University of South Bohemia in České Budějovice

Faculty of Science

Bachelor thesis

Determining the subcellular compartment in which the unique cleavage of mitochondrial F_1 ATPase subunit alpha happens

Laboratory of Functional Biology of Protists

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Annotation:

The *T. brucei*'s infectious stage is characterized by its unique metabolism which requires the mitochondrial F_oF_1-ATPase to hydrolyze ATP in order to maintain the essential mitochondrial membrane potential. This complex rotary machine has several extraordinary features including the proteolytic cleavage of the F_1-ATPase subunit alpha (α) that results in the expulsion of an internal octapeptide. This thesis is focused on whether the proteolytic processing of the essential F_1-ATPase α subunit already begins in the cytosol.

Declaration:

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

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Ana-Marija Andova

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Abstract

Trypanosoma brucei is a flagellated protist that causes a disease in humans and livestock that is often lethal if untreated. The unique metabolism of the infectious stage of this parasite requires the mitochondrial (mt) F_oF_1-ATPase to hydrolyze ATP to maintain the essential mt membrane potential. This complex rotary machine has several extraordinary features unique to Euglenozoa, one of which is the proteolytic cleavage of the F_1-ATPase subunit alpha (α) that results in the expulsion of an internal octapeptide. To determine if this cleavage is required to generate an active enzyme, we would like to identify the responsible protease(s). Since α is a nuclear gene that is translated in the cytosol before it is imported into the mitochondrion, it is possible that a protease resides in the cytosol. In fact, the cleaved octapeptide is predicted by MitoProt to be an N-terminal mitochondrial targeting signal (MTS) for the C-terminal α peptide. To experimentally test this hypothesis, we created *T. brucei* cell lines that express a jellyfish green fluorescent protein (GFP) that is fused with either the known α MTS or the α octapeptide. Using subcellular fractionation, we then assessed if the normally cytosolic reporter protein could be imported into the mitochondrion. Our results indicate that while the true α MTS targets the GFP to the mitochondrion, the α octapeptide is very inefficient at transporting this small protein into the mitochondrion. This outcome suggests that we should search for the protease(s) responsible for cleaving the α subunit within the smaller mitochondrion proteome.

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List of abbreviations

ATP – adenosine triphosphate
MTS – mitochondrial targeting sequence
T. b. – Trypanosoma brucei
PF – procyclic form
BF – bloodstream form
LS-BF – long slender bloodstream form
SS-BF – short stumpy bloodstream form
OXPHOS – oxidative phosphorylation
NADH – nicotinamide adenine dinucleotide
ADP – adenosine diphosphate
TCA – tricarboxylic acid cycle
TOM – translocase of outer membrane
TIM – translocase of inner membrane
PAM – presequence translocase-associated motor
MPP – mitochondrial processing peptidase
Oct1 – octapeptidyl aminopeptidase 1
IMP – inner membrane peptidase
Icp55 – intermediate cleaving peptidase 55
Asp – aspartic acid
Glu – glutamic acid
Ser – serine
Cys – cysteine
Thr – threonine
Asn – asparagine
GFP – Green Fluorescent Protein
dNTP - nucleotide
mNG – mNeonGreen
LB – lysogeny broth
PAGE – polyacrylamide gel electrophoresis
RNA – ribonucleic acid
DNA – deoxyribonucleic acid
SDS – sodium dodecyl sulfate
UV – ultraviolet
PCR – polymer chain reaction
TetR – tetracycline repressor
FBS – fetal bovine serum
WCL – whole cell lysate
PBS – Phosphate buffered saline
PVDF – polivinylidene fluoride
HRP – horseradish peroxidase
rpm – revolutions per minute
QS – quantum satis
ECL – substrate for enhanced chemiluminescence
Mt – mitochondrial
DHFR – dihydrofolate reductase
bp – base pair
TC – tissue culture
APRT - adenine phosphoribosyltransferase
kDA – kilodaltons
Tet – tetracycline
cyto - cytosol
org – organellar
mt - mitochondrial

1. Introduction

1.1. *Trypanosoma brucei* is a human pathogen

The flagellated protozoan *Trypanosoma brucei* is a human pathogen that causes Human African Trypanosomiasis, often referred to as sleeping sickness. This vector-borne disease is restricted to sub-Saharan Africa since it is the most suitable habitat for its transmission vector, the tsetse fly. The course of the disease has two successive stages: the haemolymphatic stage and the meningoencephalitic stage. In the first stage, the parasite is found in the peripheral circulation, but once it crosses the blood-brain barrier it invades the central nervous system, thus entering the second stage. Symptoms exhibited at the early stages of the disease include fever, headache, swollen lymph nodes, malaise, and joint ache that can easily be mistaken for a cold. Whereas once the nervous system has been infected in the later stage, there is a dysregulation of the circadian rhythm or even sleep fragmentation [1] that prevails along with neuropsychiatric disorders [2].

The parasite has three major subspecies with different rates of progression: *T. b. gambiense* – causing a slow chronic disease typically found in Central and West Africa; *T. b. rhodesiense* – causing a faster acute disease in East and Southern Africa, and *T. b. brucei* – causing an infection in livestock [2]. Almost all cases of infection are caused by *T. b. gambience* [4] with an average duration of 3 years evenly divided between both stages [3]. On the other hand, acute infections caused by *T. b. rhodesience* develop at the site of the tsetse bite. The disease quickly progresses from the first stage into the second one within 1-2 weeks of the infective bite, ultimately resulting in death within months if untreated [3, 2]. Most of the affected patients live in poor rural areas with minimal access to healthcare services that are able to diagnose and treat the disease efficiently, therefore causing a high number of undocumented infected cases and inevitably death [1].

African trypanosomiasis is often labelled a neglected tropical disease because the number of drugs available for treatment is limited. Until the recent development of Fexinidazole [5], the available drugs were not only difficult to administer but also showed frequent occurrence of serious adverse reactions. Despite recent success with new pharmaceuticals, these developing countries still face a threat from animal Trypanosomiasis, also known as nagana. Preventive measures and control activities have been implemented to control this public health issue, even though such elimination does not equal eradication and thus requires continuous efforts and innovation towards better results [4].

1.2. *T. brucei* life cycle

The parasite alternates between a mammalian host and its insert vector, the tsetse fly of the genus *Glossina*. As both the male and female flies are blood-feeders, they are both able to transfer the parasite. Interestingly, these flies are viviparous, and as such the fully developed larvae are deposited by the female fly, emerging as an adult fly a month after pupating in the soil. These newly-hatched flies have not been reported to have any trypanosome infection [2], therefore implying that the fly needs to feed on an infected mammalian host before the parasite enters its digestive tract.

The complete life cycle (Figure 1) of *T. brucei* is quite complex, but the two main life cycle stages of the parasite grown in culture are the insect derived procyclic form (PF) and the mammalian bloodstream form (BF). The BF cells found in the mammalian host are pleomorphic, existing as either the long slender bloodstream-form (LS-BF) or the cell-arrested short stumpy bloodstream-form (SS-BF) [6]. Only the latter ones are preadapted for survival in the insert vector, therefore with the bite of the tsetse fly, the SS-BF enter the food canal and start differentiating into the PF. The PF cells migrate from the fly's midgut to the proventriculus, where they differentiate into epimastigotes prior to differentiating into metacyclics in the salivary glands [7]. Due to the various environments that the extracellular parasite occupies, it must adapt its metabolism to the nutrients available. This results in significant remodeling of the mitochondrial ultrastructure and physiology of the parasite as it transitions between the two main life cycle stages. Since *T. brucei* is easy to cultivate, genetically tractable and a unicellular organism with a single mitochondrion, it serves an excellent model organism to study mitochondrial biogenesis.

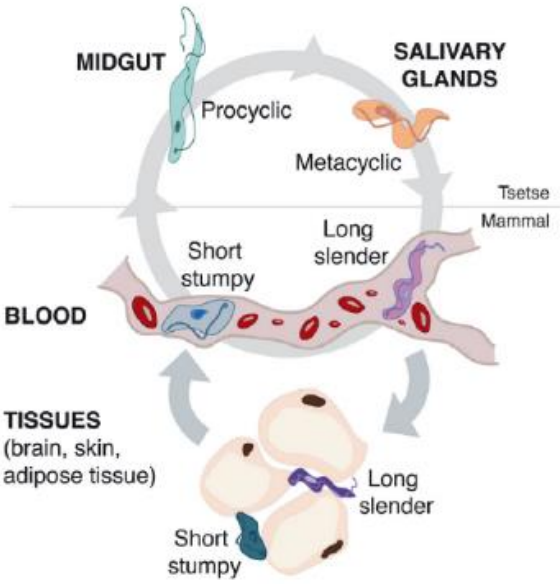

Figure 1: The complete life cycle of T. brucei. Throughout their life cycle, the parasites undergo various major changes. The LS-BF found in the mammalian host, differentiate into SS-BF that are better adapter for survival in the insect vector. The PF inside the midgut of the insect undergo metabolic and morphological changes when they migrate to the salivary glands where they differentiate into metacyclic cells capable of invading a new host. [6]

1.3. *T. brucei* bioenergetics

Mitochondria are the multifaceted organelles involved in many metabolic processes, including the biosynthesis of heme, lipids and amino acids. They also play a crucial role in intracellular signaling pathways, bioenergetics of the cell and cell death programming. The mitochondria arose from the endosymbiotic event of an Archean engulfing an α-proteobacterium [8]. Therefore, the organelle consists of an outer membrane and an invaginated inner membrane that forms cristae. Mitochondria are often called the powerhouse of the cell because chemical energy in the form of ATP is generated through a very efficient process called oxidative phosphorylation (OXPHOS) within these cristae. This pathway involves the following protein complexes: complex I (NADH coenzyme Q reductase), II (succinate dehydrogenase) III (cytochrome *bc1* complex), IV (cytochrome *c* oxidase) and complex V (FoF1 ATP synthase). Electron flow through respiratory complexes I, III, and IV are responsible for generating the mitochondrial membrane potential by pumping protons from the mitochondrial matrix into the inner membrane space. This membrane potential is then converted into ATP by the F_oF_1-ATP synthase, comprised of a hydrophobic F_o

domain and a catalytic F_1 domain. The extrinsic F_1 domain is typically comprised of 5 subunits (α_3, β_3, γ_1, δ_1, and ε_1), while the membrane intrinsic F_o domain consists of a rotary c-ring, a proton pore and a peripheral stalk that connects to the F_1 domain. By allowing the protons to flow down their concentration gradient and enter the matrix through the proton pore, potential energy is converted into mechanical energy as the c-ring rotates the central stalk of the F_1 domain to create conformational changes that synthesize ATP. The produced ATP is then exported out of the mitochondria by the ADP/ATP carrier [6].

Since the primary carbon source (Figure 2) for PF *T. brucei* in the tsetse fly is proline and glutamine, they utilize the TCA cycle to feed electrons into OXPHOS to generate most of their ATP. However, the BF parasites have abundant access to glucose and generate most of their ATP through glycolysis. Interestingly, this life cycle stage lacks functional respiratory complexes III and IV. Therefore, F_oF_1-ATP synthase must reverse its course and hydrolyse ATP to maintain the essential mitochondrial membrane potential by pumping protons into the inner membrane space [9].

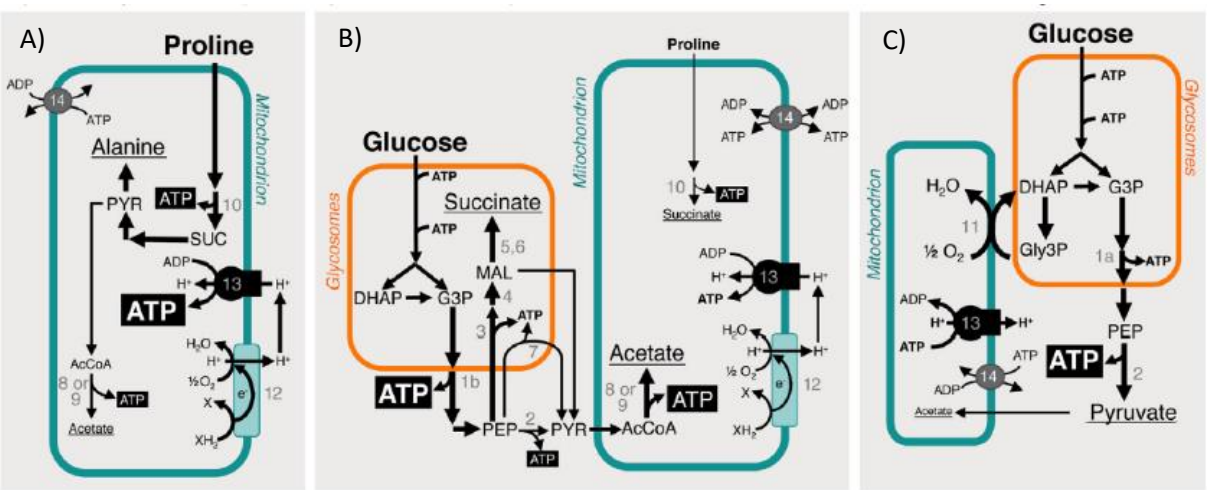

Figure 2: Main metabolic pathways for ATP production. Catabolism of the most abundant carbon sources in A) PF, grown in a glucose -depleted medium, B) PF, grown in a glucose-rich form and C) BF**.**

1.5. Mitochondrial protein import

With the endosymbiotic event, most of the bacterial genome was transferred to the host nucleus, leaving only a few genes in the organelle. Since the majority of the mitochondrial gene products are nuclear-encoded, it is crucial that they get imported into the mitochondria. This is accomplished through the mitochondrial protein import machinery by the means of the membrane potential across the inner membrane. Several different import pathways (Figure 3) and machinery exist, including the translocase of the outer membrane (TOM), the translocase of the inner membrane (TIM) and the presequence translocase-associated motor (PAM). The conventional way of import is known as the presequence pathway. Since the majority of the matrix proteins contain an N-terminal presequence, they can serve as targeting signals [8]. Such preproteins, whether cleavable or not, are imported into the mitochondria by the TOM protein complex and then into the matrix or inner membrane by the TIM complex [8]. Furthermore, PAM is an ATP-dependent machine that drives protein translocation into the matrix so that the mitochondrial processing peptidase (MPP) can cleave the presequences off.

