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Investigating the Role of RNA-Binding Protein 5 in the Life Cycle Differentiation of *Trypanosoma Brucei*

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INVESTIGATING THE ROLE OF RNA-BINDING PROTEIN 5 IN THE LIFE CYCLE
DIFFERENTIATION OF *TRYPANOSOMA BRUCEI*

A Thesis Presented

by

DAVID F. ANAGUANO PILLAJO

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INVESTIGATING THE ROLE OF RNA-BINDING PROTEIN 5 (RBP5) IN THE LIFE
CYCLE DIFFERENTIATION OF *TRYPANOSOMA BRUCEI*

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DEDICATION

This thesis is dedicated to the most important part of my life, my family, Blanca, Marcelino, Daniel, Marcelo, Mary, Emilio and Tania, who have been my support through this long pathway and have always been there when I need them. Thanks for understanding my decisions, as difficult as they might be, and for encouraging me to become a better professional, but most importantly a better person.

David

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I want to thank the Fulbright program for funding my expenses during the development of my thesis project and degree and for the great opportunity of being part of such prestigious program where I was able to meet people from all over the world with such a great potential. Finally, I would like to thank to the Microbiology department for all the help during my time as a student and for granting me the possibility of receiving high quality education.

At the end, I would like to thank everyone who somehow and somewhat offered some help or just their friendship that gave me the strength to overpass any difficulties in this road.

ABSTRACT

INVESTIGATING THE ROLE OF RNA-BINDING PROTEIN 5 IN THE LIFE CYCLE

DIFFERENTIATION OF *TRYPANOSOMA BRUCEI*

SEPTEMBER 2018

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Trypanosomatid parasites such as *Trypanosoma brucei* have unusual mechanisms of gene expression including polycistronic transcription, mitochondrial RNA editing and trans-splicing. Additionally, these protists rely mainly on post-transcriptional regulation where RNA-binding proteins (RBP) have shown to play a major role. RBP6 and RBP10 are two examples of RBPs that play crucial roles in procyclic and bloodstream form parasites differentiation respectively, by post-transcriptional regulation. Over-expression of RBP6 is enough to promote differentiation into metacyclic trypomastigotes that are infective to mice. However, continuous expression is required, and this pattern does not reflect the natural expression in the tsetse fly or the influence of other RNA-binding proteins. RBP5 is a RBP with a single RNA-recognition motif similar to RBP6 and RBP10, whose expression is upregulated during the life stages within the salivary glands of tsetse flies. We hypothesize the RBP5 facilitates metacyclogenesis in the tsetse fly. To evaluate possible contributions to *T. brucei* differentiation, we will over-express RBP5 in procyclic cells alone and in combination with RBP6. Initial screening of cells over-

expressing PTP-tagged RBP5 resulted in parasites with a moderate growing defect, and the scoring of nuclei and kinetoplasts in fixed cells showed a progressive accumulation of cells with 2 nuclei and 2 kinetoplasts (2N2K) and appearance of multinucleated cells. On the other hand, over-expression of non-tagged RBP5 generated a more severe growing defect, starting immediately after the first day of induction. The scoring of nuclei and kinetoplasts resulted in a drastic increase of 2N2K cells and a greater appearance of multinucleated cells, which suggests an irregular cell cycle progression. When developing the dual over-expression system, our cells over-expressing RBP6 were not able to differentiate into any stage, and when over-expressing RBP5 and RBP6 coordinately, no differentiation process was observed either. Together these data suggest that RBP5 might be a regulator of genes involved in the initiation of cytokinesis in *T. brucei* parasites, however a role in metacyclogenesis cannot be discarded since we were not able to obtain metacyclic parasites. This study helped us to get a better understanding of the post-transcriptional regulatory mechanisms that repress and regulate *T. brucei* cell cycle progression.

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CHAPTER 1

INTRODUCTION

Human African trypanosomiasis, also known as African sleeping sickness, is a vector-borne parasitic disease transmitted by the bite of the insect vector tsetse fly (genus *Glossina*). Sleeping sickness is curable with medication, but it could be fatal if left untreated (World Health Organization, 2013). Human African trypanosomiasis is endemic and spread across 23 countries within sub-Saharan Africa (Lejon et al., 2013; Simarro et al., 2010), where the tsetse fly is also distributed (Malvy and Chappuis, 2011). Although the disease has been reported in urban and peri-urban areas, it mainly affects people in poor and remote rural areas with limited access to health services and who depend on agriculture, fishing, animal husbandry or hunting. This, along with the fact that the actual diagnosis and treatment methods are unsatisfactory, causes a great socioeconomic impact in the affected villages and countries (Brun et al., 2010; 2013). Until today, no vaccine has been developed and most of the efforts focus on the control and surveillance of the insect vector (World Health Organization, 2013).

Since its description around the 13th century, sleeping sickness has had different cycles of decrease and resurgence. After the last increase during 2001, when the number of infected people reached alarming levels, reinforcement of control programs conducted by the World Health Organization (WHO) helped to reduce the number of reported cases below 10,000 during 2009 and to 7,106 cases in 2012 (Lejon et al., 2013; Simarro et al., 2015). In spite of this decrease, WHO reports a considerable number of underreported cases with at least 61 million of people at risk of contracting the disease in 36 countries,

making the disease one of the most neglected tropical diseases in the world (Brun et al., 2010; 2013).

The etiological agent of the African trypanosomiasis is the extracellular protozoan parasite *Trypanosoma brucei* spp., which includes two human infective subspecies *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, and one animal infective subspecies *Trypanosoma brucei brucei* (Malvy and Chappuis, 2011; Matthews, 2005). The two human infective subspecies generate different forms of the diseases with differences in epidemiology, transmission dynamics, clinical presentation and geographical distribution. *Trypanosoma brucei gambiense* mainly infects humans and animals, in a much fewer proportion, producing a chronic infection that progresses over several years and it is characterized by a low number of parasites in the blood. This form is distributed through Western and Central Africa. On the other hand, *Trypanosoma brucei rhodesiense* affects principally to livestock and game animals, while humans are only accidental hosts. It produces an acute and rapidly progressive infection characterized by a high number of parasites in the blood. This variant occurs in Eastern and Southern Africa (Franco et al., 2014; Lejon et al., 2013; Malvy and Chappuis, 2011). Finally, *Trypanosoma brucei brucei* is a non-human pathogenic subspecies that infects wild and domestic animals, especially cattle, causing the related disease, Nagana. This parasite has been widely used as experimental model of Human African trypanosomiasis (Franco et al., 2014; Lejon et al., 2013; World Health Organization, 2013).

Trypanosoma brucei spp. belong to the family Trypanosomatidae, to which parasites that cause other neglected tropical diseases such as leishmaniasis and Chagas'

disease (American trypanosomiasis) also belong (World Health Organization, 2013). Trypanosomes exhibit a complex life cycle, with different biological stages in the insect vector and the mammalian host. *T. brucei* first establishes as a proliferative procyclic form in the tsetse fly midgut following a bloodmeal from an infected mammalian host. Procyclics express EP and GPEET procyclin surface proteins and have numerous adaptations including increased mitochondrial metabolism. Next, procyclics lose GPEET expression and migrate to the salivary glands where they differentiate into epimastigote forms. Epimastigotes expressing BARP surface protein attach to the salivary gland epithelia, where a subset of parasites undergoes meiosis and mating. Finally, they differentiate into non-replicating metacyclic forms, which express Variant Surface Glycoproteins (VSG) and are prepared to re-infect a new mammalian host (Dyer et al., 2013). In mammals, metacyclics differentiate into bloodstream forms that survive and multiply freely in the blood and tissue fluids, evading the immune response through antigenic variation of the VSG's. High cell density triggers growth arrest and differentiation into stumpy forms, which then can start the cycle again when a tsetse fly takes a bloodmeal. *T. brucei* development transitions are marked by many changes in mRNA and protein abundance which drive dramatic morphological and metabolic changes (Matthews, 2005; Mugo et al., 2017; Savage et al., 2016).

Despite extensive variation in mRNA and protein abundance through the different developmental stages, even in adjacent genes, *T. brucei* does not use conventional promoter driven transcriptional regulation but instead uses an unusual mechanism. Coding regions are organized into large unidirectional gene clusters, lacking canonical RNA polymerase II (Pol II) promoters, whose transcriptional boundaries have been

shown to be defined by histone variants and epigenetic regulation (Romaniuk et al., 2016). These clustered genes, with no apparent functional connection, are co-transcribed by RNA Pol II into polycistronic pre-mRNAs, and then excised to mature monocistronic mRNAs by 5' *trans*-splicing and 3' polyadenylation, with no evident regulation at the level of individual mRNAs. So, this modulation of expression in *T. brucei* relies mainly on post-transcriptional control, where RNA-binding proteins have been shown to play a major role by assuming the burden carried by transcription factors in conventional regulatory mechanism (Kolev et al., 2014; Lueong et al., 2016; Romaniuk et al., 2016).

RNA-binding proteins (RBPs) are known to be involved in every-step of RNA metabolism, in essential processes as for example differential pre-mRNA processing, mRNA transport and subcellular localization, mRNA stability, mRNA translation, protein stability and post-transcriptional modifications (Kolev et al., 2014). In *T. brucei*, RBPs have demonstrated roles in multiple processes dependent on post-transcriptional regulation such as differentiation (Hendriks and Matthews, 2005), development (Jha et al., 2015; Kolev et al., 2012; Mugo and Clayton, 2017; Subota et al., 2011), cell cycle (Archer et al., 2009), rRNA and mRNA processing (Cirovic et al., 2017; Droll et al., 2010) and heat shock response (Droll et al., 2013). RBPs bind to structural motifs in the mRNA, mainly located in the 3' untranslated region (UTR), to form a ribonucleoprotein (RNP) complex, which, in association with other mRNA-interacting proteins, will decide the fate of the mRNA inside the cell. It is likely that different transcripts with identical motifs are regulated in a similar way (Romaniuk et al., 2016). RBPs interact with mRNA motifs through their functional RNA binding domains (RBDs), where the most relevant in trypanosomes are RNA-recognition motifs (RRMs), Zinc Finger domains, KH

domains, Pumilio domains and ALBA domains (De Gaudenzi et al., 2005; Kolev et al., 2014). In trypanosomes, over 150 proteins with conserved RBDs have been found, however recent findings suggest that other disordered sequences in RBPs could also play important roles in protein-protein interactions and in the formation of RNP complexes (Calabretta and Richard, 2015).

During *T. brucei* development, RBP10 and RBP6 are two examples of RRM-domain proteins that play crucial roles in bloodstream and procyclic form parasites differentiation respectively, by post-transcriptional regulation. Over-expression of RBP10 in procyclic forms promotes differentiation into bloodstream-form trypanosomes while the depletion of RBP10 from bloodstream forms results in cells that can grow only as procyclics (Mugo et al., 2017). Also, over-expression of RBP6 is sufficient to promote differentiation into metacyclic trypomastigotes that are infective to mice (Kolev et al., 2012). However, continuous expression is required, and this pattern does not reflect the natural expression in the tsetse fly, where peak expression occurs in the proventricular stages (Savage et al., 2016), suggesting the influence of other RNA-binding proteins in the process. RBP5 is a 130-residue RNA-binding protein with a single RRM, like RBP6 and RBP10, located toward the N-terminus. Transcriptomic analysis showed that RBP5 mRNAs have a low abundance in procyclic forms and a peak expression after the proventricular stages migrate to the salivary glands (Savage et al., 2016). Our aim is to study the role that RBP5 plays during trypanosome differentiation of procyclics to metacyclics. First, RBP5 will be over-expressed in procyclic forms to find out if it promotes any differentiation process. Second, an inducible dual expression system will be used to determine if RBP5 contributes to the RBP6-driven metacyclogenesis process.

