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Department of Molecular Biology

Characterization of Leucine aminopeptidase 1 in *Trypanosoma brucei*

Bachelor Thesis

Laboratory of Molecular Biology of Protists
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Annotation

In this work, Leucine aminopeptidase 1 in procyclic *Trypanosoma brucei* is localized, down-regulated by RNAi, and ectopically expressed with a concomitant growth phenotype, a disruption in cell cycle and mitochondrial membrane potential, which denotes a role in kinetoplastic DNA segregation.

Affirmation

I hereby declare that I have worked on the submitted bachelor thesis independently and used only the sources listed in the bibliography.

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Christian Resl

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List of papers and author's contributions

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Contribution to a part of the experimental procedures and performance of a part of the statistical analysis.

Abbreviations

dsRNA	double-stranded RNA
KFZ	Kinetoflagellar zone
LAP	Leucine aminopeptidase
mRNA	messenger RNA
mt	mitochondrial
PCR	polymerase chain reaction
PCF	procyclic form
PBS	phosphate buffered saline
RNAi	RNA interference
siRNA	small interfering RNA
TAC	tripartite attachment complex
T. brucei	Trypanosoma brucei
TMRE	Tetramethylrhodamine ethyl ester
VSG	variable surface glycoprotein

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1 *Trypanosoma brucei*

Trypanosomatids are flagellated protist eukaryotes belonging to the class Kinetoplastida. They include the genera *Trypanosoma* and *Leishmania*, causative agents of tropical diseases such as African trypanosomiasis, Chagas disease and Leishmaniasis that affect millions of impoverished populations in the developing world.

African trypanosomiasis is caused by *Trypanosoma brucei* predominantly occurring in sub-Saharan regions connected to the habitat of its insect vector, the Tsetse fly (*Glossina sp.*) (Lopes et al., 2010). Their complex life cycle with several hosts ranging from insects to vertebrates can be seen in Figure 1.

An infection of a mammalian host starts by delivering metacyclic trypanosomes from the salivary gland of a tsetse fly into the bloodstream during the blood meal of the insect. In mammalian hosts, the parasite differentiates into long slender trypomastigotes, establishing and maintaining an infection and ultimately invading the central nervous system. In preparation to be taken back up into the tsetse vector, short stumpy trypomastigotes are formed, pre-adapted for their survival in a new surrounding. Inside the insect they differentiate to procyclic forms and migrate later from the midgut to the salivary gland as epimastigotes, where they can finally develop into metacyclics, awaiting the beginning of a new transmission cycle (Langousis, & Hill, 2014).

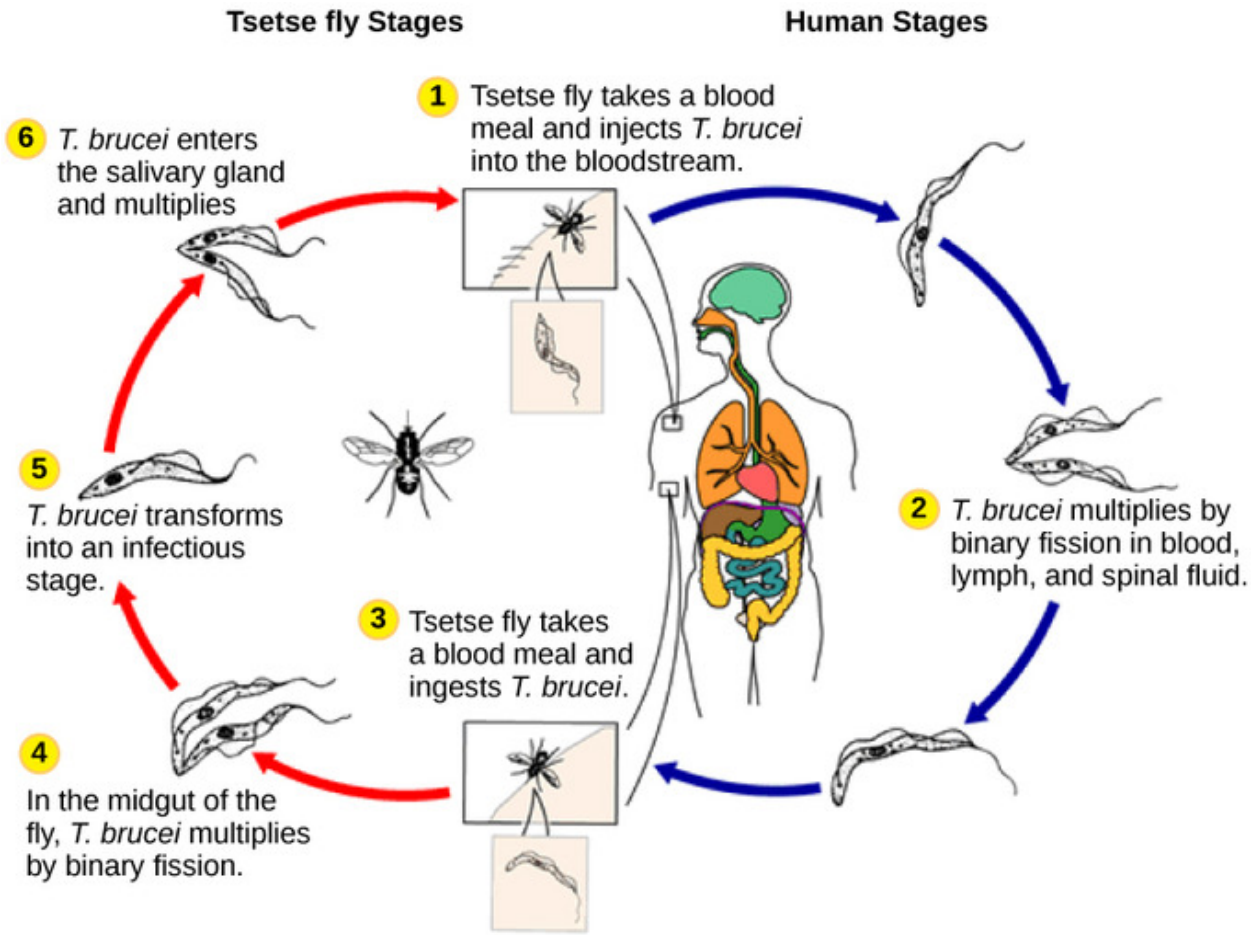

Fig. 1: Transmission cycle of *Trypanosoma brucei* taken from CDC's Division of Parasitic Diseases, http://www.dpd.cdc.gov/dpdx. When bitten by an infected tsetse fly metacyclic trypanosomes are injected into skin tissue and subsequently enter the lymphatic system and bloodstream (1). They transform into bloodstream trypomastigotes inside their mammalian host and are carried to other sites throughout the body, where they replicate by binary fission (2). After having another blood meal, the parasites are taken up by the tsetse fly (3). In the fly's midgut, they transform into procyclic trypomastigotes and start replicating by binary fission (4). They leave the midgut, and transform into epimastigotes, their infectious stage (5). After reaching the salivary gland and continuous multiplication by binary fission, they are ready to be taken up again during a new blood meal (6).

There are three different subspecies of *Trypanosoma brucei* causing different forms of trypanosomiasis. *T. brucei brucei* mainly affects livestock causing animal trypanosomiasis, also known as nagana. Although game animals and livestock are thought to be the primary pray of *T. brucei rhodesiense*, it can cause acute and chronic Trypanosomiasis in humans. The third

subspecies *T. brucei gambiense*, affecting mainly humans with a much slower onset of the disease that ultimately becomes chronic and is also known as sleeping sickness (Lopes et al., 2010).

Although the cases that were reported to World Health Organization fell below 10.000 for the first time in 2009 (Simarro, Diarra, Postigo, Franco, & Jannin, 2011) an estimated population of 65 million people is still at risk of infection, with no safe drug currently available. Without treatment, the parasite may be able to penetrate the blood-brain barrier and invade the central nervous system, causing mood changes, headaches, swollen lymph nodes, insomnia and is ultimately fatal (Stuart et al., 2008). Despite recent advances, finding an effective and reliable drug for Trypanosomiasis still remains a challenge, mainly due their variable surface glycoprotein (VSG) coat. During a process called antigenic variation, their VSG coat is continuously exchanged with new ones, which are not recognized by the host immune system and lead to the exhaustion of the host (Babokhov, Sanyaolu, Oyibo, Fabbenro-Beyioku, & Iriemenam, 2013). Their ability to prolong their proliferation time makes them valuable model organisms to study immune responses.

Apart from their relevance as pathogenic parasites, trypanosomes are also an interesting model organism for evolutionary studies, due to being at the root of the eukaryotic tree of life and therefore sharing features common in most eukaryotes (Lopes et al., 2010), but also displaying unusual peculiarities as for example in the cell cycle. The four phases that make up a typical eukaryotic cell cycle are called G_0/G_1, S, G_2 and M. During the first gap phase (G_0/G_1), the cell is metabolically active and prepares itself for a round of DNA replication and cell division. Cell growth and the availability of nutrients are often crucial factors needed for entering S phase, where DNA is replicated. Following DNA synthesis, the cell enters another gap phase (G_2) for continuation of cell growth in order to prepare itself for mitosis (M), where DNA is ultimately divided (Alberts et al., 2008). Although *T. brucei* follows in general the scheme of such a cell cycle, unique organelles and structures have to be accurately duplicated and segregated to produce viable daughter cells (Hammarton, 2007). One of them is the kinetoplast, whose location in the organism is shown in Figure 2, a network of concatenated maxi- and minicircles comprising the genome of the singular mitochondria. This very densely packaged and highly organized discoid structure gave the name to the Kinetoplastida (Lopes et al., 2010).