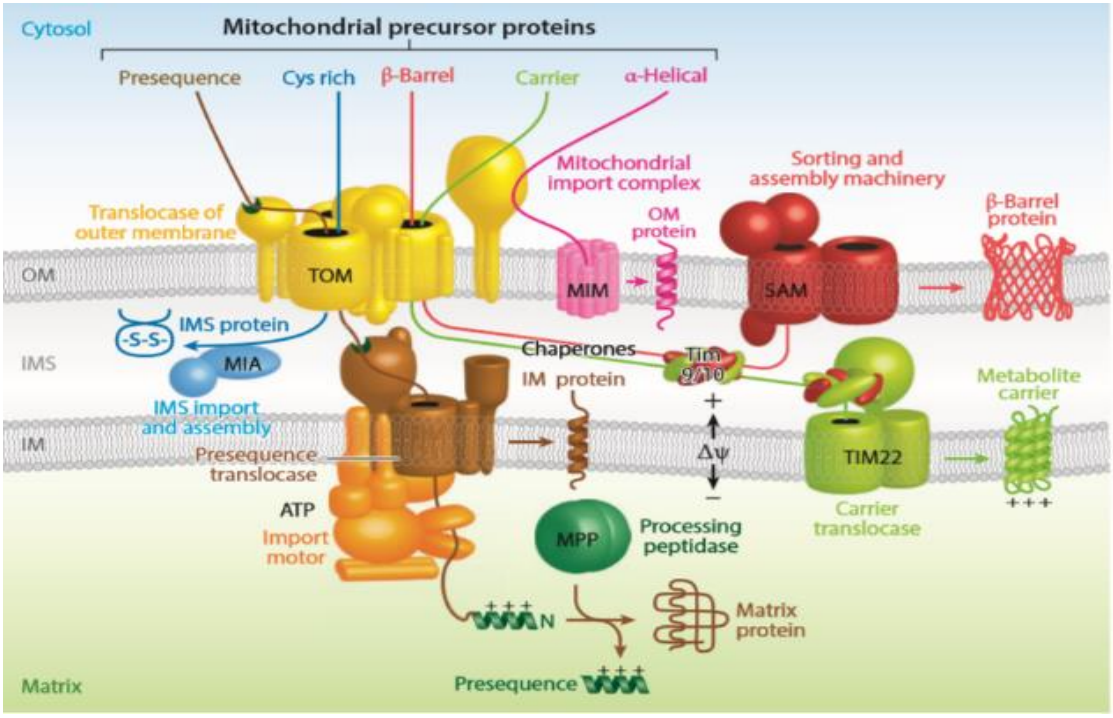

Figure 3: Protein import pathways of mitochondria. Preproteins which carry a presequence are imported by the TOM and TIM23, while proteins that have a hydrophobic signal are released into the inner membrane and the hydrophilic proteins are imported inside the matrix by PAM. The presequences are removed by the MPP [8]**.**

The mitochondrial import of the proteins generally depends on two prerequisites: the presence of a specific mitochondrial targeting sequence (MTS) and the presence of specific translocators within the mitochondrial membranes that recognize the targeting signals [11]. There exist three distinct types of MTS for mitochondrial proteins: N-terminal signals, stop-transfer or sorting signals, and internal signals [12]. The N-terminal targeting sequence is an amphipathic helix comprised of both hydrophobic and basic amino acid residues, which gets cleaved by the MPP once the preprotein reaches its final destination in the mitochondrial matrix [11] in order to make a mature matrix protein. The second type of MTS is comprised of two parts: the first being a canonical presequence accompanied by a hydrophobic patch large enough to reach the membrane. Such a signal is termed the stop-transfer signal or the sorting signal and is found in numerous proteins localized to the mitochondrial inner membrane. These sequences are recognized by a receptor sorting the protein to the correct location. Last but not least, nuclear-encoded mitochondrial proteins that do not contain an N terminal targeting signal are imported into mitochondria by means of internal targeting signals [11]. The N-terminal MTS and some internal sorting sequences are cleaved off by mitochondrial proteases once they enter the mitochondria. This processing is of great importance to protein maturation, function, and insertion into the correct location of the mitochondria. Such mitochondrial processing proteases are the mitochondrial processing peptidase (MPP), octapeptidyl aminopeptidase 1 (Oct1), inner membrane peptidase (IMP), intermediate cleaving peptidase 55 (Icp55), AAA, rhomboid protease Pcp1 and Atp23. The protease which is responsible for the N-terminal MTS cleavage from the mitochondrial proteins is the zinc-dependent metallopeptidase (MPP).

1.5. Proteases

Proteases stand as one of the most studied enzymes due to their importance in protein cleavage. These enzymes break the peptide bond that exists between two amino acids of a protein during a hydrolytic reaction. Therefore, they are involved in numerous cellular processes including cell proliferation, protein activation and maturation, protein catabolism and transport, tissue remodelling, blood coagulation, etc. In case the protease activities would get disrupted, a variety of pathological conditions would arise. Since proteases are a crucial part of the proliferation of many microorganisms, they make potential drug targets for the treatment of the disease caused by the pathogen [13].

Protease classification is based on whether the protease is identified as an endopeptidase or an exopeptidase [14], in other words, whether the protease cuts an internal peptide bond of the protein or cleaves at the carboxy (–C) or amino (–N) terminal peptide bond. Each group of proteases is named after its catalytic residue. Metalloproteases and proteases containing a catalytic aspartic acid (Asp) or glutamic acid (Glu), require water molecules to break the peptide bond of their substrate. Meanwhile, proteases with a serine (Ser), cysteine (Cys), threonine (Thr) or asparagine (Asn) use their amino acid residues for their catalytic activity [15, 16, 17].

1.6. Hypothesis

The catalytic F_1 domain of the ATP synthase is generally very conserved across life in terms of the composition and structure. However, *T. brucei* F_1-ATP synthase has two unique features - three copies of the novel subunit p18 (Figure 4) and the proteolytic cleavage of the α subunit [18]. The impetus for this research project is that the α subunit of the F_1-ATP synthase in Euglenozoans is proteolytically cleaved into a shorter N-terminal and a longer C-terminal peptide. Both peptides are assembled into the highly conserved F_1-ATPase to produce the expected structure. Previous work in the lab has shown that this cleavage occurs twice in
close proximity to remove an octapeptide.

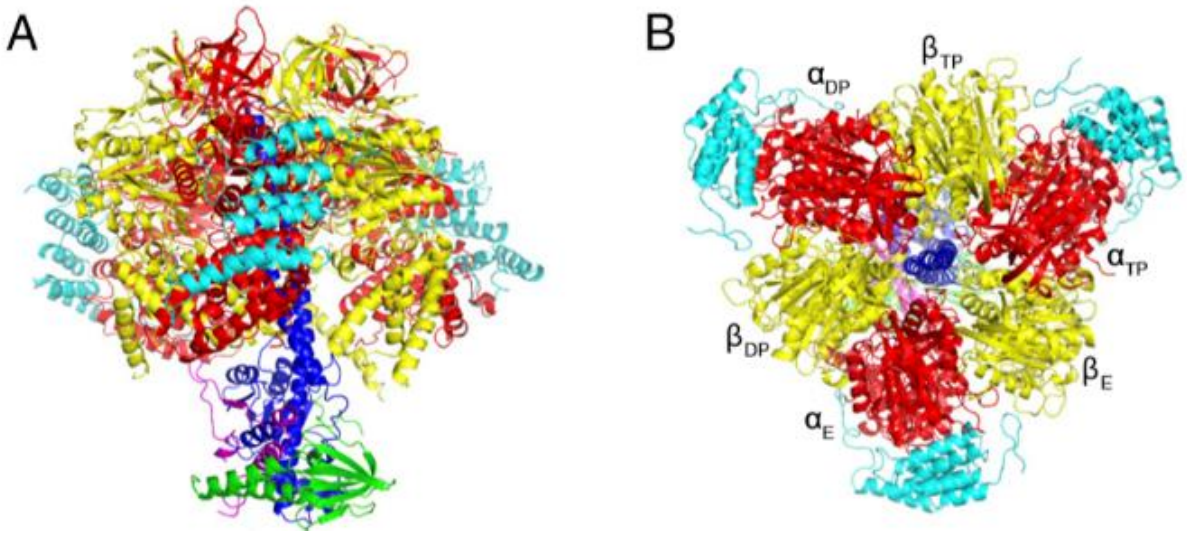

Figure 4: Structure of the F_1-ATPase in T. brucei. The α, β, γ, δ, ε, and p18-subunits are represented with red, yellow, blue, green, magenta and cyan, respectively. [19]

In order to determine the biological significance of this unique processing event, we are currently trying to identify the protease(s) that are responsible for these two cleavages. Since the alpha subunit is nuclear-encoded, it is translated in the cytosol, possibly on ribosomes located within close proximity to the mitochondria. Therefore, it is possible that the first cleavage event occurs in the cytosol and the octapeptide then acts as an N-terminal targeting signal for what will become the mature C-terminal α. Indeed, when this C-terminal α together with the octapeptide is entered into an online tool to predict mitochondrial targeting sequences, like MitoProt II, it predicts that the octapeptide might act as an N-terminal targeting sequence. Additional studies in the lab led to the observation that genetically modified PF *T. brucei* expressing a tagged C-terminal alpha with the octapeptide is predominantly localized to the mitochondrion. However, additional controls also indicated that a tagged mature C-terminal alpha was efficiently transported into the mitochondrion without the benefit of the octapeptide. Therefore, we used the GFP variant mNeonGreen (mNG) as a reporter for mitochondrial protein import. We created PF *T. brucei* cell lines that expressed mNG fused with either the known N-terminal mitochondrial targeting sequence for α or the α octapeptide sequence (Figure 5). The subcellular analysis of these cell lines will help determine if the octapeptide is sufficient to target the cytosolic mNG to the mitochondrion of the parasite.

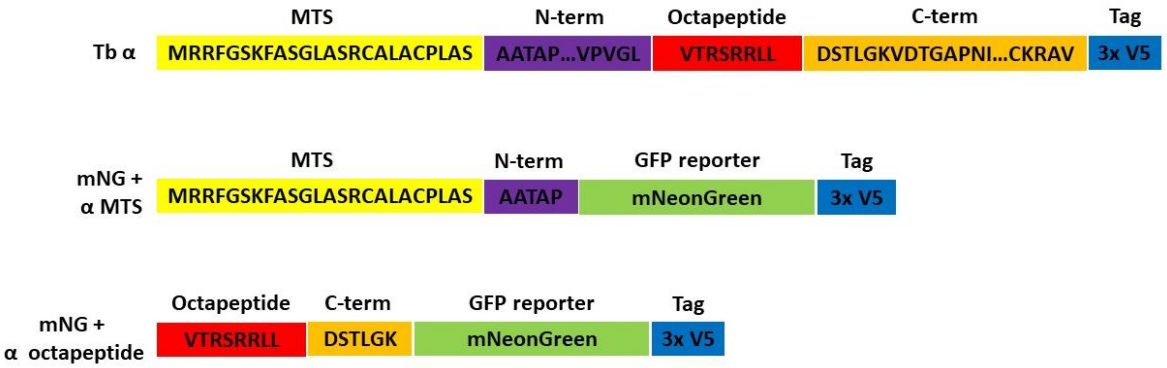

Figure 5: Schematic of important *T. brucei* F1-ATPase subunit alpha domains fused with a GFP reporter (mNeonGreen). The differently coloured blocks designate various regions of the F1-ATPase synthase subunit alpha. The primary amino acid structure has been indicated inside each of the blocks. For space saving measures, internal residues have been replaced by the ellipsis symbol. The 3x V5 tag indicates the amino acid sequence for three sequential V5 epitopes.

