University of South Bohemia in České Budějovice

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Laboratory of Molecular Biology of Protists

Institute of Parasitology

Characterization and localization of Tb1290, a putative mitochondrial protein in *Trypanosoma brucei*

Confirmation of in-situ tagging data from the TrypTag database

Bachelor Thesis

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Annotation

In this thesis methods in-situ tagging, immunofluorescence microscopy, cell fractionation are utilized for localization and RNA interference for determination of essentiality of protein Tb927.11.1290 in *Trypanosoma brucei*.

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Acknowledgment

I would like to express my gratitude to Prof. RNDr. Julius Lukeš, CSc. for letting me work in his laboratory on this thesis. Next, I would like to thank my supervisor Priscila Peña-Diaz, Ph.D. for help through the whole project and showing me how modern science is made. Further, I am thankful for advices in times of need from all members of the Laboratory of Molecular Biology of Protist. And, finally, the biggest thank you to my family and friends for endless support during my studies and the whole life.

Abbreviations:

APS	ammonium persulphate
DNA	deoxyribonucleic acid
kDNA	kinetoplast deoxyribonucleic acid
HAT	human african trypanosomiasis
DTT	dithiothreitol
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
HF	high fidelity
HMM	hidden Markov model
HRP	horseradish peroxidise
dNTP	deoxyribonicleotide triphosphate
PBS	phosphate-buffered saline
PCF	procyclic form
PEG	polyethilene glycol
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
RISC	ribonucleic acid-induced slicing complex
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RNAi	ribonucleic acid interference
lhRNAi	long hairpin ribonucleic acid interference
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
TAC	tripartite attachment complex
TEMED	tetramethylethylenediamine
Tet.	tetracycline

Table of content

1. Introduction

Trypanosoma brucei, a member of the order of Kinetoplastida, is a flagellated parasitic protist, endemic to the equatorial region of Africa, causative agent of serious diseases to humans and animals (Barrettt et al, 2003). There are 5 subspecies of *T. brucei: T. brucei brucei* causes animal African trypanosomiasis, also known as nagana, to wild and domesticated animals. Humans are immune to this subspecies due-to lysis by human apolipoprotein L1. *T. brucei rhodesiense* causes acute human African trypanosomiasis (HAT). However, most infections of HAT are caused by *T. brucei gambiense*. The last two subspecies are *T. brucei* equiperdum and *T. brucei evansi* causing dourine and surra diseases affecting mainly horses and camels (Pays, Vanhobelleke, 2008).

T. brucei contains unique organelles such as single ramified mitochondrion, in which trypanosomatids posses unusually amount of mitochondrial DNA known as kinetoplast DNA (kDNA). Kinetoplast is a disk-shaped network of circular DNA molecules adjacent to basal body of flagellum (Verner et al., 2015).

Another unique organelle is glycosom, which is modified peroxisome containing several enzymes for the glycolysis. It is disc-shaped organelle consisting of lipid bilayer and can be found throughout the cell in cytosol or attached to other organells (Parsons, 2004).

T. brucei is carried by the tsetse fly (genus *Glossina*) which infects the mammal host during a bloodmeal. *T. brucei* in metacyclic form enters the blood stream of the mammal host and transforms into trypomastigotes, where they replicate by binary fission. When the tsetse fly bites an infected mammal, it ingests bloodstream trypomastigotes that infected it; these parasites subsequently transform into procyclic trypomastigotes in the fly gut, and continue to replicate by binary fission. After leaving the midgut they transform into epimastigotes and reach the salivary glands of the fly, thus completing the cycle. The whole cycle takes approximately 3 weeks (Lopes, 2010).

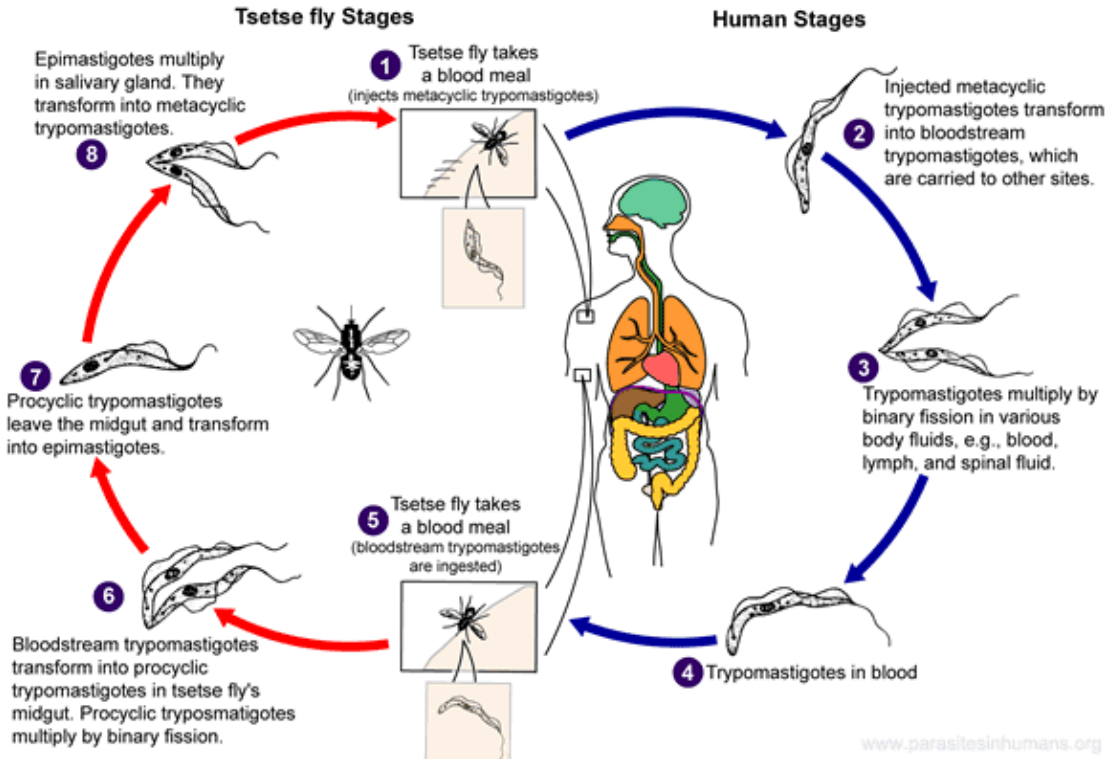

Figure 1.: Schematic life cycle of *T. brucei* adapted from http://www.parasitesinhumans.org/trypanosoma-brucei-sleeping-sickness.html

As a parasite of medical and economical importance, the genome of *T. brucei* has been sequenced to completion. It is accessible in the trypanosomatid genome database TriTrypDB (www.tritrypdb.org), which present opportunities for post-genomic research. Bioinformatics and comparative genomics define potential functions and relationships of many genes. However, the majority of the predicted genes in *T.brucei* have no known function and are currently annotated as encoding hypothetical proteins (www.genedb.org) (Panigrahi et al., 2009).

As part of the concerted approach to study trypanosomatids, another database called TrypTag (www.tryptag.org) was recently developed. This project aims to determine the localization of every protein within the cell of *T. brucei* by tagging them with a mNeonGreen tag and visualising them in live cells. Proteins with annotated signal peptide are tagged only at C-terminus end, all other proteins at both termini (Dean et al., 2017). In a concerted effort with a collaborating laboratory, a group of proteins N-terminally tagged were found to exhibit mitochondrial localisation. This phenomenon would be a rarity, as mitochondrial proteins generally exhibit N-terminal targeting sequence that would be disrupted if a tag would be

present in the same site (Kunze and Berger, 2015). One of these proteins, Tb927.11.1290, is currently predicted by TrypTag to be a conserved hypothetical protein, localized to mitochondria and near kDNA (Dean et al., 2017). In this thesis we aimed to confirm their findings and study further this phenomenon.

For this purpose, endogenous tagging was used to localize Tb927.11.1290, just as the one in the TrypTag database. However, our efforts aimed to test the localization of this protein using fixed tagged cells with a smaller tag, in both N- and C-terminus of the final protein, to determine whether there were differences in localisation. 50 nucleotides of identity are needed for homologous recombination in trypanosomes (Barnes, Mcculloch, 2007), which can be used for creating primers for long primer PCR. Commonly only part of the primer is homologous to the template for PCR and the rest is the overhang homologous to the target gene locus. A series of plasmids, called pPOT, were created to ensure a more prominent long primer PCR tagging method supporting tagging of *T. brucei* genes at both terminal ends or within the protein as well as to generate deletion mutations for investigating functions of different protein domains (Dean et al., 2015).

