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## A single-point mutation in the RNA-binding protein 6 generates *Trypanosoma brucei* metacyclics that are able to progress to bloodstream forms *in vitro*

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### Abstract

We previously established an *in vitro* differentiation system based on the inducible expression of the RNA binding protein 6 (RBP6), which initiated differentiation of *Trypanosoma brucei* non-infectious procyclics to infectious metacyclics (MFs). However, further differentiation to bloodstream forms (BFs) required infection of mice. Here we report the serendipitous isolation of a single point mutation in RBP6 (Q109K), whose expression not only generated MFs, but purified MFs continued the developmental cycle *in vitro* to BFs expressing variant surface glycoprotein-2 (VSG-2), formerly known as VSG 221. This transition occurred over a period of 11 days and by RNA-Seq, VSG-2 was first measurable on day 1, whereas metacyclic VSGs were detected up to 8 days. We further showed that inducible expression of mutant RBP6 appeared to skip the intermediate epimastigote stage and we highlight the potential involvement of RBP33 in the establishment of metacyclics and in particular in the generation of metacyclics uncharacteristically arrested at the G2/M checkpoint.

### Graphical abstract

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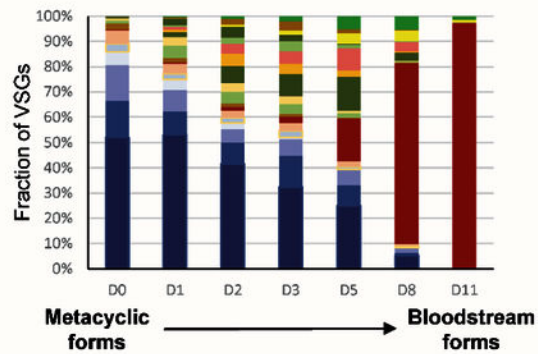
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Overexpression of a single point mutation in the *Trypanosoma brucei* RBP6 (Q109K) generates metacyclics that further differentiate *in vitro* to bloodstream forms, expressing VSG-2.

The authors declare no financial conflict of interest.



## Keywords

*Trypanosoma brucei*; metacyclic trypanosomes; variant surface glycoprotein; RNA binding protein; bloodstream form trypanosomes.

## 1. Introduction

African trypanosomes are the causative agent of sleeping sickness in humans and nagana in animals and remain a substantial public health concern in sub-Saharan Africa. The geographical restriction of the disease is due to the distribution of the tsetse fly vector (*Glossina* spp.), a blood-feeding dipteran, which transmits the protozoan parasite *Trypanosoma brucei*. To cope with the changing environment in two host systems, *T. brucei* transitions through distinct life cycle forms that have evolved to assure parasite survival and successful transmission to the next host [1, 2]. For instance, in the bloodstream of the mammal, the parasites exist mainly as proliferative slender forms, which establish parasitaemia. The differentiation of slender to quiescent, stumpy bloodstream forms (BFs) occurs in response to cell density [3] and these are the forms primed to survive the environmental change associated with the uptake by the tsetse fly [4]. In the midgut of the insect vector stumpy forms differentiate into procyclic forms that have lost their infectivity. In order to regain infectivity, parasites need to go through an intricate developmental program that generates epimastigotes and eventually leads to the establishment of infectious metacyclics in the tsetse salivary glands [5]. Metacyclics, similar to stumpy forms, are quiescent and primed for mammalian invasion [6, 7].

The two-host environments encountered by *T. brucei* requires a complex cellular differentiation program that includes changes in surface protein expression, metabolism, organelle function, and cytoskeletal architecture. For example, the trypanosome plasma membrane is covered by a distinct, densely packed surface coat consisting of GPI-anchored proteins at different stages of the life cycle. To outmaneuver the immune system when residing in the bloodstream of the mammalian host, *T. brucei* expresses a variant surface glycoprotein (VSG) coat, which is the paradigm for antigenic variation [8, 9]. In the tsetse midgut, trypanosomes cover their surface with procyclins, a family of EP (Glu/Pro repeat-containing) and GPEET (Gly/Pro/Glu/Glu/Thr repeat-containing) proteins [10, 11], whereas epimastigote forms express a family of proteins known as brucei alanine-rich proteins or

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BARPs [12]. Metacyclic trypanosomes are covered by a specific subset of VSGs, the metacyclic VSGs (mVSGs), which enables transmission to a vertebrate host [13]. In this context, differential expression of VSGs is one characteristic feature of the *T. brucei* life cycle and is based on two distinct genetic strategies. The genome of *T. brucei* has a large repertoire of VSG genes, but bloodstream-form VSG expression occurs predominantly from 1 of about 15 telomeric, polycistronic bloodstream expression sites (BES), and the active BES is sequestered and transcribed in an extranucleolar expression site body [14] by RNA polymerase I (Pol I) [15]. The mechanistic details limiting VSG expression to a single BES are not fully understood [8, 9, 16]. What has emerged is that transcription attenuation and epigenetic silencing play important roles for maintaining monoallelic VSG expression and there is recent evidence for regulation at the level of transcription initiation, involving class I transcription factor A (CITFA), the multi-subunit essential Pol I initiation factor [17].

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In contrast to a single dominant VSG being expressed on the surface of bloodstream forms at any one time, metacyclic trypanosomes are heterogeneous, displaying a number of different variants. However, each individual trypanosome expresses just one distinct mVSG, which are located at a telomere and are expressed from a monocistronic Pol I transcription unit [18–20]. Very early studies, based on monoclonal antibodies, have estimated that the repertoire of mVSGs expressed in metacyclics to be between 14 and as many as 27 [21]. Nevertheless, a subsequent survey of VSGs in the *T. brucei* Lister 427 strain challenged these numbers, since only six mVSGs with a typical metacyclic promoter were found [22]. In addition, metacyclics generated in our *in vitro* system, based on the inducible expression of the RNA binding protein 6 (RBP6), express five major mVSG transcripts, coding for mVSG-397, -653, -1954, -531 and -639 [23]. Our *in vitro* results highlighting a more limited repertoire of expressed mVSGs mirror tsetse fly transcriptome data, where four mVSGs were identified [24]. Regardless of the uncertainty about the size of the mVSG repertoire, at present little is known how mVSG gene expression is activated and how the expression is switched to bloodstream-form VSGs, once the parasite enters a mammalian host.