2. Aims of the thesis

- Create vectors for the inducible *T. brucei* expression of mNeonGreen fused with known and predicted mitochondrial targeting sequences of F_1-ATPase subunit α
- Transfect procyclic 29.13 *T. brucei* cells and select clones
- Verify regulatable expression of C-terminally V5 tagged mNeonGreen fusion proteins and determine the subcellular localization of these fluorescent reporters

3. Materials and methods

3.1. Cloning methodology

3.1.1. Midiprep of target vector

To generate a PF *T. brucei* cell line that expresses various mNG reporter proteins, we need to first clone the coding sequences of our reporters into the 5917 bp parasite specific expression vector pT7-3v5-PAC (Figure 5). The plasmid contains a part of the *T. brucei* rDNA intergenic spacer region that can be digested with NotI to generate a linearized plasmid with homologous ends that target the DNA for integration into the genome of the parasite. It contains the beta-lactamase gene for positive selection in bacteria and the PAC gene for puromycin resistance in *T. brucei*. When inserting the gene of interest into the multiple cloning region, it gets fused with a C-terminal 3x V5 epitope tag that is later used to verify successful protein expression. Independent RNA transcription of the introduced gene is driven by the T7 promoter that is recognized by the bacteriophage RNA polymerase that is constitutively expressed in the genetically modified PF 29.13 lab strain. This expression is regulated by the downstream tetracycline operator, which is bound by the heterologous expression of the Tet repressor. To initiate RNA transcription by T7 RNA polymerase, tetracycline added to the media binds the Tet repressor protein and releases it from the DNA. The transcribed mRNA contains *T. brucei* specific 5' and 3' untranslated regions to enhance the stability of the transcript

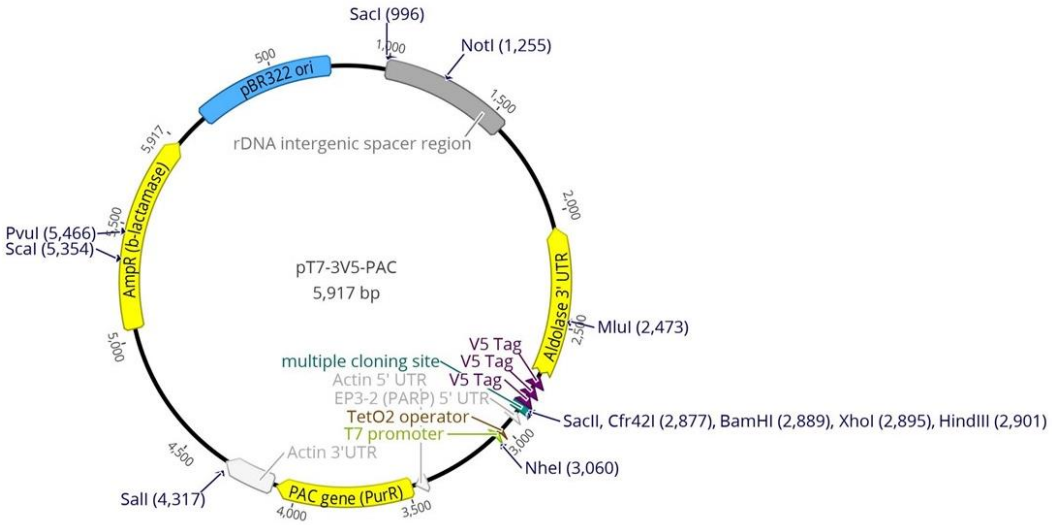

Figure 5: ***T. brucei*** **regulatable expression plasmid pT7-3V5-PAC**.

Therefore, our first task was to utilize the GenElute HP Plasmid Midiprep Kit from Sigma Aldrich (Cat. No. NA0200-1KT) to isolate enough of the plasmid DNA for downstream cloning events. First, a frozen clump of previously transformed *E. coli* cells was streaked out on a LB agar plate (Table 1) already containing the appropriate antibiotics, in this case – 100 μg/ ml ampicillin. After incubating the plate at 37°C for 12-16 hours, individual bacterial colonies were observed and the plate was removed from the incubator. Later in the afternoon, a single colony was streaked with a pipet tip onto a replica plate before being added it to a glass test tube with 5 ml LB medium (Table 2) containing 100 μg/ml ampicillin. We then proceeded with the DNA isolation according to the manufacturer's suggestions (Table 3). The liquid cultures were incubated for the next 16 hours at 37°C while shaking at 200 rpm. The bacterial cells were collected into a 50 ml conical flask and further spun in a rotating centrifuge at 5000 rpm for 10 minutes. In order to avoid enzyme degradation of the DNA from lysed cells, the centrifugation was performed at 12°C. The cells were resuspended in a buffer containing RNAse after the disposal of the supernatant and then lysed with a lysis buffer containing alkaline SDS. Since vortexing would shear the long coils of genomic DNA and therefore contaminate the purification of the plasmid, the samples were instead carefully inverted. After 3 minutes the sample was neutralized with a neutralization buffer from the provided kit. Phase separation arose with the inversion of the Eppendorf tube, where in addition to the DNA-containing aqueous phase, there was formation of white aggregate formed by the precipitated proteins and lipids. A binding solution was introduced prior to pouring the sample into a filter syringe, and the aggregate was left to rise up to the top of the liquid. Consecutively, the plunger was pressed to pass the liquid part of the solution onto the binding column, hence leaving the aggregate behind. The resulting DNA solution was passed through the membrane of the spin column, enabling the DNA to bind to the membrane while washing out all of the contaminants into the collection tube. The bounded DNA was washed with 2 different washing solutions. In order to release the plasmid from the membrane, the spin column was placed into a new collection tube and an elution buffer previously preheated to 65 °C was added. After a final spin, the resulting eluate now contained the desired extracted plasmid. An additional precipitation step was performed using sodium acetate and isopropanol, to ensure a more concentrated plasmid. After centrifuging, the resulting pellet was washed with 70% cold ethanol and then left to dry for 10 minutes in order to remove any excess ethanol. The pellet was then resuspended in 100 μl of the preheated elution buffer. As a final step,

using the elution buffer as a blank, the concentration of the resulting plasmid DNA was determined with the use of the Nanodrop, a full-spectrum, UV-Vis spectrophotometer for microvolumes.

Table 1: LB Agar Plates recipe

Reagent	FW or [Stock]	[Final]	500 ml
MiliQ			400 ml
Tryptone			5.0 g
Yeast Extract			2.5 g
NaCl	58.45	171 mM	2.5 g
pH ~ 7.3 with NaOH			
QS to:			500 ml
Agar, Bacteriological			7.5 g
Ampicillin	100 mg/ml	100 μg/ml	500 μl

Table 2: Terrific Broth Recipe

Reagent	FW	[Final]	1L
MilliQ			900 ml
Tryptone		1.2%	12 g
Yeast Extract		2.4%	24 g
Glycerol		0.4%	4 ml
KH2PO4	136.09	17 mM	2.31g
K2HPO4	174.18	72 mM	12.54 g

Table 3: Reagents used to isolate pT7-3v5-PAC plasmid according to manufacturer

Reagent	Volume
Resuspension solution/RNase	4 ml
Lysis solution	4 ml
Neutralization solution	4 ml
Binding solution	3 ml
Column Preparation Solution	4 ml
Wash Solution 1	4 ml
Wash Solution 2	4 ml
Elution Solution	1 ml
3M sodium acetate buffer solution 5.2 pH	x0.1 vol
Isopropanol	x0.7 vol
70% Ethanol	1.5 ml

3.1.2. Primer design

Before the gene of interest can be cloned into an expression vector, it first needs to be amplified from genomic or vector DNA by polymerase chain reaction (PCR). To start, primers required for cloning were designed using the program Geneious. First, the mNG gene to be amplified was inserted into the program, followed by the addition at the 5' and 3' ends of the primer specific sequence. When designing the primers, important parameters concerning the length, GC content, restriction sites and the inclusion of start and stop codons were taken into consideration. In general, the primers annealed to 20-22 bp of either the 5' or 3' end of the mNeonGreen open reading frame, the GC content was approximately 50% and when possible the 3' end included a G or C for maximal H-bonding. Since we were generating a fusion protein that combined a portion of the *T. brucei* F_1-ATP synthase α subunit with the mNG, the forward primers were quite long as they included the sequence of the α gene. In addition, the forward primers (AZ0850 – mNG + α MTS, AZ0851 – mNG + α octapeptide) included the restriction site HindIII and a start methionine, while the reverse primer (AZ0823) included a BamHI site and no stop codons to continue the in-frame translation of the C-terminal 3x V5 tag (Table 4). In order to verify that the primers were correctly constructed, Geneious was used to generate the expected

amplicons *in silico* that were then digested and ligated into the pT7-3V5-PAC plasmid. Upon successful completion of the plasmid, the primers were ordered through Generi Biotech.

Table 4: List of used primers

Primer ID	Sequence
AZ0850	ATAAAGCTTATGCGTCGCTTTGGTTCCAAGTTTGCCTCCGGGTTGGCGT CACGCTGTGCCCTGGCCTGCCCCCTAGCGAGCGCAGCAACGGCACCGG TCTCGAAAGGTGAGGAAGAC
AZ0851	ATAAAGCTTATGGTTACCCGCAGTCGCCGCCTGCTGGACAGCACGTTGG GTGTCTCGAAAGGTGAGGAAGAC
AZ0823	CGCGGATCCCTTATACAATTCGTCCATCCCC

Once the lyophilized primers were delivered to our lab, they were spun down and resuspended in a volume of MilliQ suggested from the manufacturer (Generi Biotech) to generate a 100 μM stock solution. This stock solution was further diluted 1:10 to create a 10 μM working stock used for the following PCR experiments.

3.1.3. Gradient PCR - polymerase chain reaction

Having the primers prepared, it was now possible to amplify the genes of interest from a plasmid template containing the mNG gene. This was achieved using a gradient PCR, a method that uses the least number of steps to empirically calculate the optimal annealing temperature for the primer pairs AZ0823 + AZ0850 and AZ0823 + AZ0851. To accomplish this, a total of 8 PCR reactions of 50 μl were generated (Table 5), following the directions from the AmpOne Fast-Pfu DNA polymerase protocol from GeneAll (Cat. No. 505 – 050). A master mix of +1 was created to reduce errors in pipeting. The PCR reactions were then placed on an 8 x 12 well thermocycler (T100, BioRad) with each PCR reaction treated with a different annealing temperature based on a gradient between a range of selected temperatures decided by the GC content of the primers.

Table 5: PCR reactions

Reagents	[Stock]	1x Rxn Vol.	[Final]	9x Master Mix
MilliQ		34.5 µl		310.5 µl
Fast Pfu buffer	5x	10 µl	1x	90 µl
dNTP's mix	10 mM	1 µl	200 µM	9 µl
Forward Primer	10 µM	1 µl	200 nM	9 µl
Reverse Primer	10 µM	1 µl	200 nM	9 µl
plasmid	5 ng/µl	2 µl	10 ng	18 µl
AmpOne	2.5 U/µl	0.5 µl	1.25 U	4.5 µl
Total		50 µl	50 µl	aliquot 50 µl

The thermal PCR conditions were set as in Table 6 based on the recommendation of the polymerase manufacturer. The first step is a general denaturation step. Since both of the forward 5' primers were quite long with variable GC content compared to the 20 terminal bp that annealed to the mNG gene, the first three cycles were set with a uniform reduced annealing Tm to aid in the generation of the first few amplicons generated from the template DNA. The next 32 cycles then applied the gradient annealing temperatures between 50-57 ℃, which are closer to the expected Tm of the entire primer sequence. Since the neither amplicon is longer than 810 bp, the extension time at 72 ℃ was set to 30 seconds since the polymerase can extend 1 kb/30 seconds. A final extension at 72 ℃ for 5 minutes was added to fill in any incomplete amplicons. The samples were then stored indefinitely at 4 ℃ until the samples could be retrieved.

Table 6: Thermal PCR conditions used for gradient PCR

Temperature	Time	
98 °C	30’	
98 °C	10”	3x cycles
48 °C	20”	
72 °C	30“	
98 °C	10”	32x cycles
50-57 °C	20”	
72 °C	30“	
72 °C	5’	
4 °C	infinite	

In order to determine the best annealing temperature for the primers, the amplicons were resolved on a 1% agarose gel (Table 7) containing ethidium bromide, a DNA intercalating die. When placed on a UV light source, it is possible to observe the quantity of the amplicons generated under the various temperatures. Gel electrophoresis is a method used for the size separation of the negatively charged DNA driven by an electric current that moves the DNA through the pores of the gel matrix towards a positive electrode. The approximate length of a DNA fragment can be determined when compared with the migration distances of a comercial DNA ladder (GeneRuler 1 kb Plus, Thermo Scientific) that contains a collection of known DNA fragment lengths. Before loading the samples, a 10x DNA loading dye (Table 8) was added to each, containing glycerol to ensure that the DNA would sink into the wells of the gel. Additionally, a negatively charged detergent SDS was added to help denature and charge the DNA evenly. After loading the samples, a constant voltage of 100 V was applied for 45-60 minutes, causing the migration of the DNA fragments due to the repulsion of charges from the wells located near the negative cathode towards the positive anode of the apparatus. Naturally, the shorter and smaller DNA molecules travel through the gel matrix faster than the longer ones and therefore migrate the farthest. Ultimately, the gel was visualized using the ImageLab software after applying UV light from the Chemidoc Gel Imaging Sistem (BioRad).