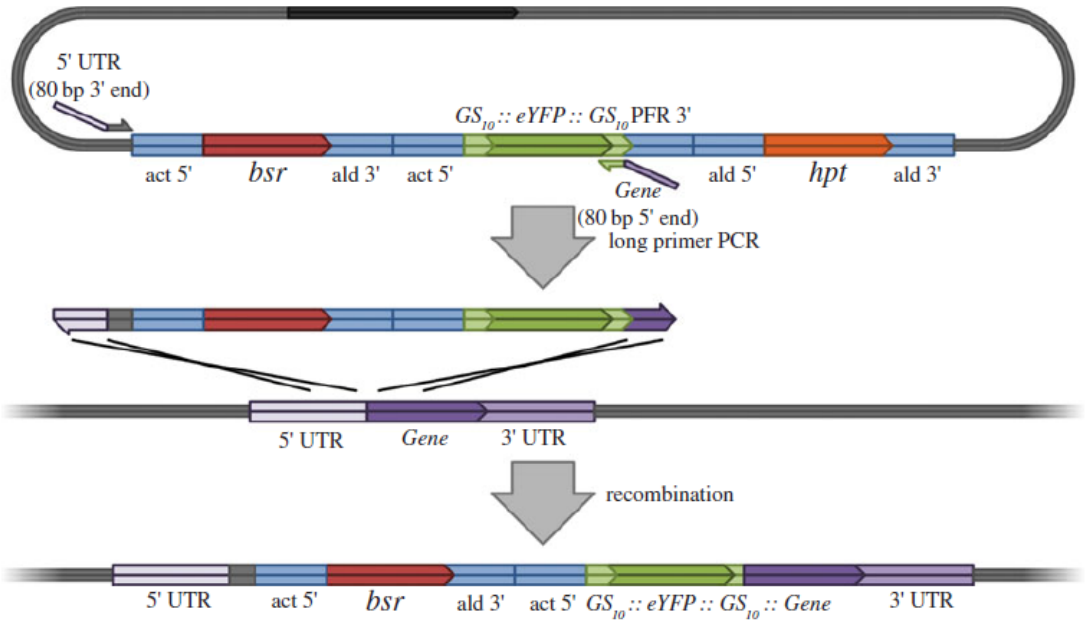

Figure 2.: Example of long primer PCR tagging N-terminal end using pPOTv4, adapted from (Dean et al., 2015).

In order to verify expression of V5-tagged Tb1290 in *T. brucei*, SDS-PAGE and Western blot methods were used. SDS-PAGE (sodium dodecylsulphate-polyacrylamide gel electrophoresis) divides negatively charged proteins by their molecular weight in polyacrylamide gel (Oswald, 2008). The gel is composed of a stacking gel and a running gel (Table 8) which differs by concentration of acrylamide and pH. 12% gel is ideal for proteins with molecular weight between 10–200 kDa (Sino Biological, 2013)-which our protein is expected to have. Proteins get negative charge and their secondary and tertiary structures are disassembled due to presence of SDS. They ran in an electric field from cathode to anode in SDS-PAGE running buffer (Oswald, 2008).

Western blot analysis was used for the detecting protein of interest resolved by SDS-PAGE analysis and transferred to polyvinylidene difluoride membranes (PVDM). Afterwards, the membrane was immunodecorated. A primary antibody is used to bind onto a specific epitope of the protein of interest (Yang and Mahmood, 2012) or in my case onto the V5 tag, which will express as a fusion with the protein of interest. A secondary antibody a horseradish peroxidase (HRP), specifically binds onto the primary antibody. Adding luminol, substrate for HRP, triggers the chemiluminescent reaction emitting detectable light.

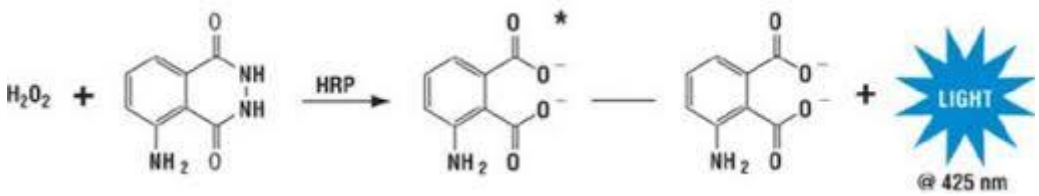

Figure 3: The chemiluminescent reaction of luminol with HRP in presence of peroxide. Adapted from https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

Immunofluorescence microscopy was used for visualization of Tb927.11.1290 in fixed cells. A primary antibody binds to a specific epitope on the V5 protein and a secondary antibody containing a fluorophore, binds to the primary antibody. When a fluorophore is illuminated by light with a specific wavelength, the molecules are excited to a high-energy state, then decay to the lowest single excited state; during the transition to ground energy state they emit light with higher wavelength and lower energy than the light used for excitation. The emitted light

then goes through a mono-wavelength filter into oculars or camera (Mandrel, et al, 1988), (Spring and Davidson, retrieved 2018-11.18).

RNA interference (RNAi) pathway, which is present in many eukaryotes including *T. brucei*, is a biological process against RNA viruses, which inhibits gene expression, and was originally discovered in the nematode *Caenorhabditis elegans*. The nuclease Dicer cleaves double stranded RNA into small non-coding RNA molecules, from which antisense strands are incorporated into RISC (RNA-induced slicing complex) and guide it to the complementary messenger RNA (mRNA), which is then cleaved by the enzyme Argonaute 2 in RISC, thus preventing the translation of the protein of interest resulting in the loss of function (Fire et al., 1998). RNAi is also the name of the technique applied for the same purpose and which was used as a method to determine the essentiality of the protein Tb927.11.1290 in *Trypanosoma brucei*.

RNAi is a long-standing molecular technique used to test gene essentiality in *T. brucei* (McAllaster et al., 2016). For this purpose, long hairpin RNAi (lhRNAi) method was chosen for inducing RNAi and loss-of-function. TheTbPLK pTrypSon plasmid (Figure 4) contains the sequence for triggering RNAi and stuffer between two cloning sites bearing the same target region. Tetracycline induces expression of the gene of interest and creates a hairpin structure, which results in double stranded RNA (McAllaster et al., 2016).

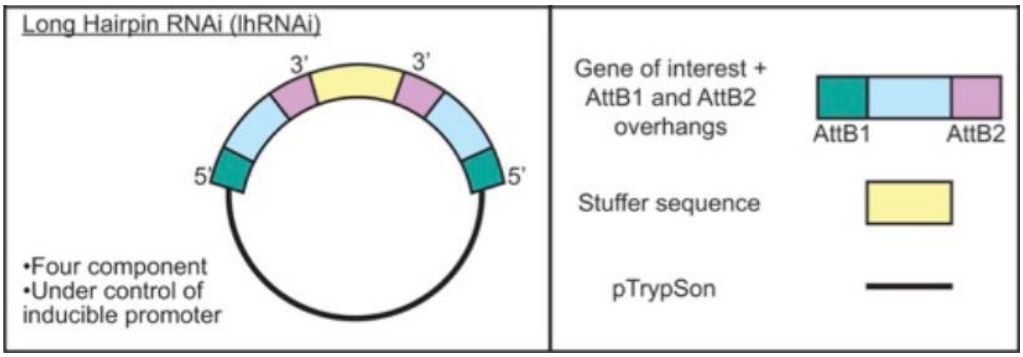

Figure 4.: Schematic representation of TbPLKpTrypSon plasmid for lhRNAi, adapted from (McAllaster et al., 2016).

The plasmid for inducing lhRNAi was assembled by Gibson assembly allowing rapid assemble of multiple overlapping DNA fragments in a single reaction. The assembly is conducted in 3 steps. First, the T5 exonuclease chews-back linear DNA in 5′→3′ direction,

but closed circular molecules are not affected. Then complementary overlapping sequences of fragments anneal together, where the phusion polymerase fills the gaps, and lastly, a Taq DNA ligase seals the nicks (Gibson et al., 2009).

2. Aim of the thesis

The aim of this thesis was to localize gene Tb927.11.1290 (which is termed Tb1290 throughout the manuscript) in procyclic form *Trypanosoma brucei* by in-situ tagging on both termini, crude cellular fractination and immunofluorescence microscopy. We also tested the essentiality of this gene using RNAi.