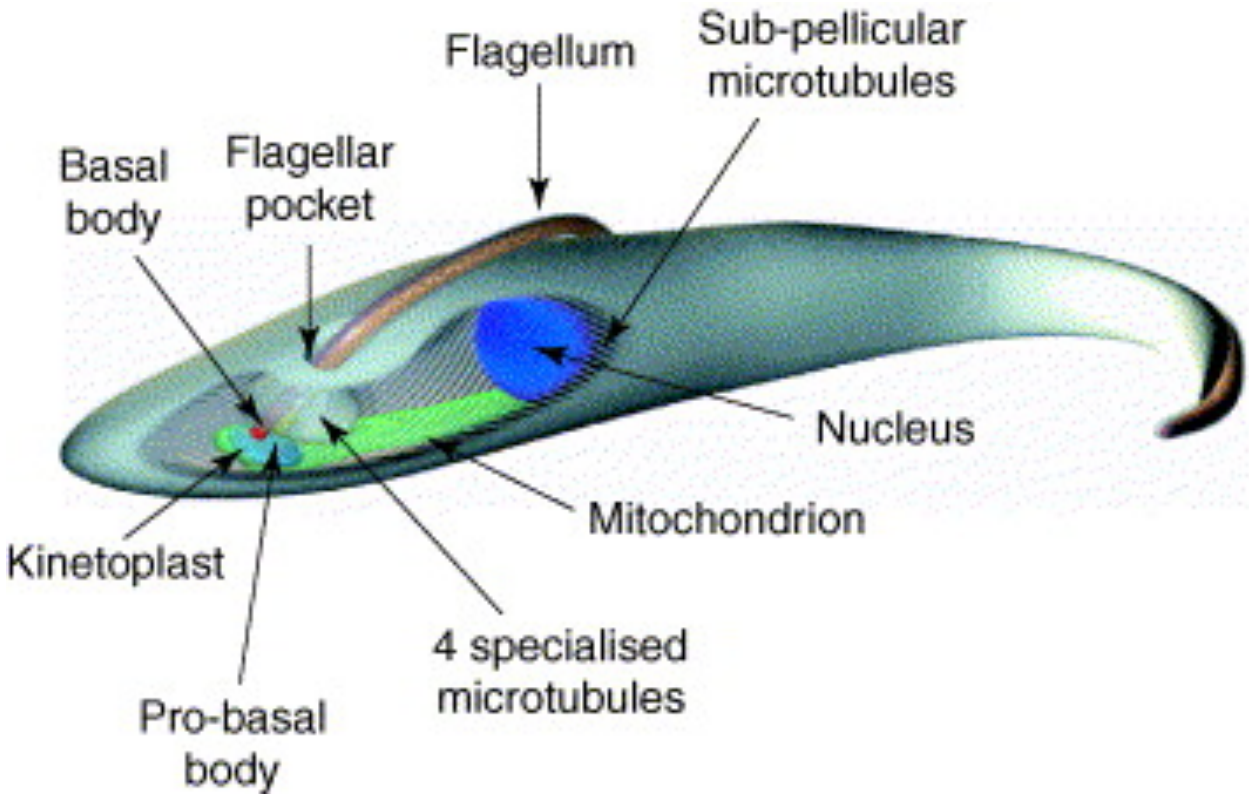

Fig. 2: Cellular structure of *Trypanosoma brucei* (Vaughan, & Gull, 2003). The relative location of the nucleus (blue), kinetoplast (turquois), mitochondrion (green), basal body (red) and flagellum (brown) are shown.

The beginning of a new cell cycle in *T. brucei* is recognized by the elongation of the pro-basal body and the beginning of the assembly of a new flagellum (McKean, 2003). Entering the S phase, discrete phases of nuclear and kinetoplast DNA replication are exhibited, despite starting chronologically very close together (Woodward, & Gull, 1990). As shown in Figure 3, the kinetoplast S phase is initiated immediately before the nuclear S phase, though lasting significantly shorter and completing kinetoplast segregation before the onset of mitosis (McKean, 2003).

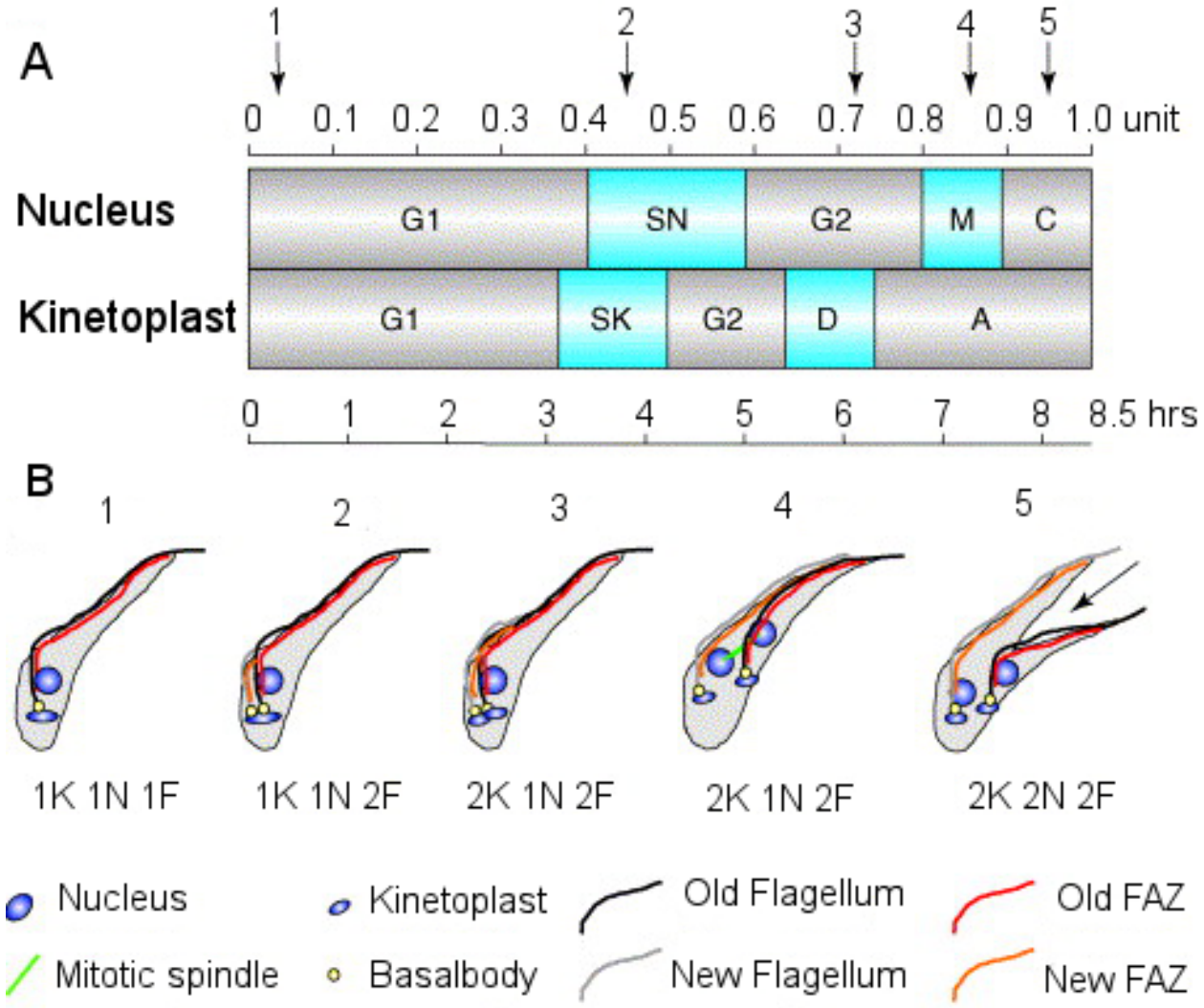

Fig. 3: Diagram representing the major morphological changes of procyclic *T. brucei* during the cell cycle (McKean, 2003). (A) The events of the cell cycle are separated into nuclear and kinetoplast phases. Kinetoplast S phase is initiated slightly earlier than nuclear S phase, but is shorter and kinetoplast segregation occurs before mitosis. (B) Schematic representation of trypanosomes taken from various time points throughout the cell cycle. The abbreviations K, N and F represent the kinetoplast, nucleus and flagellum respectively.

The current replication model of kDNA is shown in Figure 4. Covalently closed minicircles are released from the network into the kinetoflagellar zone (KFZ), where they initiate replication as theta structures. Unlike most other organisms, RNA primers for Okazaki fragments are not immediately removed, instead replicated minicircles migrate from the KFZ to the antipodal site, where primer removal, gap filling and sealing of most of the nicks occur. Minicircles are then

attached to the network periphery. At the end of replication minicircles containing at least one gap are repaired in the network.

The last step in kDNA replication is the division of the double-sized network. In *T. brucei*, at the start of kinetoplast S phase, maxicircles are uniformly distributed throughout a network of covalently closed minicircles. As the minicircles are released from the network, and as their gapped progeny are reattached to the poles of the network, the maxicircles are passively concentrated in the shrinking central zone of covalently closed minicircles. When the last, unreplicated minicircle is released, the two progeny minicircle networks are effectively segregated (Jensen, & Englund, 2012). The physical separation of the progeny kDNA networks has been associated with the formation of a filament-resembling structure, termed nabelschnur or umbilicus (Gluenz, Shaw, & Gull, 2007).