Table 7: Reagents used to make 1% Agarose Gel

Reagents	Amount
TAE buffer	70 ml
Agarose	0.7 mg
Ethidium Bromide	1 µl

Table 8: 10x DNA loading Dye

Reagent	[Stock]/FW	[Final]	10ml
MilliQ			4.2 ml
Glycerol	80%	39%	4.9 ml
SDS	10%	0.5%	500 µl
EDTA	0.5 M	10 mM	200 µl
Xylene cyanol	2%	0.1%	250 µl

3.1.4. DNA gel extraction

After determining an ideal annealing temperature of 54.3 °C for both primer pairs, two PCR reactions were performed for each primer pair to produce enough material for downstream cloning steps. The resulting PCR products were resolved on a 1% TAE agarose gel and then extracted using the GenElute Gel Extraction Kit from Sigma-Aldrich (Cat. No. NA1111), following the recommendations of the manufacturer. After extracting the DNA fragment of interest from the gel, it was solubilized using the gel solubilization solution from the kit and incubated at 50-60 °C for 10 minutes. After making sure the entire gel had dissolved, isopropanol was added. The sample was then applied to the binding column and centrifuged to remove the solubilized agarose in the flow-through liquid. After washing the DNA sample with a wash solution, the binding column was transferred to with a new collection tube and eluted with 50 µl elution solution. The resulting concentration and purity of each amplicon was measured using the NanoDrop.

3.1.5. Restriction digest of PCR amplicons and target vector

After generating the DNA sequences that we would like to express in the pT7-3V5-PAC plasmid, we need to ligate them into the parental vector. To achieve this, we will digest one end of the amplicon with HindIII and the other end with BamHI (Table 10). This will produce a specific 5'overhang that acts as a sticky end that can anneal to a similar overhang generated from the same digestion of the plasmid. Since the orientation of the generated amplicon DNA in the expression plasmid is essential, using two different restriction sites allows for directional cloning. After the respective sticky ends of the amplicon and the plasmid anneal, the molecules can easily be ligated into a circular plasmid.

Table 10: Restriction enzymes used to generate sticky ends

Restriction enzyme	sequence
HindIII	5'-A|AGCTT-3' 3'-TTCGA|A-5'
BamHI	5'-G|GATCC-3' 3'-CCTAG|G-5'.

The digestion of the plasmid DNA and amplicons was performed in a similar manner, but they were each prepared for the downstream ligation in different ways. Therefore, we will first specifically describe how the digested plasmid was generated. A total of 10 µg of plasmid DNA was digested by mixing the following components and incubating at 37 °C for 2 hours:

Table 11: Reagents used to digest the vector

Reagents	Final
MilliQ	up to 41 µl
DNA	10 µg
10x Fast Digest green buffer	5 µl
HindIII restriction enzyme	2 µl
BamH I restriction enzyme	2 µl
Total	50 µl

For the verification of the successful linearization of the plasmid DNA, both the digested and undigested plasmid were resolved on a 0.8% TAE agarose gel (Table 12) with larger pore sizes to resolve the high molecular weight plasmids. Since the 10x Fast Digest buffer already contains glycerol and a loading dye, the samples could be directly run on the gel with a constant voltage of 100 V applied for 1 hour to allow the maximum resolution of the DNA molecules. The observed linearized plasmid DNA bands were then extracted from the agarose using the previously described GenElute Gel Extraction Kit.

Table 12: Reagents used to make 0.8% agarose gel

Reagents	Volume
TAE buffer	70 ml
Agarose	0.56 mg
Ethidium Bromide	1 µl

The gel extracted amplicons purified on the spin columns were digested in a similar manner (Table 13). Due to the limiting amount of DNA, all of the eluted amplicon was digested in a 50 µl reaction volume and incubated for 2 hours at 37 ℃. The digested amplicon was then isolated from the restriction enzyme and the digested nucleotide ends using the GenElute PCR Clean-up kit from Sigma-Aldrich (Cat. No. NA1020). We again followed the manufacturer's instruction to perform this simple procedure using a similar spin column format. Finally, the concentration and purity of the linearized vector and digested PCR fragments was measured on the NanoDrop.

Table 13: PCR amplicon digest

Reagents	Final
MilliQ	11 µl
DNA	30 µl
10x Fast digest buffer	5 µl
HindIII restriction enzyme	2 µl
BamH I restriction enzyme	2 µl
Total	50 µl

One of the final steps for constructing the desired recombinant plasmid is to ligate the insert DNA with the compatible digested vector. This step is called the ligation, where the DNA ligase enzyme catalyzes the linkage between the phosphates to join the nucleotides together. To determine if there is any undigested plasmid contaminating our ligations, a negative control ligation without any insert DNA was conducted as well. Using the obtained concentrations, the molarity of each DNA molecule was calculated in order to set the ligation reactions in a 3:1 molar ratio of insert to vector in a total volume of 10 µl. The ligation reactions (Table 14) were incubated at 4 ℃ overnight.

Table 14: Reagents used for ligation reaction

Reagent	Rxn volume mNG + α octapeptide	Rxn volume mNG + α MTS
MilliQ	up to 7.0 µl	up to 7.0 µl
10x Ligase buffer	1µl	1µl
Linearized Vector	1µl	1µl
Insert	3.9 µl	3.7 µl
T4 DNA ligase	1µl	1µl
Total	10 µl	10 µl

3.1.6. Bacterial transformation and miniprep

The ligated product was transformed into chemically competent XL1-blue *E. coli* by mixing 3 µl of the ligation reaction with 50 µl of the cells. To allow the DNA to coat the cells, they were incubated on ice for 10 minutes. After subjecting the mixture to a heat shock at 42 °C for 45 seconds, it was left to recover on ice for 2 minutes. 250 µl fresh SOC media was added to the cells before incubating them on a shaking platform for 45 minutes at 37 °C. 200 µl of the transformed cells were then spread on an LB plate containing ampicillin and finally incubated at 37 °C overnight. After a 16 hour incubation, the plates were removed from the incubator before the individual colonies became overcrowded with satellite colonies that lack any plasmid. The plates could then be stored for up to 4 weeks at 4 ℃.

To determine if the positively selected bacterial colonies contained the pT7-3V5-PAC plasmid with the mNG insert, we needed to isolate plasmid DNA. Several individual colonies were picked from the plate and replica plated before innoculating 5 ml of LB media containing 100 µg/ml ampicillin. The individual colonies were allowed to grown overnight at 37 °C on a shaking platform. After 16 hours of growth, the bacterial culture was dense and the plasmid DNA was isolated using the GenElute HP Plasmid Miniprep kit from Sigma-Aldrich (Cat. No. NA0140). To increase the plasmid yields, 4.5 ml of the bacterial culture was harvested by spinning consecutive 1.5 ml aliquots for 1 minute at 12,000 xg. The resulting supernatant was aspirated, while the cell pellet was resuspended in 200 µl of prechilled resuspension buffer.

Subsequently, the cells were lysed by adding 200 µl lysis buffer followed by a gentle tube inversion before incubating for no longer than 5 minutes. 350 µL of neutralization buffer was added while repeating the gentle inversion, trying not to shear any of the genomic DNA. The sample was spun for 10 minutes at 12,000 xg. These consecutive steps resulted in a precipitation of the cellular membranes and proteins, leaving the DNA in the supernatant. The columns were equilibrated with 500 µl of preparation solution before the cleared lysate was transferred to the spin column and centrifuged for 1 minute at 12,000 xg. At this point, the plasmid of interest was bound to the membrane of the column and the flow-through liquid was discarded. Several wash spins were carried out while removing the flow-through each time. The final washes were conducted with 500 µl of wash buffer 1 and 750 µl of wash buffer 2 using the above-mentioned centrifugation parameters. At last the column was spun without further addition of any wash buffers to remove any leftover traces of ethanol. Next, the spin column was transferred to a new collection tube and 50 µl of the elution buffer preheated to 65°C was added to elute the plasmid DNA by spinning for 1 minute at 12,000 xg. Finally, the concentration and purity of each of the isolated DNA plasmids were determined with the Nanodrop.

Next, we digested the isolated plasmids to determine if they contained the appropriate mNG insert (Table 15). By digesting with the same enzymes we used to clone the plasmid, we should generate two DNA fragments with predicted sizes that can be determined on an agarose gel. The larger band represents the linearized backbone vector, while the other is the DNA insert. Therefore, 1 µg of plasmid DNA was incubated with 0.5 µl of each restriction enzyme for 15 minutes at 37 °C. A master mix was generated to reduce errors in pipeting. The samples were

directly loaded onto a 0.8% agarose gel and resolved for 30-45 minutes along with a DNA ladder. The ethidium bromide stained DNA was then visualized with a UV light on the Chemidoc.

Table 15: Plasmid DNA verification by restriction digest

Reagents	Amounts	10x Master Mix
MilliQ	5.4 µl	59.4 µl
10x Fast Digest green buffer	2.5 µl	27.5 µl
plasmid DNA	1 µg	-
HindIII	0.5 µl	5.5 µl
BamH I	0.5 µl	5.5 µl
Total	10 µl	100 µl

3.1.7. Sequencing of cloned plasmids

Once it was determined that a clone contained a DNA insert of the expected size, the plasmid DNA was submitted to SEQme to determine the precise sequence of the inserted DNA. This was crucial to determine if any PCR errors resulted in any frame shift errors of the mNG fusion proteins. Each plasmid was sequenced with a plasmid specific primer that would sequence the insert from both the 5' end (T7 SEQme provided primer) and the 3' end (AZ0468 lab specific primer). The samples were prepared according to SEQme specifications: 500 ng of plasmid DNA in 5 µl and 5 µl of 5 µM lab primer. The samples were placed in an onsite dropbox and the sample information was uploaded to the SEQme webpage (http://www.seqme.eu). After 3-4 days the sequencing results could be downloaded and imported into Geneious where they could be aligned with the *in silico* constructs.

3.1.8. Glycerol stocks

Bacterial colonies from the replica plates that were verified to contain the correct plasmid were grown overnight at 37 °C in 5 ml cultures with 100 µg/ml ampicillin. Care was taken not to let the cultures get overgrown, which could possibly degrade all the antibiotic and allow for bacteria without the plasmid to overgrow the population of cells. Therefore, after 16 hours of growth, aliquots of the bacterial culture were thoroughly mixed with 80% glycerol (Table 16) and cooled on ice for 30 minutes. Then the glycerol stocks were stored long term in the -80 ℃.

Table 16: Reagents used to create Glycerol stocks

Reagent	1ml Cryovial
Bacterial culture	812 µl
80% sterile glycerol	188 µl

3.2. *T. brucei* transfection and selection

3.2.1 Preparing linearized plasmid

In order to incorporate the recombinant DNA into the parasite genome, linearization of the recombinant plasmids was required. A Not1 restriction site was incorporated into the middle of a *T. brucei* rDNA spacer region of each plasmid, generating a linearized plasmid with ends for homologous recombination into the genome. This ensures a more efficient homologous recombination process that promotes the successful genomic integration of the recombinant DNA. To promote thorough linearization, 20 µg of plasmid DNA was incubated with 5 µl of NotI and incubated for 3 hours at 37 °C (Table 17). This was followed by the precipitation of the linearized DNA with the addition of 3M sodium acetate, pH 5.2 and 96% ethanol. Upon vortexing the mixture, a white precipitate was evident. To promote maximum precipitation, the sample was incubated at -80 °C for 30 minutes before centrifuging at 16,000 xg for 30 minutes at 4 ℃. After aspirating the supernatant, the DNA was washed in 70% ethanol and then air dried for no more than 10 minutes in a sterile environment. The DNA was then resuspended in 30 µl sterile MilliQ warmed to 65 ℃. Then a small aliquot is used for quantification on the Nanodrop and another aliquot resolved on an agarose gel to verify it has been linearized.

Table 17: Plasmid digest for *T. brucei* transfections

Reagent	Volume
Plasmid	20 µg
MilliQ	up to 49 µl
10x FD buffer	6 µl
NotI	5 µl

3.2.2. Cell culture and growth

Throughout the entire experimental work, a crucial part was the maintenance of the PF *T.brucei* cell cultures from the PF 29.13 parental line. This genetically modified cell line constitutively expressed the T7 RNA polymerase and the bacterial tetracycline repressor (TetR). This allows for the regulated protein expression of heterologous genes that are under the control of a T7 promoter followed by a TetR binding site downstream. The addition of tetracycline to the media binds the TetR and causes a conformational change that allows the T7 polymerase to transcribe the downstream gene.

The parasite cultures are normally grown in 10 ml cultures in 25 cm^2 plastic flasks in a 27 °C incubator. The PF *T. brucei* culture typically doubles every 8 hours in SDM-79 media supplemented with 10% FBS (Table 18). The cultures were split 1:10 with fresh media whenever the cell density reached 1-2x10e^7 cells/ml. PF *T. brucei* do not like to be diluted much below 0.5x10e^6 cells/ml. Genetically modified cell lines were grown in the presence of selectable markers (Table 19) to ensure that they did not lose any genetic information. The morphology of the cultures was visually monitored using an inverted light microscope. Healthy cultures ought to contain several dividing parasites within one field of view. This occurrence can be noted as two flagellated parasites still attached at their posterior ends. It is also important to monitor for signs of stress that manifest as long slender cells with an uneven movement and potentially large rosetta-like aggregations of parasites. It is also important to scan for contamination from bacteria or fungi. To eliminate the possibility of contamination, aseptic methods and techniques are strictly adhered when culturing the cells in the tissue culture hood.