CHAPTER 2

MATERIALS AND METHODS

Plasmids

Over-expression of RBP5-PTP: The coding sequence of RBP5 (Tb927.11.12100) was PCR-amplified from *T. brucei* 927 genomic DNA. The PCR-amplified fragment was ligated into the HindIII and XbaI sites of pLEW100-PTP-PURO using Gibson cloning to create the pLEW100-RBP5-PTP-PURO vector (Appendix B1). The integrity of the over-expression plasmid was verified using restriction enzyme digestion with HindIII, XbaI, NruI and NotI. The restriction fragments were then visualized and analyzed using 0.8% agarose gel electrophoresis (Appendix B2). RBP5-PTP sequence was verified by sequencing.

Dual over-expression of RBP5 and RBP6: First, the same coding sequence of RBP5 was PCR-amplified and the fragment was ligated into the BglII and HindIII sites of pJ1271 (a kind gift of Dr. Jack Sunter) using Gibson cloning to create the pJ1271-RBP5 vector (Appendix B3). The integrity of the expression plasmid was verified using restriction enzyme digestion with NotI, NruI and EagI. The restriction fragments were then visualized and analyzed using 0.8% agarose gel electrophoresis (Appendix B4).

Second, the coding sequence of RBP6 (Tb927.3.2930) was PCR-amplified from *T. brucei* 927 genomic DNA. The PCR-amplified fragment was ligated into the HindIII and XbaI sites of pLEW100.v5 (Addgene, USA) using Gibson cloning to create the pLEW100.v5-RBP6 vector (Appendix B5). The integrity of the over-expression plasmid

was verified using restriction enzyme digestion with HindIII, BamHI, NotI and EcoRI. The restriction fragments were then visualized and analyzed using 0.8% agarose gel electrophoresis (Appendix B6). RBP5 and RBP6 sequences were verified by sequencing.

***T. brucei* cell lines**

Lister 427, 29-13 and SMUMA procyclic cells were cultured at 27°C in SDM-79 medium containing 15% heat-inactivated fetal bovine serum with the corresponding drug selection. Cell density was determined using a Beckman Coulter Z2 particle counter. All transfections were carried out using the AMAXA Nucleofactor (Lonza) according to manufacturer's protocol.

29-13 procyclic cells (Wirtz et al., 1999) carrying the T7 RNA polymerase and the tetracycline repressor (tetR) were used for induced over-expression of RBP5-PTP. The RBP5-PTP cell line was established by transfecting NotI-digested pLEW100-RBP5-PTP-PURO into 29-13 cells. Stable RBP5-PTP transfectants were selected with 1 µg/ml puromycin (Puro) followed by limiting dilution as previously described (Klingbeil et al., 2002).

SMUMA (Single Marker UMASS) procyclic cells carrying the T7 RNA polymerase, tetR and vanillic acid repressor (vanR) were used for induced over-expression of RBP5 and RBP6. This new cell line was generated by transfecting HindIII-digested pJ1173 plasmid (Sunter, 2016) (Appendix B7) into Lister 427 procyclic cells. Stable SMUMA transfectants were selected with 1 µg/ml Puro followed by limiting dilution as previously described (Klingbeil et al., 2002). SMUMA clonal cell lines were analyzed for expression of tetR and vanR by Western blotting and qPCR, respectively (Appendix A7, A8).

The SM-RBP5 and SM-RBP6 cell lines were established by transfecting NotI-digested pJ1271-RP5 and NotI-digested pLEW100.v5-RBP6, respectively, into SMUMA cells. Stable SM-RBP5 transfectants were selected with 10 $\mu\text{g/ml}$ blasticidin and SM-RBP6 transfectants were selected with 2.5 $\mu\text{g/ml}$ phleomycin each followed by limiting dilution as previously described (Klingbeil et al., 2002). And, finally, the SM-RBP6-RBP5 cell line was established by transfecting NotI-digested pJ1271-RBP5 into SM-RBP6 cells. Stable transfectants were selected with 10 $\mu\text{g/ml}$ blasticidin followed by limiting dilution as previously described (Klingbeil et al., 2002)

Inducible expression of RBP5-PTP, RBP5 and RBP6

RBP5-PTP and SM-RBP6 cells were induced with 1 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ of tetracycline, respectively, for 10 days. Cells were counted daily and diluted every 48 h in medium containing tetracycline. Tetracycline was supplemented with half the required concentration on non-dilution days. Samples for western blotting and immunofluorescence were collected every 48 h.

SM-RBP5 cells were induced with 250 μM of vanillic acid for 4 days. Cells were counted every 24 h and diluted daily in medium containing vanillic acid. Samples for western blotting and immunofluorescence were collected every 24 h.

Immunofluorescence assays

RBP5-PTP and SM-RBP5 cells were harvested for 5 min at 1000 X g, resuspended in 1X phosphate-buffered saline (PBS) and adhered to poly-L-Lysine (1:10) coated slides for 5 min. Cells were fixed for 5 min using 4% paraformaldehyde and

washed three times (5 min) in PBS containing 0.1 M glycine (pH 7.4) followed by overnight methanol permeabilization at -20°C. Cells were then washed three times in PBS (5 min), followed by a 90 min incubation with anti-protein A (1:3000) or rabbit antibody anti-RBP5 (1:500) (GenScript, USA) and rat monoclonal antibody YL1/2 (1:3000) (Abcam, USA) diluted together in PBS containing 1% bovine serum albumin (BSA). Cells were then washed three times (5 min) in PBS containing 0.1% Tween 20 and incubated for 60 min with the secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-rat for diluted together in PBS containing 1% BSA. Cells were then washed three times (5 min) in PBS containing 0.1% Tween 20 and incubated for 5 min with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (1µg/mL) for DNA staining. Finally, slides were washed twice (5 min) in PBS prior to mounting in Vectashield (Vector Laboratories, USA). Cells were analyzed using a Nikon Eclipse E600 fluorescence microscope (USA) and images were captured and processed using the SPOT imaging software. Different cell cycle stages (1N1K, 1N2K, 2N2K, 2N1K) in RBP5-PTP and SM-RBP5 cells were scored by analyzing nuclear and kDNA content in three hundred cells at each time point.

Western Blot Analysis

Cells were harvested for 15 min at 1000 X g (4°C) and resuspended in PBS supplemented with 10X protease inhibitor cocktail (Roche Diagnostics, Germany). Cells were lysed for 4 min at 94°C using 4X Laemmli sample buffer containing 5% beta-mercaptoethanol. Proteins were separated by SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane overnight

at 90 mA in transfer buffer containing 10% methanol. The PVDF membrane was incubated in blocking reagent (5% nonfat dry milk in PBS) overnight at 4°C followed by incubation with peroxidase anti-peroxidase (PAP) (1:2000), anti-RBP5 (1:500) or anti-RBP6 (1:1000) antibodies diluted in 0.5% blocking reagent (90 min). Membranes were then washed four times (10 min each) in Tris-buffered saline (TBS) containing 0.1% Tween 20, followed by a 60 min incubation with Pierce goat anti-rabbit IgG-horseradish peroxidase (1:5000) (Thermo Scientific, USA). Finally, membranes were washed four times (10 min each) in TBS containing 0.1% Tween 20. Clarity Western ECL Substrate (Bio-Rad, USA) was used for protein detection, following manufacturer's protocol. Detection was carried out in an ImageQuant LAS 4000 Mini apparatus (General Electric Health, USA).

For additional antibody detections, the membrane was stripped twice (5 min each) using stripping buffer (1.5% glycine, 0.1% SDS, 1.0% Tween 20, pH 2.2), washed twice in PBS (10 min) and twice in TBS containing 0.1% Tween 20, blocked overnight, and reprobated. Specific antibodies included: anti-Metacyclic invariant surface protein (MISP) (1:2000) (a kind gift of Dr. Acosta-Serrano) or anti-calflagin (1:1000) (a kind gift of Dr. Engman) followed by Pierce goat anti-rabbit IgG-horseradish peroxidase (1:5000), and anti-alpha tubulin (1:20000, Sigma) or anti-trypanosome alternative oxidase (TAO) (1:100) (a kind gift of Dr. Chaudhuri) followed by goat anti-mouse IgG-horseradish peroxidase (1:5000) (Thermo Scientific, USA).

CHAPTER 3

RESULTS

Subcellular localization of RBP5-PTP in *T. brucei* procyclic forms

RNA-binding protein 5 (RBP5) is part of a group of post-transcriptional regulatory proteins in *T. brucei* which are differentially expressed during their development (Kolev et al., 2014). RBP5 mRNAs is transiently down-regulated during procyclic stages, while it is up-regulated during the salivary gland-stages (Savage et al., 2016). However, specific roles for most of the RNA-binding proteins are still unknown. Since low expression of RBP5 is found in procyclic forms, we induced over-expression of RBP5 tagged at the C-terminus with the PTP epitope (Schimanski et al., 2005) to determine the subcellular localization of RBP5 in procyclic cells, and evaluate phenotypic changes. Two clonal cell lines with different RBP5-PTP expression levels were selected and protein expression was analyzed by Western blotting using Peroxidase Anti-Peroxidase (PAP) antibody complex. The band detected corresponded to the estimated 34 kDa mass (RBP5 = 14.8 kDa; PTP = 18.9 kDa) (Figure 1). RBP5-PTP subcellular localization was determined by immunofluorescence microscopy using anti-protein A antibody. RBP5-PTP showed a punctuate pattern throughout the cytosol at all cell cycle stages (Figure 2). RBP5-PTP was also detected in the nucleus, but in much lower levels (Figure 2). These observations suggest that RBP5 is a cytosolic protein that might need to enter the nucleus to carry out a regulatory function.

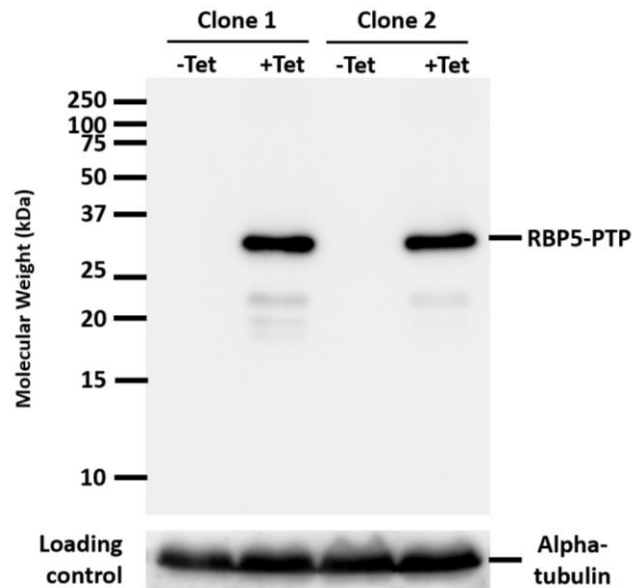


Figure 1. Western blot detection of PTP-tagged RBP5. RBP5-PTP was detected with PAP antibody (1:2000). Membrane was stripped and reprobed for detection of alpha-tubulin as loading control.

Over-expression of RBP5-PTP in procyclic forms causes a moderate growth defect

To determine the function of RBP5 in *T. brucei*, over-expression of PTP-tagged RBP5 was induced in procyclic forms. RBP5-PTP expression throughout the induction was monitored by Western blotting. In clone 1, the expression levels are significantly reduced after 8 days of induction; while in clone 2 the expression is constant through the entire induction (Figure 3). The over-expression of RBP5-PTP resulted in a moderate loss of fitness that began after the second day of induction for each of the clones (Figure 4). Clone 1 was slightly more affected than the clone 2 after 10 days of induction. This result suggests a defect in the progression of the cell cycle.