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Although inducible expression of RBP6 in *T. brucei* procyclics initiates a developmental progression to metacyclics [23], multiple attempts to continue the life cycle *in vitro* to bloodstream forms were unsuccessful. Here, we report the serendipitous isolation of a single point mutation in RBP6 (Q109K), whose expression not only generated MFs, but allowed further differentiation *in vitro* to BFs expressing VSG-2, formerly known as VSG 221.

## 2. Material and methods

### 2.1. *T. brucei* cell culture, production and purification of MF cells

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Inducible expression of an RBP6 (Tb927.3.2930) transgene in the vector pLew100.v5 was carried out in the *T. brucei* Lister 427(29–13) procyclic strain as described [7, 23]. Different trypanosome cell types (procyclics, epimastigote and metacyclics) were scored after staining of DNA in live cells with DAPI. Cells were assigned to a type based on their size, shape, position of the kinetoplast relative to the nucleus, and position of the kinetoplast relative to the posterior end of the cell. Metacyclics were purified at room temperature on 0.1 mm diameter zirconia/silica beads columns prepared in Pasteur pipettes in BBSG buffer

(50 mM bicine, 50 mM NaCl, 5 mM KCl, 70 mM glucose, pH 8.0) [25]. Purified metacyclics were cultured in BF conditions in HMI-9 media supplemented with 10% FBS at 37°C with 5% CO<sub>2</sub>. The transformation to BF cells is not very efficient and few cells survive the selection procedure, i.e. around day 5 the culture has less than 10<sup>3</sup> parasites per ml.

## 2.2 RNA preparation, RNA-Seq, read processing, data analysis and RT-PCR

Total RNA was prepared from 2 × 10<sup>7</sup> PF, purified MF, and BF cells. Biological replicates represent experiments performed at least two weeks apart. The RNA was prepared with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Four independent samples, i.e. biological replicas, were prepared from each life cycle stage and from each time point in a time course and isolation of poly(A)<sup>+</sup> mRNA, library preparation and sequencing on an Illumina HiSeq2500 platform were performed at the Yale Center for Genome Analysis. Reads of 75 nt in length were mapped to the *T. brucei* 11 megabase chromosomes (GeneDB version 5) using the Lasergene 14.1.2 software package from DNASTAR as described [7], except that the minimum aligned length was increased to 70 nt from 35 nt. RPKM (reads assigned per kilobase of target per million mapped reads) was used for normalization and the calculation of RPKM values was restricted to predicted open reading frames. Differential gene expression analysis was done with the Lasergene software package from DNASTAR [7] and differentially expressed genes were analyzed for functional annotation using the Gene Ontology (GO) enrichment tool on the TriTrypDB webserver (<http://tritrypdb.org/>) and GO terms were condensed by submitting to REVIGO [26].

Since VSG genes share substantial sequence similarities, in particular towards the 3' end of the gene, read coverage was visually inspected using the DNASTAR SeqMan NGen program, and only genes with a uniform read coverage were included in the data analysis.

Semi-quantitative RT-PCR was performed to confirm RNA-Seq results for selected mRNAs with SuperScript III reverse transcriptase (Invitrogen) and Platinum Pfx DNA polymerase (Invitrogen) following manufacturer's instructions. First strand cDNA synthesis was performed with random hexamers and the sequences of the PCR primers for the individual transcripts analyzed are available upon request.

RNA-Seq data from this study have been submitted to the NCBI Sequence Read Archive - SRA at <http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi> with accession numbers: SRP153824, SRP153562 and SRP152737.

## 3. Results and Discussion

### 3.1. The RBP6 mutant Q109K promotes an accelerated development to metacyclics

We have previously shown that the *T. brucei* RNA binding protein RBP6 is a master regulator for the transformation of non-infectious procyclic forms into infectious metacyclic forms [23]. RBP6 is 239 amino acids (aa) long and contains two RNA Recognition Motifs or RRM, separated by a 42 aa linker containing 11 consecutive glutamine residues (Fig. 1). To begin to understand the mechanism of RBP6 function, we generated N- and C-terminal epitope-tagged versions of the protein. However, this abolished RBP6's ability to trigger

developmental progression, indicating that intact termini are required for its function. Since the *T. congolense* and *T. vivax* proteins have a shorter or an absent stretch of glutamines in the linker between the two RRM, respectively (Fig. 1B), and over-expression of either protein in *T. brucei* procyclics generated metacyclics with a time course similar to the one induced by the *T. brucei* protein (unpublished data), we explored the possibility of introducing an epitope tag in place of the stretch of glutamines. This led to the serendipitous isolation of a mutant RBP6 where the first glutamine (Q109) of the stretch of 11 glutamines was changed to a lysine (Fig. 1B).

Inducible expression of wild-type RBP6 (wtRBP6) takes about 6 days to produce 30–40% metacyclics. However, inducible expression of mutant, untagged RBP6 (mutRBP6) generated metacyclic cells in 2–3 days with about 10% of the cells going through this transformation. Visual inspection by microscopy after DNA staining of the culture during the transition from procyclics to metacyclics appeared to suggest that the intermediate epimastigote stage was skipped, since we did not observe epimastigote cells. To confirm this observation, we monitored the levels of a diagnostic marker for short epimastigotes, namely the brucei alanine-rich protein or BARP [12] in cultures after inducibly expressing either the wild-type or mutant RBP6 for two days. As expected, in cultures where wtRBP6 was induced we observed approximately 20% epimastigotes and the levels of both BARP mRNA and protein were highly upregulated as compared to un-induced cells (Fig. 2, lane 2). In contrast, induction of mutRBP6 revealed a minor increase (less than 2-fold) in the level of BARP mRNA and no detectable increase of BARP protein as compared to un-induced procyclic cells (Fig. 2, lane 4). Thus, the mutRBP6 induced a developmental program generating metacyclic trypanosomes in 2–3 days without going through the intermediate epimastigote stage.