Table 18: SDM-79 media + 10% FBS

Reagent	5 L
MilliQ	4.25 L
premixed SDM-79 powder	127.4 g
Pen/Strep (10^4 U/ml), 100x	50 ml
adjust pH to 7.3 with 10M NaOH	
Hemin (2.5 mg/ml)	15 ml
QS with MilliQ	4.5 L
FBS	500 ml

Table 19: Concentration of antibiotics used for PF *T. brucei*

Antibiotic	[Final]
G418	15 µg/ml
Hygromycin	25 µg/ml
Puromycin	1 µg/ml
Tetracycline	1 µg/ml

3.2.3. Cell counting

The density of cell cultures can be easily counted with the Z2 Coulter Counter. This machine functions by forcing the parasites through a small aperture to form a single-file line in a microchannel. A voltage is then introduced through the microchannel and a cell is counted when the voltage drops due to a cell's passage. Before counting, the samples were prepared by carefully mixing 100 µl of the Trypanosomatid Cell Fix solution (Table 20) with 100 µl of the cell culture in a 1.5 ml test tube. The formaldehyde present in the solution fixes the proteins of the cell, keeping them intact and making sure the counter does not become contaminated with any pathogens. Subsequently, 50 µl of the fixed cells were introduced to a cuvette containing 5 ml isotonic Hemosol. The cuvette was further loaded onto the platform, followed by submerging of the aperture into the solution. After the counting was initiated, the computer calculated a final concentration in cells/ml based on the dilution factor previously entered in the program.

Table 20: Trypanosomatid Cell Fix

Reagent	[Stock]	Amount	[Final]
dH_2O		85 ml	
SSC	20x	5 ml	1x
Formaldehyde	36%	10 ml	3.6%

3.2.4. Transfection of *T. brucei* PF 29.13

The Tb PF 29.13 cell line can be transfected when a high-electric voltage is applied to the culture in the presence of the DNA. Due to electroporation, the cell membranes generate pores which allow a certain portion of the cells to take up the DNA and integrate it into the genome. Besides using the mNG + α MTS and the mNG + α octapeptide linearized plasmids, an additional mock transfection was included using MilliQ instead of the plasmid to serve as a negative control. Since homologous recombination is more prominent when the cells are actively replicating, it was of utmost importance that the cells were in mid-log phase of growth (0.4-0.8x10e^7 cells/mL) when harvested for the transfections. To complete 2 transfections, 1x10e^8 cells were harvested in 50 ml conical flasks spun at 1,300 xg for 10 minutes at room temperature. Each pellet was washed with Cytomix buffer (Table 21) and later resuspended in 1 ml of Cytomix buffer. 0.5 ml of the cell suspension was added to the 0.2 cm gap sterile cuvettes previously loaded with 12 μg of linearized sterile DNA. The cells were then electroporated with the BTX ECM 630 with the following settings: 1600 V, 25 Ω and 50 μF. After completing the electroporation, the cells were resuspended in a flask containing 6 ml SDM-79 media supplemented with 10% FBS. After 16 hours of incubation, the selectable antibiotic was applied to the cultures by adding an extra 6 ml of SDM-79 containing 10% FBS and 2x the concentration of the antibiotic that selects for the newly integrated DNA. 2ml of these transfected cultures were then plated into the top row of a 24 well plate. These wells were then serially diluted down each column of the plate, trying to find the maximum dilution that will still generate a well of transfected cells. Since PF *T.brucei* parasites cannot tolerate harsh dilutions, the last row with positively selected transfectants are more likely to have derived from just a few parasites.

Table 21: Cytomix Buffer

Reagent	FW	500 ml	[Final]
MilliQ		400 ml	
EGTA, pH 7.6	380.35	0.380 g	2 mM
$MgCl_2 \cdot 6H_2O$	203.30	0.508 g	5 mM
KCl	74.56	4.47 g	120 mM
Glucose	180.16	2.5 g	0.5% (27 mM)
$CaCl_2$	147.02	0.011g	0.15 mM
BSA		50 mg	100 μg/ml
K_2HPO_4/KH_2PO_4, pH 7.6	100 mM	50 ml	10 mM
Hypoxanthine	100x	5 ml	1 mM
HEPES	238.30	2.98 g	25 mM

A mock plate of the parental cells transfected without any of the linearized DNA was also created. After 7-10 days these cells form clusters and other signs of stress as they start to die due to the presence of the selectable antibiotic. Meanwhile, the wells populated by *T.brucei* transfected cells with good motility and morphology are maintained at high densities but provided with fresh media containing more selectable antibiotics. After a dense well is split 1:1 into a new well, it takes 2-3 days to become dense again. The dense wells are then split 1:3 and allowed to recovery for 2-3 days before they are split 1:5 into a new well. Finally, a dense well is expanded into a 25 cm^2 flask by diluting it 1:10. Subsequently, these flasks were split several times and after concluding a consistent growth rate, long-term stabilates were created. Each population of cells was mixed with a trypanosome freezing solution (Table 22) in a cryovial in a 1:1 ratio. The vials were cooled on ice for 30 minutes before being placed in the -80 °C freezer for 3 days. Finally, these stabilates were transferred to a liquid nitrogen storage tank.

Table 22: Trypanosome stabilate freezing solution

Reagent	FW	[Final]	100ml
MilliQ			70 ml
Glucose	180.16	100 mM	1.86 g
NaCl	58.44	70 mM	420 mg
Tri-Sodium Citrate (dihydrate)	294.10	5mM	150 mg
BSA		0.1%	100 mg
Glycerol		15%	18.75 ml
QS with MilliQ			100 ml

3.3. Verification of transgenic cell lines by Western blot analysis

3.3.1. Western blot analysis of WCL

To determine if the genetically modified cell lines were capable of regulatable expression of the V5 tagged mNG reporters, we analysed the whole cell lysates (WCL) generated from these cultures by western blot analysis. Cells from the same culture were divided into two flasks and one was treated with 1 µg/ml tetracycline for three days to induce heterologous expression. A determined number of cells from the non-induced and induced cultures were then harvested during a 10 minute spin at 4 °C at 1,300 xg. The supernatant was decanted and the cell pellet was resuspended in 1ml of PBS-G (Table 23) and transferred to a 1.5 ml eppendorf tube. The cells were spun again for 10 minutes at 1,300 xg at 4 °C. The supernatant was aspirated and the cell pellet was resuspended in PBS (Table 24) and 3x SDS loading dye so that 30 µl would equate for $1x10e^7$ cells. The lysed samples were then incubated at 97 °C for 7 minutes. After careful inversion, the samples were cooled to room temperature before being stored at -20 °C.

Table 23: PBS-G

Reagents	FW	Final	500 ml
MilliQ			450 ml
PBS	10x	1x	50 ml
Glucose	180.16	6 mM	0.541 g

Table 24: Potassium phosphate buffer, pH 7.6

Reagents	FW	100 Mm
1 M K_2HPO_4	228	4.35 ml
1 M KH_2PO_4	136.09	650 µl

3.3.2. SDS-PAGE

SDS-PAGE uses the negatively charged SDS detergent to coat proteins with a negative charge, thus denaturing them so they can be resolved on a polyacrylamide gel according to their molecular weight. The charged proteins are subjected to an electric field, causing them to migrate through the matrix created by the cross-linked acrylamide polymers. Hence, larger molecules would migrate more slowly than smaller ones. Uniform precast acrylamide gels (BioRad Mini-PROTEAN® TGX Stain-Free™ Precast Gels, 4-20%) were submerged in SDS-PAGE running buffer (Table 25) in an BioRad electrophoresis unit. The WCL's obtained previously were loaded onto the gel next to a 4ul PageRuler pre-stained protein ladder to allow the determination of the molecular weight of the proteins when detected by western blot analysis. A constant voltage of 130 V was applied to gels until the bromophenol blue dye in the SDS PAGE loading buffer ran out of the bottom of the gel.

Table 25: 10xSDS Page Running buffer

Reagent	FW	[Final]	1L
MilliQ			700 ml
Tris	121.14	250 mM	30.3 g
Glycine	75.07	1.92 M	144.4 g
SDS	280.4	1%	10 g
QS to:			1 L

Electroblotting: Transfer of proteins to PVDF membrane

Using a wet electroblotting apparatus, the proteins from the SDS PAGE gel were transferred onto a more stable platform to probe the resolved proteins with specific antibodies. For this transfer, the gel and the PVDF membrane were assembled into a sandwich containing a layer of blotting paper and a fiber pad on both sides and finally a plastic support grid. First the PVDF membrane was activated by submerging it into methanol for 45 seconds, followed by a 2 minute rinse in MilliQ water and finally soaking it in the transfer buffer (Table 26) to equilibrate. All the other layers necessary for the electroblotting were also equilibrated with the transfer buffer. Once the gel was removed from the tank and the wells cut off, it was submerged into the transfer buffer as well. To assemble the transfer stack, the support grid closest to the cathode was placed down, followed by the fiber pad soaked in transfer buffer while rolling out the air bubbles. Then the filter paper, gel and transfer membrane were added. Similarly, the filter paper, fiber pad and lastly the support grid to be placed near the anode were assembled. This way the negatively charged proteins would migrate from the gel onto the membrane due to the assembly of the cathode and anode. As a last step, the transfer stack was placed into a tank filled with transfer buffer and run for 90 minutes at 90 V. To avoid damage due to the generated heat, the transfer tank was kept cold in a 4 °C refrigerator. Once the transfer was completed, the gels and membranes were analysed.

Table 26: 10xTransfer buffer

Reagent	FW	[Final]	1L
MilliQ			700 ml
Tris	121.14	480 mM	58.15 g
Glycine	75.07	390 mM	29.27 g
QS to:			**1 L**

In order to avoid any drying out, the membranes were immediately placed in 50 ml conical flasks containing 5% skim milk in PBS-T (Table 27), which acts to block the membrane with the abundant casein. This helps to limit any non-specific binding with the antibodies. Special care is taken that the membrane side with the proteins is facing the interior of the tube. After one hour of blocking, the membrane is stored overnight at 4 °C. The following day, the overnight milk

solution was replaced with 5 ml fresh milk. The anti V5 tag mouse monoclonal antibody was added directly to the milk in a 1:1000 dilution. We also added the polyclonal rabbit antibody that recognizes the cytosolic adenine phosphoribosyltransferase (APRT, 1:500 dilution), an enzyme involved in the purine salvage pathway. APRT will serve as a loading control for our samples. These primary antibodies are incubated with the membrane for 1 hour on the lambada rotator at room temperature. The milk was then discarded and the membrane rinsed with a series of PBS-T washes consisting of two short PBS-T washes, a 15-minute and two more 5 minute washes, each with a volume of 25 ml. These washes were conducted to remove any excess unbound antibodies, thus avoiding any non-specific binding. To amplify the binding of the primary antibody to the target protein, a 1-hour membrane incubation with a secondary antibody was carried out in the same manner as described for the first antibody. In this case we added both horseradish peroxidase (HRP) conjugated goat anti-mouse or anti-rabbit antibodies, both diluted 1:2000. These secondary antibodies recognize any mouse or rabbit IgG antibodies. Since they are conjugated with HRP, they can further amplify the signal by generating chemiluminescence when the ECL reagent is added. After the probing with secondary antibodies, the PBS-T washes were performed in the same manner as after the primary antibody. Before visualizing the membrane, two substrates from the Clarity ECL kit (BioRad) were mixed in a 1:1 ratio, applying 500 µl of the combined ECL solution to each membrane. Any excess solution was removed and finally the blots were visualized using the ImageLab software on the Chemidoc.

Table 27: PBS-T

PBS-T Wash Buffer	**1 L**
ddH_2O	800 ml
10x PBS	100 ml
Tween 20	500 µl
QS to:	1 L

3.4.1. Determining subcellular localization

To determine where are mNG reporters are targeted within the PF *T. brucei* parasite, we need to perform a crude subcellular fractionation that utilizes the detergent digitonin to poke holes in the plasma membrane so that we can separate the cytosolic fraction from the organellar fraction. First, 1x10e^{8} *T.brucei* cells were harvested in 50 ml conical tubes, followed by a 10-minute spin at 1,300 xg at 4 °C. After discarding of the media, the cells were kept on ice for the consecutive steps. The cell pellet was resuspended in 1 ml PBS-G before being transferred into an Eppendorf tube and further spun using the same parameters as before. After carefully discarding the supernatant, the cell pellet was resuspended in a mixture of 500 µl SoTE buffer (Table 28) while pipetting up and down to ensure thorough mixing. Then 500 µl SoTE/0.03 % digitonin (Table 29) was added and the tube was inverted just a single time before being placed on ice for 5 minutes. The conical tube was spun at 7,000 rpm for 3 minutes at 4 °C. The resulting supernatant contained the cytosol fraction of the cells and was therefore transferred into a new Eppendorf tube. 50ul 3x SDS PAGE dye was added to the cytosolic fraction, while the organellar pellet was resuspended in 1 x PBS of the same volume. Having the organellar fraction transferred to a new 1.5 ml Eppendorf tube, 50ml 3 x SDS PAGE dye was added. Both samples were incubated at 97 °C for 7 minutes to promote denaturation of the proteins.