3. Materials and methods

3.1. Cell lines

All experiments were performed in procyclic form (PCF) *Trypanosoma brucei* SMOX cells grown in SDM–79 medium supplemented with 10% fetal bovine serum (FBS) at 27 °C, containing 0,5 μg/ml of puromycin to maintain the expression of single marker construct, which expresses T7 polymerase and tet repressor for inducible expression of genes.

Table 1.: Composition of 1 l of SDM-79 medium with 10% FBS.

Solution	Amount
SDM-CGGGPPTA	22,05 g
Sodium bicarbonate	2 g
Sodium acetate	10 mg
L-Glutamic acid	22 mg
L-Glutamine	500 mg
L-Proline	600 mg
L-Threonine	400 mg
Sodium Pyruvate	100 mg
Glucose	1 g (pH 7,3)
Glucasamin	50 mg
Hemin	0,75 ml
Pen-Strep	10 ml
Distilled H_2O	Bring to 900 ml
FBS	100 ml

3. 2. In-situ tagging and cloning of RNAi construct

3.2.1. Preparation of gene-tagging cassette

In order to tag the gene Tb1290 in situ, 100 nucleotides primers were designed. The primers contained 20 nucleotides homologous to the vector pPOTV4-V5 and 80 nucleotides homologous to the gene Tb1290 were designed (Table 2) to insert a cassettes with an antibiotic resistance gene, with a V5 tag into either termini of the gene (Dean et al., 2015). The modified vector pPOTV4 was used containing V5 tag instead of yellow fluorescent protein (Peña-Diaz et al., 2017).

PCR was performed (Tables 3 and 4) and PCR products were verified by agarose gel electrophoresis. Whole unpurified PCR products were transfected into procyclic *Trypanosoma brucei* SMOX cell line.

Table 2.: N- and C-terminus primers used for inserting V5 tag into Tb1290 in 5′→3′ direction.

Primer	Sequence
N–terminus forward	TTGTTTGCTCATACAGACACACACACACAAAAAAAAAGGCCGG AGCCACGATAAATTATACACGTGCATGCTGGAATAGTATAATGCA GACCTGCTGC
N–terminus reverse	TCAGAATGGCTCGTCCCACGGGAAGGAAGCGTGCCGGCCTCCGCT AAAGTGTTTGATAGGAACCGTTGTGACAATTTCATACTACCCGATC CTGATCC
C–terminus forward	TGAATGTTGGACCTCGTCTTCCCATCGATGATCTAAAGGTATTACC CGTGTACCCTCTTTTGGTGCGTCAAACACGTTAGGGTTCTGGTAGT GGTTCC
C–terminus reverse	ATACCTTTCTATCCTCATGACGGCGGATGAGAAACATTCGCCCCTA ATAACTAAGAAAAACAACAGCAACAACAACAACACCAATTTGAGG ACCTGTGC

Table 3.: Composition of PCR mix for amplifying V5-tagging cassette for Tb1290 for both N- and C-terminus.

Solution	Volume
Final volume	50 µl
Q5 2x master mix	25 µl
pPOTv4V5 (25 mM)	1 µl
Forward primer (10 mM)	1 µl
Reverse primer (10 mM)	1 µl
MilliQ H_2O	22 µl
Final volume	50 µl

Table 4.: PCR condition for amplifying N- and C-terminus V5-tagging cassette into Tb1290.

Steps	Temperature	Time	Cycles
Preheating	94 °C	5 min	1
Denaturation	94 °C	15 sec	30
Annealing	60 °C	15 sec	
Extension	72 °C	2 min	
Final extension	72 °C	7 min	1

3.2.2. Preparation of RNAi plasmids

TheTbPLK pTrypSon plasmid (McAllaster et al., 2016) was used for creating tetracycline-inducible RNAi cell lines. A PCR of a stuffer (Tables 6 and 7) and the Tb1290 RNAi region of interest (Tables 5 and 7) was performed and the molecular weight of the DNA fragments was verified by agarose gel electrophoresis. Primers were designed based on the TrypTag resource project. The pTrypSon vector was digested with the HindIII restriction enzyme. The stuffer, the Tb1290 insert and the plasmid backbone were purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel, Germany) and subsequently mixed in a ratio 3:3:1 with 20 µl aliquot of 2× home-made Gibson Assembly Master mix (699 µl MilliQ H_2O, 320 µl 5x isothermal reaction buffer [500 mM Tris-HCl, 250 mg/ml PEG–8000, 50 mM $MgCl_2$, 50 mM DTT, 1 mM Each of four dNTPs], 0,64 µl T5 exonuclease, 20 µl phusion DNA polymerase, 160 µl Taq DNA ligase). The mix was incubated for 3 hours at 50°C.

Table 5.: Composition of the PCR for the Tb1290 RNAi region of interest for Gibson assembly.

Solution	Amount
2x Q5 Master mix	25 µl
Forward primer (10 mM)	1 µl
Reverse primer (10 mM)	1 µl
MilliQ H_2O	22 µl
Final volume	50 µl

Table 6.: Composition of the PCR for the stuffer for creating long hairpin in Gibson assembly.

Solution	Amount
2x Q5 Master mix	25 µl
ATTB 2 primer (10 mM)	1 µl
pTrypSon vector (647,8 ng/µl)	1 µl
MilliQ H_2O	22 µl
Final volume	50 µl

Table 7.: PCR conditions for Tb1290 RNAi region of interest and stuffer for Gibson assembly.

	Temperature	Time	Cycles
Preheating	98 ˚C	30 sec	1
Denaturation	98 ˚C	15 sec	30
Annealing	60 ˚C	30 sec	
Extension	72 ˚C	1 min	
Final extension	72 ˚C	5 min	1

3.2.2.1. *Escherichia coli* transformation with Gibson assembly product

The Gibson assembly product was transformed into chemically competent *E. coli* XL-1 Blue. Chemically competent *E. coli* was mixed with the Gibson assembly product and incubated on ice for 20 min, prior to exposing the mix to heat shock at 42 °C for 90 sec, subsequently incubating on ice for 5 min. The transformation mix was then combined with 0,5 ml of SOC medium (Thermo Fisher Scientific) and allowed to recover at 37 °C for 1 hour in shaking. Clones containing the plasmid were verified by colony PCR. Positive clones were transferred into LB media containing ampicillin. Plasmid was purified using NucleoSpin mini prep. kit (Macherey-Nagel, Germany). Linearization of the plasmid was done with NotI-HF restriction enzyme prior to transfection into procyclic *Trypanosoma brucei*.

3.3. Transfection of procyclic form *Trypanosoma brucei* with the AMAXA Nucleofactor II (VPA–1002 Lonza)

$2x10^7$ PCF *T. brucei* cells were harvested by centrifugation at 1800 ×g at room temperature. Cells were resuspended in 82 µl of human nucleofactor, 18 µl of supplement, and PCR product or 10 µg of linearised vector. The mix was transferred into Amaxa cuvettes and electroporation was performed using setting X-014. Cells were recovered in 6 ml SDM-79 medium over night. After approximately 18 hours, the selective antibiotic was added. 10 µg/ml blasticidin was used for N-terminus tagged cells and 50 µg/ml hygromycin was used for C-terminus tagged cells, and 2,5 µg/ml phleomycin was used to select for RNAi constructs. SMOX cell lines were used as a growth control, treated with the same antibiotics as cells above. Cultures were diluted according to Figure 5 into 24-wells plates. Resistant clones emerged after 10 days.

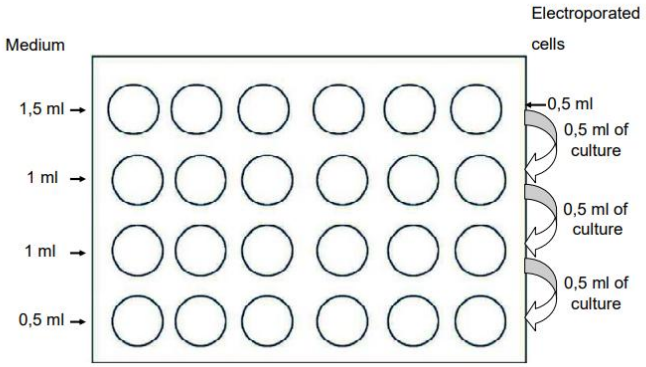

Figure 5.: Dilution of electroporated cells (Adapted from Novotná, 2010).

