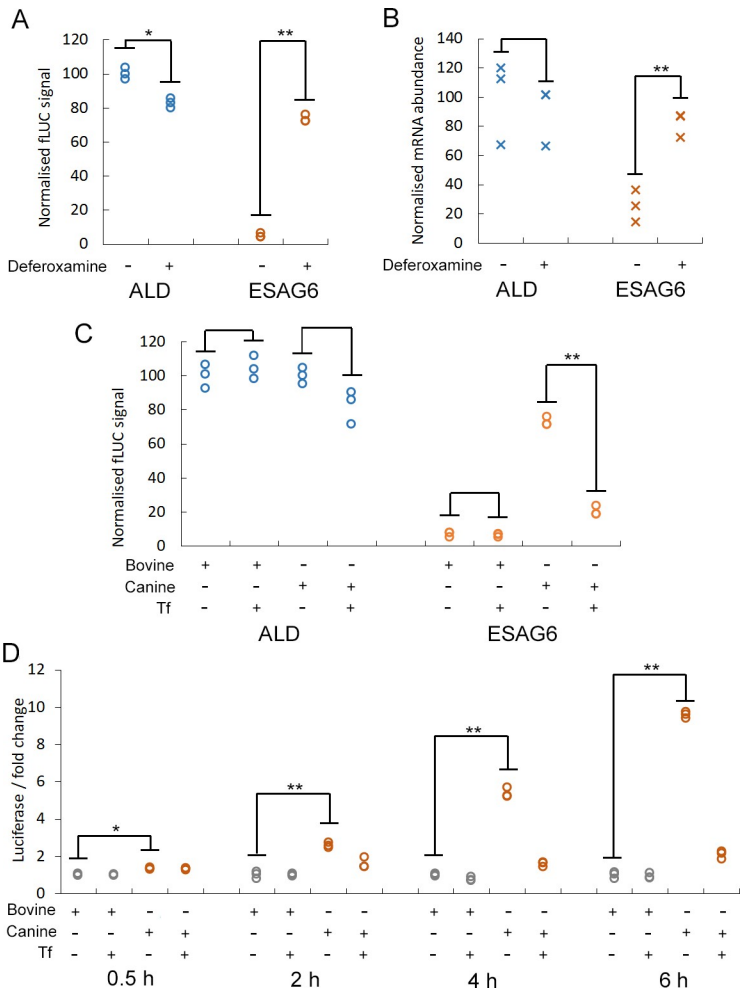

Fig 4. Luciferase expression in the *ESAG6*-3'UTR reporter increases under iron starvation conditions. A. Luciferase activity of *fLUC* reporter cells treated with 25 μM deferoxamine for 5 h, normalised to untreated ALD signal. B. *fLUC* mRNA levels in reporter cells treated with 25 μM deferoxamine for 5 h, normalised to untreated ALD mRNA level. C. Luciferase activity of *fLUC* reporter cells switched to HMI11-T + 10% serum (bovine or canine as indicated) for 5 h with or without the addition of 200 μg/ml bovine transferrin (Tf), normalised to untreated ALD signal. D. Time course of luciferase activity of *fLUC ESAG6*-3'UTR reporter cells switched to HMI11-T + 10% serum (bovine or canine as indicated) with or without the addition of 200 μg/ml bovine Tf, normalised to signal in HMI11-T + 10% FCS. ALD–aldolase 3'UTR, ESAG6 –ESAG6 3'UTR. Data point for three biological replicates shown, * $p < 0.05$, ** $p < 0.001$.

https://doi.org/10.1371/journal.pone.0206332.g004

luciferase activity was 73 ± 3% of that measured in the untreated *ALD*-3'UTR reporter cells (Fig 4A). Analysis of the fLUC reporter mRNA by qRT-PCR confirmed that the effect was also evident at the level of mRNA abundance, with the *ALD*-3'UTR reporter mRNA level reduced (0.9-fold) and that of the *ESAG6*-3'UTR reporter increased by 3.2-fold so that the later was 83 ± 8% of untreated *ALD*-3'UTR level (Fig 4B). Although both the protein and mRNA levels of the luciferase in deferoxamine treated *ESAG6*-3'UTR reporter cells are increased to ~80% of the level in the untreated *ALD*-3'UTR reporter cells, the discrepancy in the fold-change at mRNA and protein level suggests that the iron starvation response may affect translation efficiency via the *ESAG6*-3'UTR.