These WCL, cytosolic and organellar fraction samples were run on a 12% SDS PAGE gel in a 4°C refrigerator with a constant voltage of 130 V, before transferring the proteins onto a PDVF membrane with electroblotting using 90 V for 90 minutes in the same manner as mentioned above. The fractionation quality was analysed by probing the samples with organellar and cytosolic specific antibodies on separate membranes, hence the subcellular localization of the protein of interest was determined. One Western Blot was performed for enolase - a cytosolic antibody and another one for Hsp70 - a mitochondrial antibody. At last, each membrane was analysed using Chemidoc.

Table 28: SoTE buffer

Reagent	[Stock]/FW	[Final]	50ml
MilliQ			40 ml
Tris-HCl, pH 7.5	1 M	20 mM	1 ml
Sorbitol	182.2	0.6 M	5.47g
EDTA	0.5 M	2 mM	200 µl
Qs w/MilliQ			50 ml

Table 29: SoTE with 0.03% digitonin

Reagent	[Stock]/FW	[Final]	50ml
MilliQ			40 ml
Tris-HCl, pH 7.5	1 M	20 mM	1 ml
Sorbitol	182.2	0.6 M	5.47 g
EDTA	0.5 M	2 mM	200 µl
Digitonin	100%	0.03%	15 µl
Qs w/MilliQ			50 ml

Table 30: Dilutions of used antibodies

Antibody	Dilutions
Anti-V5 monoclonal (mouse)	1:1000
Anti-APRT (rabbit)	1:500
Anti-Hsp70 (mouse)	1:1000

4. Results

4.1. Generation of PF *T. brucei* cell lines expressing mNG reporters fused with various regions of the F_1-ATPase alpha

4.1.1. Preparation of the pT7-3v5 pac plasmid for the mNG reporter cloning

The pT7-3V5-PAC vector was isolated from recombinant *E. coli* cultures using the instructions from the manual of GenElute HP Plasmid Midiprep Kit (Cat. No. NA0200S) as explained in the methods. The resulting concentration of the plasmid was 2419.9 ng/µl when measured with Nanodrop. The purity of the DNA sample was confirmed using the A260/A280 ratio, which is based on the absorption wavelengths of the DNA and contaminants such as RNA or proteins. Since our sample is in the ratio of 1.8-2.0, it is considered as sufficiently pure. (Table 31)

Table 31: Nanodrop quantification of the DNA concentration and purity of the isolated pT7-3V5-PAC plasmid

Plasmid	Concentration	A260/A280
pT7-3V5-PAC	2419.8 ng/µl	1.89

To successfully prepare the plasmid for cloning with the mNG reporters, 2.1 µg was first linearized using a digestion reaction with 2 µl of the restriction enzymes BamHI and HindIII . The reaction was incubated for 2 hours at 37 ℃ to ensure completion of the reaction before resolving the final digested plasmid on an 0.8% TAE agarose gel at 100 V for 1 hour (Figure 6). The gel contained both the non-linearized and linearized plasmid for comparison.

As expected, the undigested plasmid produced 3 bands on the gel – circular, supercoiled and nicked plasmids. Importantly these bands were not detected in the digested plasmid, suggesting it has been successfully linearized. According to the DNA ladder, the predominant band observed for the digested plasmid was about the expected size of 5909 bp. Next, the linearized plasmid was extracted from the gel using the Gen Elute Gel Extraction Kit (Cat. No. NA1111) yielding 110.5 ng/µl (Table 32).

Table 32: Concentration and purity of pT7-3V5-PAC after restriction digest with BamHI and HindIII restriction enzymes

Linearized plasmid	Concentration	A260/A280
pT7-3V5-PAC	110.5 ng/µl	1.95

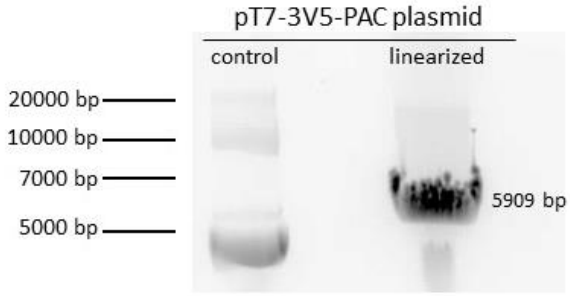

Figure 6: The pT7-3V5-PAC plasmid digested with BamHI and HindIII restriction enzymes to prepare the plasmid for downstream cloning events. The sample labelled control was undigested, while the linearized sample has been digested. The expected size of the digested plasmid is 5909 bp.

4.1.2. Determining the correct annealing temp to amplify the mNG + α MTS and mNG + α octapeptide amplicon

To find the optimal annealing temperature for the primer pairs used to amplify the mNG + α MTS and mNG + α octapeptide amplicons, a gradient PCR was performed. As stated in the methods, the annealing temperature for the various samples ranged between 50-57 ℃. Having the first three cycles set with a gradient around the expected TM of the specific sequence of the primer that anneals the DNA template, while the following 27 cycles set with a gradient around the expected TM of the entire primer sequence. In total, 8 PCR reactions were performed for each amplicon, which were then resolved on a 1% TAE agarose gel after applying 100 V for 45 minutes.

The ethidium bromide stained DNA gel was then visualized with the Chemidoc using a UV light source. Each PCR sample was observed to have a single band of the expected size, 810 bp for mNG + α MTS and 765 bp for mNG + α octapeptide (Figure 7).

To ensure that we generated enough of the amplicons for downstream cloning, we performed two 50 µl reactions of each amplicon. As expected, a single band of the expected sizes was resolved on an 1% agarose gel stained with EtBr (Figure 8). The experiment was run for 40

minutes at 100V. The DNA bands were extracted from the gel using the Gen Elute Kit (Cat. No. NA1111), which produced DNA concentrations of 90 ng/µl (Table 33). The purity for the extracted DNA is important for downstream enzymatic reactions and it can be estimated from the ratio of the absorbance at 260 nm to 280 nm. Our isolated DNA samples appeared to be of good quality since the A_{260}/A_{280} was between 1.8-2.0.

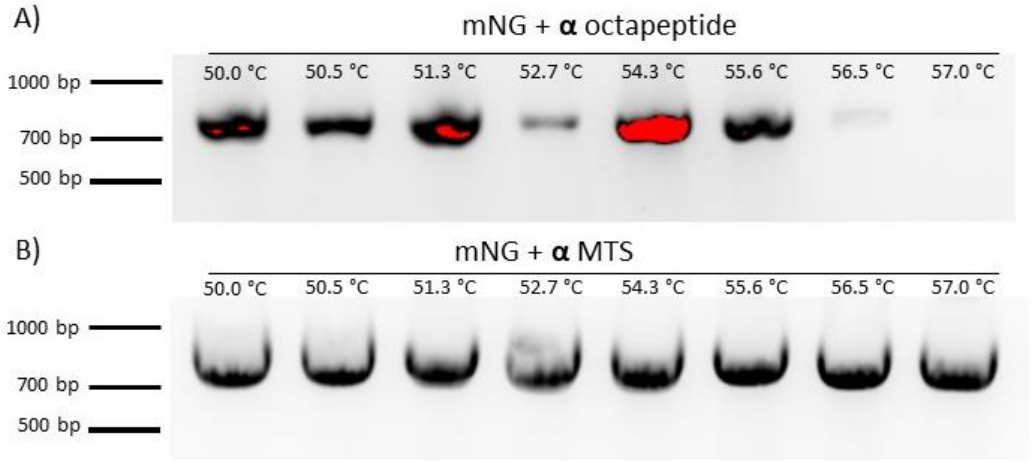

Figure 7: A) Mng + α octapeptide and B) mNG + α MTS gradient PCR amplicons resolved on a 1% agarose gel, to conclude the optimal annealing temperature for the primer pairs. The annealing temperatures for each sample are indicated above the amplicon. The expected amplicon sizes are 810 bp for mNG + α MTS and 765 bp for mNG + α octapeptide. The red color within the DNA bands indicate saturated pixels.The results were variable for mNG + α octapeptide, but the temperature that resulted in the highest levels of the amplicon was 54.3 ℃. Since the output for the mNG + α MTS was fairly consistent, we also chose 54.3 ℃ so both PCR reactions could be run at once on the same thermocycler.

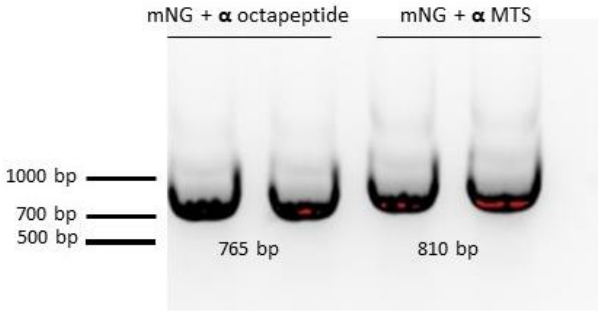

Figure 8: mNG + α octapeptide and mNG + α MTS PCR amplicons resolved on a 0.8% agarose gel.

Table 33: Concentration and purity of the gel extracted amplicons for mNG + α MTS and mNG + α octapeptide

Amplicon	Concentration	A260/A280
mNG + α MTS	90.3 ng/µl	1.89
mNG + α octapeptide	90.5 ng/µl	1.91

4.1.3. Preparing the amplicons for cloning into digested pT7-3V5-PAC

After the gel extraction, 60 µl of each amplicon was digested with 2 µl of BamHI and HindIII restriction enzymes, in order to create sticky overhangs that can be used to anneal to the same overhangs generated in the plasmid backbone. To remove the enzymes and the digested short nucleotide ends, the restriction digest was cleaned using the GenElute PCR clean-up kit (Cat. No. NA1020-1KT). The resulting yields of ~66% of the starting amplicons is quite good and the DNA contains few impurities as the A_{260}/A_{280} values were fine (Table 34).

Table 34: Concentration and purity of the mNG + α MTS and mNG + α octapeptide amplicons digested with BamHI and HindIII.

Amplicon	Concentration	A260/A280
mNG + α MTS	59.6 ng/µl	1.96
mNG + α octapeptide	62.5 ng/µl	1.94

4.1.4. Ligations of mNG amplicons into pT7-3V5-PAC

Following the digestion of the DNA amplicons and the vector, ligation reactions were performed in a 3:1 molar ratio of insert to vector in a total volume of 10 µl to promote the circularization of the plasmid with the amplicon insert. First, to calculate the molarity of each DNA molecule, Equation (1) was used.

$$(1) \quad Molarity = \frac{DNA\ concentration\ \left(\frac{ng}{\mu l}\right)}{nt\ average\ weight\ \left(\frac{g}{mol}\right) * length\ (number\ of\ nucleotides)}$$

$$\mu M_{pT7-3V5-PAC} = \frac{110\,\frac{ng}{\mu l}}{0.325\,\frac{g}{mol} * 5909\ bp} = 0.057 \mu M = 57\ nM$$

$$\mu M_{mNG+\,\alpha\,MTS} = \frac{59.6\,\frac{ng}{\mu l}}{0.325\,\frac{g}{mol} * 810\ bp} = 0.22\ \mu M = 22\ nM$$

$$\mu M_{mNG+\,\alpha\,octapeptide} = \frac{62.1\,\frac{ng}{\mu l}}{0.325\,\frac{g}{mol} * 765\ bp} = 0.24\ \mu M = 24\ nM$$

In order to maintain a 3:1 ratio of insert to vector in a 10 µl reaction, the plasmid was first diluted 1:1 with MilliQ water, resulting in a molarity of 28.5 nM. Then the amount of DNA required for each insert was calculated using Equation (2).

$$(2) \quad Volume = \frac{plasmid\ molarity * molar\ ratio}{molarity\ of\ insert}$$

$$V_{mNG+\,\alpha\,octapeptide} = \frac{28.5\ nM * 3}{24\ nM} = 3.56\ \mu l$$

$$V_{mNG+\,\alpha\,MTS} = \frac{28.5\ nM * 3}{22\ nM} = 3.89\ \mu l$$

4.1.5. Restriction digest analysis to verify plasmids in positively selected bacterial clones

To determine if these bacterial clones actually contained the pT7-3V5-PAC plasmid with either the mNG + α MTS or mNG + α octapeptide amplicons, a restriction digest was performed on isolated DNA and then resolved on an agarose gel to determine the sizes of the digested DNA. First, five colonies from each transformation plate were replica plated before innoculating a 5 ml overnight LB culture. From this liquid bacterial culture, plasmid DNA was isolated the following morning using the GenElute Miniprep kit (Cat. No. NA0140). Fairly robust quantities of DNA were successfully isolated from most clones, with the lone exception being clone 3 of the pT7-3V5-PAC mNG + α MTS (Table 35). However, since we only needed 1 µg plasmid DNA for the restriction digest analyses, we had obtained enough high quality DNA to continue.