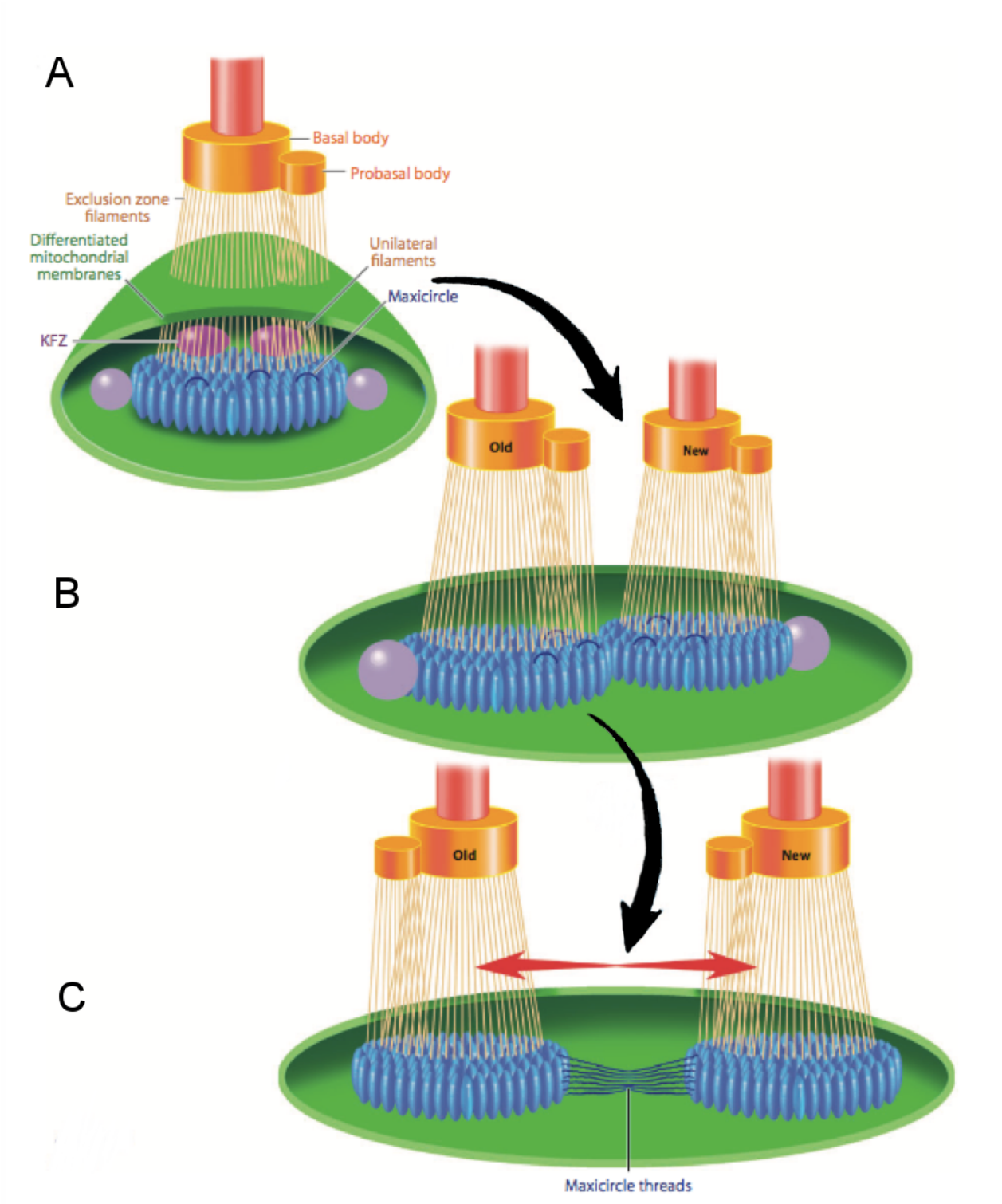

Fig. 4: Current kDNA replication model (Jensen, & Englund, 2012). The mitochondrial tubule, connected via various filaments of the tripartite attachment complex (TAC) to the flagellar basal body, shows the kDNA disk, which consists of minicircles catenated to each other and to maxicircles (A). Kinetoplast, flanked by antipodal sites, undergoing replication. The flagellar

system and TAC have duplicated, and a new basal body is shown (B). The two connected kDNA networks are pulled apart, but remain joined via a thin thread of maxicircles until cleaved by the nabelschnur (C).

To elucidate the function of proteins and their influence on cellular processes such as kDNA replication, RNA interference (RNAi) has established itself as a powerful tool silence to their respective genes. Described first in *Caenorhabditis elegans* (Fire et al., 1998) it acts as a natural defense mechanism against intruding RNA viruses. As shown in Figure 5, the first step, called RNA initiation step, involves binding of a family member of the Ribonuclease III family known as DICER to double-stranded RNA (dsRNA) (A) and its subsequent cleavage into fragments with a length of 21 to 23 nucleotides, called small interfering RNA (siRNA) (B). These siRNA combine with protein subunits to form a multinuclease complex, called RNA-induced silencing complex (RISC), where siRNA gets denatured and its sense strand is degraded, exposing anticodons and thus activating the complex (C). RISC uses the anti-sense RNA to bind to the corresponding target messenger RNA (mRNA). Once bound the target breaks apart, thereby successfully silencing gene expression (D). If the antisense siRNA and the mRNA are not exactly complementary, the RISC complex stays bound to the target and interfering with the ability of ribosomes to translate the mRNA (Sen, & Blau, 2006).

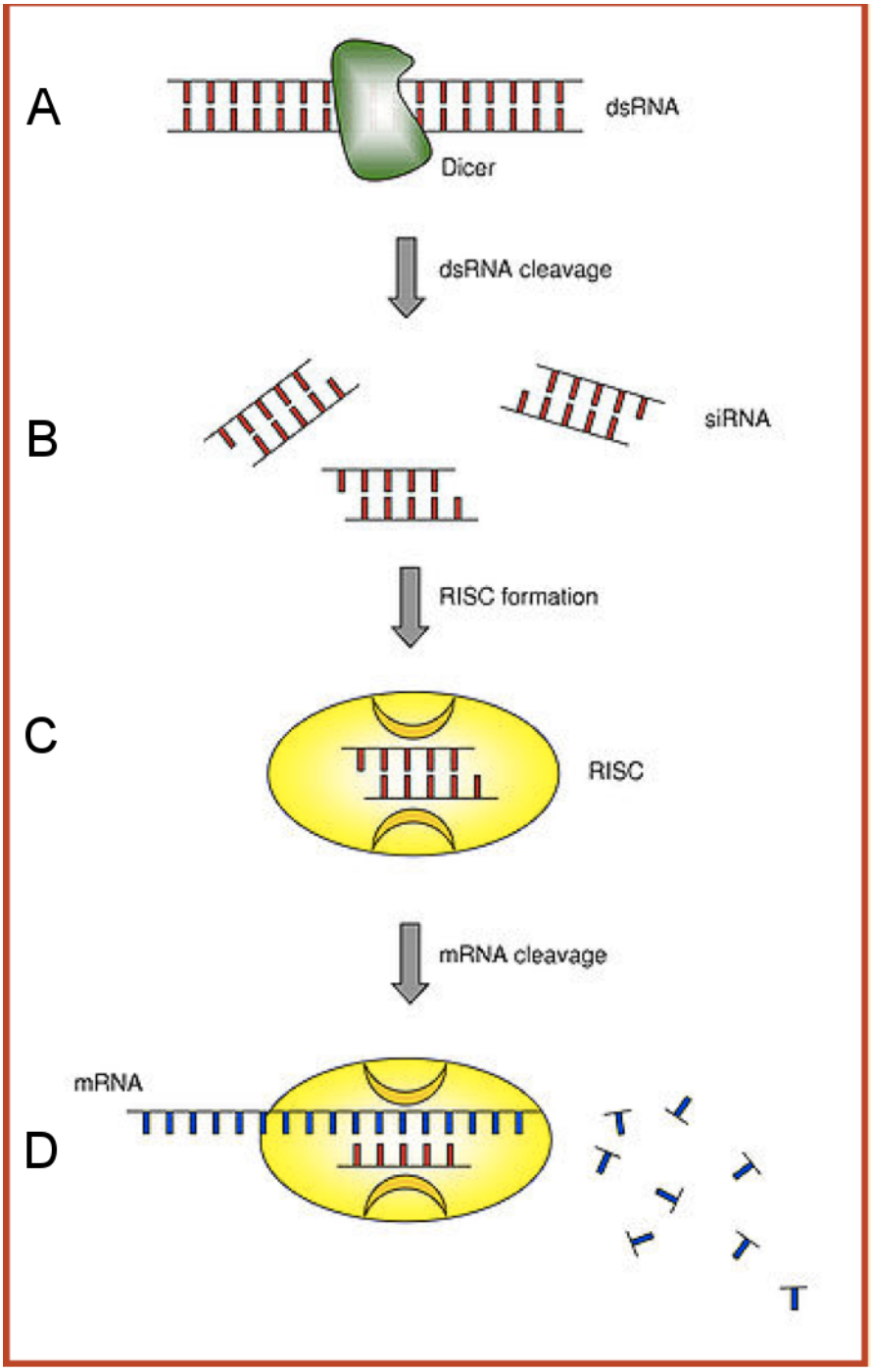

Fig. 5: Schematic representation of the pathway cascade of RNA interference within a cell (Mocellin, & Provenzano, 2004).

Though the classical approach for identifying the function of a gene begins with knockdown studies that enable loss-of-function mutations, some functions might still remain undetected. Experiments and observations applying ectopic expression indicated that simply increasing the number of gene copies could cause mutant phenotypes (Birchler, & Veitia, 2007),

which ultimately lead to the development of overexpression for further elucidation of the function of genes.

In this thesis a leucine aminopeptidase (LAP), a metallopeptidase known to cleave N-terminal amino acids and preferentially leucine residues from protein and peptides, is further investigated. They form homohexamers, belonging to either the M1 or the M17 protease family. The catalytic domains of M1 and M17 family members are distinguished by the presence and absence of a HEXXH motif, respectively (Rawlings & Barret, 1995). Though LAPs play an important role in protein degradation and are often viewed as maintenance enzymes (Matsui, Fowler, & Walling, 1990), they perform a range of moonlight functions in different organisms. Amongst these functions are regulating meiotic events in fungi (Ishizaki et al., 2002), involvement in the glutathione metabolism to prevent damage to cellular constituents and maintain cell viability (Cappiello et al., 2006), regulating biotic and abiotic stresses (Walling, 2004) in plants, as well as regulating hormonal levels of e.g. oxytocin and vasopressin in serum during pregnancy in humans (Nomura et al., 2005). Though LAPs provide a vast diversity of moonlight functions, impacting cellular and physiological activities, their exact mechanisms remains unclear.

2 Aim of the thesis

The aim of the thesis was the characterization of the leucine aminopeptidase 1 in the procyclic stage of *Trypanosoma brucei*, using RNAi and ectopic expression cell lines to screen for phenotypes, as well localization analysis via immunolocalization of overexpressed *in situ* tagged peptidase.