3.4. SDS-PAGE analysis

Visualisation of the V5-tagged Tb1290 was performed using SDS-PAGE for further Western blot (next section). For this purpose, cells were harvested by centrifugation at 1800 ×g for 10 min. The pellet was resuspended in 2× Laemmli buffer (65,8 mM Tris-HCl pH 6,8, 26,3% (v/v) glycerol, 2,1% (w/v) SDS, 0,01% bromophenol, 50 mM DTT) to a concentration of $5x10^5$ cells/µl, boiled at 98 °C for 10 min, sheared with a 25-gauge syringe, and 10 µl was loaded into 0,75 mm 12% polyacrylamide gel. Proteins ran in SDS running buffer (25 mM Tris, 192 mM glycine, 0,1% SDS) at 50 mA/gel, until the dye reached gel front, for approximately 1 hour.

Table 8.: Composition of 12% polyacrylamide gels used for SDS–PAGE.

Solution	Running gel (10 ml) Amount	Stacking gel (3 ml)
MilliQ H_2O	3,3 ml	2,1 ml
Acrylamide/Bis-acrylamide (29%/1% w/v)	4 ml	0,5 ml
1,5M TRIS–HCL pH8,8	2,5 ml	0,38 ml
SDS 10% (w/v)	0,1 ml	0,03 ml
APS 10% (w/v)	0,1 ml	0,03 ml
TEMED	0,004 ml	0,003 ml

3.5. Western blot analysis

Samples submitted to SDS-PAGE were blotted into PVDF (polyvinylidene difluoride) membranes using a set up shown in Figure 6 at 100 V, free amperage for 1 hour in SDS-PAGE blotting buffer (0,0387 mM glycine, 0,0479 mM Tris, 20% (v/v) methanol). Membranes were blocked by 5% milk in PBS (0,137 M NaCl, 0,0027 M KCL, 0,01 M Na_2HPO_4, 0,0018 M KH_2PO_4) for 30 min. Immunodecoration with primary antibody was done using a monoclonal anti-V5 antibody at dilution of 1:1000 over night and washed with PBS 0,1%-Tween 20. Secondary antibody (anti-mouse made in rabbit containing horseradish peroxidise [HRP]) at dilution of 1:1000 was incubated for 90 min and washed with PBS 0,1% -Tween 20. Visualization was done by chemiluminescence of HRP in ChemiDoc (Bio Rad) using luminol substrate in presence of peroxide reagent (Clarity from Bio Rad).

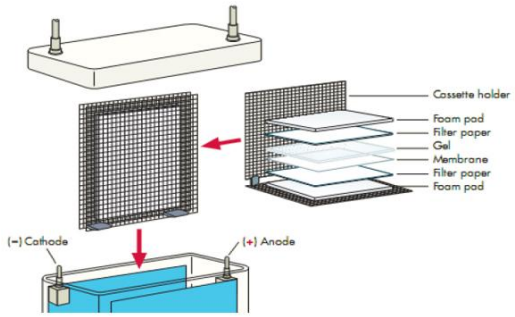

Figure 6.: Schematic representation of Western blot apparatus set up (Adapted from: https://www.bosterbio.com/protocol-and-troubleshooting/western-blot-principle)

3.6. Immunofluorescence microscopy

Visualisation of whole cells for localisation of Tb1290 was performed using immunofluorescence microscopy. $2x10^6$ procyclic *Trypanosoma brucei* cells were harvested by centrifugation at 1800g for 10 min. Cells were resuspended in 5 ml SDM-79 medium with 2 µl 100µM MitoTracker Red. The samples were incubated at 27 °C for 10 min covered from light. The rest of the procedures were done the same for all samples in light sensitive conditions. Cells were fixed in 1 ml 4% (w/v) paraformaldehyde in PBS and incubated at room temperature without shaking for 18 min. Immediately after that paraformaldehyde was eliminated by centrifugation at 1800 ×g for 3 min. Cells were resuspended in fresh PBS and pipetted into a charged microscope slide (Huida, China) and were permeabilized in 0,2% Triton X–100 in PBS for 20 min. Primary antibody (see Table 9 for names and dilutions) was added to each slide and was allowed to incubate over-night in PBS/gelatin. Slides were thoroughly washed with an excess of PBS after which, an appropriate secondary antibody was added and incubated for 1 hour at room temperature. ProLong-antifade reagent with DAPI was used for staining of DNA and as a mounting solution. Samples were visualized in Zeiss Axioplan 2 fluorescence microscope using an APOCHROMAT 100× objective with immersion oil.

Table 9.: Primary and secondary antibodies used for immunofluorescence assay.

Sample	Primary antibody	Dilution	Secondary antibody	Dilution
N–Terminus/ C–Terminus with MitoTracker	Anti-V5 made in mouse	1:200	Anti-mouse green ALEXA 488	1:2000
N–Terminus/ C–Terminus	Anti-V5 made in rabbit	1:500	Anti-rabbit green ALEXA 488	1:2000
	Anti TAC made in mouse	1:1000	Anti-mouse red ALEXA 583	1:2000
N–Terminus/ C–Terminus	Anti-V5 made in mouse	1:200	Anti-mouse green ALEXA 488	1:2000
	Anti-enolase made in rabbit	1:1000	Anti-rabbit red ALEXA 583	1:2000
N–Terminus/ C–Terminus	Anti-V5 made in mouse	1:200	Anti-mouse green ALEXA 488	1:2000
	Anti-hexokinase made in rabbit	1:1000	Anti-rabbit red ALEXA 583	1:2000

3.7. Crude cellular fractionation

PCF *T. brucei* bearing N- and C-terminus V5-tagged Tb1290 were grown to $1x10^7$ cells/ml in 100 ml and were harvested by centrifugation at 1800 ×g, 4 °C for 10 min. Cells were resuspended in Hank's balanced salt solution (HBSS; 1,26 mM $CaCl_2$ 5,33 mM KCl, 0,44 mM KH_2PO_4, 0,81 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$, 0,3 mM Na_2HPO_4, 5,6 mM glucose, pH 7,3) to a concentration of $5x10^9$cells/ml. Aliquots containing 1 mg of protein, determined by Bradford Assay, were diluted with HBSS buffer and digitonin (10 mg/ml in MilliQ H_2O) was added to reach final concentration of 0,4 µg/µl. The solutions were vortexed shortly and incubated at room temperature for 5 min, afterwards centrifugation at 14000 ×g for 2 min and supernatant containing soluble cytosolic fraction was collected. After washing with HBSS buffer, the suspension was solubilised with 0,1% (v/v) Triton X–100. The mix was incubated on ice for 5 min, centrifuged at 14000g for 2 min and the supernatant containing mitochondrial fraction was collected. The pellet containing insoluble proteins was resuspended in HBSS buffer. Each fraction was mixed with Laemmli buffer and boiled at 98˚C for 10 min. SDS-PAGE and Western blot was performed to analyse samples.

4. Results

4.1. Gene tagging

Cassettes containing V5 tag and antibiotic resistance gene (blasticidin for N–terminus and hygromycin) were obtained by PCR (Figure 7). Note that the size of cassette for C–terminus end is larger in size then insert for N-terminus end. That is due to the size of the gene for resistance to hygromicin, which is larger than the gene for resistance to blasticidin. After transfection, resistant clones were found in some wells after 10 days. The control cell lines died out after a few days.

The clones were tested for the presence of the V5 tag. In Figure 8, the presence of the V5 tag in clone 2 for N-terminus culture and clone 1 for C-terminus tagging is shown. In further experiments we worked with those clones exclusively.