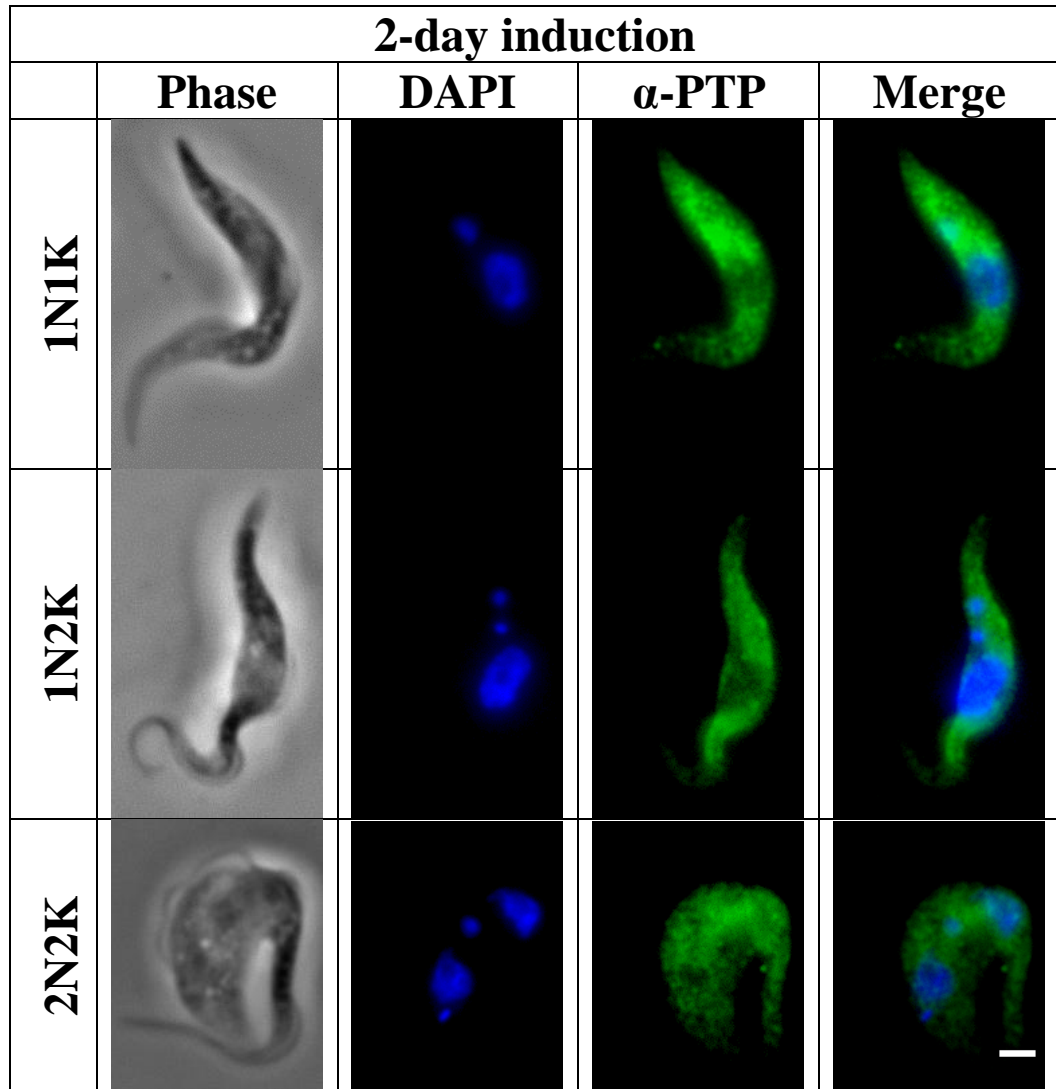


Figure 2. Subcellular localization of PTP-tagged RBP5 in procyclic forms of *T. brucei*. RBP5-PTP expression was tetracycline-induced (1 μ g/mL) and visualized by immunofluorescence microscopy. DAPI (1 μ g/mL) and anti-protein A (1:3000) were used for DNA and PTP-tag detection, respectively. Bar, 2 μ m. Images correspond to clone 1.

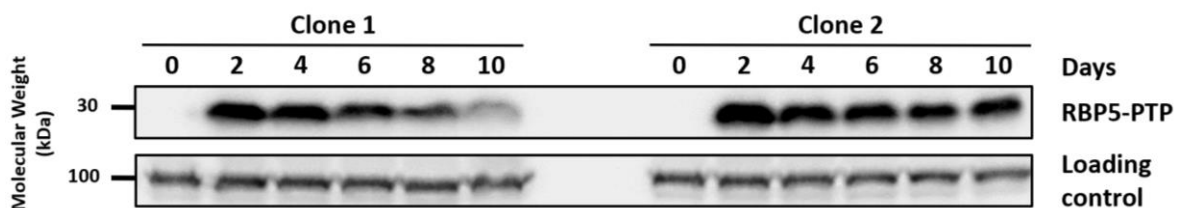


Figure 3. Western blot detection of PTP-tagged RBP5 during 10 days of induction. RBP-PTP was detected with anti-RBP5 (1:1000). A non-specific cross-reacting band was used as loading control.

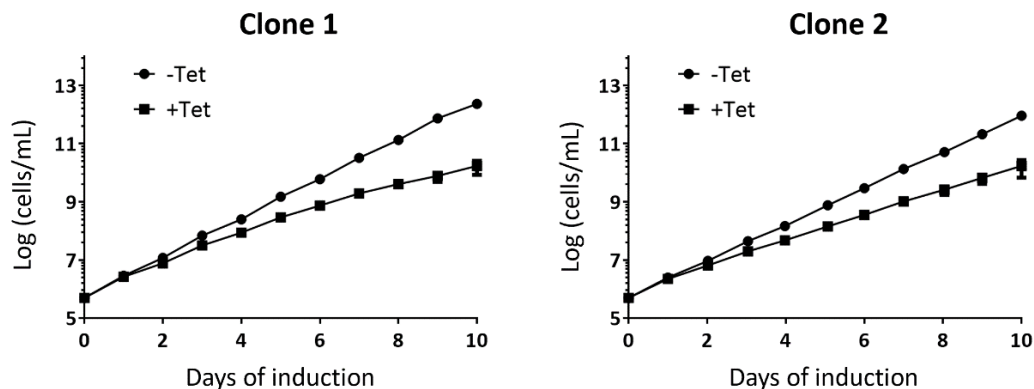


Figure 4. Growth curves of over-expression of PTP-tagged RBP5 in procyclic forms of *T. brucei*. Parasites were diluted every other day to 5×10^5 cells/mL. RBP5-PTP over-expression was induced with tetracycline (1 μ g/mL) for 10 days. Cell density was plotted as the product of cell number and total dilution. Values are the mean (\pm standard deviation) from three independent replicates.

Using DAPI imaging of the nucleus and kDNA in individual procyclic cells, cell cycle progression in *T. brucei* can be monitored (Siegel et al., 2008). To determine if the over-expression of RBP5-PTP was causing a cell cycle arrest, we scored the number of nuclei (N) and kinetoplasts (K) in 300 cells from randomly selected fields at each time point of induction. Under RBP5-PTP over-expression, the number of 1N1K cells decrease gradually from ~90% at day 0 to ~50% at day 10 in clone 1, while in clone 2 the reduction was lower from ~70 to ~50%. This reduction was accompanied by a gradual increase in the number of 2N2K cells from ~5% at day 0 to ~30% at day 10 in clone 1 and from ~10% to ~30% in clone 2. Also notable was the emergence of abnormal multinucleated cells (2N1K, >2N and >2K). The number of zoids, or anucleated cells, showed a slight increase to ~4% in both clones (Figure 5-6). The increase of 2N2K cells and the emergence of multinucleated cells suggests that the over-expression of RBP5-PTP causes a cytokinesis defect in procyclic forms.

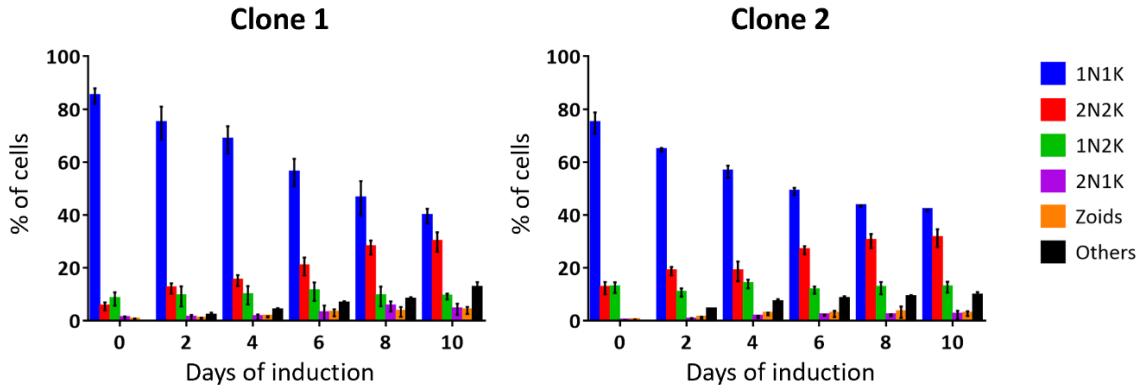


Figure 5. Quantification of cells with different nuclei and kinetoplasts during the 10 days of over-expression of PTP-tagged RBP5. DAPI-stained cells were scored for the number of nuclei and kDNA. The data are presented as the mean percentages \pm standard deviation of 300 cells scored for each time point from three independent replicates using a fluorescence microscope.

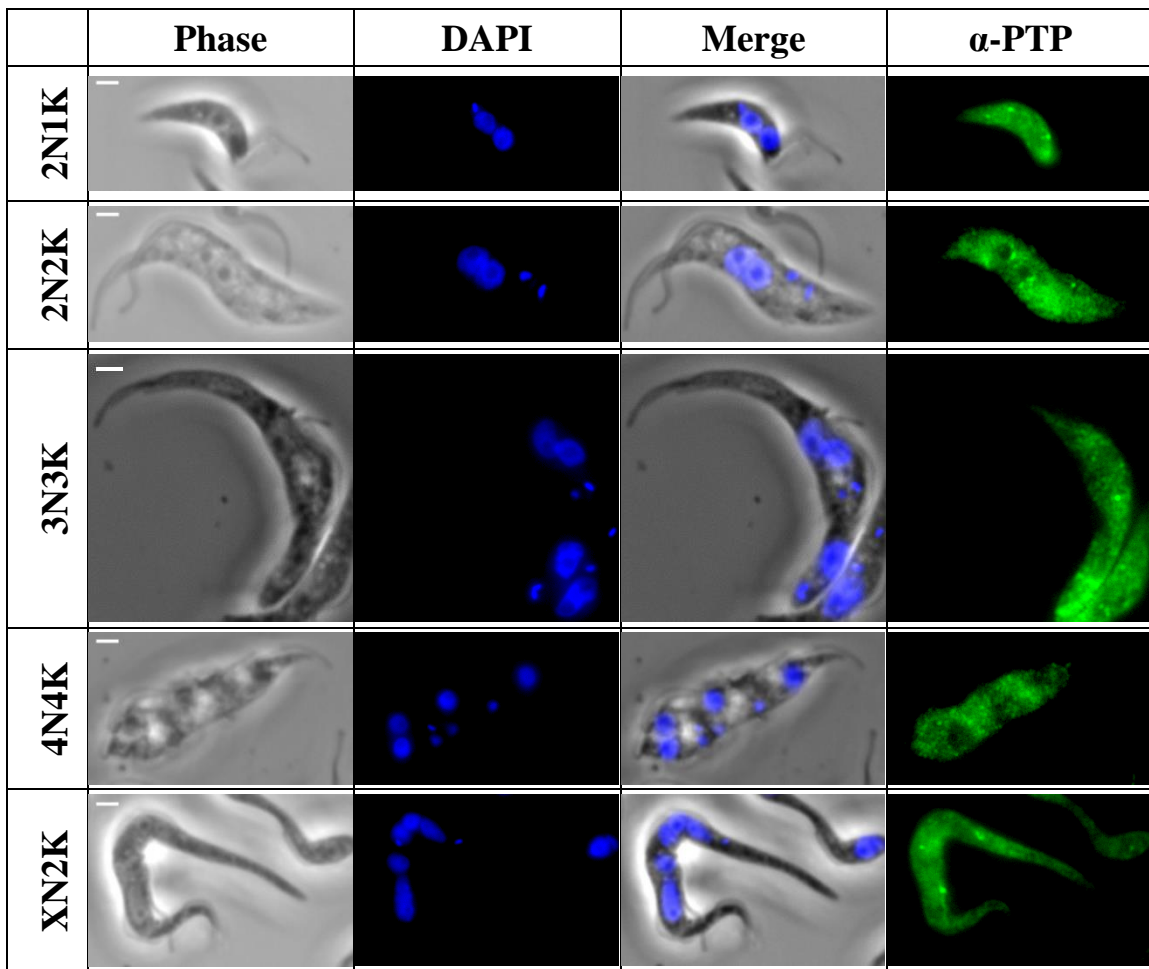


Figure 6. Morphology of procyclic cells with multiple nuclei and kinetoplast after over-expression of PTP-tagged RBP5. RBP5-PTP expression was tetracycline-induced (1 $\mu\text{g}/\text{mL}$) and visualized by immunofluorescence microscopy. DAPI (1 $\mu\text{g}/\text{mL}$) and anti-protein A (1:3000) were used for DNA and PTP-tag detection. $X > 2$. Images correspond to clone 1. Bar, 2 μm .