Switching *T. brucei* cells with an active BES1 from growth in media supplemented with 10% bovine serum to growth in media supplemented with 10% canine serum results in rapid 2.5–

5-fold upregulation of *ESAG6* and *ESAG7* at the mRNA and protein level, as the *Tb*TfR expressed from BES1 has a particularly low affinity for canine Tf [4, 10, 11, 27]. The addition of excess bovine Tf to media supplemented with 10% canine serum prevents the upregulation of *Tb*TfR as cells are no longer starved of iron, validating the specificity of the response [4, 5]. Switching the fLUC reporter cell lines to canine-serum-supplemented media for 5 hours increased the luciferase activity of the *ESAG6*-3'UTR reporter cells by ~3.5-fold compared to growth in canine-serum-supplemented media with excess bovine Tf (Fig 4C). In contrast, the addition of excess bovine Tf to bovine-serum-supplemented media for 5 hours had no significant effect on the luciferase activity of either reporter cell line compared to growth in bovine-serum-supplemented media alone (Fig 4C).

The *fLUC ESAG6*-3'UTR reporter cell line was used to investigate the temporal profile of the response to serum switching. In the absence of additional bovine Tf, a time-dependent increase in luciferase activity was observed in cells grown in canine-serum-supplemented media compared to cells grown in bovine-serum-supplemented media (Fig 4D), with a significant 1.4-fold increase observed as early as 30 minutes after serum switching and increasing to ~10-fold after 6 hours. Addition of bovine Tf to the canine-serum-supplemented media reduced the magnitude of the increase to ~2-fold after 6 hours but did not completely negate the effect, suggesting that the amount of additional bovine Tf used here is not sufficient to completely restore iron intake.

These data demonstrate that fusion of the *ESAG6*-3'UTR to a reporter gene is sufficient to confer dynamic regulation of reporter at both the mRNA and protein level in response to iron starvation conditions, and that up-regulation is more rapid than previously thought. The changes in the luciferase activity observed here are broadly consistent with the magnitude of increase in TfR expression previously observed for the iron starvation response, with levels as high as 10-fold reported [27, 28]. The slightly larger changes observed with the fLUC reporter compared *Tb*TfR might be due to the superior quantitation and dynamic range of the bioluminescence assay compared to western blotting with polyclonal anti-*Tb*TfR antibodies.

Discussion

Our data demonstrate that fusion of the *ESAG6*-3'UTR to reporter genes is sufficient to confer a specific response to iron starvation conditions that increases the mRNA and protein level of the reporter with a magnitude and temporal profile consistent with that previously observed for *Tb*TfR upregulation. Since the reporter gene is located in a *RRNA* locus, this provides evidence that the dynamic regulation of *Tb*TfR is independent of transcription within the BES site or location at the telomere, and decouples the rapid dynamic regulation of *Tb*TfR from subsequent VSG switching events [4, 5, 12]. To our knowledge, this is the first time that a *T. brucei* 3'UTR has been demonstrated to be involved in dynamic post-transcriptional regulation of a gene in response to a specific nutritional stimulus, rather than mediated through irreversible developmental regulation in response to lifecycle changes.

We have demonstrated that fusion of the *ESAG6*-3'-UTR causes the expression of the gene product to be repressed under basal conditions, which is consistent with the low level of *ESAG6* mRNA present despite being located on the highly transcribed *VSG* polycistronic transcriptional unit. Under iron starvation conditions the repressive effect of the *ESAG6*-3'UTR is reduced, resulting in an increase of expression that is driven at least in part by an increase in transcript abundance, just as reported for *Tb*TfR [10, 11]. Whether the effect is driven by an increase in mRNA stability and/or increased translation efficiency remain to be investigated. The involvement of the 3'UTR in iron starvation response suggest that it contains secondary structural elements or sequence motifs that are recognised by RNA binding protein(s) (RBPs).

We performed simultaneous folding and alignment of the 13 *ESAG6*-3'UTRs to predicted a minimum free energy structure of the consensus sequence [29, 30], which revealed a high level of structure (Figure C in S1 File) suggesting that such features are more extensive than the 16-mer regulatory sequence identified in VSG mRNAs [31]. Further experiments will be required to identify any regulatory features present in the *ESAG6*-3'UTR, and whether they have a positive or negative regulatory role.