### 3.2. Differential mRNA expression during the development from procyclic to metacyclic cells

To begin to understand events triggered by wild-type or mutant RBP6, we first performed RNA-Seq analysis after a 24-hour induction. wtRBP6 changed the level of 184 transcripts, with 152 and 32 transcripts up- and down-regulated 2-fold or more, respectively (Table 1 and suppl. Table S1). Most increased in mRNA abundance was an atypical VSG of unknown function (Tb927.5.3990, 140-fold). In addition, up-regulated transcripts included the mitochondrial alternative oxidase (Tb927.10.7090, 7.1-fold), BARP (Tb927.9.15530, 5.8-fold), six RNA binding proteins (DRBD6A/DRBD6B, Tb927.3.3960/Tb927.3.3930; RBP26, Tb927.7.3730; DRBD12, Tb927.7.5380; PUF5, Tb927.7.4730; and ZC3H45, Tb927.11.8470), two cyclins (Tb927.8.6350 and Tb927.6.5020), as well as several nucleoside and amino acid transporters. Finally, three mRNAs encoding proteins associated with differentiation (PAD, carboxylate-transporter family), implicated as sensors of environmental stimuli and triggers of differentiation [27], were up-regulated, namely PAD4, PAD6 and PAD8 (Tb927.7.5960, 5980 and 6000). Functional categorization by Gene Ontology (GO) analysis of the up-regulated transcripts revealed statistically significant cellular components, including membrane and integral component of the membrane, whereas the down-regulated transcripts were enriched for transporter activity (suppl. Table S2).

In contrast, inducible expression of mutRBP6 changed abundance levels of 22 transcripts after 24 hours, with 21 and 1 transcripts up- and down-regulated, respectively (Table 1 and suppl. Table S3). 21 of the 22 transcript changes were also observed in the background of wtRBP6 induction, with the transcript for the flagellar calcium-binding 44 kDa protein (Tb927.8.5460) only appearing in the mutant RBP6 induction. However, this transcript increased 2-fold after 4 days of induction of wtRBP6. The low number of detected transcript changes is most likely a reflection of a more heterogeneous population in mutRBP6-induced cells, since only about 10% undergo the differentiation process, as compared to 40–50% in wtRBP6-induced cells.

We next monitored transcript abundance changes during 6 and 5 days of induced expression of wild-type and mutant RBP6, respectively (Table 1 and suppl. Tables S4 and S5). 80 transcripts changed in abundance 2-fold or more during the induction of mutRBP6, as compared to 601 transcripts in wtRBP6-induced expression, and the majority of transcripts changing in mutant RBP6 cells (72 out of 80) were part of the set in wtRBP6 cells. The 8 transcripts changing abundance only in mutRBP6-induced cells are coding for a zinc finger protein (Tb927.10.12760, ZC3H36), an RNA binding protein (Tb927.11.12100, RBP5), a mitochondrial carrier protein (Tb927.10.12840, MCP12), a putative STE/STE11 serine/threonine-protein kinase (Tb927.6.2030), a receptor-type adenylate cyclase (Tb927.6.430, GRESAG 4), a putative protein with a PSP1 C-terminal conserved region (Tb927.9.9370), and two hypothetical proteins (Tb927.4.4940, Tb927.10.4340). Finally, monitoring transcripts encoding VSGs, and in particular the 5 known metacyclic VSGs (VSG-1954, VSG-397, VSG-531, VSG-639, and VSG-653), did not show any significant differences with the 5 known mVSGs becoming the most abundant transcripts around day 2 and 3 in both wild-type and mutant RBP6 inductions.

### 3.3. Metacyclics arrest outside the G1/G0 checkpoint

In *T. brucei* the nucleus and kinetoplast (mitochondrial kDNA) have distinct replication cycles, with the kinetoplast beginning replication first [28]. Cells in the G1/G0 phase have 1 kinetoplast and 1 nucleus (1K1N), cells with 2 kinetoplasts and 1 nucleus (2K1N) have segregated the kinetoplast and are at the end of S phase, whereas cells with 2 kinetoplasts and 2 nuclei (2K2N) have undergone mitosis but have not yet undergone cytokinesis. As predicted for an asynchronously growing cell population, procyclic parasites are mainly in the 1K1N configuration with around 20–25% of cells representing dividing cells with either a 2K1N or 2K2N configuration [7]. We have shown previously that purified metacyclics (99% pure) generated by inducibly expressing wtRBP6 are quiescent cells with no evidence of dividing cells and arrested in G1/G0 [7]. In contrast, inducible expression of mutRBP6 resulted in metacyclics, where 75% of the cells were in the 1K1N configuration, and 2K1N and 2K2N configurations represented 6% and 13% of the cells, respectively (Fig. 3). In addition, abnormal cell types were increased to 6% with cells with multiple kinetoplasts and/or nuclei, >2K>2N cells, being the most prevalent. The increased number of 2K2N and >2K>2N cells could indicate a defect in kinetoplast replication/segregation and/or cytokinesis. Since the metacyclics are non-dividing, this result indicated that inducible expression of mutRBP6 not only skipped the epimastigote stage, but also caused cell cycle arrest outside the G1/G0 phase.