Over-expression of non-tagged RBP5 in procyclic forms causes a more drastic growth defect

RBPs regulatory functions depend on their ability to bind mRNAs, so altering their structure by adding large epitope tags might disturb their activity (Kimple et al., 2013). We used the SM-RBP5 cell line to induce over-expression of untagged RBP5 in procyclic forms by adding vanillic acid (250 μM). Toxicity of vanillic acid was tested in SMUMA cells, and it showed no considerable effect (Supplemental Figure B9). RBP5 protein levels using SM-RBP5 were lower compared to the over-expression of RBP5-PTP, so Western blotting conditions and antibody concentrations were adjusted to detect RBP5 throughout the induction. RBP5 expression was high after 24 h of induction and decreased during the 4 day induction. Also, the new conditions allowed for the detection of basal levels of RBP5 at day 0. Oddly, the sample from day 2 showed problems in all three replicates and in multiple blots (Figure 7). Like RBP5-PTP over-expression, RBP5 over-expression caused a loss of fitness in the procyclic cells, however the phenotype was much more severe (Figure 8). This result reaffirms the initial suggestion that RBP5 affects the normal progression of the cell cycle and confirmed our idea that the PTP tag interferes with the normal function of the protein.

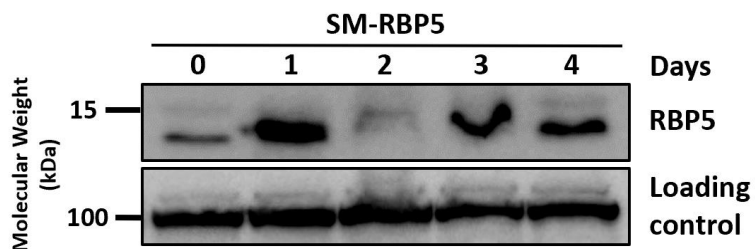


Figure 7. Western blot detection of non-tagged RBP5 during 4 days of induction. RBP was detected with anti-RBP5 (1:500). A non-specific cross-reacting band was used as loading control. RBP5 was detected at a longer exposure time.

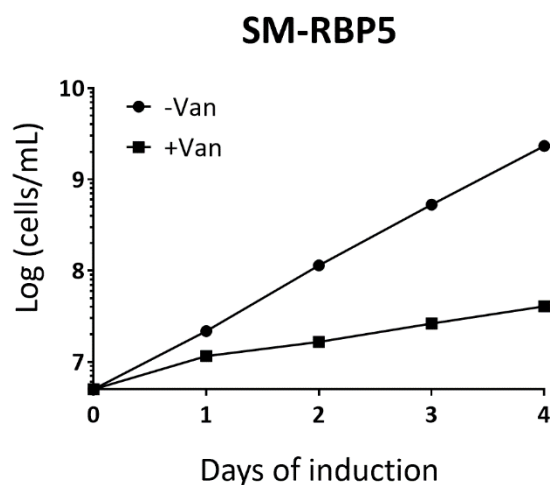


Figure 8. Growth curve of expression of non-tagged RBP5 in procyclic forms of *T. brucei*. Parasites were diluted every day to 5×10^6 cells/mL. RBP5 expression was induced with vanillic acid (250 μ M) for 4 days. Cell density was plotted as the product of cell number and total dilution. Values are the mean (\pm standard deviation) from three independent replicates.

To confirm that RBP5 over-expression induced a cell cycle defect in procyclic cells, we scored the number of nuclei (N) and kinetoplasts (K) in 300 cells from randomly selected fields at each time point of induction. Surprisingly, RBP5 over-expression caused the percentage of 1N1K cells to decrease from ~80% to ~40% and the percentage of 2N2K cells increase from ~10% to ~50% after only 1 day of vanillic acid induction. The percentages of 1N1K and 2N2K cells remained high during the induction,

however the number of multinucleated cells (Others) clearly increased throughout the induction until reaching ~20% on the last day. The number of zoids did not show a significantly increase, so we can discard any DNA replication defect involved with the over-expression of RBP5 (Figures 5,6). The dramatic increase of 2N2K cells and the emergence of multinucleated cells confirms our previous idea that RBP5 might be involved in the regulation of cytokinesis.

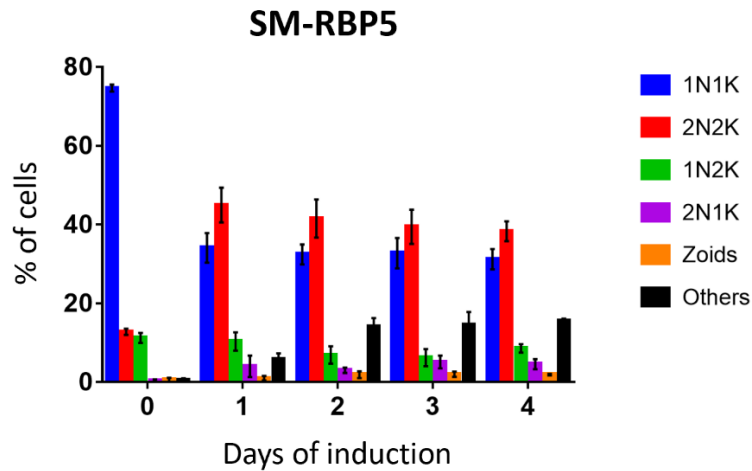


Figure 9. Quantification of cells with different nuclei and kinetoplasts during the 4 days of over-expression of non-tagged RBP5. The data are presented as the mean percentages \pm standard deviation of 300 cells scored for each time point from three independent replicates using a fluorescence microscope.

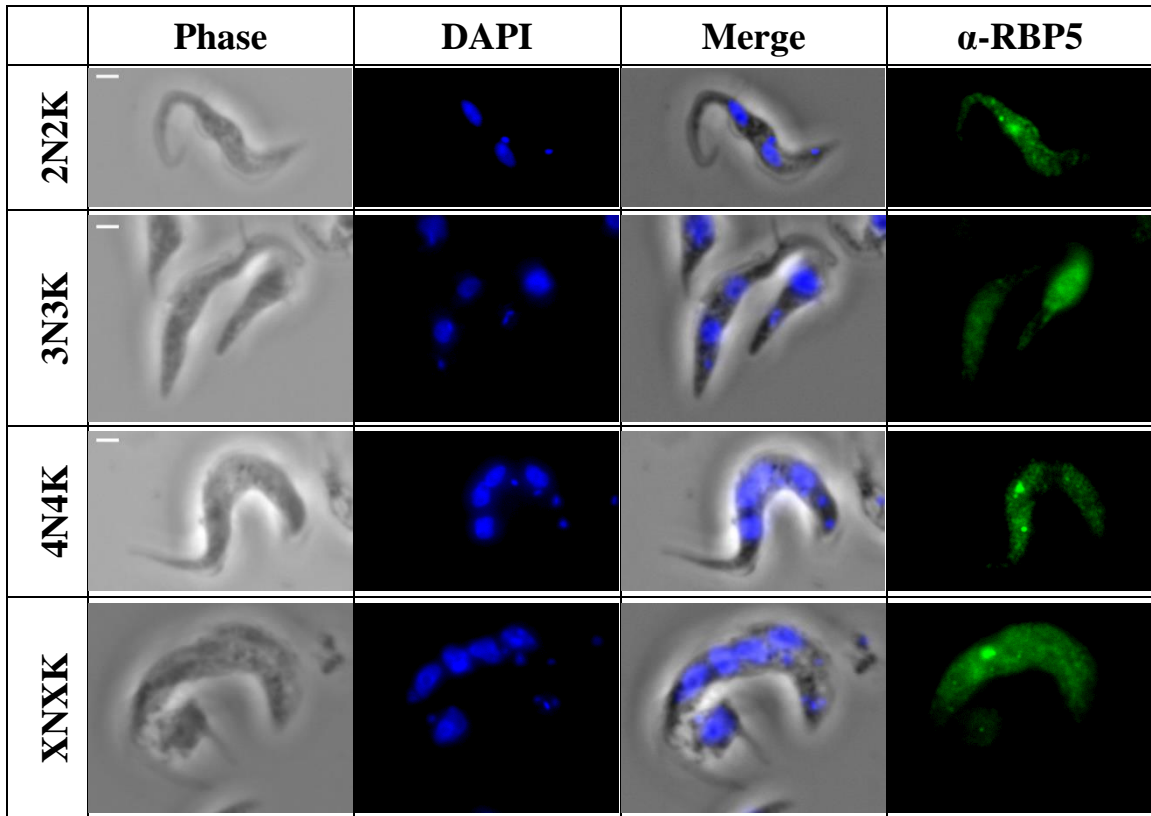


Figure 10. Morphology of procyclic cells with multiple nuclei and kinetoplast after over-expression of non-tagged RBP5. RBP5 expression was induced with vanillic acid (250 μ M) and detected with anti-RBP5 antibody (1:500). DNA was detected with DAPI. X > 2. Bar, 2 μ m.

RBP6 over-expression in SMUMA cells is not able to drive metacyclogenesis

As mentioned before, RBP5 transcripts are mainly up-regulated at the salivary gland stages, where metacyclic and epimastigote forms are found (Savage et al., 2016). RBP6 is another RNA-binding protein whose induced over-expression in procyclic forms could drive metacyclogenesis by itself, with the respective formation of long and short epimastigotes (Kolev et al., 2012). Therefore, to investigate better the function of RBP5 we developed an inducible dual-expression cell line which over-expresses RBP6 and

expresses RBP5 under the regulation of tetracycline repressor (tetR) and vanillic acid repressor (vanR), respectively.