*Tb*TfR is a multi-gene family that occurs in a promoter proximal position in the 15 BES, and Ansorge *et al*. have previously used RT-PCR analysis to propose that ~20% of *ESAG6* mRNA is transcribed from the 14 silent BES due promoter-proximal de-repression of silencing [32]. Recent analysis of the surface proteome in culture adapted monomorphic cell line by Ghadella *et al*. [33] detected VSGs and ESAGs from multiple BES in addition to the major active BES, which the authors suggest is due to the occurrence of low abundance of a subpopulation of cells that have switched their active BES. Analysis of our own global proteomic data sets [20, 34, 35] supports their conclusion, as although the most abundant ESAGs detected correlate with the BES of the most abundant VSG, several other VSGs from 'silent' BES are detected at lower abundance along with their corresponding ESAGs. Therefore we propose that the transcription of *Tb*TfR from supposedly 'silent' BES can at least in part be explained by the occurrence of a low abundance of subpopulation of cells that have switched their active BES, and that any contribution of de-repression of silent BES to the rapid dynamic regulation of *Tb*TfR expression is likely to be minimal.

Conclusions

Taken together, the data presented here demonstrate that fusion of the *ESAG6*-3'UTR to a reporter gene is sufficient to confer a specific response to iron starvation conditions that increases the mRNA and protein level of the reporter with a magnitude and temporal profile consistent with that previously observed for *Tb*TfR upregulation. The effect is independent from transcription within the expression site or location at the telomere, and is therefore decoupled from VSG switching events. The *fLUC-ESAG6*-3'UTR reporter system is experimentally tractable and provides a simple and reproducible signal-and-response system in cultured monomorphic cells with which to elucidate a signalling pathway essential for the clinically-relevant bloodstream form parasite.

Supporting information

S1 File. Supporting information containing Figures A–C. Figure A—Sequence alignment of the BES ESAG6 3'UTRs. Figure B—Sequence alignment of the BES and putative genomic ESAG6 3'UTRs. Figure C—Extensive secondary structure is predicted in the *ESAG6*-3'UTR. (PDF)

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References

1. Salmon D, Geuskens M, Hanocq F, Hanocq-Quertier J, Nolan D, Ruben L, et al. A novel heterodimeric transferrin receptor encoded by a pair of VSG expression site-associated genes in *Trypanosoma brucei*. Cell. 1994; 78:75–86. PubMed Central PMCID: PMC8033214. PMID: 8033214
2. Steverding D, Stierhof YD, Fuchs H, Tauber R, Overath P. Transferrin-binding protein complex is the receptor for transferrin uptake in *Trypanosoma brucei*. J Cell Biol. 1995; 131:1173–82. PMID: 8522581
3. Taylor MC, Kelly JM. Iron metabolism in trypanosomatids, and its crucial role in infection. Parasitology. 2010; 137(6):899–917. https://doi.org/10.1017/S0031182009991880 PMID: 20152063.
4. Bitter W, Gerrits H, Kieft R, Borst P. The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*. Nature. 1998; 391:499–501. https://doi.org/10.1038/35166 PMID: 9461219