3 Materials and methods

3.1 Construction of cell lines

For the construction of TbLAP1 RNAi cell lines the p2T7^{Ti}-177 vector was used, which consists of a 177 bp sequence for targeting to *T. brucei* minichromosomes, two tetracyclin-inducible head-to-head T7 promoters in a multiple cloning site, and a phleomycin resistance gene

(Wickstead, Ersfeld, & Gull, 2002). A PCR amplified 400 bp-long region of the TbLAP1 gene was cloned into the vector between the restriction sites *Bam*HI and *Hind*III to replace the GFP fragment. The final construct was then linearized with *Not*I and transfected into procyclic 29-13 (Wirtz, Leal, Ochatt, & Cross, 1999) and SMOXP9 *T. brucei* (Poon, Peacock, Gibson, Gull, & Kelly, 2012), which express the tet repressor and T7 RNA polymerase respectively.

For overexpression, the full-size TbLAP1 gene was first amplified with primers AAAAGTAAAATTCACGGGCCCATGCTCAAGAGAGT and CAGATTTTCGTTTCTGGTACCTCAATTGCCAGACCT and then cloned into the p2623 vector (Kelly et al., 2007). The vector consists of a 177 bp repeat sequence for targeting to *T. brucei* minichromosomes, a hygromycin resistance gene and 2 tandem TEV:6 tandem HA as a tag.

TbLAP1 was *in situ* tagged using the long PCR approach with V5 to its C-terminus. The pPOTv4 vector (Dean, Sunter, Wheeler, Hodkinson, Gluenz, & Gull, 2015), in which the yellow fluorescent protein (eYFP) was replaced by the V5 tag, was used to amplify a PCR product, which was then transfected into procyclic *T. brucei* 427 cells (Oberholzer, Morand, Kunz, & Seebeck, 2005) using the following primers 5'-AGTAATGCGCCACGGGGCTGCATGTAACCCTGTTGATGTCATTGAGAACTATCTGGAGGACAAACTCGATGAAATCGACATATGGGTGGGTACCGGGCCCCCCCTCGAG-3' and 5'-CACATCCTGATGTGTTGCTTCTCGCCGCACCTAGCACGGTGAAGGCCGTGAGCATGTATGTGTAGTGCAGAAGAGTAAAGAGCGTTTTGGCGGCCGCTCTAGAACTAGTGGAT-3'.

3.2 Cell culture and transfection

All experiments were carried out in SDM79 medium supplemented with 10% fetal bovine serum. Procyclic 29-13 and SMOXP9 cell lines were used as parental lines for RNAi against TbLAP1 and PCF 13-13 cell line for the ectopic expression of TbLAP1-HA.

Procyclic trypanosomes were cultivated at 27°C in SDM79 media. The 29-13 cell line was grown in the presence of hygromycin (50 μg/mL) and geneticin or G418 (15 μg/mL) and the SMOXP9 cell line in the presence of puromycin (0.5 μg/mL) for the stable expression of T7 polymerase and tetracycline (Tet) repressor.

PCF 13-13 trypanosomes were grown in the presence of phleomycin (5 μg/mL) for the maintenance of stable expression of Tet repressor (Alibu, Storm, Haile, Clayton, & Horn, 2005).

Transfections were performed according to a well-established protocol (Wang, Morris, Drew, & Englund, 2000). PCF *T. brucei* cells (2 x 10^7) were washed once with 2 mL CytoMix (23) and then resuspended in 0.5 mL of CytoMix containing either 10 μg of linearized vector or PCR product. The mixed sample was loaded in a 2-mm electroporation cuvette and transfection was carried out using a BTX electroporator applying one pulse with the BTX settings 1500 V, 50 microfarads and 500 ohms. The cells were immediately transferred into fresh SDM-79 medium containing G418 and hygromycin. Phleomycin (2.5 μg/mL) was added after 18 hours to select positive transfectants. After 10 days of cultivation, phleomycin-resistant cells were used for assessment of RNAi. Induction of RNAi and overexpression was initiated by the addition of 1 μg/mL Tet to the cultures. Cell numbers were monitored using a Beckman Coulter Z2 counter. All growth curves were started with 2 x 10^6 cells/mL and subcultured to the same cell number every 24 hrs.

3.3 Immunofluorescence and DAPI staining

Ectopically expressed and in situ tagged TbLAP1 was followed by immunofluorescence assay as performed by Colasante et al. (2009), with minor modifications. Expression was induced with 1 μg /mL Tet and samples were taken at several time points, with parental cell lines used as controls.

T. *brucei* cells were centrifuged from culture medium at 1800 x*g*, and immediately re-suspended and fixed for 15 min with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS). They were permeabilized with 0.2% (v/v) TX-100 in PBS on microscopy slides and then probed with primary antibodies in PBS/gelatin. Polyclonal anti-TbLAP1 and anti-HA antibodies

produced in rabbits (Sigma-Aldrich) and polyclonal enolase antibodies were used at 1:5000 dilution. Rabbit anti-V5 antibody (Sigma-Aldrich) was used at a dilution of 1:8000. Monoclonal anti-TAC102 was used at 1:2500 dilution. As secondary antibodies, Alexa Fluor 488 anti-rabbbit and Alexa Fluor 555 anti-mouse (Life Technologies) were used. DNA was visualized using ProLong antifade reagent with DAPI (Life Technologies) and DAPI counts were performed using 200 cells per time point. Immunofluorescence analysis was performed using a Zeiss microscope Axioplan 2, equipped with an Olympus DP73 digital camera and detection was carried out with cellSens software (Olympus). Image analysis was performed using ImageJ. All zoomed image sections are approximately 1µm x 1µm.

3.4 Western blot

Cell lysates were prepared in SDS-PAGE LDS sample buffer using 5 x 10^6 cells per lane separated on Bolt 4-12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to a Amersham Hybond P PVDF membrane (GE Healthcare). The membrane was blocked with 5% non-fat milk in 1xTBST for 60 min at room temperature and subsequently hybridized with polyclonal anti-HA (Sigma) antibodies at 1:5000 dilutions in blocking buffer for 60 min at room temperature. After washing three times each for 10 min, the membrane was incubated with anti-rabbit antibody conjugated with horseradish peroxides (Sigma) at 1:200 for 60 min at 4 °C. Following hybridization, the membrane was washed four times each for 10 min and Clarity ECL substrate (Bio-Rad) was used to visualize the proteins.

3.5 Fluorescence-activated Cell Sorting (FACS) analysis for the measurement of mitochondrial membrane potential

FACS analysis was performed using a Canto II flow cytometer (BD Biosciences). For mitochondrial (mt) membrane potential measurement, 5 µL of MitoTracker Red CMXRos or Tetramethylrhodamine ethyl ester (TMRE) was added to 5 x 10^6 cells procyclic trypanosomes overexpressing TbLAP1. The sample solution was incubated at 27°C for 30 min in SDM79 and afterwards spun down at 1300 x*g* for 10 min at room temperature. The supernatant was pipetted off without disturbing the pellet and gently and quickly re-dissolved in 1 mL PBS. The sample

solution was transferred to a FACS tube and diluted with 1 mL PBS. Ten thousand cells were counted in each measurement. Data were analyzed using Flowing Software program (Turku Centre for Biotechnology).

4 Results

4.1 Subcellular localization of TbLAP1

In situ tagging of TbLAP1 at the C-terminus with V5 revealed that the protein was associated with the kinetoplast throughout the cell cycle, as shown in Figure 6. In order to monitor the movement of the tripartite attachment complex, a structure that connects kDNA with the basal body and mediates kDNA segregation (Ogbadoyi, Robinson, & Gull, 2003), with respect to the kDNA network one of its components, namely TAC102 (Trikin et al., 2016), was immunolocalized.

In the beginning of the cell cycle, subcellular localization of TbLAP1 showed that the protein was co-localized with the kDNA, but not with TAC102 (A). Following the replication of the basal body, TAC and kDNA undergo DNA synthesis simultaneously (Ogbadoyi, Robinson, & Gull, 2003). Minicircle duplication and their distribution to the antipodal sites, causes the kDNA to become elongated and to adopt a dumbbell-like structure (Jensen, & Englund, 2012), to whose respective ends TbLAP1 was localized. The TAC102 signal displayed as well an elongated structure found next to the point of segregation (B). The segregation of the kDNA into two daughter networks was accompanied by the appearance of a characteristic structure called nabelschnur, made visible by the TbLAP1 signal between the two foci. With the formation of the nabelschnur, TAC102 remained visible as two dots indicating a separation into two distinct structures, which co-migrated with the kDNA/TbLAP1 association (C-E). After the successful segregation, the signal correlating to the nabelschnur diminished and TbLAP1 remained confined to spots overlaying the newly synthesized kDNA discs (F-G).