At first, we induced RBP6 over-expression to prove that the protein can drive metacyclogenesis in our cell line. However, a 12 day induction following Kolev et al. (2012) experimental conditions neither metacyclics nor epimastigotes were detected during the induction. Metacyclics are non-diving cells whose cell cycle is arrested at G1 phase, so a growth defect was expected in the cells as a sign of metacyclogenesis, however cells over-expressing RBP6 did not suffer from a significant loss of fitness (Figure 11). To confirm the presence or absence of metacyclics in our collected samples, we used antibodies against two different metacyclic markers, MISP and calflagin, MISP, metacyclic induced surface protein, is a GPI-anchored surface protein which is highly expressed in metacyclics with lower abundance in epimastigotes (Casas-Sánchez et al., 2017). Calflagin is a calcium signaling protein also highly expressed in metacyclics and with low abundance in procyclics (Emmer et al., 2010). After 12 days of RBP6 over-expression, no metacyclics were detected by microscopy (data not shown) and no difference in expression was observed by Western blotting using either of the markers (Figure 12). Two more replicates were analyzed and neither of them showed any indication of the differentiation process. TAO, an enzyme involved in the reoxidation of NADH to NAD⁺ in cells that utilize glycolysis for generation of ATP (Smith et al., 2017), was also used as a marker in the original publication. We detected the expression of TAO and it was clear that the over-expression of RBP6 increases the expression levels of TAO during the 12 days of induction (Figure 12). However, this difference in expression was not reflected by any morphological differentiation change.

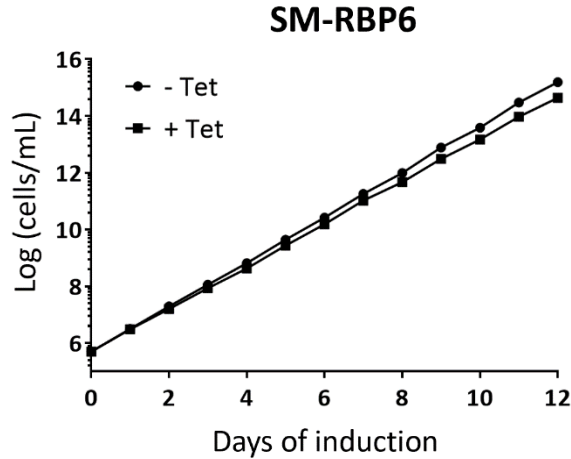


Figure 11. Growth curve of over-expression of RBP6 in procyclic forms of *T. brucei*. Parasites were diluted every other day to 5×10^5 cells/mL. RBP6 over-expression was induced with tetracycline (10 μ g/mL) for 12 days. Cell density was plotted as the product of cell number and total dilution. Values are the mean (\pm standard deviation) from three independent replicates.

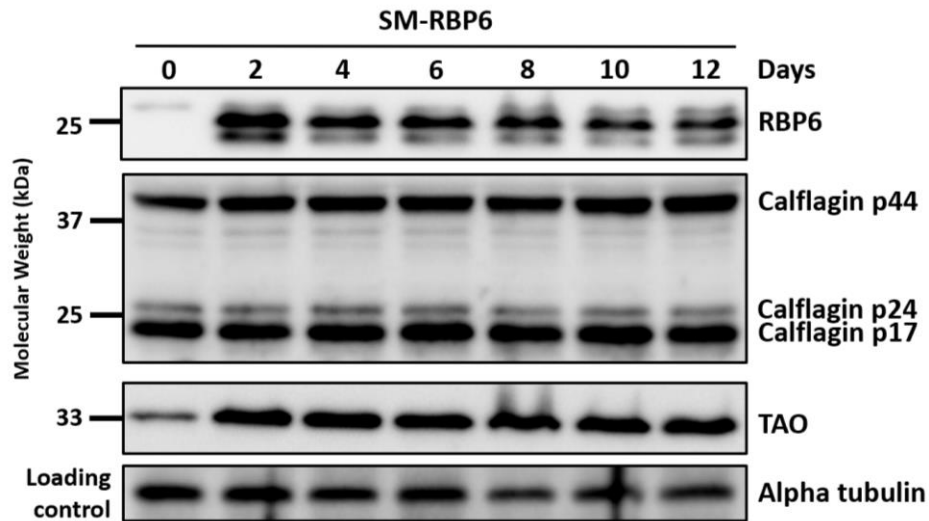


Figure 12. Western blot to detect metacyclogenesis markers during the 12 days of RBP6 induction. RBP6 expression was detected using anti-RBP6 (1:1000). Differentiation markers were detected using their respective antibodies, anti-calflagin (1:1000) and anti-TAO (1:100). Alpha tubulin was used as loading control.

For additional antibody detections, the membrane was stripped twice (5 min each) using stripping buffer (1.5% glycine, 0.1% SDS, 1.0% Tween 20, pH 2.2), washed twice in PBS (10 min) and twice in TBS containing 0.1% Tween 20, blocked overnight, and

reprobed. Specific antibodies included: anti-Metacyclic invariant surface protein (MISP) (1:2000) (a kind gift of Dr. Acosta-Serrano) or anti-calflagin (1:1000) (a kind gift of Dr. Engman) followed by Pierce goat anti-rabbit IgG-horseradish peroxidase (1:5000), and anti-alpha tubulin (1:20000, Sigma) or anti-trypanosome alternative oxidase (TAO) (1:100) (a kind gift of Dr. Chaudhuri) followed by goat anti-mouse IgG-horseradish peroxidase (1:5000) (Thermo Scientific, USA).

Dual over-expression of RBP6 and RBP5 in procyclic forms does not help in the generation of metacyclic forms

Since no metacyclogenesis was detected in SMUMA cells after 12 days of RBP6 over-expression, we analyzed whether the combined over-expression of RBP6 and RBP5 could help induce the process. We tried two different approaches to test this idea. First, we induced over-expression of RBP6 alone for 3 days and on day 3 we then induced over-expression of RBP6 and RBP5. For the second, we simultaneously induced over-expression of RBP6 and RBP5 for 4 days. Similar to previous results, the induction of RBP6 did not affect cell growth, while the dual-induction of RBP6 and RBP5 showed a severe growing defect that can be mainly attributed to the over-expression of RBP5, as shown previously (Figure 13). We analyzed samples for the expression of the metacyclic markers, MISP and calflagin, by Western blotting. MISP was not detected in any of the time points (Data not shown) while calflagin did not show any variation between the uninduced and induced samples (Figure 12). These results together suggest that RBP5 does not cooperate with RBP6 in the process of metacyclogenesis, but instead it might be

involved in the regulation of cell cycle stall in non-dividing metacyclic forms, specifically in cytokinesis arrest.

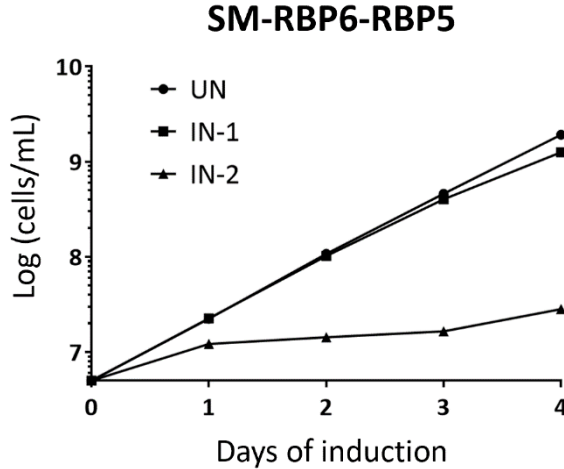


Figure 13. Growth curve of over-expression of RBP6 and RBP5 in procyclic forms of *T. brucei*. Parasites were diluted every day to 1×10^7 cells/mL. RBP6 over-expression was induced with tetracycline ($10 \mu\text{g/mL}$) and RBP5 over-expression was induced with vanillic acid ($250 \mu\text{M}$). UN: Uninduced; IN-1: RBP6 induced for 4 days and RBP5 induced at day 3; IN-2: RBP6 and RBP5 induced for 4 days. Cell density was plotted as the product of cell density and total dilution. Values represent a single experiment.

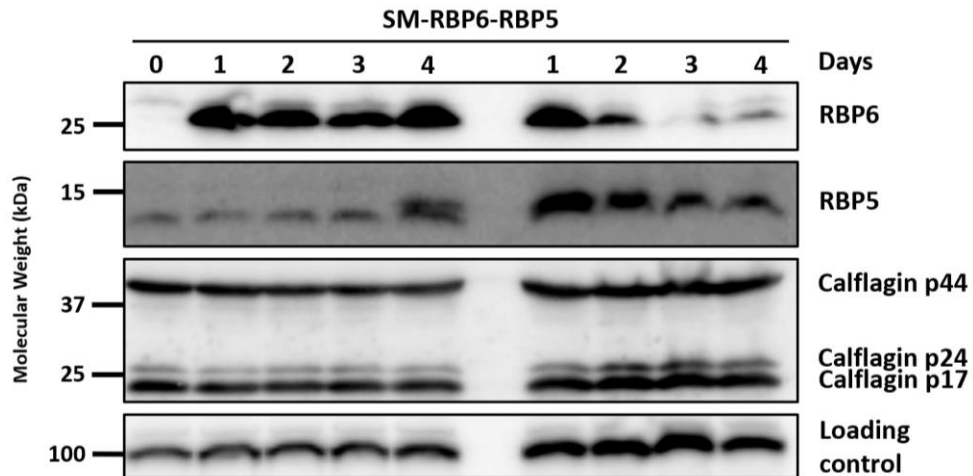


Figure 14. Western blot to detect metacyclogenesis markers during the 4 days of RBP6 and RBP5 induction. Dual-expression was detected using anti-RBP5 (1:500) and anti-RBP6 (1:1000). Calflagin was used as differentiation marker and detected using anti-calflagin (1:1000). Alpha tubulin was used as loading control.

CHAPTER 4

DISCUSSION

In this work we studied the function of RNA-binding protein 5 (RBP5) and demonstrated its involvement as a regulator of cytokinesis in *T. brucei* procyclic forms. In procyclic forms, over-expression of RBP5-PTP and RBP5 caused a growth arrest due to defective cell abscissions, which resulted in the increase of 2N2K cells and the appearance of multinucleated cells (Figure 5, 6, 9 and 10). However, a difference in the phenotype was observed between RBP5-PTP and RBP5 over-expression, which can be attributed to the size of the tag, which is larger to the size of the protein itself (RBP5 = 14.8 kDa; PTP = 18.9 kDa). Protein tagging has been recently argued to interfere with protein stability (Dave et al., 2016), trafficking and protein-protein interaction (Lotze et al., 2016), especially when large tags are used (Gunzl and Schimanski, 2009; Kimple et al., 2013; Mohanty and Wiener, 2004). This supports our suggestion that the PTP-tag might be interfering with the correct binding of RBP5 to the corresponding mRNA to regulate its function. Also, this would explain why higher expression levels of RBP5-PTP (Figure 3) generated a milder growth defect compared to the lower expression of untagged RBP5 (Figure 7).

RBP5 over-expression generated a majority population of 2N2K cells and a smaller, but considerable, population of multinucleated cells, which indicates they are defective in cytokinesis. The low percentage of zoids and the stable percentage of 1N2K cells throughout the induction argues against a potential role of RBP5 in the regulation of DNA replication or mitosis (Figure 5 and 9).

Cytokinesis in *T. brucei* initiates from the anterior end of the new flagellum attachment zone (FAZ) and the cleavage furrow ingresses unidirectionally along the longitudinal axis towards the posterior end of the cell (Vaughan and Gull, 2008), without any evidence of the formation of an actomyosin contractile ring, an essential and well conserved cytokinesis structure in other higher eukaryotes (Balasubramanian et al., 2004). However, the mechanism controlling the initiation or termination of this unusual mode of cytokinesis remains poorly understood. Our data showed the presence of 2N2K and multinucleated cells without the formation of the furrow (Figure 6 and 10), which suggests that RBP5 over-expression might be inducing a defect in cytokinesis initiation, rather than on the completion of the process. It would be beneficial to observe the population of cells using higher resolution electron microscopy to confirm the absence of any furrow ingression.