5. Gerrits H, Mußmann R, Bitter W, Kieft R, Borst P. The physiological significance of transferrin receptor variations in *Trypanosoma brucei*. Mol Biochem Parasitol. 2002; 119:237–47. PMID: 11814575
6. Cross GAM. Antigenic variation in trypanosomes: secrets surface slowly. Bioessays. 1996; 18:283–91. https://doi.org/10.1002/bies.950180406 PMID: 8967896
7. Hertz-Fowler C, Figueiredo LM, Quail MA, Becker M, Jackson A, Bason N, et al. Telomeric expression sites are highly conserved in *Trypanosoma brucei*. PloS one. 2008; 3(10):e3527. https://doi.org/10.1371/journal.pone.0003527 PMID: 18953401; PubMed Central PMCID: PMCPMC2567434.
8. Salmon D, Hanocq-Quertier J, Paturiaux-Hanocq F, Pays A, Tebabi P, Nolan DP, et al. Characterization of the ligand-binding site of the transferrin receptor in *Trypanosoma brucei* demonstrates a structural relationship with the N-terminal domain of the varient surface glycoprotein. EMBO J. 1997; 16:7272–8. https://doi.org/10.1093/emboj/16.24.7272 PMID: 9405356
9. Mehlert A, Wormald MR, Ferguson MA. Modeling of the N-glycosylated transferrin receptor suggests how transferrin binding can occur within the surface coat of *Trypanosoma brucei*. PLoS Pathog. 2012; 8 (4):e1002618. https://doi.org/10.1371/journal.ppat.1002618 PMID: 22496646; PubMed Central PMCID: PMCPMC3320590.
10. Fast B, Kremp K, Boshart M, Steverding D. Iron-dependent regulation of transferrin receptor expression in *Trypanosoma brucei*. Biochem J. 1999; 342:691–6. PMID: 10477281
11. Mußmann R, Janssen H, Calafat J, Engstler M, Ansorge I, Clayton C, et al. The expression level determines the surface distribution of the transferrin receptor in *Trypanosoma brucei*. Mol Microbiol. 2003; 47 (1):23–35. PMID: 12492851
12. van Luenen HG, Kieft R, Mussmann R, Engstler M, ter Riet B, Borst P. Trypanosomes change their transferrin receptor expression to allow effective uptake of host transferrin. Mol Microbiol. 2005; 58 (1):151–65. https://doi.org/10.1111/j.1365-2958.2005.04831.x PMID: 16164555.
13. Steverding D. The significance of transferrin receptor variation in *Trypanosoma brucei*. Trends Parasitol. 2003; 19(3):125–7. https://doi.org/10.1016/s1471-4922(03)00006-0 PMID: 12643995
14. Jefferies D, Tebabi P, Pays E. Transient activity assays of the *Trypanosoma brucei* variant surface glycoprotein gene promoter: control of gene expression at the posttranscriptional level. Mol Cell Biol. 1991; 11(1):338–43. PMID: 1986230
15. Roditi I, Furger A, Ruepp S, Schurch N, Butikofer P. Unravelling the procyclin coat of *Trypanosoma brucei*. Mol Biochem Parasitol. 1998; 91:117–30. PMID: 9574930
16. Mayho M, Fenn K, Craddy P, Crosthwaite S, Matthews K. Post-transcriptional control of nuclear-encoded cytochrome oxidase subunits in *Trypanosoma brucei*: evidence for genome-wide conservation of life-cycle stage-specific regulatory elements. Nucleic Acids Res. 2006; 34(18):5312–24. https://doi.org/10.1093/nar/gkl598 PMID: 17012283; PubMed Central PMCID: PMCPMC1636420.
17. Monk SL, Simmonds P, Matthews KR. A short bifunctional element operates to positively or negatively regulate ESAG9 expression in different developmental forms of *Trypanosoma brucei*. J Cell Sci. 2013;