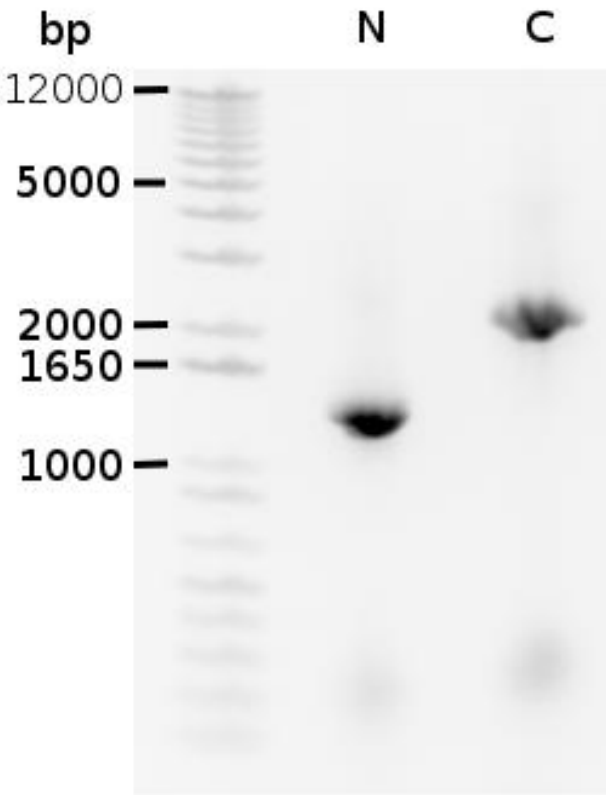

Figure 7.: PCR of Tb1290 V5-tagging cassettes amplified from pPOTV4-V5 vector for N- and C-terminal tagging, respectively.

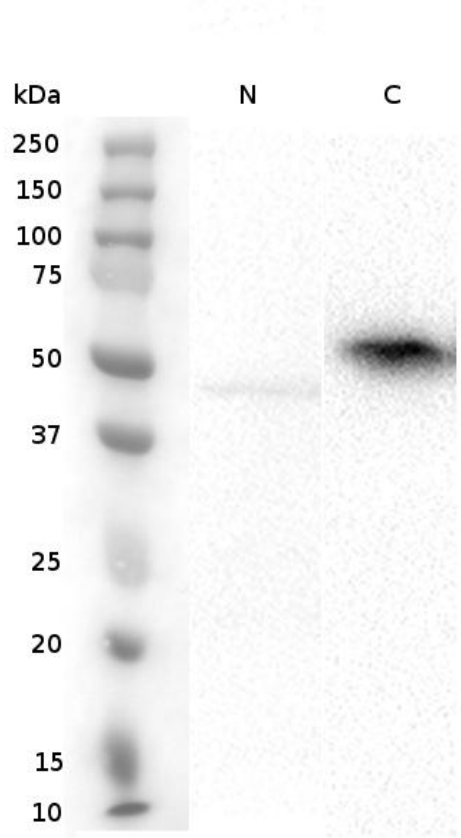

Figure 8.: Western blot of *Trypanosoma brucei* cells of Tb1290 V5-tagged on N- and C-termini.

4.2. Sequence$_{(398aa)}$ analysis of Tb1290 by mitochondrial targeting prediction softwares

The predicted protein sequence of Tb1290 was submitted to bioinformatics softwares (TargetP, iPSORT, and MitoProt II). The results suggest that there is approximately 65% likelihood of presence of mitochondrial targeting sequence.

Another predictor, Phobius, was used to find out whether Tb1290 contained any transmembrane domains. It is based on a hidden Markov model (HMM) that models the different sequence regions of a signal peptide and the different regions of a transmembrane protein in a series of interconnected states (Käll, Krogh and Sonnhammer, 2004). It showed a probability of a transmembrane domain in Tb1290 (Figure 9).

4.3. Localisation of Tb1290

Two techniques were used for localisation of Tb1290. The first of them was the immunofluorescence assay. Tb1290 was compared to MitoTracker (Figure 10), which is a mitochondrial dye; to TAC102 (Figure 11), a component of the TAC (tripartite attachment complex), near kDNA; to enolase, a classical cytosolic marker (Figure 12); and to hexokinase (Figure 13), a marker of glycosomes.

In Figure 10, the signal for N-terminally V5-tagged Tb1290 (in green) is distributed in globular clusters, but C-terminally V5-tagged Tb1290 shows more continuous pattern. They are partially colocalised with MitoTracker (in red), which is a mitochondrial dye. The yellow colour in composite image shows the exact spots of colocalisation. The colocalisation between those two signals is more prominent for C-terminally tagged cells.

In Figure 11, there is no colocalisation of the signals for N-terminally V5-tagged Tb1290 (in green) and for anti-TAC102 marker.

Figure 12 depicts the signal for V5 (in green) in a patchy distribution and only partially colocalises to enolase (in red), a classical cytosolic marker.

In Figure 13, the signal for V5 (in green) is similar for both N- and C- terminally tagged Tb1290. It is distributed throughout the cell in three main clusters with the highest intensity, from which the signal fades throughout other parts of the cell. It is only partially colocalised

with hexokinase (in red), a marker of glycosomes, which exhibits more discontinuous clusters as shown in the composite image (in yellow).

Altogether, our observation of the V5-tagged TB1290 by immunofluorescence microscopy, are inconclusive, as no real colocalisation with the organelle is observed and no merge pattern was homogenously distributed throughout the cell.

Because the immunofluorescence results did not lead us to any solid conclusion, we attempted a crude cellular fractionation (Figure 14) to determine if it was possible to pinpoint the localisation of Tb1290. Both N- and C-terminally V5-tagged Tb1290 were compared to anti-Hsp70, a mitochondrial marker, and to enolase, the cytosolic marker. Figure 14 shows that the cytosolic fraction was not appropriately separated from the mitochondrial fraction in both N- and C-terminally tagged cell lines, which is why a faint signal is observed in the mitochondrial fraction with the anti-enolase antibody. The mitochondrial fraction was separated completely from the insoluble fraction, only in the C-terminally tagged cell line there was a faint signal observed in the insoluble fraction with anti-Hsp70 antibody. The V5 signal, corresponding to Tb1290, is shown in the mitochondrial fraction in both N- and C-terminally tagged cell lines.

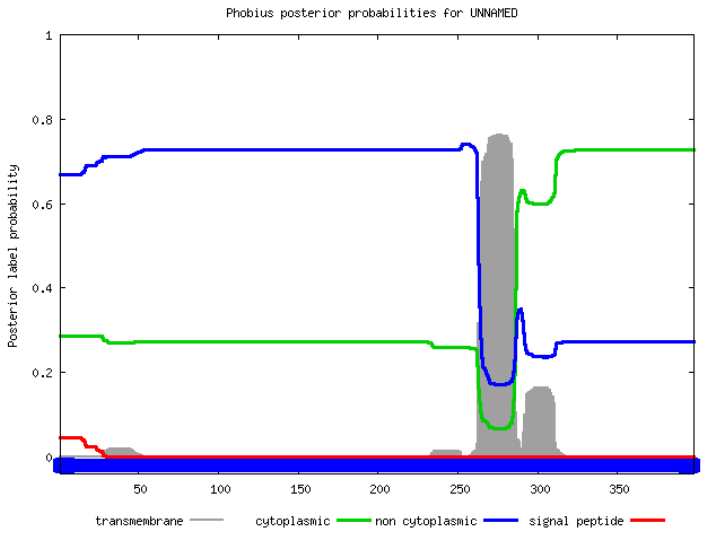

Figure 9.: Prediction of transmembrane sequence in Tb1290 using Phobius predictor.

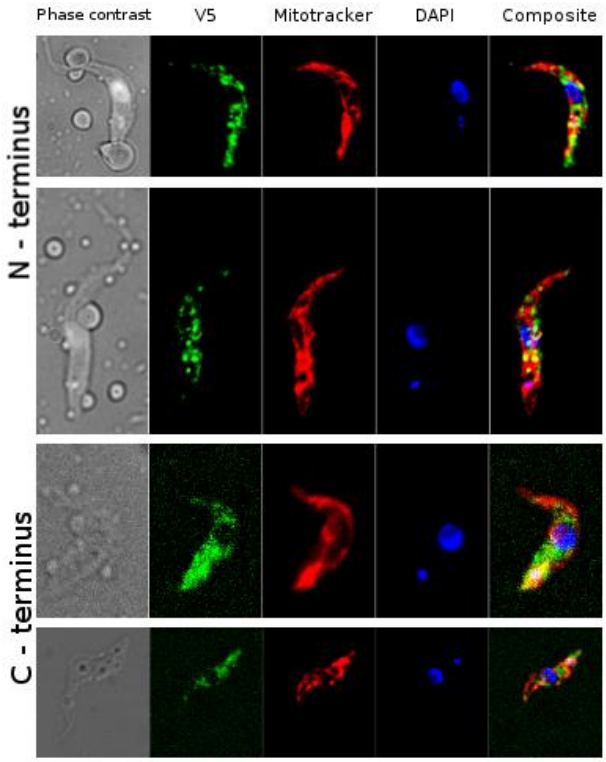

Figure 10.: Immunofluorescence assay showing N- and C-terminal V5-tagged Tb1290 (in green), compared to MitoTracker (in red). DAPI is shown in blue. The composite shows the merge of the three channels.