### 3.4. RNA-Seq analysis of metacyclics

Since the RNA-Seq time course analysis described above monitored a heterogeneous population with only about 10% of cells becoming metacyclics after mutRBP6 induction, we next interrogated the transcriptome of purified metacyclics. This revealed 1,392 differentially expressed transcripts in metacyclics when compared to un-induced procyclic trypanosomes, with 660 and 732 transcripts up- and down-regulated, respectively (Table 1 and suppl. Table S6). Overall, metacyclics derived from inducible expression of mutRBP6 had a predominantly bloodstream-form type transcriptome with the largest changes occurring in genes encoding cell surface components. This result mirrored our previous transcriptome analysis of metacyclics obtained after induction of wtRBP6 [7] and thus, the majority of changes (1,097, 79%) were in common. Nevertheless, there were noteworthy differences, in particular in the set of transcripts down-regulated only in mutant metacyclics (231 transcripts). 13 subunits of eukaryotic translation initiation factors 2, 3 and 4 were less abundant in metacyclics than in procyclics, with 8 transcripts encoding subunits of eukaryotic initiation factor 3 (eIF3), the largest and most complex initiation factor. Furthermore, 8 aminoacyl-tRNA synthetases were down-regulated, thus highlighting translation initiation and accurate mRNA translation. GO analysis also identified components of the mitochondrial membrane as significantly down-regulated in this data set (suppl. Table S2). Conversely, the 30 transcripts only down-regulated in metacyclics generated by wtRBP6 induction were enriched for ribosomal proteins (7 out of 30). Thus, although both metacyclic transcriptomes clearly highlight a down-regulation of transcripts encoding components of the translation machinery, there appears to be a subtle difference in what components are affected in the respective transcriptomes, which will require further investigations. Finally, among the 63 transcripts only up-regulated in metacyclics following mutRBP6 induction was RBP33 (suppl. Table S6) and this transcript was also one of the most highly expressed mRNAs in the metacyclic transcriptome (number 112; suppl. Table S7). This is an intriguing result, since it was previously shown that overexpression of RBP33 leads to a rapid growth arrest in G2/M, i.e. accumulation of 2K2N cells [29], which is one of the configurations enriched in metacyclics (13%; Fig. 3). Overexpression of RBP33 also resulted in a decrease of total mRNA [29], an observation we noted previously in metacyclics when compared to procyclics [7]. Thus, RBP33 could play a role in the establishment of metacyclics and in particular in the generation of metacyclics uncharacteristically arrested at the G2/M checkpoint.

The similarity of the transcriptomes of the two metacyclic populations was also true for the mRNAs encoding VSGs. Although using 0.1% of VSG mRNA as a cutoff, there were 23 VSG transcripts in the wild-type and 9 VSGs in the mutant RBP6 metacyclics, the overall representation of the 5 known metacyclic VSGs was comparable (Fig. 4). As shown previously [7], VSG-397 transcript was the most abundant representing about 50% of the VSG mRNAs in both populations, and for the transcripts derived from the 5 known metacyclic VSG expression sites comprised between 86 and 90 % of VSG mRNAs.

### 3.5. Metacyclics generated by inducibly expressing mutRBP6 can progress to bloodstream forms *in vitro*

Although inducible expression of wtRBP6 triggered differentiation of procyclics to metacyclics in culture, further differentiation to bloodstream forms required infection of mice, despite numerous attempts to continue the development *in vitro*. The unusual characteristics of mutRBP6, especially the apparent arrest of some cells at a different checkpoint, as compared to metacyclics generated by wtRBP6, led us to test whether this cell population is able to differentiate into bloodstream forms *in vitro*. We therefore incubated enriched metacyclics in bloodstream form media at 37°C and within two weeks an exponentially growing culture was obtained, which by visual inspection had characteristics of bloodstream form (BF) trypanosomes. We next investigated the identity of the expressed VSG(s) in the established BF culture, i.e. did the cells switch expression from metacyclic VSGs to a BF VSG? We performed RT-PCR with common primers and following cloning and sequencing the prominent VSG in the *in vitro* generated BF cells was VSG-2. The appearance of VSG-2 can be explained by the observation that BF trypanosomes expressing VSG-2 have a shorter doubling time *in vitro*, compared to cells expressing other VSGs [30]. This result was confirmed with gene-specific primers, which detected VSG-2 mRNA at day 2 after transfer to BF conditions and on day 11 only VSG-2, but not VSG-397 was detected (Fig. 5), providing strong evidence that the metacyclic VSGs were silenced and the BF VSG-2 was activated during this transformation process. In order to more accurately monitor the VSG switching event, we performed high-throughput mRNA sequencing for cells collected at similar time points. Our RNA-Seq data revealed that multiple VSGs were being expressed during this transition. Using 0.1% of VSG mRNA as an arbitrary cutoff, 23 VSGs were expressed on day 0 with the known five mVSGs being the most prominent VSGs (>80%; Fig. 6). The number of different expressed VSGs increased to 67 at day 3 and then decreased to 38 at day 11. Many of these VSGs, e.g. VSG-2, -21, -3, -8, -17, -11 and -13 are known to be present in BESs in Lister 427 [31]. Whereas the mVSG gradually decreased, VSG-2 was above the threshold on day 1 (0.4%) and steadily increased till it represented more than 90% of VSG mRNAs on day 11, but it was only at day 8 that VSG-2 became the most prominent VSG.

Monitoring the ESAGs revealed that the mRNAs for ESAG7 and ESAG6, the two subunits of the transferrin receptor and the first two genes in a BSF expression site, were upregulated from the first day of transition, reached a peak at day 3 and then decreased (Fig. 7). It is intriguing to note that the ESAG7/6 mRNA accumulation mirrors the increased diversity of VSGs expressed, suggesting that multiple expression sites were activated in the population initially, but this will require further investigations.

Including the changes in VSG and ESAG transcript abundance, 560 and 73 transcripts were up- and down-regulated, respectively, during the day 0 to day 11 time course (suppl. Table S8). GO analysis revealed a significant enrichment of transporter activity and membrane components in the up-regulated transcripts. A similar representation emerged when the metacyclic transcriptome (day 0) was compared to the bloodstream form one (day 11), with 449 and 61 transcripts up- and down-regulated, respectively (suppl. Table S9). Consistent with our previous observation that the transcriptome of metacyclics was similar to that of



bloodstream forms, the two transcriptomes showed a very good correlation ( $R^2=0.97$ ), and a majority of the up-regulated transcripts (71 out of 449) encoded putative VSG- and ESAG-related proteins. Notable highly up-regulated transcripts include chromosomal passenger complex 2 (11.5-fold; Tb927.11.14840), two procyclic-enriched flagellar receptor adenylate cyclases (7-fold; ACP1, Tb927.11.17040 and ACP2, Tb927.10.16190), PUF nine target 1 (4.9-fold; Tb927.11.6550), and a family of 5 almost identical proteins (Tb927.5.2160, Tb927.5.2170, Tb927.5.2200, Tb927.5.2230, and Tb927.5.2260), up-regulated between 7- and 10-fold. The 5 transcripts encode a 116 aa-long protein with a predicted META domain, a small domain of about 100 aa found in proteins of unknown function. Nevertheless, in *Leishmania major* the syntenic protein meta-1 localized to the flagellar pocket [32], was mainly expressed in infective metacyclics [33] and overexpression in *L. amazonensis* generated a more virulent strain [33]. Intriguingly, the *T. brucei* transcripts encoding a META domain-containing protein were significantly down-regulated in metacyclics as compared to procyclics (between 8.6- and 11-fold; suppl. Tables S6 and S9) in both the wt and mutRBP6 background, which was also evident at the protein level in wtRBP6 metacyclics [7]. Thus, further experiments are warranted to elucidate the function of these META domain-containing proteins in the *T. brucei* life cycle.