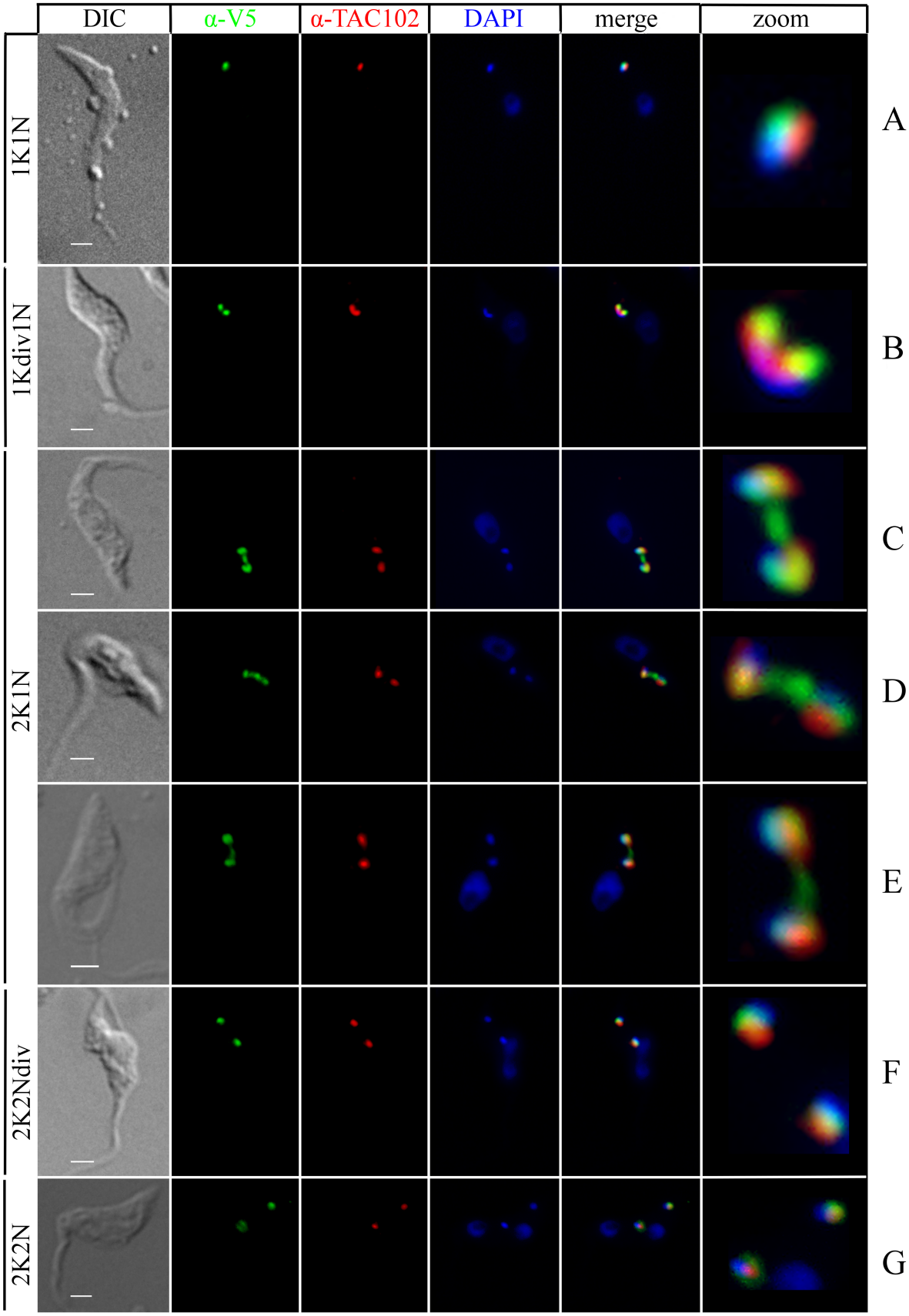
DIC
α-V5
α-TAC102
DAPI
merge
zoom
1K1N
1Kdiv1N
2K1N
2K2Ndiv
2K2N
A
B
C
D
E
F
G

Fig. 6: Immunofluorescence of PCF 427 cell line expressing *in situ*-tagged TbLAP1-V5 protein, labeled with Rabbit anit-V5 (green). Anit-TAC102 (red) was used to visualize the TAC. DAPI (blue) visualizes the location of the nucleus and kDNA. Zoom shows an enlargement of the kDNA together with associated components from the merge. (A) The V5 signal is co-localized with the kinetoplast, but not with the TAC102 signal. (B) At the end of kDNA replication, minicircles accumulated at the antipodal sites giving the kDNA a dumbbell-like shape, to which TbLAP1 is located at two foci, each at the tip of the structure. (C) During kDNA segregation, TbLAP1 is associated with the nabelschnur and is still co-localized to the kDNA disc. (E) After kDNA segregation is finished, the nabelschnur fades correlating with a weakening V5 signal and TbLAP1 remains co-localized with the kDNA. (F) The TAC signal co-migrates with the kDNA/TbLAP1 association (G) TbLAP1 and TAC102 show similar localizations as in 1K1N cells.

4.2 Downregulation of TbLAP1 by RNAi

PCF 29-13 cell line containing the construct for the expression of double stranded RNA was induced with tetracycline in SDM79 media and was grown for 12 days. No significant growth defect could be observed with an RNAi-mediated down-regulation of TbLAP1 protein, shown in Figure 7.

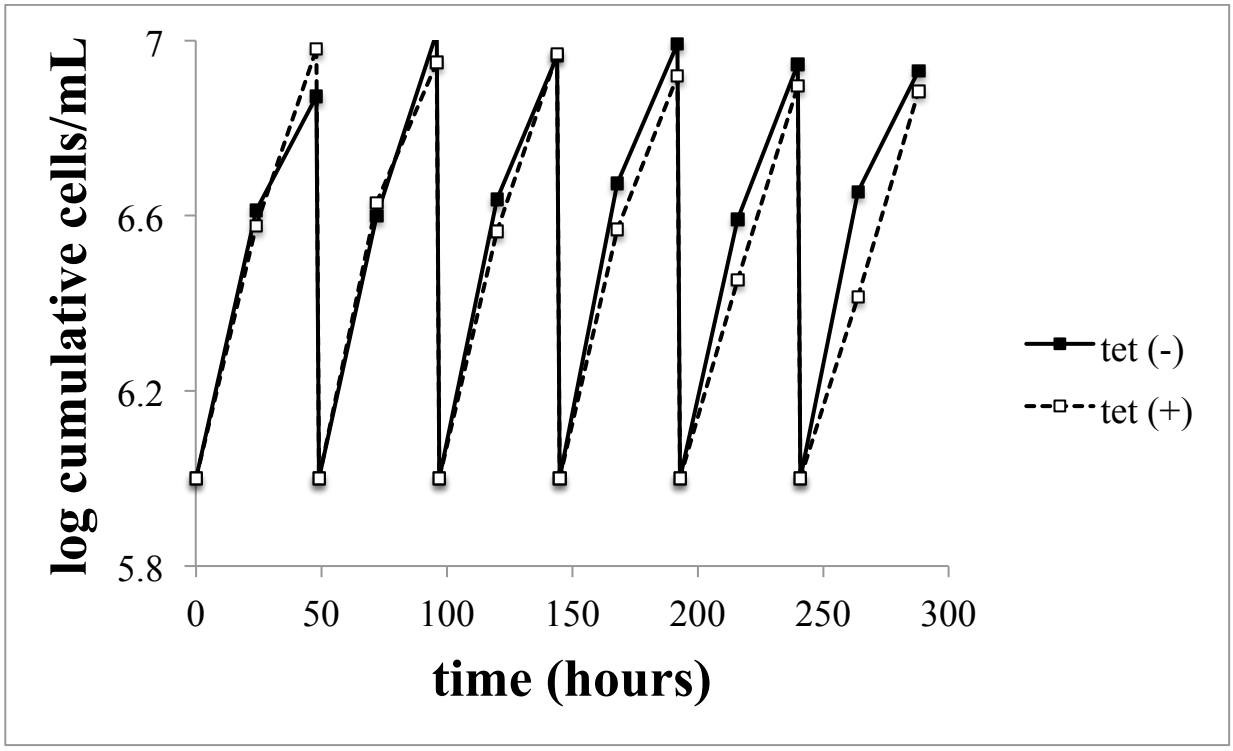

Fig. 7: Growth curve of PCF 29-13 cell line in the presence (white, dashed lines) and absence (black, unbroken lines)) of tetracycline to induce TbLAP1 RNAi silencing, over a 12-day period. Cultures were diluted to 2 x 10^6 cells /mL and cell number was measured every 24 hours.

Western blot analysis, following the expression of TbLAP1 protein (Figure 8), confirmed a successful tetracycline induced down-regulation after five days.

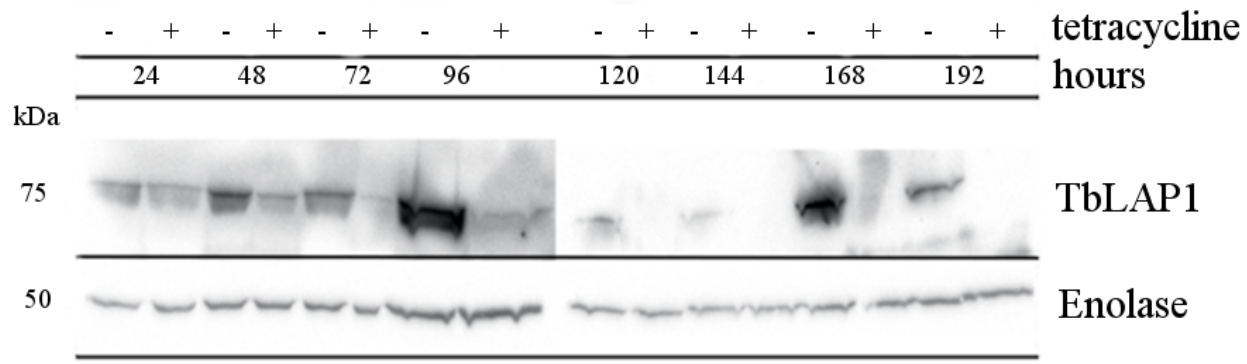

Fig. 8: Western blot of RNAi-mediated, down-regulated TbLAP1 using anti-enolase antibody as a loading control. 5 x 10^6 PCF 29-13 cells per sample were loaded per well.

Due to the possible leakiness of 29-13 cell lines, the down-regulation of TbLAP1 was repeated using the SMOXP9 cell line. As can be seen in Figure 9 and 10, a non-lethal growth defect was induced, which caused the accumulation of trypanosomes having two kinetoplasts, as well as two nuclei (2K2N).