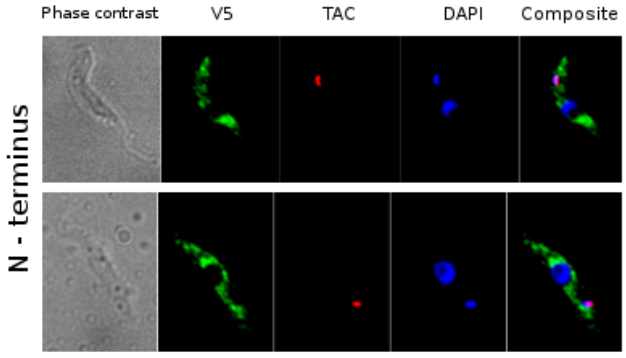

Figure 11.: Immunofluorescence assay showing N-terminal V5-tagged Tb1290 (in green), compared to TAC102 (in red). DAPI is shown in blue. The composite shows the merge of the three channels.

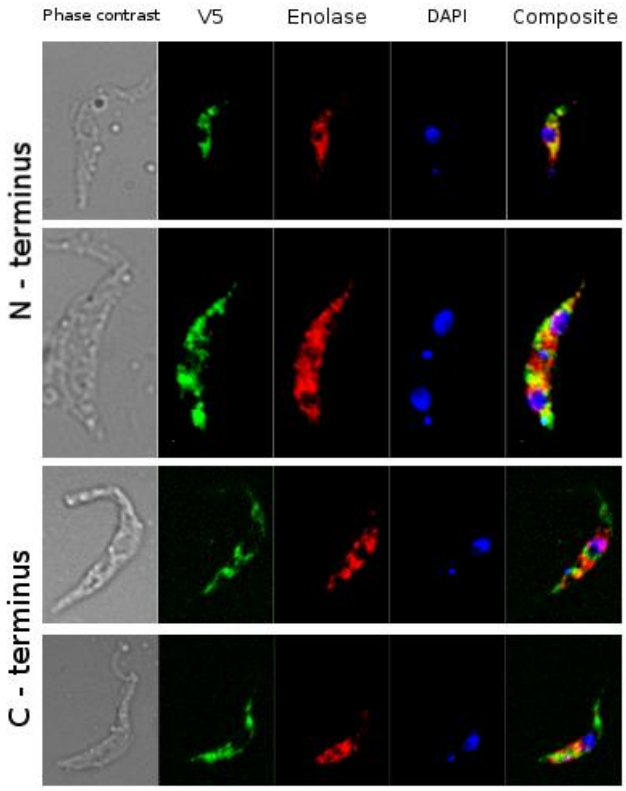

Figure 12.: Immunofluorescence assay showing N- and C-terminal V5-tagged Tb1290 (in green), compared to enolase (in red). DAPI is shown in blue. The composite shows the merge of the three channels.

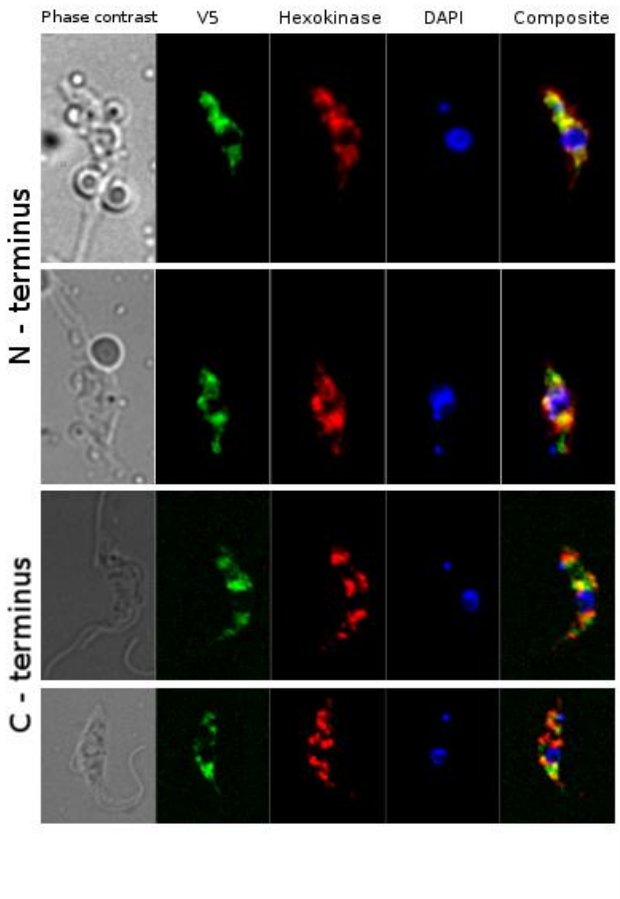

Figure 13.: Immunofluorescence assay showing N- and C-terminally V5-tagged Tb1290 (in green), compared to hexokinase (in red). DAPI is shown in blue. The composite shows the merge of the three channels.

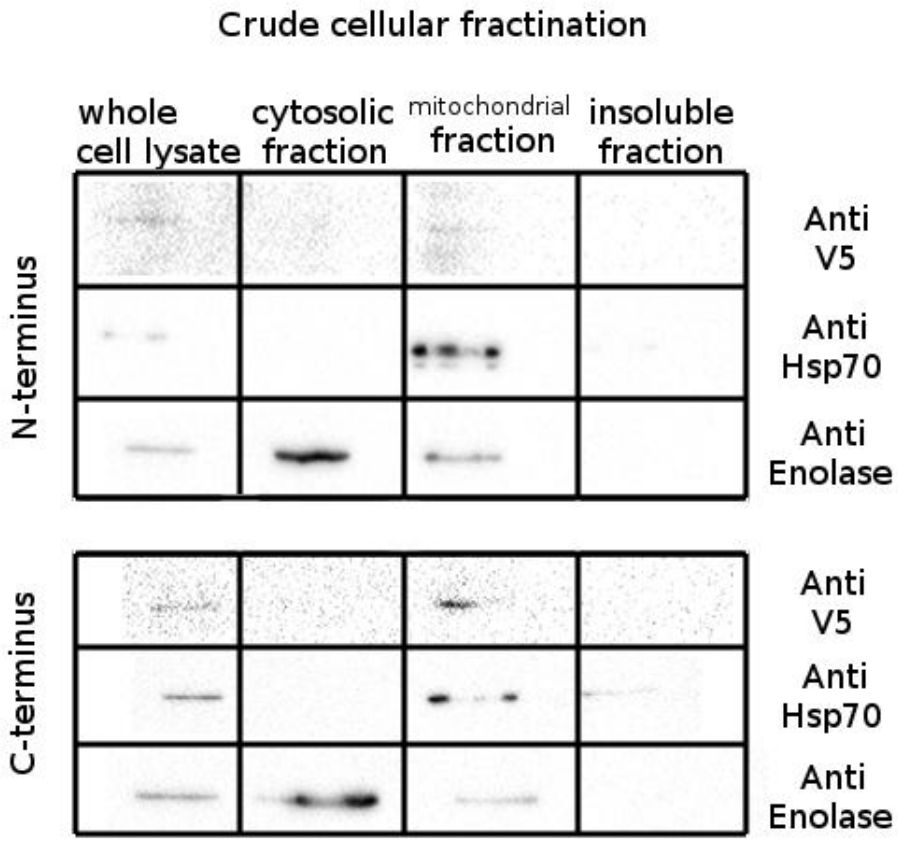

Figure 14.: Western blot of fractions from crude cellular fractination of PCF *T. brucei* Tb1290 N- and C-terminally V5-tagged. Monoclonal anti-V5 was used to detect N- and C-terminal V5-tagged Tb1290; anti-mtHsp70 was used as a mitochondrial marker. Anti-enolase was used as a cytosolic marker.