Table 35: Concentration and purity of isolated plasmid DNA from bacterial colonies transformed with either pT7-3V5-PAC mNG + α octapeptide or mNG + α MTS.

Plasmid	Clone	Conc. (ng/µl)	A_{260}/A_{280}
pT7-3V5-PAC mNG + α MTS	1	296.0	1.84
	2	327.8	1.85
	3	62.8	1.78
	4	332.0	1.82
	5	325.8	1.85
pT7-3V5-PAC mNG + α octapeptide	1	299.8	1.78
	2	347.7	1.84
	3	158.2	1.80
	4	150.2	1.77
	5	257.6	1.87

The isolated DNA was digested with BamHI and HindIII restriction enzymes to remove any inserted DNA fragment that would be identified based on its size on an agarose gel. While the size of the pT7-3V5-PAC digested backbone should be 5909 bp irrelevant of the inserted amplicon, the released insert should be 802 bp for mNG + α MTS and 757 bp for mNG + α octapeptide. Indeed, all five clones selected from each transformation contained the expected DNA fragment sizes (Figure 9), with the mNG + α octapeptide inserts migrating slightly farther through the gel.

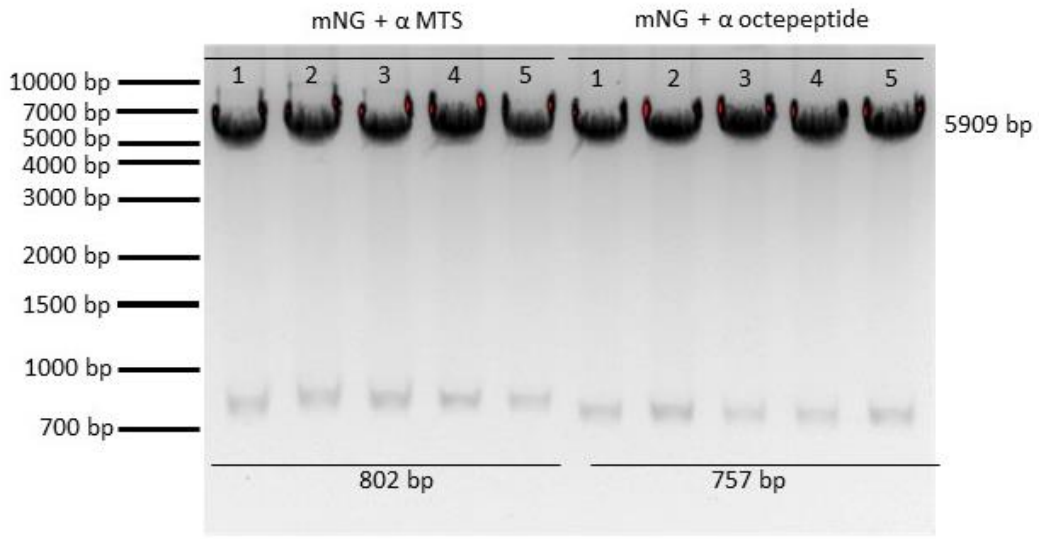

Figure 9: Isolated plasmid DNA verified by restriction digest analysis. Clones 1-5 for each pT7-3V5-PAC plasmid are indicated under the heading mNG + α MTS or mNG + α octapeptide. The digested plasmid backbone is predicted to run at 5909 bp, while the sizes of the two different inserted DNA fragments are indicated at the bottom.

4.1.6. Sequencing results from a single clone of each plasmid

While all of the analysed clones for both plasmids produced DNA fragments of the expected sizes, we needed to confirm that there were no mistakes in the inserted DNA sequence that could potentially lead to a frame shift mutation. Therefore, a single clone of each plasmid was sequenced (SeqMe s.r.o.) through the region of the plasmid that encodes for the fusion reporter protein. Each plasmid was sequenced in the forward direction with a primer that anneals to the T7 promoter, along with a lab specific primer (AZ0468) designed to sequence the inserted DNA in the reverse orientation. This dual sequencing information would allow us to determine if any errors were just sequencing problems or real mutations introduced into the plasmid DNA.

The resulting ABI chromatograms were imported into Geneious and aligned with the *in silico* generated plasmids (Figure 10). The sequencing results provided clear, discernable peaks for each of the nucleotides, observing no mismatches within the protein coding sequence from either the forward or reverse sequencing reactions. We can observe the start methionine and the 3' TAG stop codon. The fused *T. brucei* F1-ATPase subunit α sequences are in-frame with the coding region of the mNG protein. The C-terminal 3xV5 tag is also in-frame with the encoded protein. These results would predict the correct expression of the mNG reporter proteins.

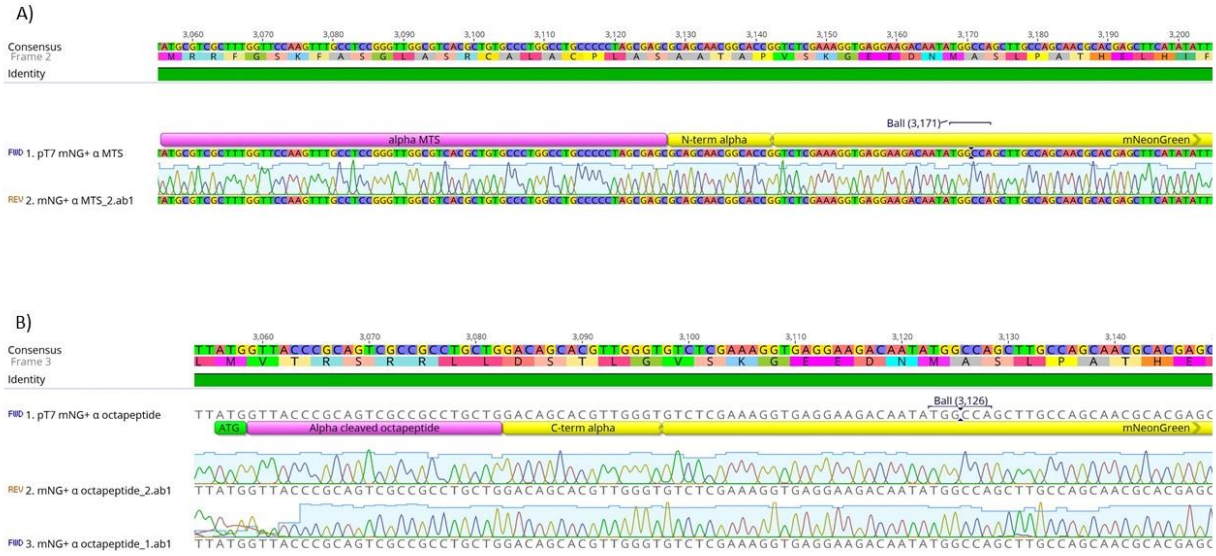

Figure 10: Sanger sequencing results of A) the pT7-mNG + α MTS plasmid and B) Sanger the pT7-mNG + α MTS plasmid. The upper row represents the consensus nucleotide sequence where the green bar represents regions of the aligned molecules with identical nucleotides. The consensus sequence is also translated to depict the encoded amino acids in the single letter code. The sequence immediately beneath the consensus sequence is the Geneious generated *in silico* plasmid with the indicated protein domains of the expressed protein. The last two rows are the sequencing generated ABI files that provide the chromatograms that can be analysed for correct nucleotide assignment. These images represent merely a portion of the entire protein coding sequence as the alignment was zoomed in to visualize the details of the chromatogram.

4.1.7. Plasmid preparation for *T. brucei* transfections

In order to increase the rate of homologous recombination of the plasmid DNA into the *T.brucei* genome, the plasmid needs to be linearized within the rDNA intergenic spacer region. Typically ~20 µg of plasmid DNA is digested with excess NotI, an enzyme that cuts only once within the rDNA region of the plasmid. The digested plasmid is then precipitated and resuspended in 30 µl of sterile MilliQ water. A small sample of the linearized plasmids were resolved on an agarose gel (Figure 11). Not only did they migrate differently from the uncut plasmids, but they also produced a single band at the expected sizes. Additionally, the concentration and purity of these linearized plasmids were determined on the Nanodrop (Table 36). 12 µg of this pure DNA was then used to transfect PF *T. brucei*.

Table 36: Concentration and purity of DNA used to transfect PF T. brucei.

Amplicon	Concentration	A260/A280
mNG + α MTS	961.7 ng/µl	1.81
mNG + α octapeptide	861.9 ng/µl	1.79

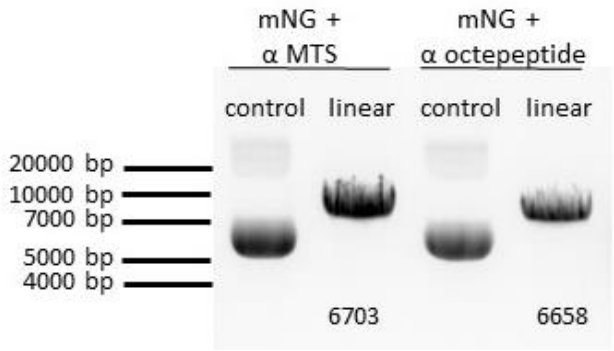

Figure 11: NotI linearized plasmids prepared for PF *T. brucei* transfections. The control samples represent undigested plasmids, whereas the linearized samples are the NotI digested plasmids used for transfections. The linearized pT7-3V5-PAC plasmids migrated at their expected values: mNG + α MTS is 6,703 bp, while mNG + α octapeptide is 6,658 bp.

4.2. Characterization of generated *T. brucei* cell lines

4.2.1. Expression levels of mNG reporter proteins determined by western blot analyses

Now that the cell lines have been generated, we need to determine if they are able to express the mNG reporter proteins in a regulated manner. The addition of tetracycline to the media will bind the tetracycline repressor protein (TetR) and cause a conformational change that releases the inhibitory protein from the DNA, allowing the transcription of the mNG reporter by the introduced T7 polymerase. Several positively selected clonal cell lines transfected with either of the mNG reporter proteins were split into two flasks, with one flask of each clone induced with tetracycline for three days. After this induction period, $7x10e^7$ cells were harvested from both the induced and uninduced cell cultures. The harvested cells were resuspended in a standard SDS-PAGE loading dye to generate whole cell lysates. The denatured proteins from the cell equivalents of $1x10e^7$ trypanosomes were resolved on a 4-20% polyacrylamide gel (BioRad Mini-PROTEAN® TGX Stain-Free™ Precast Gels Cat. No. 4568094) before being transferred to a PVDF membrane. The blots were probed with a 1:500 dillution of the APRT antibody and a 1:1000 dilution of the V5 antibody. Using ECL reagent (BioRad, cat. no. 1705060EM), the antigens were detected on the BioRad Chemidoc using the ImageLab software (Figure 12). The bands observed were of the expected sizes: APRT is 26 kDa and the V5 tagged mNG proteins are between 34 - 35 kDa.

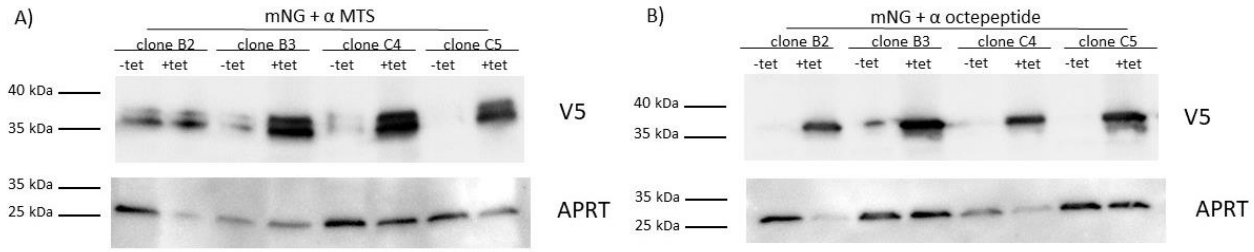

Figure 12: **Western blot analysis of A) mNG + α MTS and B) mNG + α octapeptide protein expression.** The uninduced (-tet) and induced (+tet) samples of each clone are depicted. The upper panel is the blots probed with the V5 antibody (1:1000 dilution), while the lower panel was probed with APRT (1:500 dilution). The sizes of the relevant protein markers are indicated on the left.

Most of the cells lines tested demonstrated good levels of protein expression in the tetracycline induced cells. However, it is interesting that the mNG + α octapeptide protein is detected as a signal band at the expected size, but the mNG + α MTS proteins appear as a tight doublet. Next, we would like to determine if there is tight regulation of the mNG protein expression, in which we would expect to observe a V5 signal only in the samples that had been induced with tetracycline. Such was the case for the cell lines expressing the mNG + α octapeptide (Figure 12B), where most clones did not detect any signal in the -tet samples. On the other hand, we observed a V5 signal for most of the mNG + α MTS uninduced samples (Figure 12A), except for clone C5.