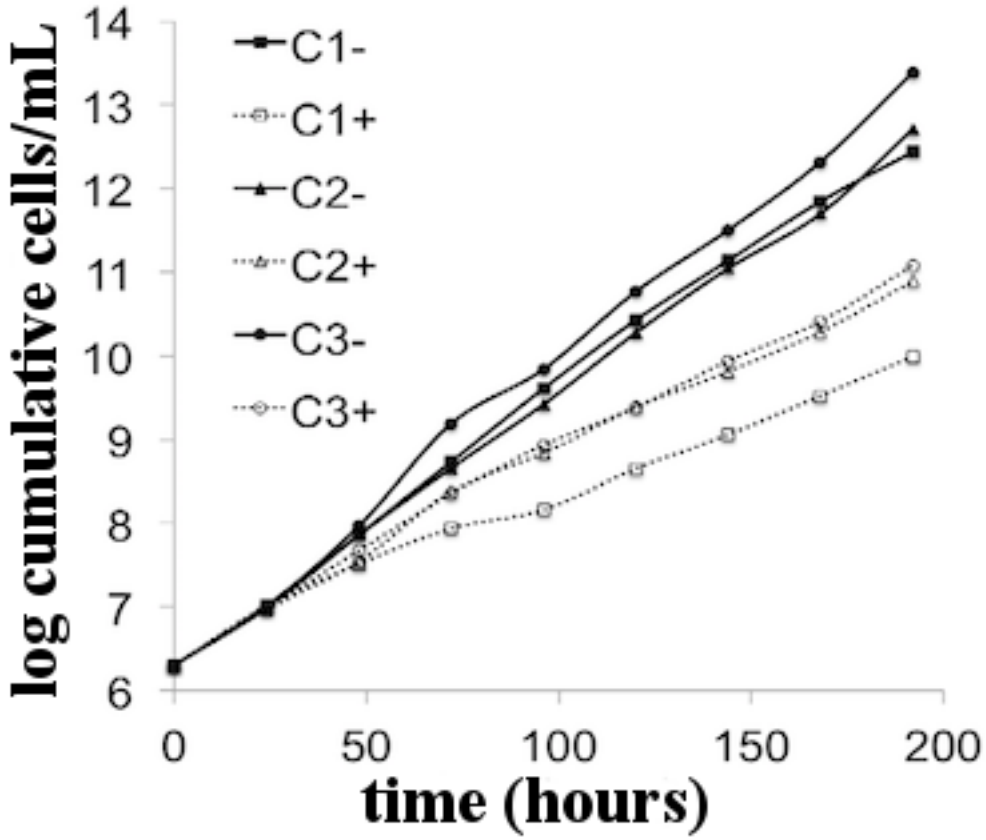

Fig. 9: Growth curve of PCF SMOXP9 cell line harbouring the TbLAP1 RNAi construct (p2T7-177). Tetracycline presence (white, dashed lines) and absence (black, unbroken lines) induced expression over an 8-day period. Three clones were used for the analysis. Cultures were diluted to 2 x 10^6 cells /mL and cell number was measured every 24 hours.

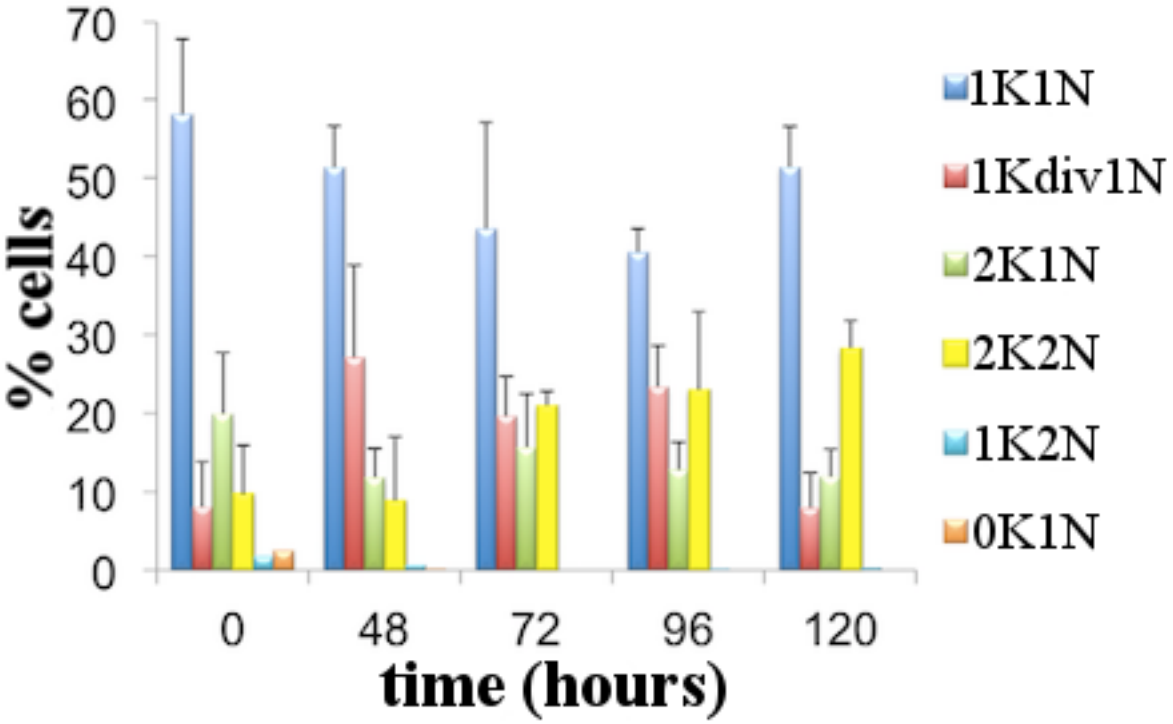

Fig. 10: DAPI counts of TbLAP1 RNAi induced and uninduced PCF SMOXP9 cell line. Two hundred cells per time point were counted and used for the analysis. The abbreviations N and K represent the nucleus and kinetoplast respectively.

4.3 Effects of inducible ectopic expression of TbLAP1-HA

Ectopic expression of TbLAP1-HA in procyclic *T. brucei* induced a significant growth defect noticeable after 24 hours and shown in Figure 11. The defect manifested itself by the accumulation of cells having only one nucleus (0K1N), shown in Figure 12. The loss of kDNA was observed right after 2 hours post-induction with tetracycline, as shown by the significantly increased proportion of 0K1N cells, almost complete disappearance of cell duplets, represented by 2K2N, after 48 hours, and the increase of cells having two nuclei after 72 hours. An immunofluorescence assay, shown in Figure 14, using an anti-HA antibody for the detection of the overexpressed protein displayed TbLAP1 being localized into the kinetoplast.

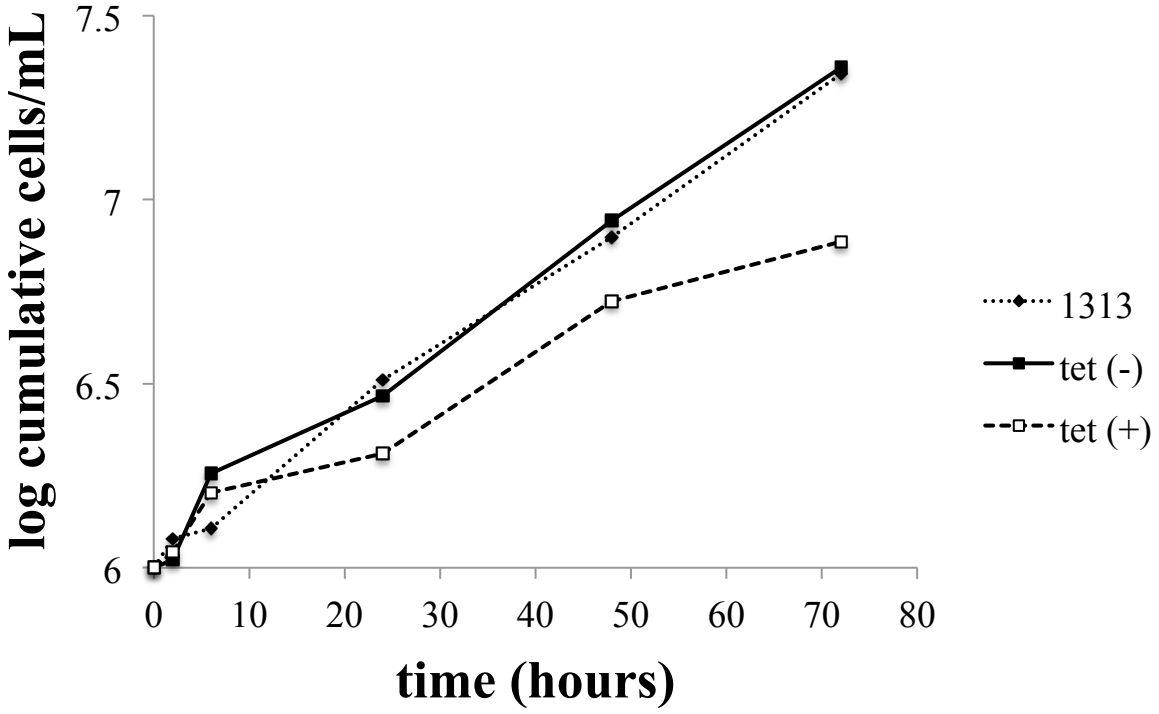

Fig. 11: Growth curve of PCF 13-13 *T. brucei* cell line ectopically expressing TbLAP1-HA in the presence (white, dashed lines) and absence (black, unbroken lines) of tetracycline (Tet). Parental PCF 1313 cell line was used (black, dashed line) as a control.