4.4. Preparation of an RNAi construct for the downregulation of Tb1290 in PCF *T. brucei*

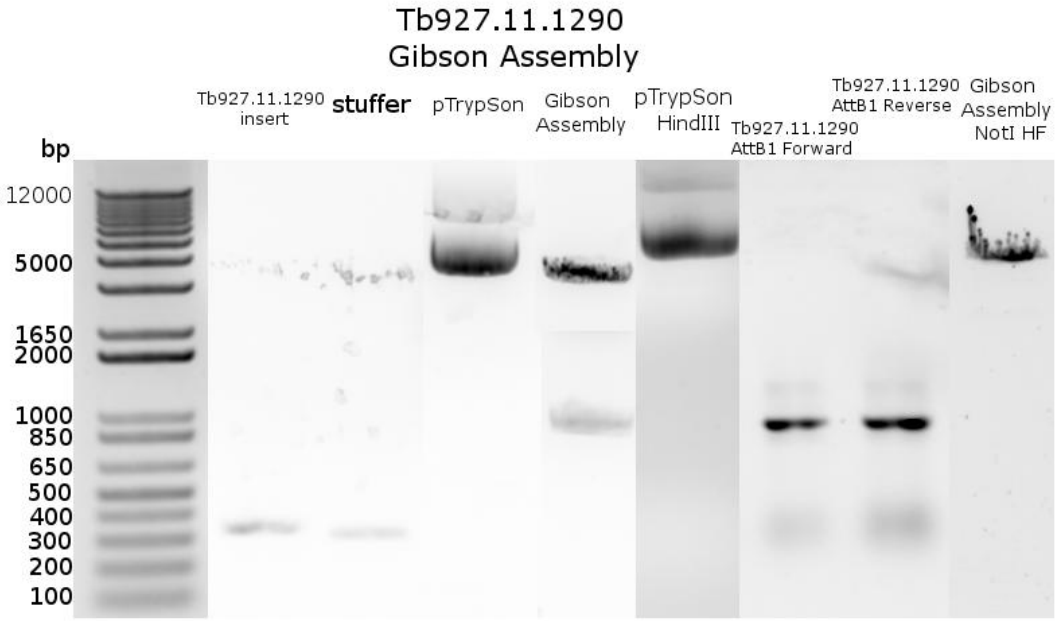

Figure 15.: Agarose gel electrophoresis of all components necessary for creating a tetracycline inducible construct of RNAi to transfect into tagged *T. brucei* cell lines. From the left: PCR of *Trypanosoma brucei* gene Tb1290; PCR of the stuffer for creating hairpin; midiprep of pTrypSon vector; digestion of pTrypSon vector by HindIII enzyme; whole Gibson Assembly construct; PCR of bacterial colony containing the highest amount of Gibson Assembly construct using forward sequencing primer; PCR of bacterial colony containing highest amount of Gibson Assembly construct using reverse sequencing primer; digested Gibson Assembly construct using NotI HF enzyme.

4.4.1. Growth curve of Tb1290 RNAi cell lines

As shown in Figure 16, there was a decrease in number of cells in the RNAi induced Tb1290 N-terminus-tagged culture, as compared to the uninduced culture (tetracycline (-)). On the other hand, Figure 17 shows growth curve of C-terminus cell line where induction with tetracycline did not result in growth impairment of the cells.

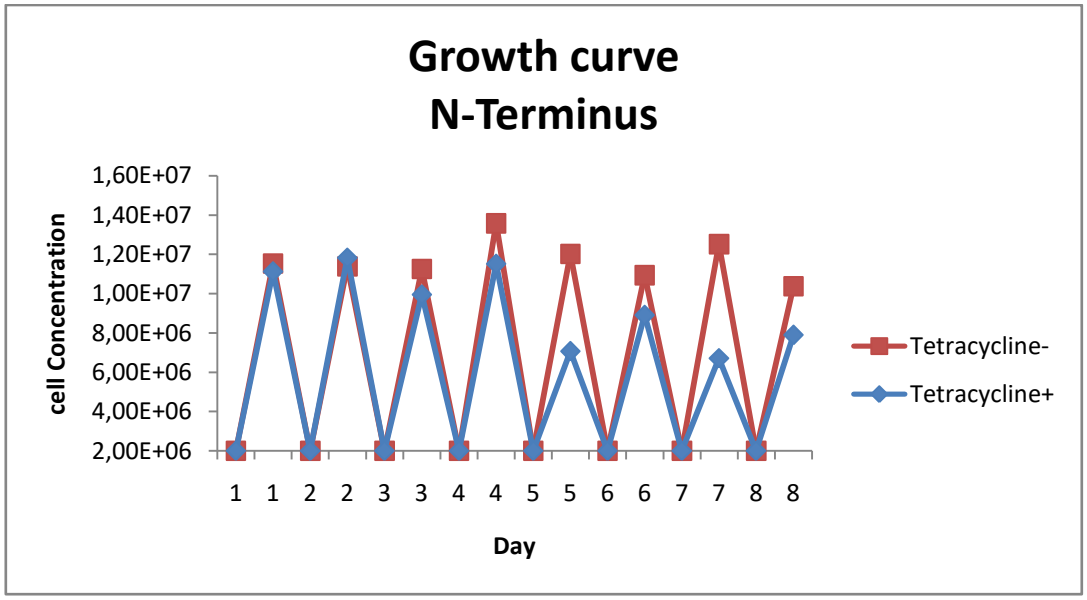

Figure 16: Growth curve of Tb1290 RNAi in a N-terminally V5-tagged cell line. Tetracycline (-) indicates absence and (+) presence of the drug, respectively. Cultures were systematically diluted to $2x10^6$ cells every 24 hours for 8 days.

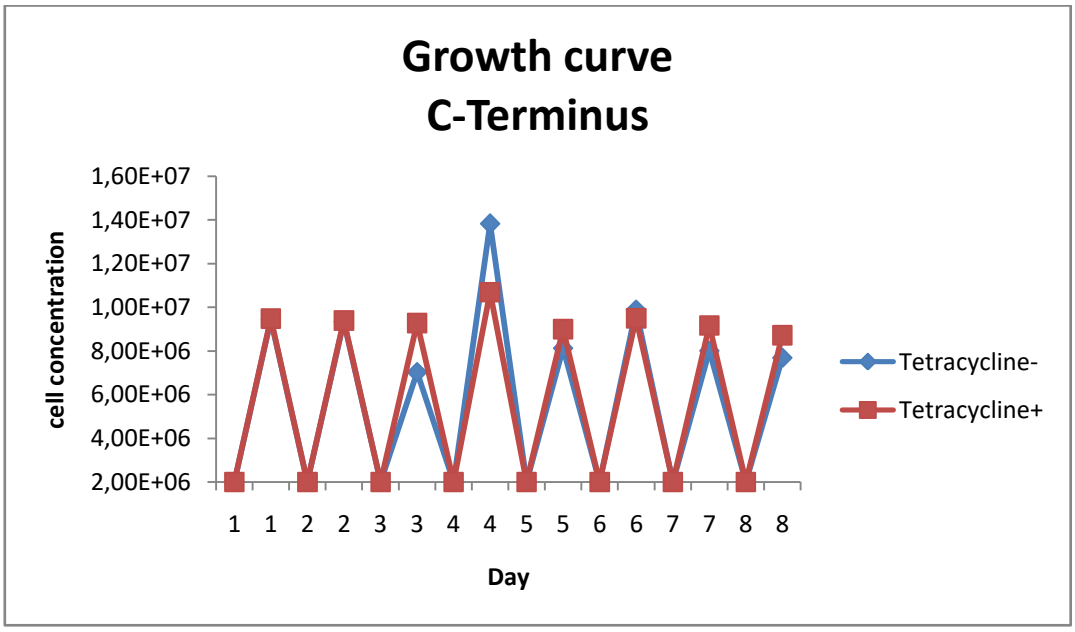

Figure 17: Growth curve of Tb1290 RNAi C-terminally V5-tagged cell line. Tetracycline (-) indicates absence and (+) presence of the drug, respectively. Cultures were systematically diluted to $2x10^6$ cells every 24 hours for 8 days.

4.4.2 Confirmation of downregulation by RNAi of Tb1290

After each growth curve measurement, samples were taken and prepared to determine the levels of downregulation of Tb1290 by RNAi. $1x10^7$ cells/ml were submitted to SDS-PAGE and blotted to PVDF by Western blot. The tagged protein was detected with anti-V5 antibody; anti-tubulin antibody was used as a loading control. The curve showed a differential efficiency depending on whether the V5 tag was placed on the N-terminus or the C-terminus of the protein. The N-terminally-tagged Tb1290 cell line showed complete disappearance of the protein observed by Western blot from the first day of treatment with tetracycline. The protein remained absent over the course of 8 days of the growth curve (Figure 18).