126(Pt 10):2294–304. https://doi.org/10.1242/jcs.126011 PMID: 23524999; PubMed Central PMCID: PMCPMC3672941.

18. Rico E, Ivens A, Glover L, Horn D, Matthews KR. Genome-wide RNAi selection identifies a regulator of transmission stage-enriched gene families and cell-type differentiation in *Trypanosoma brucei*. PLoS Pathog. 2017; 13(3):e1006279. https://doi.org/10.1371/journal.ppat.1006279 PMID: 28334017; PubMed Central PMCID: PMCPMC5380359.
19. Alsford S, Kawahara T, Glover L, Horn D. Tagging a *Trypansoma brucei* RRNA locus improves stable transfection efficiency and circumvents inducible expression position effects. Mol Biochem Parasitol. 2005; 144(2):142–8. https://doi.org/10.1016/j.molbiopara.2005.08.009 PMID: 16182389; PubMed Central PMCID: PMCPMC3833055.
20. Urbaniak MD, Martin DM, Ferguson MA. Global quantitative SILAC phosphoproteomics reveals differential phosphorylation is widespread between the procyclic and bloodstream form lifecycle stages of *Trypanosoma brucei*. Journal of proteome research. 2013; 12(5):2233–44. Epub 2013/03/15. https://doi.org/10.1021/pr400086y PMID: 23485197; PubMed Central PMCID: PMC3646404.
21. Soysa R, Carter NS, Yates PA. A dual luciferase system for analysis of post-transcriptional regulation of gene expression in Leishmania. Mol Biochem Parasitol. 2014; 195(1):1–5. https://doi.org/10.1016/j.molbiopara.2014.05.002 PMID: 24878002; PubMed Central PMCID: PMCPMC4142068.
22. Schumann Burkard G, Jutzi P, Roditi I. Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. Mol Biochem Parasitol. 2011; 175(1):91–4. Epub 2010/09/21. https://doi.org/10.1016/j.molbiopara.2010.09.002 PMID: 20851719.
23. Siegel TN, Hekstra DR, Wang X, Dewell S, Cross GA. Genome-wide analysis of mRNA abundance in two life-cycle stages of *Trypanosoma brucei* and identification of splicing and polyadenylation sites. Nucleic Acids Res. 2010; 38(15):4946–57. https://doi.org/10.1093/nar/gkq237 PMID: 20385579; PubMed Central PMCID: PMC2926603.
24. Kolev NG, Franklin JB, Carmi S, Shi H, Michaeli S, Tschudi C. The transcriptome of the human pathogen *Trypanosoma brucei* at single-nucleotide resolution. PLoS Pathog. 2010; 6(9):e1001090. Epub 2010/09/15. https://doi.org/10.1371/journal.ppat.1001090 PMID: 20838601; PubMed Central PMCID: PMCPMC2936537.
25. Stothard P. The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. BioTechniques. 2010; 28:1102–4. PubMed Central PMCID: PMCPMID: 10868275.
26. Notredame C, Higgins D, Heringa J. T-Coffee: A novel method for multiple sequence alignment. J Mol Biol. 2000; 302:205–17. https://doi.org/10.1006/jmbi.2000.4042 PMID: 10964570
27. Mussmann R, Engstler M, Gerrits H, Kieft R, Toaldo CB, Onderwater J, et al. Factors affecting the level and localization of the transferrin receptor in *Trypanosoma brucei*. J Biol Chem. 2004; 279(39):40690–8. https://doi.org/10.1074/jbc.M404697200 PMID: 15263009.
28. Schwartz KJ, Peck RF, Tazeh NN, Bangs JD. GPI valence and the fate of secretory membrane proteins in African trypanosomes. J Cell Sci. 2005; 118(Pt 23):5499–511. Epub 2005/11/18. https://doi.org/10.1242/jcs.02667 PMID: 16291721.
29. Will S, Joshi T, Hofacker IL, Stadler PF, Backofen R. LocARNA-P: accurate boundary prediction and improved detection of structural RNAs. RNA. 2012; 18(5):900–14. Epub 2012/03/28. https://doi.org/10.1261/rna.029041.111 PMID: 22450757; PubMed Central PMCID: PMCPMC3334699.
30. Lai D, Proctor JR, Zhu JY, Meyer IM. R-CHIE: a web server and R package for visualizing RNA secondary structures. Nucleic Acids Res. 2012; 40(12):e95. Epub 2012/03/22. https://doi.org/10.1093/nar/gks241 PMID: 22434875; PubMed Central PMCID: PMCPMC3384350.
31. Ridewood S, Ooi CP, Hall B, Trenaman A, Wand NV, Sioutas G, et al. The role of genomic location and flanking 3'UTR in the generation of functional levels of variant surface glycoprotein in *Trypanosoma brucei*. Mol Microbiol. 2017; 106(4):614–34. Epub 2017/09/15. https://doi.org/10.1111/mmi.13838 PMID: 28906055; PubMed Central PMCID: PMCPMC5698767.
32. Ansorge I, Steverding D, Melville S, Hartmann C, Clayton C. Transcription of 'inactive' expression sites in African trypanosomes leads to expression of multiple transferrin receptor RNAs in bloodstream forms. Mol Biochem Parasitol. 1999; 101:81–94. PMID: 10413045
33. Gadelha C, Zhang W, Chamberlain JW, Chait BT, Wickstead B, Field MC. Architecture of a Host-Parasite Interface: Complex Targeting Mechanisms Revealed Through Proteomics. Mol Cell Proteomics. 2015; 14(7):1911–26. Epub 2015/05/02. https://doi.org/10.1074/mcp.M114.047647 PMID: 25931509; PubMed Central PMCID: PMCPMC4587319.
34. Urbaniak MD, Guther MLS, Ferguson MAJ. Comparative SILAC Proteomic Analysis of *Trypanosoma brucei* Bloodstream and Procyclic Lifecycle Stages. PloS one. 2012; 7(5):e36619. https://doi.org/10.1371/journal.pone.0036619 PMID: 22574199

35. Domingo-Sananes MR, Szoor B, Ferguson MA, Urbaniak MD, Matthews KR. Molecular control of irreversible bistability during trypanosome developmental commitment. J Cell Biol. 2015; 211(2):455–68. https://doi.org/10.1083/jcb.201506114 PMID: 26483558; PubMed Central PMCID: PMCPMC4621835.