In conclusion, inducible expression of a mutant RBP6 where the first glutamine (Q109) of a stretch of 11 glutamines between the two RRM domains was changed to a lysine not only offers an experimental system to investigate how trypanosomes receive instructions to begin synthesizing the mVSG coat, and how each cell expresses a single mVSG, but more importantly will also provide insights how mVSG expression is repressed and switched to the expression of bloodstream-form VSGs. The effect of this single amino acid change is quite remarkable, considering that removing the entire stretch of 11 glutamines did not impact RBP6 function. The next important question to be tackled is the mechanism of action of this mutant RBP6 protein.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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## Abbreviations:

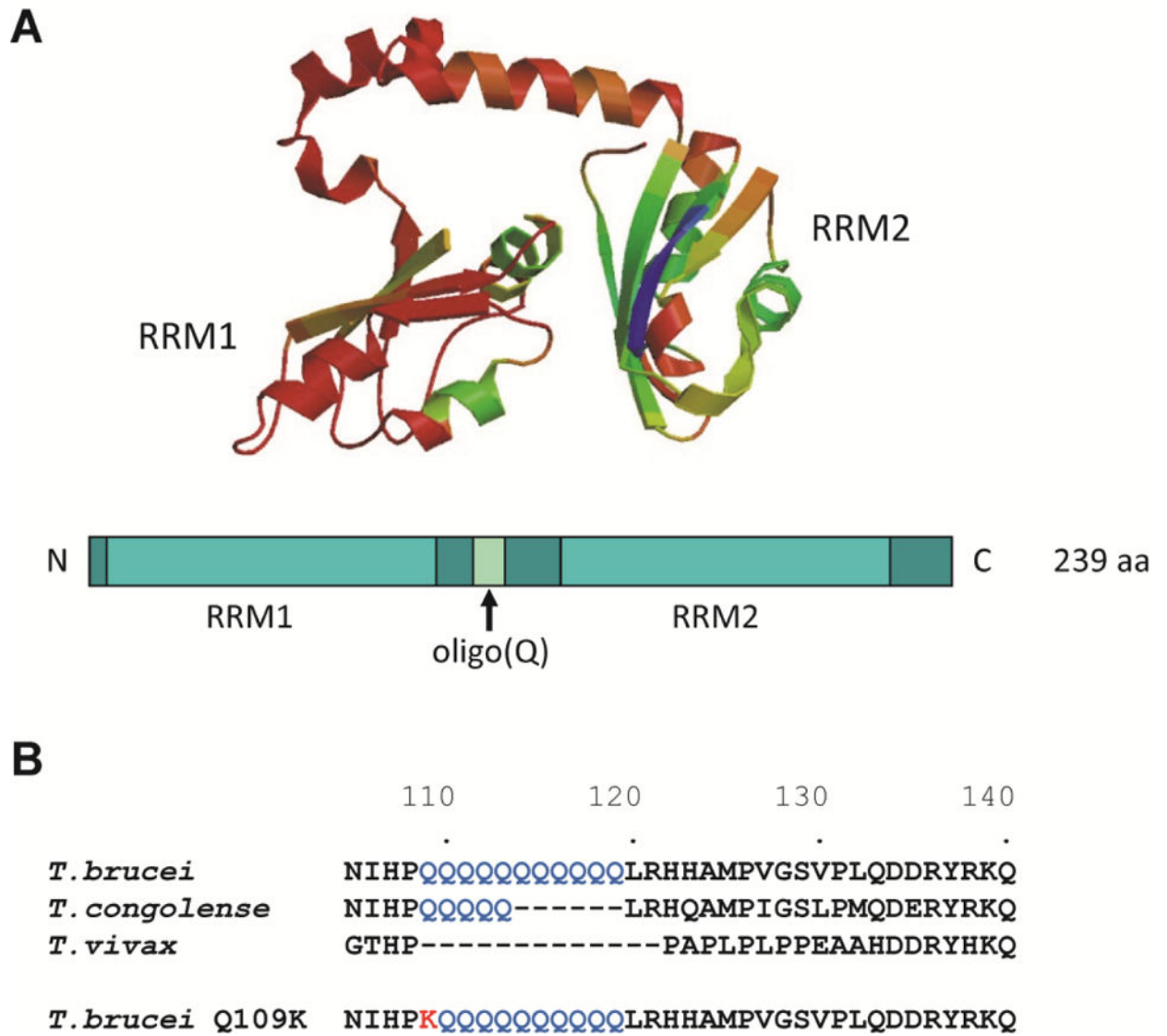
<b>BARP</b>	brucei alanine-rich protein
<b>BES</b>	bloodstream expression site
<b>BF</b>	bloodstream forms
<b>GO</b>	Gene Ontology

<b>MF</b>	metacyclic forms
<b>mVSG</b>	metacyclic variant surface glycoprotein
<b>Pol I</b>	RNA polymerase I
<b>PF</b>	procyclic forms
<b>RBP6</b>	RNA binding protein 6