The signaling pathway that initiates and promotes cytokinesis is better understood and five proteins have been identified and characterized; two protein kinases, the Pololike kinase (TbPLK) (Hammarton et al., 2007) and the Aurora B Kinase (TbAUK1) (Tu et al., 2006), and three trypanosome-specific proteins, CIF1, also known as TOEFAZ1 (Hu et al., 2015; McAllaster et al., 2015), CIF2 (Zhou et al., 2016) and CIF3 (Kurasawa et al., 2018). Both kinases are likely to act as upstream regulators for cytokinesis initiation and are required for cytokinesis initiation, while the CIF proteins are likely to be downstream regulators which form complexes to regulate the functions of TbPLK and TbAUK1 (Kurasawa et al., 2018). RNAi data on these proteins involved on cytokinesis initiation showed accumulation of 2N2K and multinucleated cells, as well as growth arrest, however these phenotypes were not nearly as severe as the one observed after over-

expression of RBP5. This suggests RBP5 might be a master repressor of multiple genes, which leads to an exacerbated phenotype.

RNA-binding proteins can modulate gene expression by binding to specific motifs within the 3'-UTR of mRNAs (Romaniuk et al., 2016). It has been shown that many mRNAs are coordinately regulated in trypanosomes and in some cases, these regulatory elements are shared by these coregulated genes and bound by a specific RNA-binding protein (Archer et al., 2009; Das et al., 2012; Droll et al., 2013; Mugo and Clayton, 2017). These observations and the fact that RBP5 has a reduced expression in procyclic cells (Figure 7 and Supplemental Figure B10) make us conclude that RBP5 could be involved in the repression of multiple genes that coordinately regulate the initiation of cytokinesis, however further work needs to be done to determine the sequence of the motifs to which RBP5 binds, to find out the genes that are being repressed. A motif search analysis using MEME software (Bailey and Elkan, 1994) found a consensus motif among TbPLK, TbAUK1, CIF1, CIF2 and CIF3, which is located at the 3'UTR and corresponds to YKTGTYTKCTTKTTTTTTTT. This analysis provides an initial idea of where RBP5 could bind, but binding assays are necessary to elucidate this hypothesis.

Also, in this work, we analyzed whether RBP5 contributes to the differentiation of procyclic forms into metacyclic forms in *T. brucei*. However, our developed cell line, SMUMA, was not able to induce any differentiation process under over-expression of RBP6 for 12 days, as reported by Kolev et al. (2012). The only plausible explanation for this, relates with the cell line used in each experiment, Kolev et al. (2012) used 29-13 cells to induce metacyclogenesis, while we used our cell line SMUMA. Both cell lines

are derivatives of Lister 427 (Wirtz et al., 1999), however 29-13 cells have a long history of culturing and sub-culturing that might have altered their metabolism, as it was showed by Herder et al. (2007), who demonstrated that the cell line is not able to complete their development within tsetse flies. So, the use of two different cell lines could explain why even though our cell line supported the expression of RBP6 (Figure 12), it could not drive metacyclogenesis. Finally, we showed that neither the dual over-expression of RBP6 and RBP5, or the sequential over-expression could induce the metacyclic differentiation process (Figure 14). However, we cannot completely discard the participation of RBP5 in metacyclogenesis since our cell line was never capable to generate epimastigotes or metacyclics under the over-expression of RBP6 and transcriptomic data show that RBP5 transcripts are up-regulated in salivary gland forms (Savage et al., 2016).

Future work would help us understand better the role of RBP5 and the interaction with its main targets. For example, RIP-Seq (RNA immunoprecipitation sequencing) will help us find the mRNA-binding targets as well as RNA-seq in cells over-expressing RBP5 would let us narrow the targets which are being repressed by RBP5.

APPENDIX A

GENERAL EXPERIMENTAL PROTOCOLS

A1. Quantitative Real Time-PCR

SMUMA cells were harvested for 15 min at 1000 X g (4°C) and resuspended in 1X PBS. Total RNA was purified from the harvested cells using TRIzol reagent. Isolated RNA was treated with DNase I (New England Biolabs, USA) and then concentrated to remove any DNA contamination. First-strand cDNA was then synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). A 99-bp cDNA fragment from VanR coding region was amplified by real-time PCR using the cDNA of SMUMA as template, and the SYBR green PCR master mix (Bio-Rad, USA) according to manufacturer's protocol. Telomerase reverse transcriptase (TERT) transcripts were used as positive control. Three replicates were run simultaneously in a MX3005P qPCR System (Agilent, USA), and the results were analyzed using the MXPro software.

APPENDIX B

SUPPLEMENTARY DATA

Table B1. List of primers used in this study.

Gene	Primer	Sequence (5' - 3')
Over-expression of RBP5-PTP		
RBP5	Forward	TAAAATTCACAAGCTTATGTTCAACCAGTTTTTCGAAGATGCCCT
	Reverse	CTTCCAGCGGTCTAGAAGCAGTAATACACGTCGGTGGGTA CAGAAC
Over-expression of RBP5 and RBP6		
RBP5	Forward	GGGATCGCTAAGATCTTTAAGCAGTAATACACGTCGGT
	Reverse	TAAAATTCACAAGCTTATGTTCAACCAGTTTTTCGAAGATGC
RBP6	Forward	TAAAATTCACAAGCTTATGTTCTACCCCAACAGCCCG
	Reverse	TAAATGGGCAGGATCCTCAACCAGCGGCACCGCG
qRT-PCR of Vanillic Acid repressor		
VanR	Forward	TGGTGTGCTTGAGGGTTTC
	Reverse	GCCTCACCTCTGCAATAAG

RBP5: RNA-binding protein 5, RBP6: RNA-binding protein 6; VanR: Vanillic Acid repressor

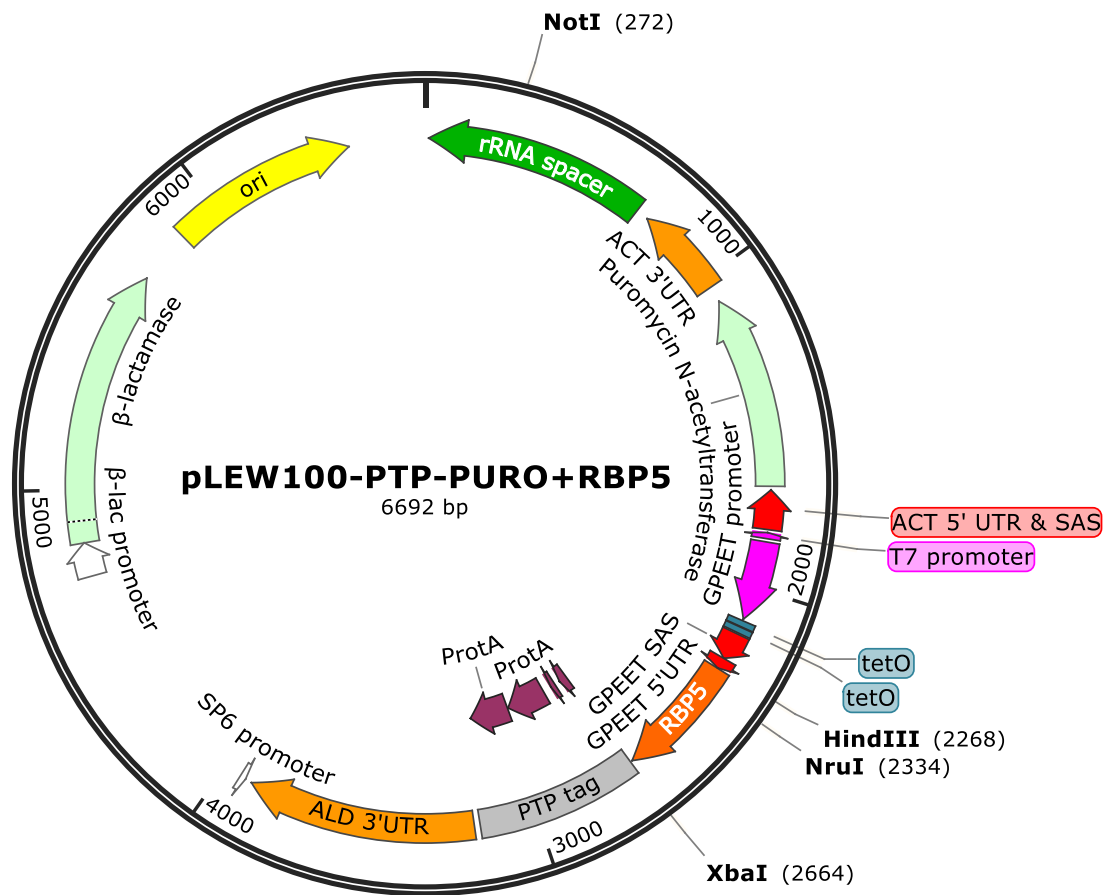


Figure B1. Scheme of the over-expression plasmid pLEW100-RBP5-PTP-PURO. The coding sequence of RBP5 has a PTP tag at the N-terminal. Protein over-expression is regulated by a T7 promoter and 2 tetO operators. A puromycin resistance cassette is present for drug selection.

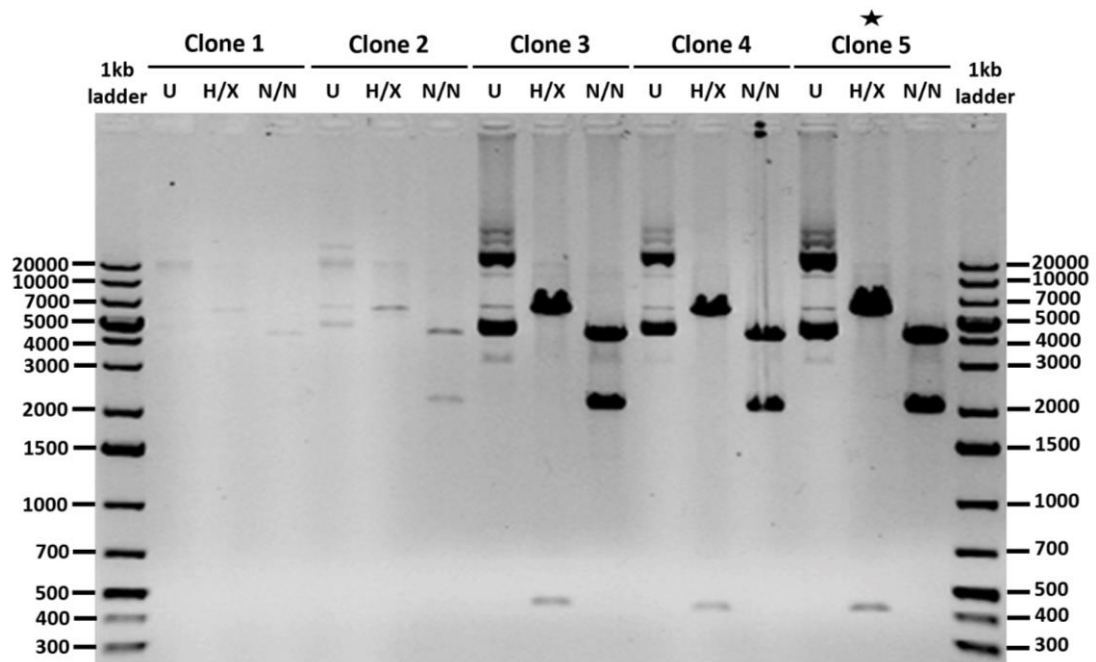


Figure B2. Restriction digestion fragments of the plasmid pLEW100-RBP5-PTP-PURO. Six cloned plasmids were analyzed for integrity. U: Uncut. H/X: HindIII + XbaI, N/N: NruI + NotI. Expected sizes: HindIII-XbaI: 6296 bp and 396 bp. NruI-NotI: 4630 bp and 2062 bp. *Clone selected for transfection.