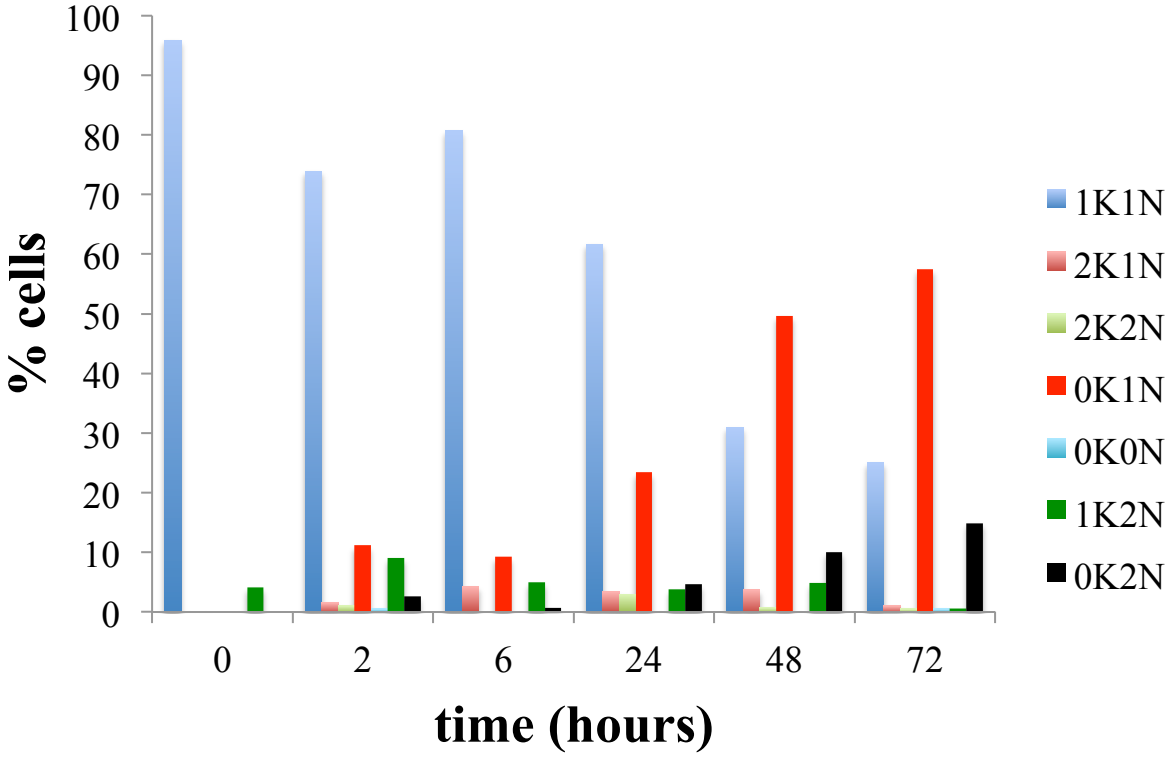

Fig. 12: DAPI counts in Tet induced PCF 13-13 cell line for ectopic expression of TbLAP1-HA. Two hundred cells per time point were counted and used for analysis. The abbreviations N and K represent the nucleus and kinetoplast respectively.

Ectopic expression of TbLAP1-HA was monitored by Western blot using anti-HA antibody, shown in Figure 13.

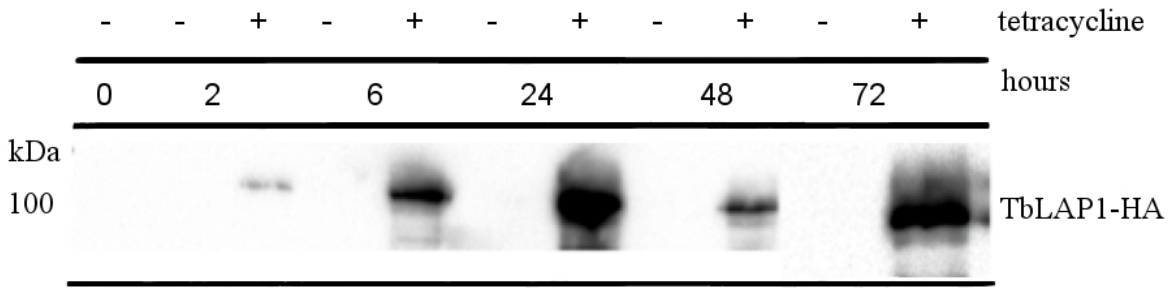

Fig. 13: Western blot analysis of ectopically expressed TbLAP1-HA of PCF 13-13 cells. 5 x 10^6 cells per sample were loaded per well.

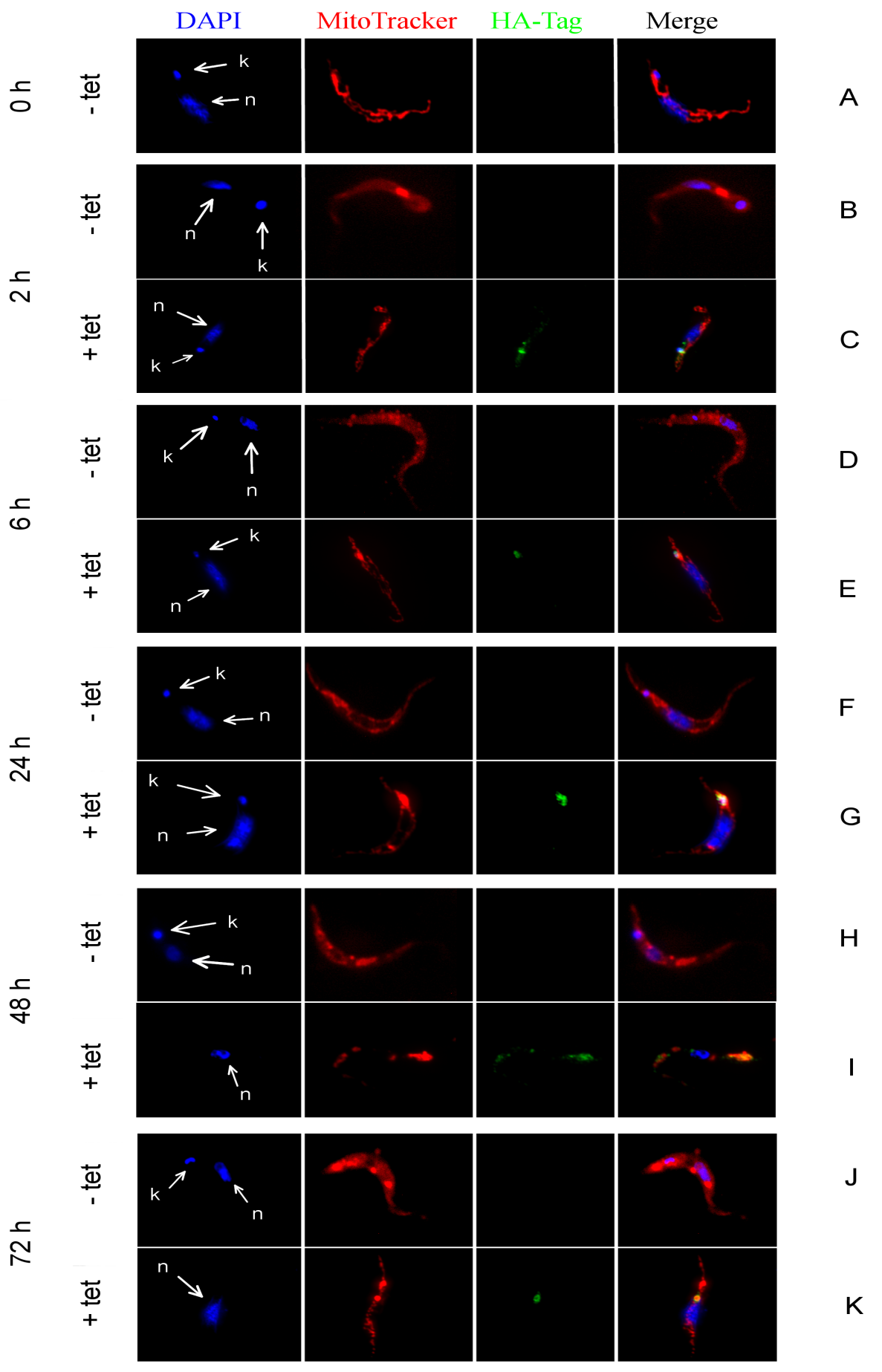
DAPI
MitoTracker
HA-Tag
Merge
0 h
2 h
6 h
24 h
48 h
72 h
- tet
+ tet
A
B
C
D
E
F
G
H
I
J
K
k
n

Fig. 14: Immunofluorescence of PCF 13-13 cell line ectopically expressing TbLAP1-HA protein, labeled with polyclonal anti-HA (green). MitoTracker (red) was used to show the mitochondrion. DAPI (blue) visualizes the location of the nucleus and kDNA, indicated via arrows and labeled with n and k respectively. (A-E) Up to 2 hours post-induction with tetracycline, the vast majority of cells consisted of one nucleus and one kinetoplast. (G, I, K) At 24 hours of ectopic expression of TbLAP1-HA, the appearance of cells containing only one nucleus and showing a loss of kinetoplast became evident, whose number continued to increase up until the end of the experiment.

4.4 Fluorescence-activated cell sorting

The staining of procyclic *T. brucei* expressing TbLAP1-HA with MitoTracker resulted in an uneven, patchy distribution of the probe throughout the reticulated mitochondrion, as can be seen in Figure 15 after 24 hours of induction with tetracycline. Due to the dye being membrane potential-dependent, a defect in mt membrane potential as a secondary effect of kDNA loss was suggested and a FACS analysis was conducted, due to similar phenotypes being found in cases of kDNA polymerase depletion (Bruhn, Sammartino, & Klingbeil, 2011).

Cellular suspensions were incubated for a certain time period with a cationic fluorescent dye, localized into the mitochondrion due to its attraction towards the negative potential across the mitochondrial membrane (Cottet-Rousselle, Ronot, Leverve, & Mayol, 2011). The injection of the suspensions into the flow cytometer was followed by the excitation of the fluorophore via lasers at specific wavelengths, resulting in the emission and scattering of light recorded as cells pass optical detectors one by one (Kalisky, & Quake, 2011). The dye TMRE is retained in metabolically active mitochondria (Shapiro, 2000), but less will accumulate once the organelle experiences mt depolarization (Kholmukhamedov, Schwartz, & Lemasters, 2013) (Perry, Norman, Barbieri, Brown & Gelbard, 2011), which manifests as decreased fluorescence intensity.

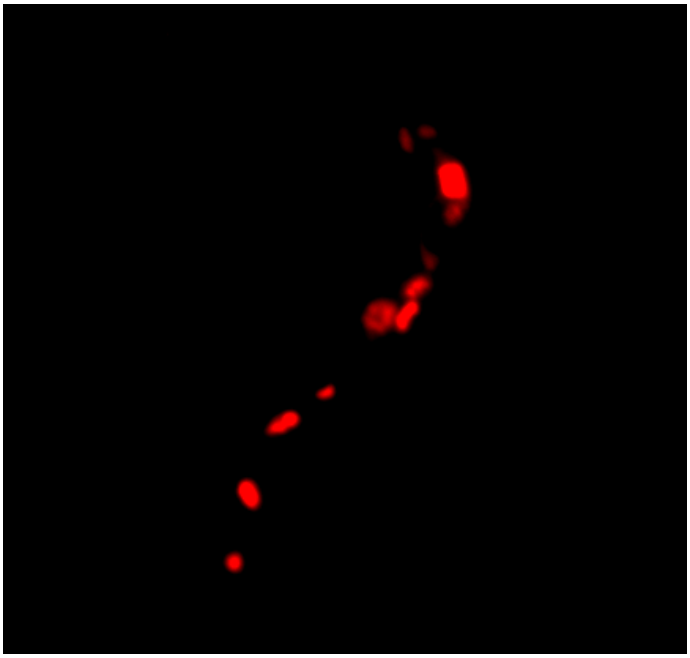

Fig. 15: Immunofluorescence assay of PCF 13-13 cell line ectopic expression with TbLAP1-HA incubated with MitoTracker (red). Accumulation of patches throughout the reticulated mitochondrion was noticed.