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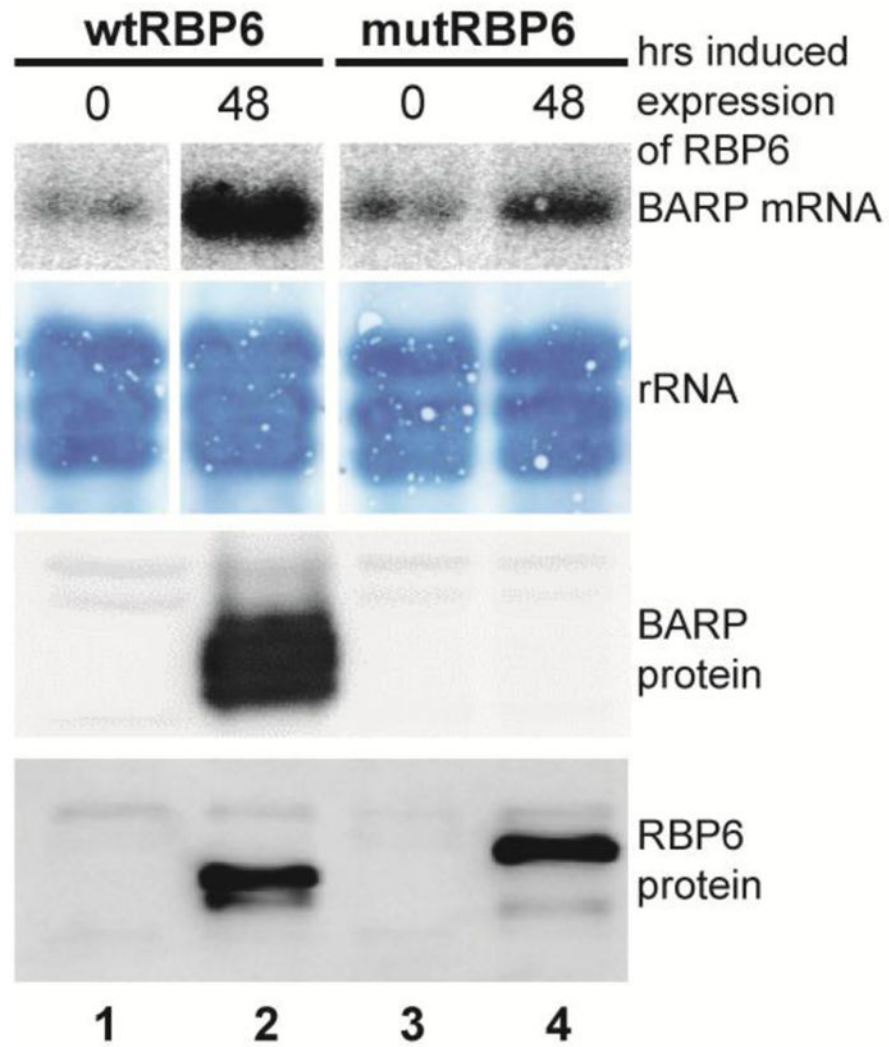
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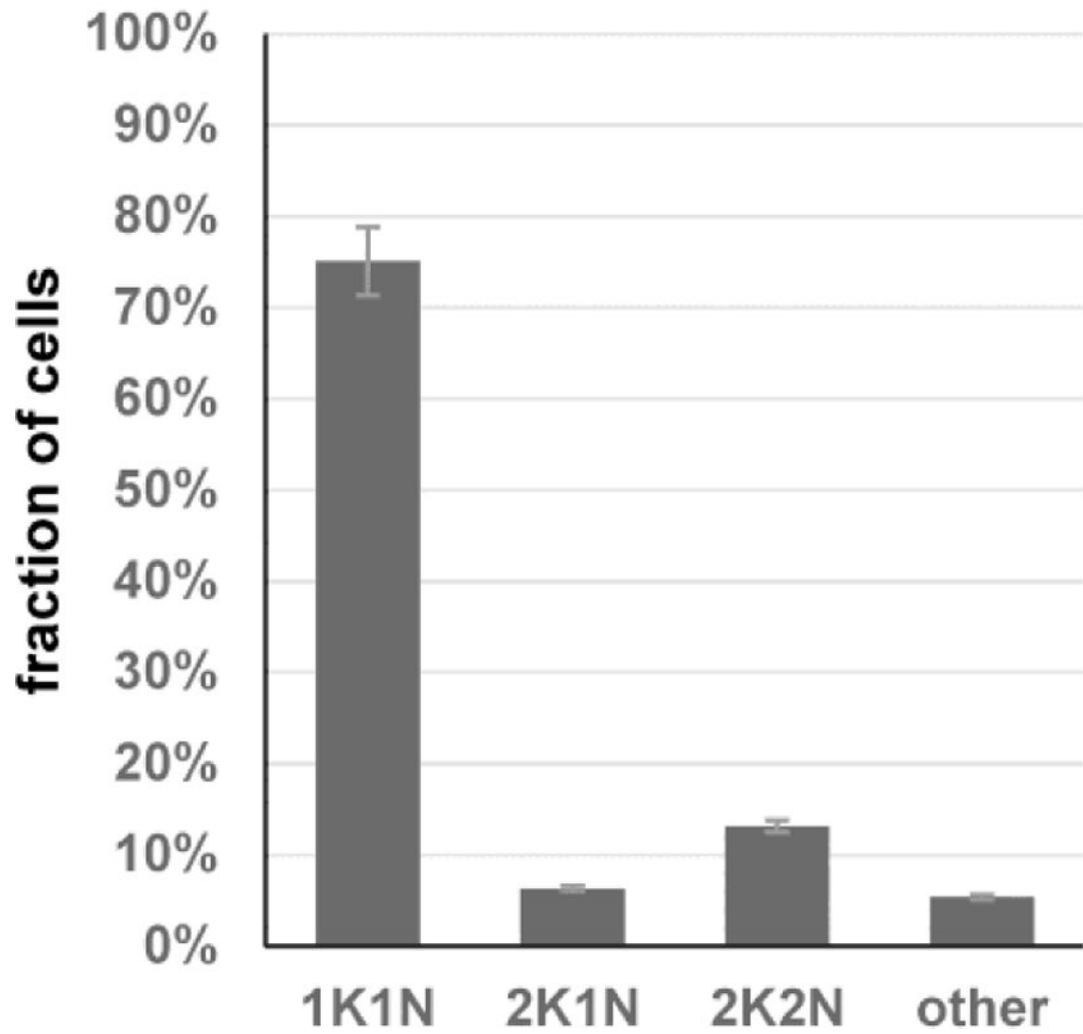
**Fig. 1. Bioinformatics analysis of RBP6.**

(A) Tertiary structure model of the *T. brucei* RBP6 (Tb927.3.2930) using the alignment interface of SWISS-MODEL [34] and schematic diagram below. RRM, RNA recognition motif. (B) Sequence alignment of *T. brucei*, *T. congolense* (TcIL3000\_0\_03630), *T. vivax* (TvY486\_0302270) and the mutant *T. brucei* Q109K RBP6. Note that the alignment was restricted to the oligo(Q) region.