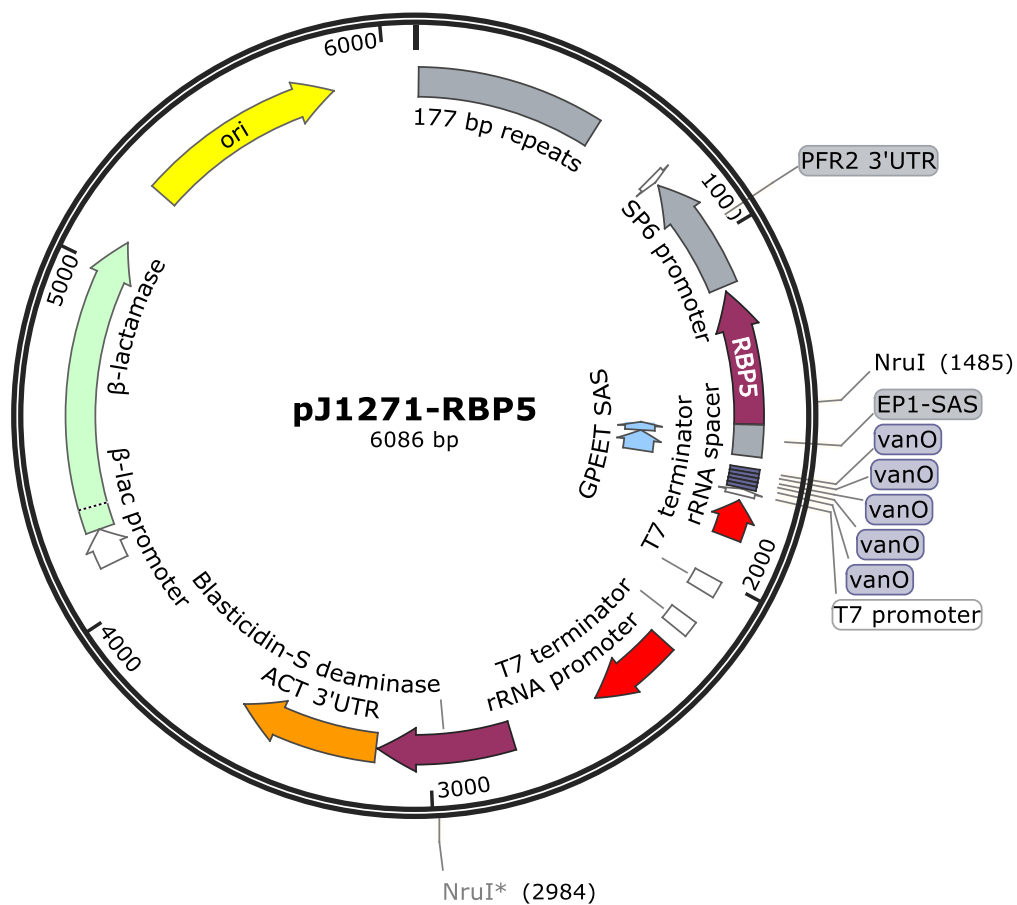


Figure B3. Scheme of the expression plasmid pJ1271-RBP5. Protein expression is regulated by a T7 promoter and 5 vanO operators. A blasticidin resistance cassette is present for drug selection.

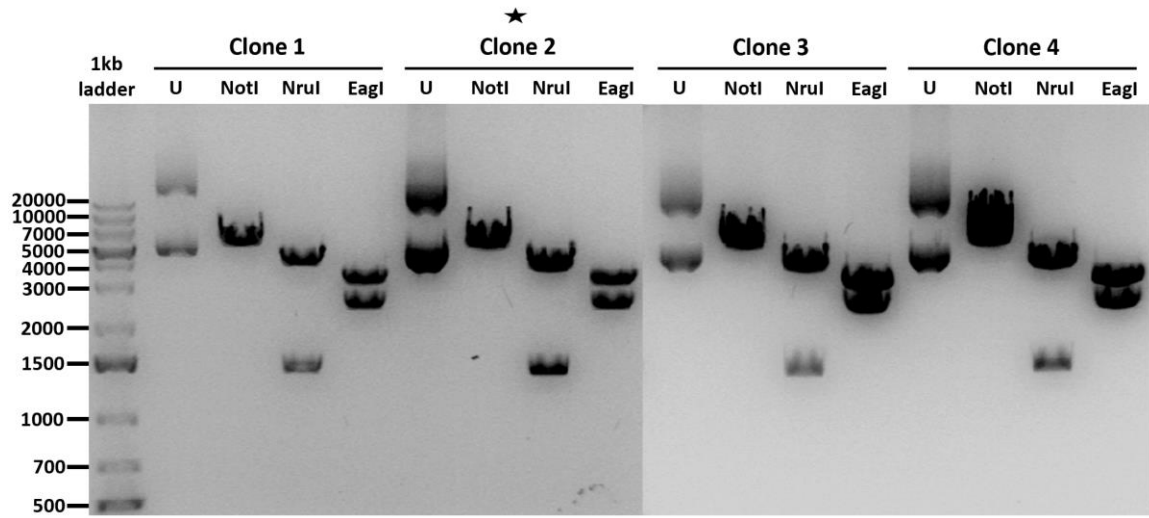


Figure B4. Restriction digestion fragments of the plasmid pJ1271-RBP5. Four cloned plasmids were analyzed for integrity. U: Uncut. Expected sizes: NotI: 6086 bp. NruI: 4587 bp and 1499 bp. EagI: 3502 bp and 2584 bp. *Clone selected for transfection.

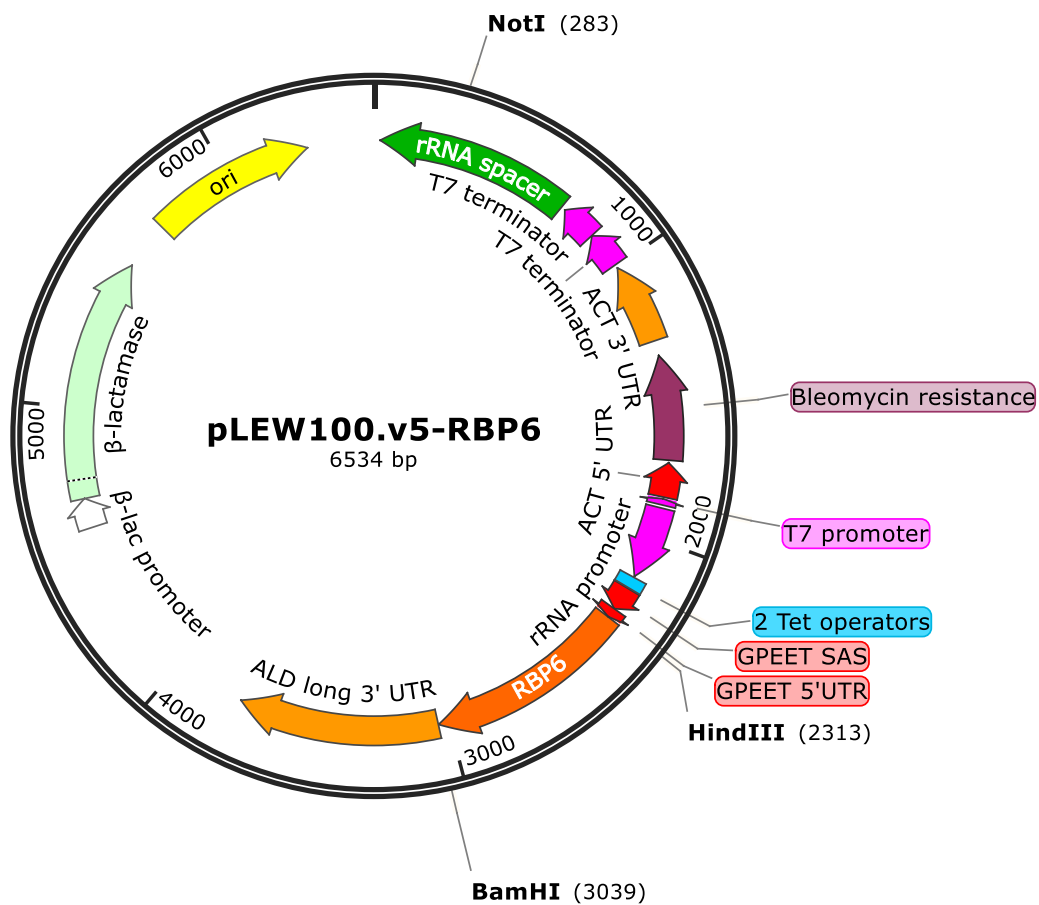


Figure B5. Scheme of the expression plasmid pLEW100.v5-RBP6. Protein expression is regulated by a T7 promoter and 2 tetO operators. A bleomycin resistance cassette is present for drug selection.

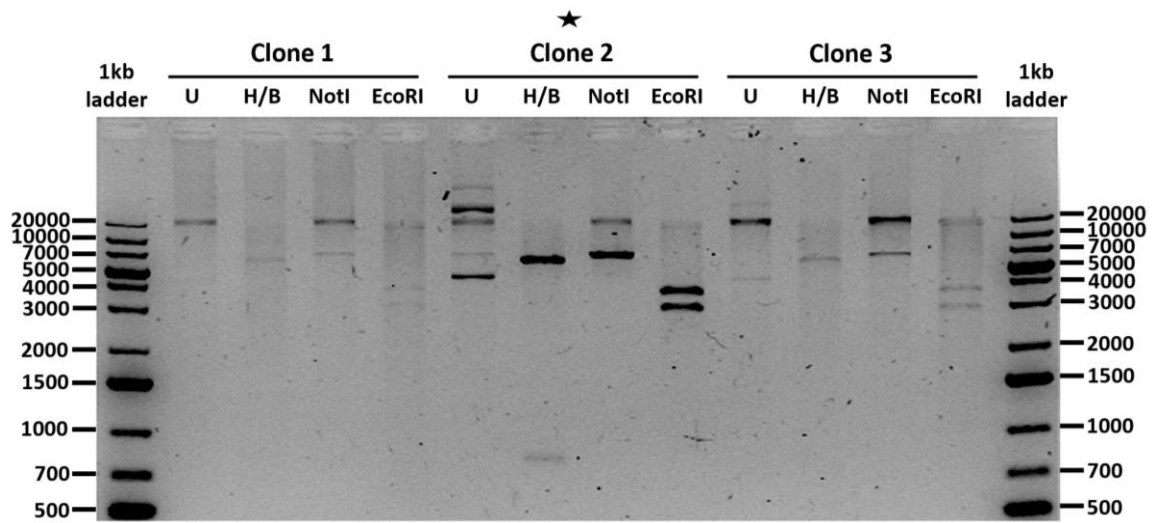


Figure B6. Restriction digestion fragments of the plasmid pLEW100.v5-RBP6. Three cloned plasmids were analyzed for integrity. U: Uncut, H/B: HindIII + BamHI. Expected sizes: HindIII-BamHI: 5808 bp and 726 bp. NotI: 6534 bp. EcoRI: 3607 bp and 2927 bp.
*Clone selected for transfection