As can be seen in Figure 16, over-polarization of the mt membrane continued until 48 hours of induction, after which the mt membrane potential decreased and fell below values in uninduced cells.

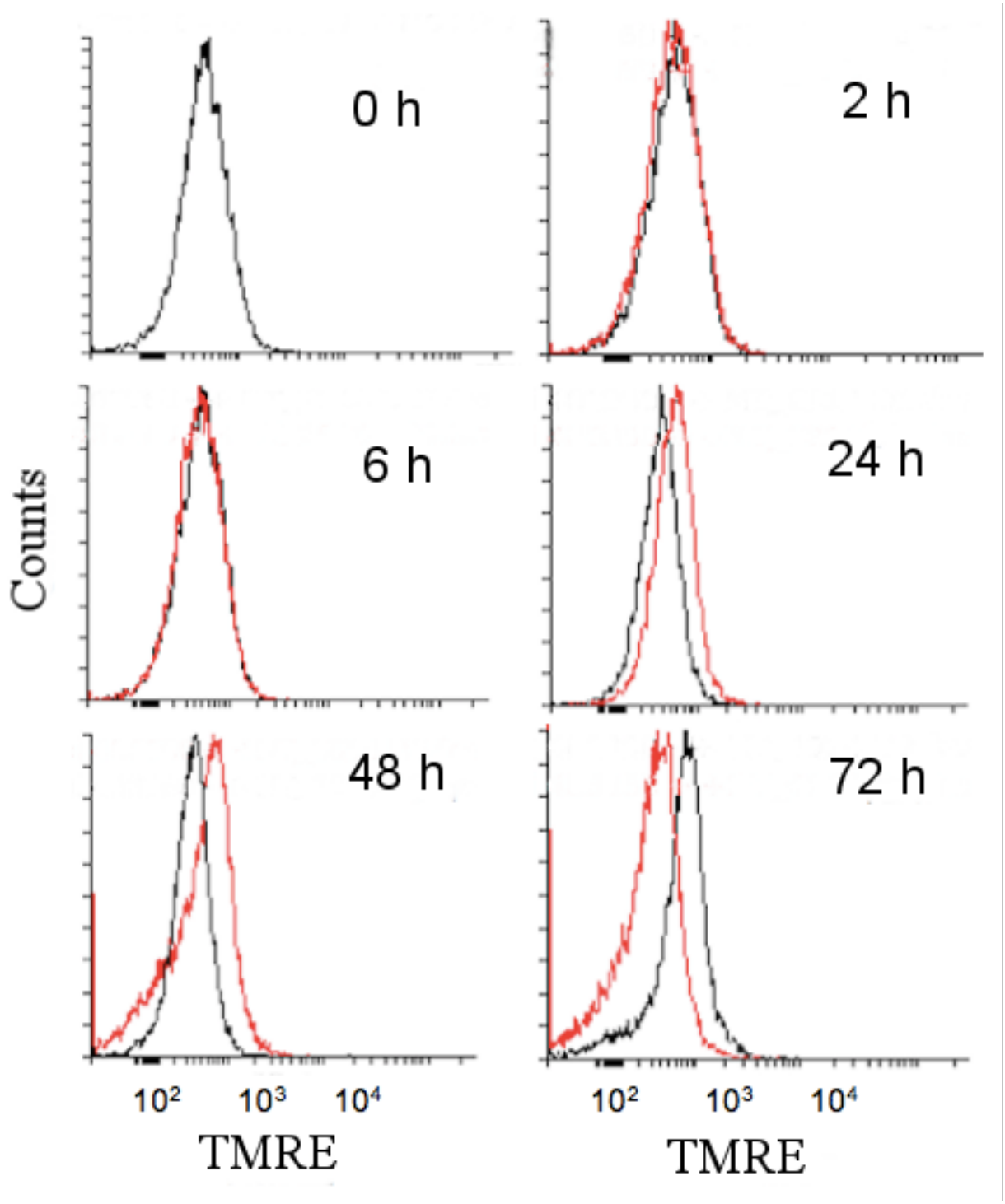

Fig. 16: Mitochondrial membrane potential measurement using PCF 13-13 cells expressing TbLAP1-HA stained with TMRE. Measurements were made using a Canto II FACS. Black and red line represent uninduced and induced cells respectively (2,6, 24, 48 and 72 hours of induction).

5 Discussion

As shown in the results section TbLAP1 was identified as a kinetoplast-localized protein having a surprising influence and relevancy regarding the cell cycle in *T. brucei*.

Throughout the cell cycle the protein was localized to the kinetoplast, though the association did not cover the entire network, as could be seen during G_1 phase or right after the cells have undergone kDNA segregation. During the segregation process TbLAP1 seemed to be associated with a newly assembled structure functioning as a link between the two emerging daughter networks, the nabelschnur, whose appearance and subsequent unlinking is believed to mark the final stage of kDNA segregation (Gluenz, Povelones, Englund, & Gull, 2011). Afterwards the protein remained confined to two foci representing the newly emerged kDNA networks during the division process of the nucleus. Following its completion, cytokinesis partitioned the cytoplasm, forming two daughter cells and thereby ending the round of the cell cycle.

The knockdown RNAi experiment manifested a growth phenotype, denoting the essentiality of the protein for normal growth in vitro. Following the depletion of TbLAP1 in the SMOXP9 cell line, DAPI staining indicated an aberration in the processes of the cell cycle, evident by the accumulation of 2K2N cells. Such a configuration indicated a delay in cytokinesis, regardless of the successful replication of both the kinetoplast and nucleus. Since no multinucleated cells appeared during the timescale of the experiment one can assume that the terminating step was simply postponed rather than completely aborted, which was further confirmed by the observation of cells having a 2K2N configuration undergoing already kinetoplast replication prior to cytokinesis.

TbLAP1 ectopic expression affected kDNA segregation resulting in the loss of kDNA structure. Although proteins of importance in kDNA segregation and involved in its maintenance showed a similar effect upon overexpression, as for example the helicase TbPIF2, where ~30% of cells had no kinetoplast and <10% suffered from kinetoplast shrinkage (Liu et al., 2009), or a cytochrome b_5 reductase-like protein, where 63% of the cells lost their kinetoplast after 9 days (Motyka, Drew, Yildirir, & Englund, 2006), it never occurred in such a rapid fashion. TbLAP1-HA accumulated around the kinetoplast and thereby disrupted its segregation. As nuclear

division and segregation are independent of the mechanism of kDNA segregation (Robinson, & Gull, 1991) (Ploubidou, Robinson, Docherty, Ogbadoyi, & Gull, 1999) the cell cycle continued and the progenies were divided via cytokinesis, resulting in cells with either a nucleus and a kinetoplast still trying to undergo kDNA segregation or a 0K1N configuration. Regardless of the loss of kinetoplast, 0K2N cells still formed, although their viability was seriously hampered, emphasizing that nuclear division was not affected by the overexpression of TbLAP1 as kDNA was.

The collapse of the mt membrane potential observed by flow cytometry after 72 hours has been suggested to be a side effect of kDNA loss, as a similar result previously observed in an experiment involving the depletion of three mitochondrial DNA polymerase (Bruhn, Sammartino, & Klingbeil, 2011).

Although at this point no conclusions about the exact mechanism of how TbLAP1 works could be drawn, the observed phenotypes during the knockdown and ectopic expression experiments brought forth the evidence for its involvement in the segregation process of kDNA in procyclic *T. brucei*.

The high concentration of TbLAP1-HA resulting from overexpression was able to hamper segregation of kDNA progeny, an indication that the protein must vacate a site at a certain point of the cell cycle, probably by having their expression levels regulated. TbLAP1 might have acquired a moonlight function involved in segregation of kDNA, but this process appears strictly regulated and timely related to the cell cycle. The concentration of TbLAP1, or its location must be regulated to fulfill its assignment as a similar conclusion could be drawn from the overexpression of TbPIF5, a helicase involved in the replication of minicircles causing the loss of the kinetoplast (Liu, Wang, Yildirir, & Englund, 2009).

Absence of the protein, resulting in a delay of cytokinesis, suggests that TbLAP1 may interact with a possible regulator molecule, which indicates beginnings and ends of cell cycle phases, as similar phenotypes were observed with other proteins involved in cytokinesis, such as a member of the centrin-family (Selvapandiyan et al., 2007) or katanins and spastin (Benz, Clucas, Mottram, & Hammarton, 2012). However, the fact that division of either kDNA or nuclei

was never aberrant nor halted indicates that TbLAP1 may be regulated during the cell cycle, but not vice versa.

6 Summary

The main goal of this thesis was to localize TbLAP1 and assess phenotypes resulting from silencing and inducible ectopic expression of TbLAP1-HA in procyclic *T. brucei*.

The subcellular localization experiment showed an association of the protein with the kinetoplast as well as with the nabelschnur during kDNA segregation.

Two RNAi experiments with different cell lines were conducted in which it was possible to successfully knock down the protein of interest. A growth phenotype could be observed in the SMOX cell line and its respective DAPI count analysis showed the appearance of cell duplets consisting of two nuclei and two kinetoplasts. It seemed that the successful down-regulation of TbLAP1 affected the separation of the cell duplets, but did not affect their division process.

Ectopic expression of TbLAP1 induced a growth phenotype and a complete loss of kinetoplast. The collapse of mt membrane potential, brought to attention by the accumulation of patches throughout the reticulated mitochondrion when stained with MitoTracker dye, could have been a potential secondary effect of kDNA loss.

7 Literature

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