**Fig. 2. BARP expression.**

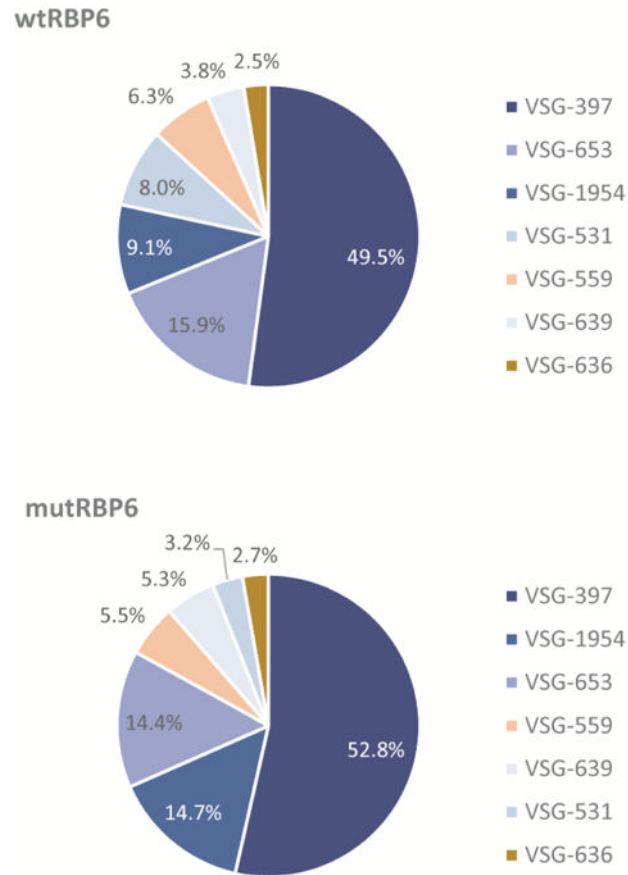
wt and mutRBP6 were induced in procyclic cells for 48 hrs, lanes 2 and 4, and BARP mRNA and protein were monitored by Northern and Western blot, respectively. RBP6 protein expression was monitored by Western blot. Note that mutRBP6 is Flag-tagged.



**Fig. 3. Karyotype of metacyclics following induction of mutRBP6.**

Purified metacyclics were scored for the presence of 1 kinetoplast and 1 nucleus (1K1N), 2 kinetoplasts and 1 nucleus (2K1N), 2 kinetoplasts and 2 nuclei (2K2N) or other configuration (other).





**Fig. 4. Metacyclic VSG expression.**

Presented are the percentages for the seven most abundant VSGs. Metacyclic VSGs are shown in shades of blue.

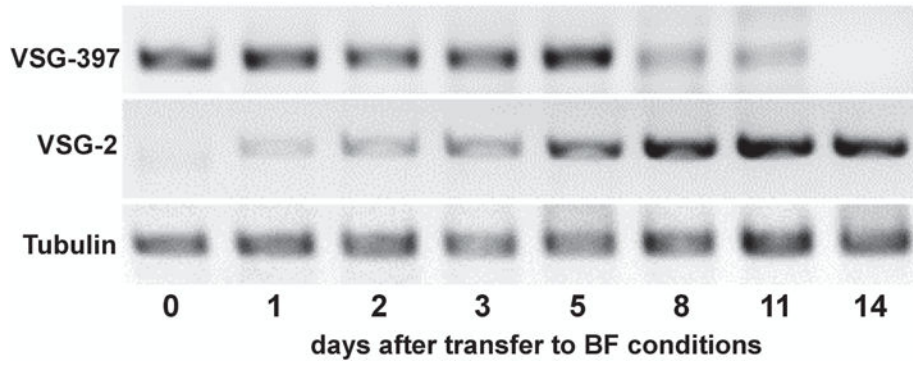


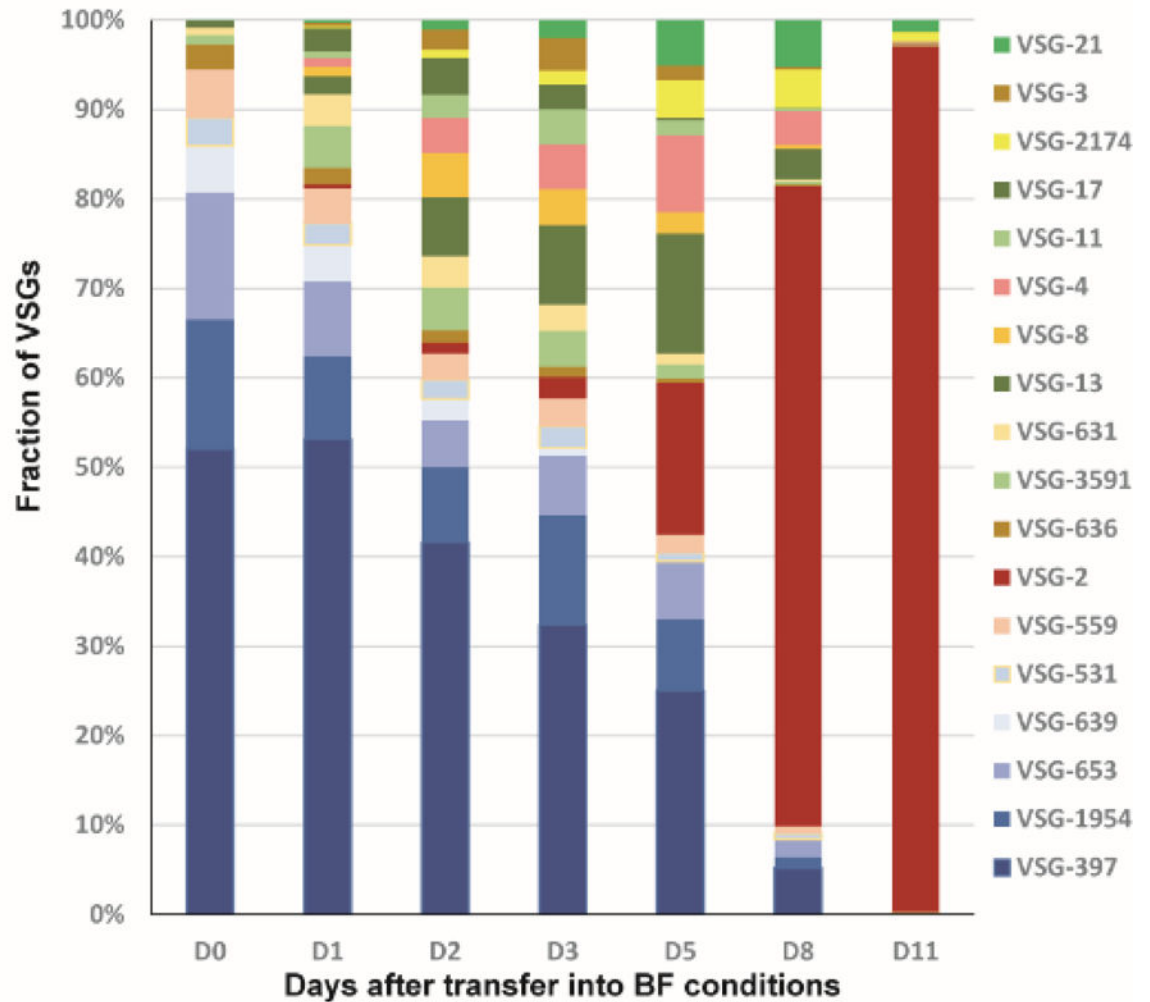
Fig. 5. RT-PCR using primer specific for VSG-397, VSG-2 and  $\alpha$ -tubulin.