To make sure that the difference in the mNG protein expression levels was not affected by the amount of sample loaded, we used the expression levels of the cytosolic APRT as a loading control. The expression levels of this protein should remain unchanged between the samples if everything was loaded consistently. However, we observed very uneven expression levels of APRT between the samples. The most problematic loading controls concerned clone B2 of both the mNG + α MTS and the mNG + α octapeptide cell lines. Since the levels of APRT differed significantly between the uninduced and induced samples for these clones, we cannot confidently assign the levels of mNG protein expression in these clones. Therefore, we selected the clone that had the most consistent levels of APRT expression and the least amount of V5 signal in the uninduced cells for further characterization (B3 of mNG + α MTS and C5 of mNG + α octapeptide).

4.2.2. Subcellular *T. brucei* localization of the mNG reporter proteins

Now that we have generated and verified the cells lines expressing the mNG reporter proteins, we can determine if the F_1-ATPase alpha octapeptide can act as a mitochondrial targeting signal. The selected clones of the mNG + α MTS and mNG + α octapeptide cell lines were again split into flasks, with one of each induced with tetracycline for 3 days. After the induction period, some cells were harvested from each condition and used to generate whole cell lysates. Additional cells were also treated with SoTe + 0.03% digitonin before a differential centrifugation step isolated the cytosolic fraction from the organellar fraction. Equal amounts of the protein samples based on original amount of cells were resolved on multiple 4-20% polyacrylamide gels before being transferred onto a PVDF membrane. Each blot was probed

either with the APRT antibody (1:500 dilution), mtHSP70 antibody (1:1000 dilution) or the V5 antibody (1:1000 dilution) (Figure 13).

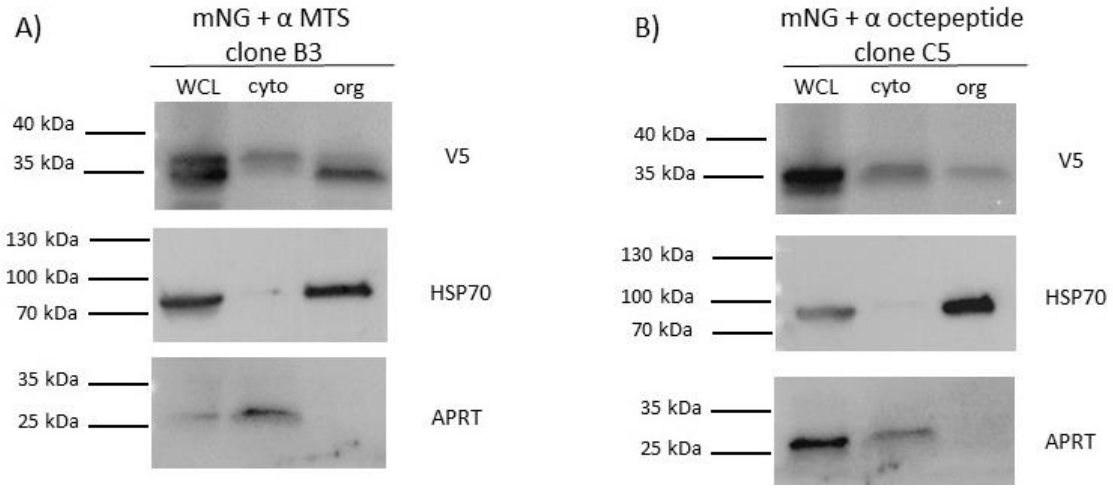

Figure 13: **Western blot analysis of A) mNG + α MTS and B) mNG + α octapeptide subcellular localization.** The top panel was probed with V5 antibody (34-35 kDA, 1:1000 dilution), the middle panel with mtHSP70 (70 kDA, 1:1000 dilution) and the bottom panel with cytosolic APRT (26 kDa, 1:500 dilution). The relevant protein size markers are indicated on the left. WCl- whole cell lysates, cyto – cytosolic fraction, org – organellar fraction.

To verify how well the subcellular fractionation was executed, we observe that the cytosolic APRT is confined to the cytosolic fraction as it is undetected in the organellar fraction. The vast majority of the mitochondrial HSP70 is detected in the organellar fraction, although there is a very faint band also present in the cytosolic fraction. This would indicate that a small percentage of the mitochondria were also lysed during the 0.03% digitonin treatment. Due to the strict confinement of the control proteins to their respective compartments, we can interpret how well the mNG reporter proteins were targeted to the mitochondrion. Again, we detect a doublet of the mNG + α MTS in the whole cell lysate, with the upper and lower bands being the same intensity. However, it is predominantly only the upper band detected in the cytosolic fraction and the lower band in the organellar fraction. The ratio of the V5 signal between the two fractions appears to suggest that most of the mNG is targeted to the mitochondrion. However, we observe a different pattern for the mNG + α octapeptide, where the single band of expected size is predominantly localized in the cytosolic fraction. The small amount of V5 signal detected in the organellar fraction does indicate that the F_1-ATPase alpha octapeptide is able to target some of the normally cyctosolic mNG to the mitochondrion.

5. Discussion

5.1. The subcellular fractionation analysis

The main aim of this thesis was to determine if the cleaved α octapeptide can target a cytosolic reporter protein to the mitochondrion of *T. brucei*. A positive result would indicate that the proteolytic processing of the nuclear encoded F_1-ATPase α subunit might already begin in the cytosol. Since the mNG + α octapeptide reporter was very inefficiently targeted to the mitochondrion, our preliminary results would suggest that the proteolytic cleavages of subunit α likely occur within the mitochondrion. However, since the data was not completely black and white, this set of experiments did not completely rule out the possibility that subunit α is first cleaved at the N-terminus of the octapeptide in the cytosol and then both the N-terminal α and C-terminal α are recruited to the mitochondrion via an N-terminal targeting sequence that is further processed once imported into the mitochondrial matrix. If more time was available, it would be optimal to repeat this experiment with a few more important controls. The following discussion will try to further interpret the observed results and suggest future experiments that can decipher the ability of the octapeptide to act as an N-terminal targeting sequence.

The subcellular localization protocol used in this thesis is well established in the lab. However, there are critical aspects of the procedure that were previously optimized and should be discussed briefly so our data can be interpreted properly. The principle of the subcellular fractionation is to lyse the plasma membrane with a mild detergent without disrupting the internal compartments of the cell, thus keeping the mitochondria intact. This lysis is followed by a differential centrifugation step that separates the cytosolic material from the organelles. Important variables in the assay include the type of detergent used, the amount of detergent for a specific number of parasites, the length of incubation and the centrifugation parameters. To verify the quality of the two main subcellular fractions, the samples were probed with antibodies to proteins that are strictly localized within determined cellular compartments. As a cytosolic marker, we utilize an antibody against adenine phosphoribosyltransferase (APRT), an enzyme involved in the purine salvage pathway of the parasite, while the multifunctional heat shock protein mtHSP70 is used as a mitochondrial marker. If the ratio of detergent to the number of parasites was too small, it would result in the incomplete lysis of the plasma membrane and the differential spin would generate an organellar fraction containing many intact parasites. Western blot analyses of these improperly lysed samples would detect the APRT signal also in the organellar fraction, which is not the case

in our experiment. Another possibility is that the ratio of digitonin to the number of parasites would be too large, resulting in the lysis of some mitochondria. In this scenario, an mtHSP70 signal in the cytosolic fraction would be noticeable. While our data indicate that the mtHSP70 signal is predominantly in the organellar fraction, a very faint band can be detected in the cytosolic fraction. This will probably have a negligible effect on our interpretation of the localization of the V5 tagged mNG proteins, but it should be noted that a very small amount of V5 signal detected in the cytosol might be due to some mitochondrial contamination. Due to the overall high quality of the controls, we can be fairly confident that our subcellular fractionation worked well and that the observed localization of our mNG reporter represents the ability of the protein to be imported into the mitochondria of *T. brucei.*

5.1.1. mNG + α MTS

In Figure 12A and 13A, we observe a double band in the whole cell lysates (WCL) of the mNG + α MTS samples. This could indicate that about 50% of the expressed protein remains intact as the full-length version, while the rest of the protein enters the mitochondria where the N-terminal targeting signal is proteolytically removed to produce a mature matrix protein. This is further supported by the observation that the V5 signal in the cytosolic fraction is predominantly just the upper band, while the protein in the mitochondrial fraction is mostly the lower band. Even though a significant proportion of the expressed mNG protein is localized to the mitochondrial fraction, some of it can still be found in the cytosolic fraction. This could simply be due to the partial mislocalization of a heterologous protein that is significantly overexpressed within the parasite [20]. Another possibility is that in addition to an N-terminal targeting sequence, some *T. brucei* mitochondrial matrix proteins may also require an internal mitochondrial targeting sequence for efficient import [21]. However, this is usually the case for proteins that interact with the mitochondrial inner membrane.

Since the mitochondrial targeting signal (MTS) of subunit α has been verified by mass spectrometry, our results might indicate that there are some intrinsic properties of the mNG protein that make its mitochondrial import inefficient in *T. brucei*. For example, GFP and its variants have a β-barrel structure [22]. Typically proteins with this secondary structure (VDAC and TOM) are usually inserted into the mitochondrial outer membrane due to a beta-hairpin motif [23]. Since most experiments concerning GFP targeted to the mitochondria rely on simple fluorescent image overlays with proteins of known mitochondrial localization, it is fairly difficult

to determine the if the GFP is targeted to the mitochondrial outer membrane, inner membrane space, inner membrane or the matrix. However, several reports of GFP targeted to specific mitochondrial locations have been confirmed by biochemical assays that include susceptibility to protease digestion [24].

5.1.2. mNG + α octapeptide

Interestingly, we also observe a double band in the WCL for mNG + α octapeptide in Figure 12 and 13B. However, only a slight amount of the protein is detected at the lower molecular weight, which is not present in either the cytosolic or mitochondrial fractions. This double band might just be an artifact of the western blot or sample preparation and would thus need to be repeated to further verify. For example, it is possible that the lower band in the WCL is detected simply because more material was loaded for this sample. To make a comparison between the amount of detected material in the subcellular fractions and the WCL, we attempt to load volumes of our subcellular fractions that match the number of cells loaded in the WCL. However, there is no good loading control to account for minor differences between these samples. It is possible that repeating this experiment would lead to a greater signal detection in the mitochondrial fraction that would then reveal the presence of a minor proportion of the processed V5 tagged reporter.

It appears that a significant amount of this mNG reporter is localized in the cytosolic fraction, suggesting that the α octapeptide is not as efficient at targeting the reporter to the mitochondrion. Nevertheless, some of the protein does get localized to the mitochondrial fraction. The lack of a proper negative control certainly makes the interpretation of this result difficult, which is why we would need to include the subcellular fractionation data of a *T. brucei* cell line expressing only the mNG protein. This would determine whether this small proportion of mitochondrial localization is just the default of over-expressing the heterologous mNG protein. If this control did, in fact, prove to be negative, then it would suggest that the α octapeptide or the first 5 amino acids of the mature C-terminal α enhance the mitochondrial localization of mNG. Since we do not detect the smaller mature protein of this reporter in the mitochondrial fraction, it would suggest that the octapeptide targeting sequence was not proteolytically removed. This might provide further evidence that the mNG protein targeted to the mitochondria does not enter the matrix but is in fact localized to either the mitochondrial outer membrane or the mitochondrial inner membrane space. This outcome would lead to a false positive since we are testing if the α

octapeptide can target the mNG to the mitochodnrial matrix. To further resolve this possibility, we would need to treat mitochondria isolated with or without the outer membrane with proteinase K and determine if the signal for the V5 tagged mNG reporter persisted.

We included in our α octapeptide mNG reporter some additional residues from the mature C-terminal α to ensure that enough of the substrate recognition site remained to be recognized by the mitochondrial protease upon entering the matrix. Therefore, another necessary control would be the mNG fused with only the first 5-10 amino acids of the mature C-terminal α. If this reporter was equally efficient at mitochondrial import as the mNG + α octapeptide, it would suggest that maybe there is some intrinsic mitochondrial targeting sequence within the N-terminus of the mature C-terminal α. This would again cast doubt about the role of the octapeptide to act as an N-terminal mitochondrial targeting sequence for the mature C-terminal α subunit. Indeed, experiments performed by other members of the lab indicate that a V5 tagged mature C-terminal α subunit is predominantly localized to the *T. brucei* mitochondrion.

To exclude any complications of using a beta-barrel protein as a reporter for mitochondrial protein import, it might be better to utilize the mouse cytosolic DHFR protein as it has been used previously for this purpose [25, 26, 27]. Since *T. brucei* DHFR is a mitochondrial-targeted protein, it appears that there are no inherent structures that would hinder its import into the mitochondrion. Using the mouse cytosolic DHFR that lacks a mitochondrial target signal, we can better determine if different regions of the α subunit might be involved in the mitochondrial targeting of the mature C-terminal α. This would add a small piece of evidence about the localization of the protease that cleaves at the N-terminus of the endogenous α octapeptide.

6. Conclusion

While further studies involving more controls of a potentially better reporter protein for mitochondrial import are required, these preliminary results would suggest that the α octapeptide cannot efficiently target the mature C-terminal α subunit to the mitochondria by itself. This would suggest that all of the F_1-ATPase subunit α proteolytic maturation events occur within the mitochondrion of *T. brucei*.

7. References

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