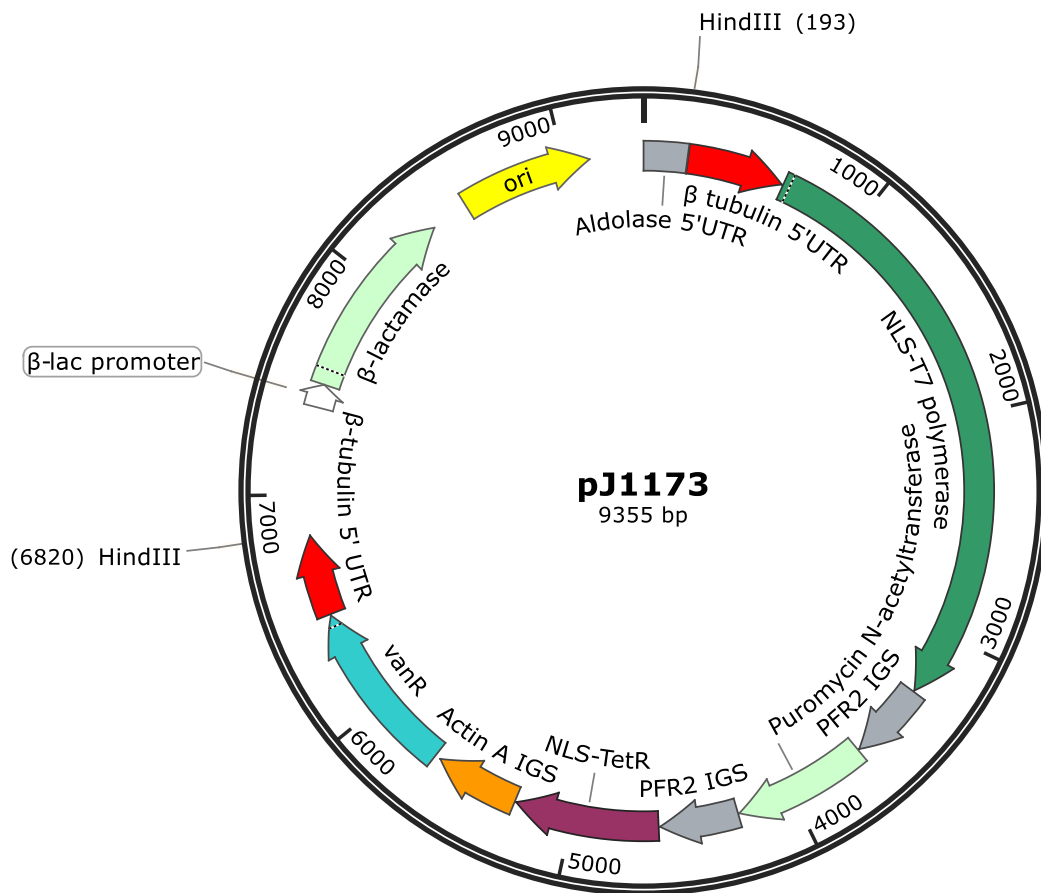
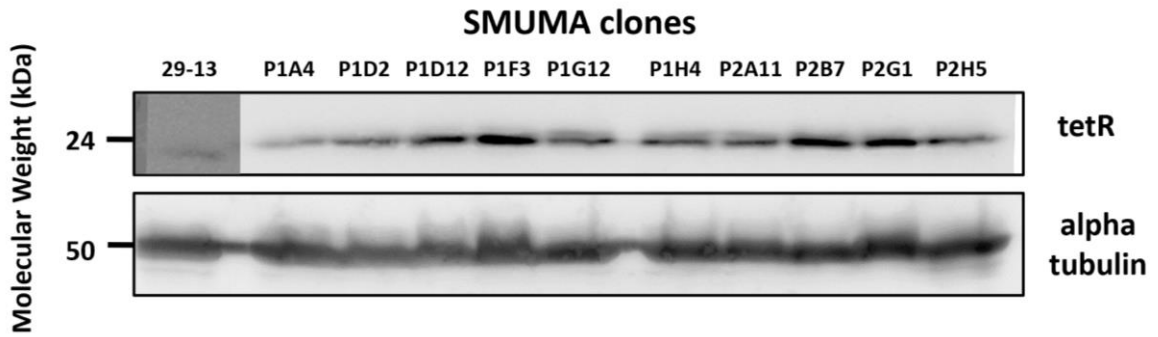


Figure B7. Scheme of the plasmid pJ1173. The coding sequences of T7 polymerase, tetracycline repressor (tetR) and Vanillic acid repressor (vanR) have a nuclear localization signal (NLS) at the N-terminal. A puromycin resistance cassette is present for drug selection.

A.



B.

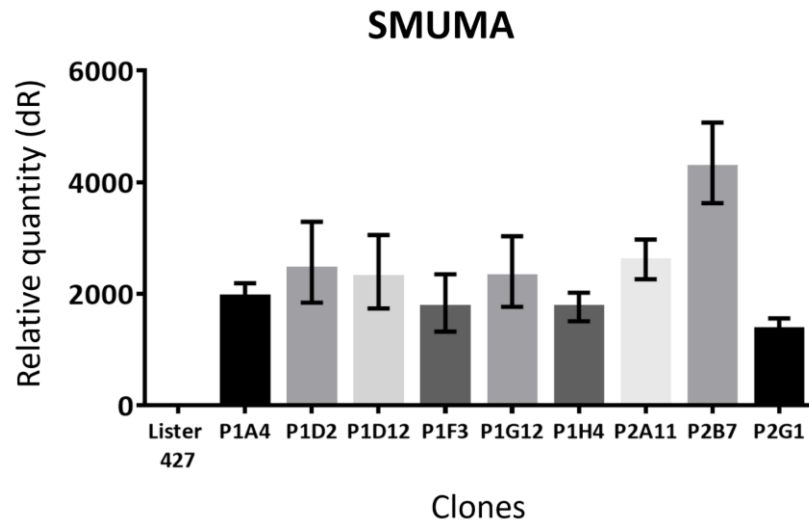


Figure B8. Analysis of expression of tetR and vanR expression in SMUMA clones. A) Western blot to detect expression of tetR. Membrane was reprobbed using anti-alpha tubulin as loading control. 29-13 lane was detected at a higher exposure time. B) Relative expression of vanR transcripts. Lister 427 cDNA was used as negative control.

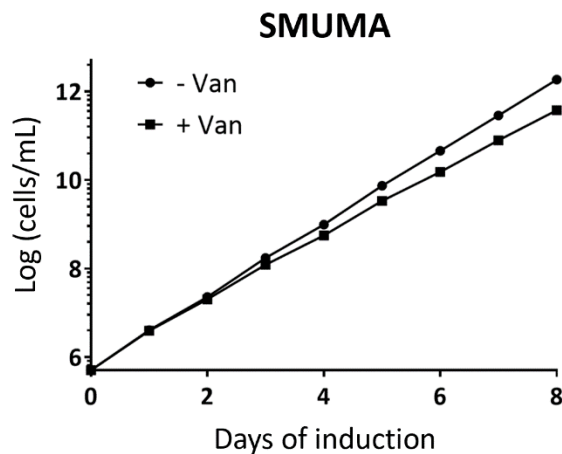


Figure B9. Effect of vanillic acid on SMUMA procyclic forms of *T. brucei*. Parasites were diluted every other day to 5×10^5 cells/mL. Vanillic acid ($250\mu\text{M}$) was added to the medium every day for 8 days. Cell density was plotted as the product of cell number and total dilution. Values are the mean (\pm standard deviation) from three independent replicates.

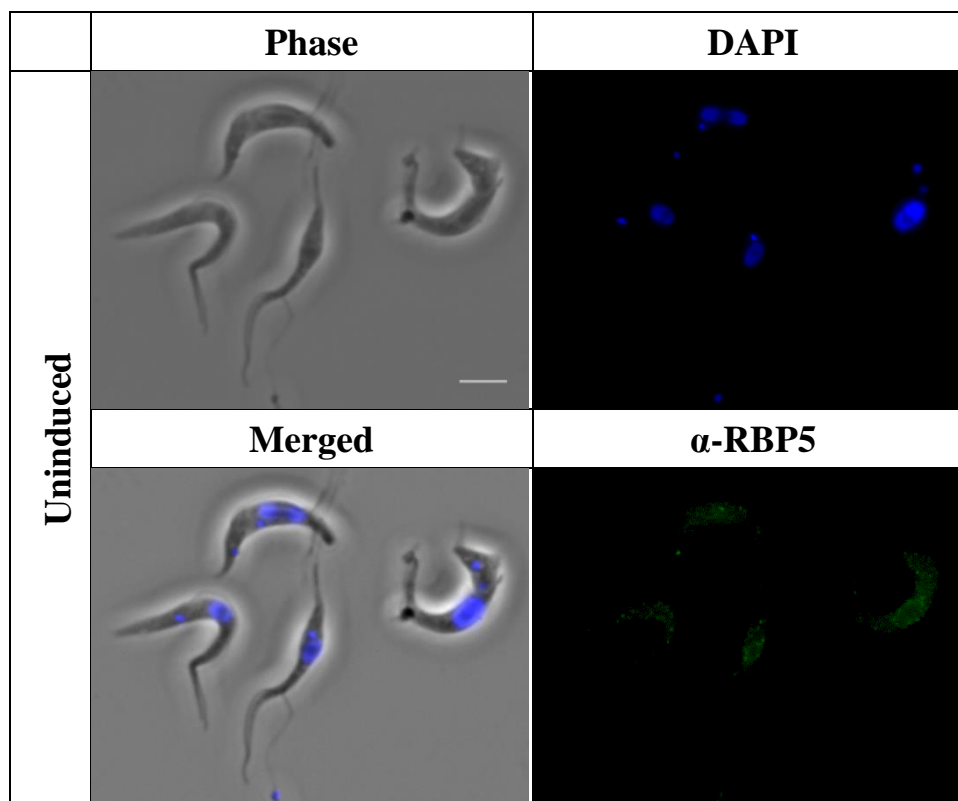


Figure B10. Morphology of procyclic cells with no over-expression of RBP5. RBP5 expression was detected with RBP5 specific antisera (1:500). DNA was detected with DAPI ($1\mu\text{g/mL}$). Bar, $5\mu\text{m}$.

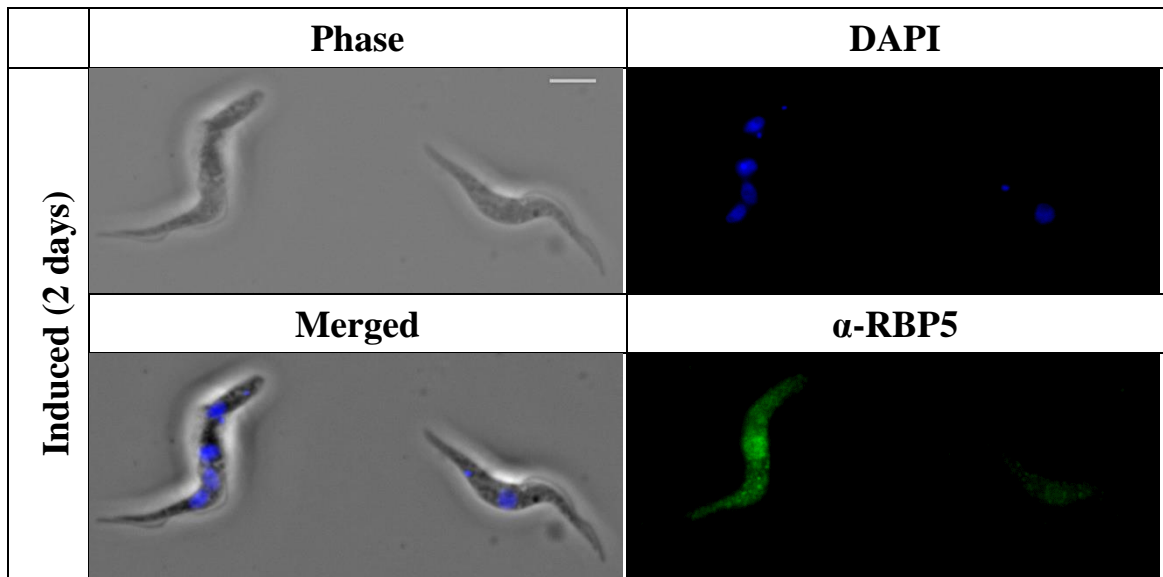


Figure B11. Morphology of two procyclic cells, one which over-express RBP5 and other which does not. RBP5 expression was detected with RBP5 specific antisera (1:500). DNA was detected with DAPI (1 μ g/mL). Bar, 5 μ m.

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