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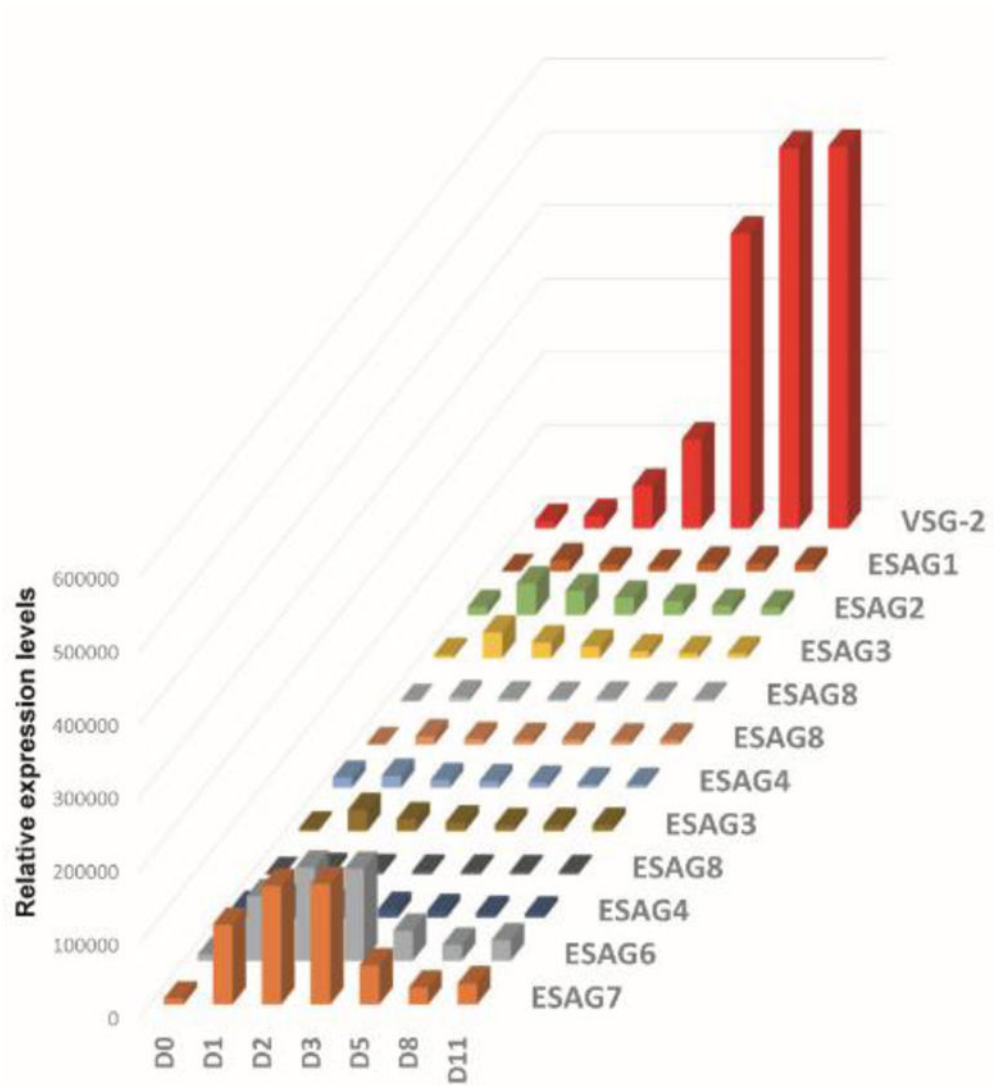


number of different VSG transcripts (0.1% cutoff)

Day	Number of different VSG transcripts (0.1% cutoff)
D0	23
D1	57
D2	64
D3	67
D5	60
D8	54
D11	38

**Fig. 6. Dynamics of VSG expression during transformation from metacyclics (D0) to bloodstream forms (D11).**

Each colored box represents an individual VSG's presence in the population. Only variants present at greater than 0.1% of the population at each time point are shown. Metacyclic VSGs are shown in shades of blue.



**Fig. 7.** Dynamics of ESAG and VSG-2 expression during transformation from metacyclics (D0) to bloodstream forms (D11).

**Table 1.**

Differentially expressed transcripts.

	wtRBP6			mutRBP6		
	24 h induction	6 days induction	Purified metacyclics	24 h induction	5 days induction	Purified metacyclics
<b>Significantly regulated transcripts</b>	184	601	1,302	22	80	1,392
<b>Up-regulated</b>	152	504	771	21	71	660
<b>Down-regulated</b>	32	97	531	1	9	732

All changes are compared to un-induced cells.

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