The C-terminus V5-tagged Tb1290 RNAi cell line also showed a decline in the amount of protein for the first 4 days of treatment, though not as significant as the one observed in the N-terminus cell line. Over the course of the next 4 days, the amount of protein was returning to similar concentration as tetracycline untreated control (Figure 19).

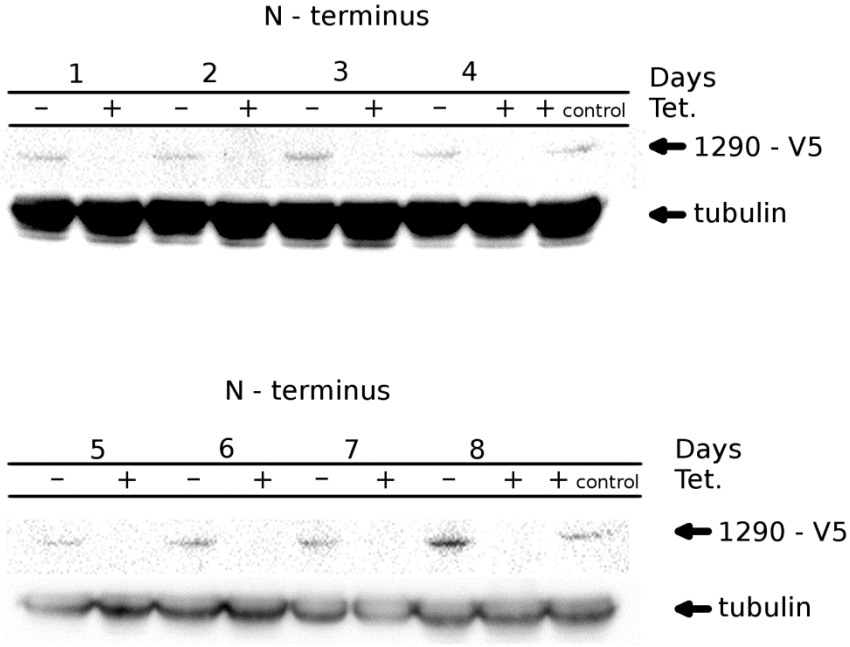

Figure 18: Western blot of N-terminally V5-tagged Tb1290 used for RNAi cell line. $1x10^7$ cells/well were loaded in the gel. Anti-tubulin antibody was used as loading control. Tetracycline (-) and (+) denote absence and presence of drug, respectively.

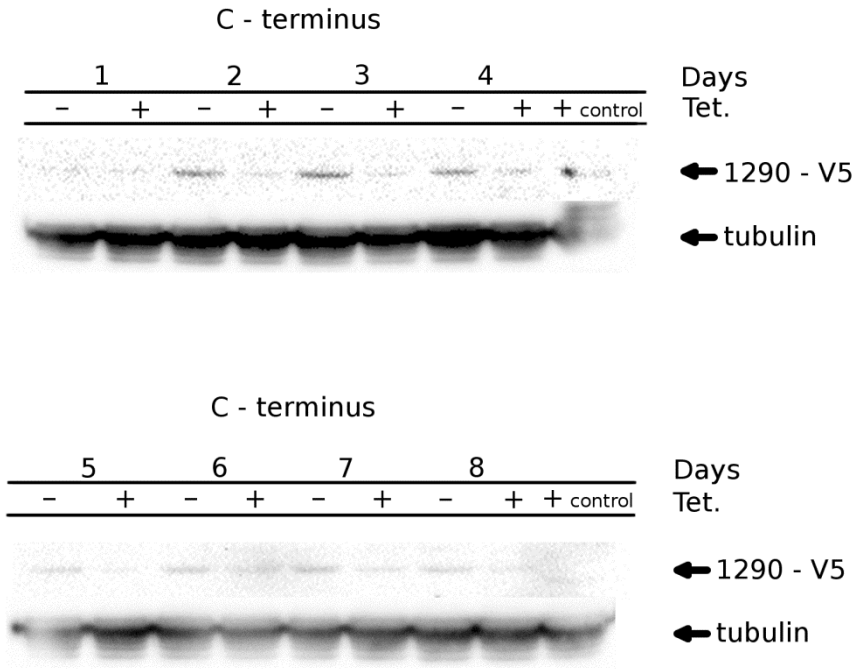

Figure 19: Western blot of C-terminally V5-tagged Tb1290 used for RNAi cell line. $1x10^7$ cells/well were loaded in the gel. Anti-tubulin antibody was used as loading control. Tetracycline (-) and (+) denote absence and presence of drug, respectively.

5. Discussion

To confirm the findings of theTrypTag project regarding the protein Tb1290, immunofluorescence microscopy was performed using organelle markers such asMitoTracker staining (Figure 10), and anti-TAC antibodies (Figure 11). There was no colocalisation between Tb1290 and anti-TAC antibody in neither N- nor in C-terminally V5-tagged cell lines; the same was observed for the signal of MitoTracker. Since this is the initial report of TrypTag for Tb1290, it would suggest a possible artefact in the result for this particular protein. In view of our lack of colocalization with the expected markers mentioned above, we attempted visualisation with enolase (Figure 12) and hexokinase (Figure 13), which did not lead to any colocalization either.

However, the crude cellular fractionation (Figure 14) showed both N- and C-terminally V5-tagged Tb1290 located in the organellar fraction which is detected with a mitochondrial marker mtHsp70. These findings suggest that Tb1290 could indeed be bound to or associated to the mitochondrion, but its detection may have been difficult due to the exposure of the tag. Figure 9 shows that Tb1290 has a predicted transmembrane domain, which could make the detection difficult without prior standardisation.

The protein does, however, show a high probability of bearing an N-terminal targeting sequence, which would explain why there is a differential phenotype of RNAi efficiency depending on whether the protein is tagged on the N- or the C terminus. If the protein is unable to translocate to mitochondria because of the presence of the tag, RNAi would be efficient, as much of the protein will be anyway eliminated by the cell, or accumulated elsewhere but in the mitochondrion. In contrast, RNAi in the C-terminally tagged cell line could barely reduce the concentrations of the protein, suggesting this protein may be essential for the parasite.

Unfortunately, the lack of growth phenotype did not allow us to predict the role of this protein in the cell. Yet its localisation seems like a challenge that may be approached with different methods, for example, its overexpression for better visualisation. The same technique may allow determining if its concentration levels are being regulated and therefore, the overexpression may produce an evident growth phenotype.

6. Summary

The main goal of this thesis was to localise gene Tb927.11.1290 in procyclic form *Trypanosoma brucei* by in-situ tagging it on both N- and C- terminal ends, and then use immunofluorescence microscopy and crude cellular fractination techniques for its localisation. Tb1290 was previously localised in mitochondria and near kDNA by live imaging using mNeonGreen by TrypTag project. To confirm their findings regarding this protein, immunofluorescence microscopy was performed using organelle markers such as MitoTracker and anti-TAC102. There was no colocalisation between Tb1290 and anti-TAC antibody in neither N- nor in C-terminally V5-tagged cell lines; the same was observed for the signal of MitoTracker. Since this is the initial report of TrypTag for Tb1290, it would suggest a possible artefact in the result for this particular protein. In view of our lack of colocalization with the expected markers mentioned above, we attempted visualisation with hexokinase and enolase, which did not lead to any colocalization either. However, the crude cellular fractionation technique showed both N- and C-terminally V5-tagged Tb1290 located in the organellar fraction which is detected with a mitochondrial marker mtHsp70. These findings suggest that Tb1290 could indeed be bound to or associated to the mitochondrion, but its detection may have been difficult due to the exposure of the tag.

The N-terminally-tagged Tb1290 cell line showed complete disappearance of the protein observed by Western blot from the first day of treatment with tetracycline. The protein remained absent over the course of 8 days of the growth curve

Knockdown studies of Tb1290 were performed using long hairpin RNAi. The growth curve showed a differential efficiency depending on whether the V5 tag was placed on the N-terminus or the C-terminus of the protein. The N-terminally-tagged Tb1290 cell line showed complete disappearance of the protein observed by Western blot from the first day of treatment with tetracycline and it lasted throughout the growth curve measurement. In contrast, RNAi in the C-terminally tagged cell line could barely reduce the concentrations of the protein, suggesting this protein may be essential for the parasite. Unfortunately, the lack of growth phenotype did not allow us to predict the role of this protein in the cell.

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