The University of South Bohemia in České Budějovice

Faculty of Science

BACHELOR THESIS

2014 **Adéla Křížová**

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Functional Analysis of Protein MRB8620 of the Mitochondrial RNA Binding Complex 1 of *Trypanosoma brucei*

Bachelor Thesis

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ANNOTATION

The function of MRB8620, a protein subunit of the putative mitochondrial RNA binding complex(MRB1) found in parasitic protist *Trypanosoma brucei* was studied by reverse genetics.

DECLARATION

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

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ABSTRACT

RNA editing in *Trypanosoma brucei* mitochondria (mt) is a post transcriptional processing mechanism, either incorporating or deleting the uridine (U) residues from the pre-edited precursor messenger (m)RNAs, eventually creating frame shifts to the start and stop codons, or for mt encoded mRNAs. The mt genome is comprised of ~ 50 maxicircles and thousands of minicircles. Catenated maxi- and minicircles are stuffed into a dense structure called kinetoplast (kDNA). Maxicircles transcribe 2 mt rRNA and 3 classes of pre-mRNA categorized as never edited (no U-insertion/deletion), partially edited (few U-insertion/deletion), and pan edited (large U-insertion/deletion). Minicircles transcribe majority of guide RNAs (gRNAs). There can be a lot of editing events at a single pre-edited mRNA, interspersed by few nucleotide (nt). Guide RNA binds to 3' of pre-edited transcript, providing the template for U- insertion/deletion. Guide RNA mediated editing also encompasses large repertoire of proteins constituted by several protein complexes. One such well studied RNA editing core complex (RECC), comprising of 20 proteins providing a role of catalyst in enzymatic cascade reactions like cleaving and ligation of pre mRNA during editing. The other emerging complex is Mitochondrial RNA binding complex (MRB1), regulating various critical steps in RNA editing with undefined mechanisms in most cases. MRB1 core is constituted by GAP1, GAP2, MRB3010, MRB5390, MRB8620 and MRB11870 proteins. All the core proteins are being worked out in terms of RNAi knock downs (kd) except MRB8620. Current study involves probing the essentiality of MRB8620 in mt RNA editing using RNAi kd and by making single knockout and double knockout. This preliminary work on MRB8620 by RNAi kd in glucose-free media shows the negative growth phenotype on 5th day onwards. Similarly, efforts to generate double knockout of MRB8620 were not successful as loci of MRB8620 always shifted to other location in Trypanosome genome. These two results indicate towards the essentiality of MRB8620 in mt RNA editing. To substantiate the results further qPCR were down on various pre and post edited transcripts in mt genome. qPCR results further validate the indispensable nature of MRB8620 as post edited transcripts seen large decline in relative abundance

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1. INTRODUCTION

1.1 Introduction to *Trypanosoma brucei*

Trypanosoma brucei, a unicellular protozoan parasite, is noted to cause several human and animal diseases mainly affecting sub-Saharan African countries such as human African trypanosomiasis (commonly known as sleeping sickness) and Nagana in animals, respectively. The impact of sleeping sickness on the African population is severe as confirmed by numbers provided by the World Health Organization. More than 90% of reported cases of sleeping sickness are caused by the sub-species *T. brucei gambiense*, leading to chronic infection. *T.brucei rhodesiense* is known to mainly impact East Africa. Another sub-species, *T. brucei brucei* causes Nagana in animals, therefore it is used as a model organism in laboratory research because it is not pathogenic to humans.

The order Kinetoplastida, to which *T. brucei* belongs, derives its name from the extraordinarily large kinetoplast, which represents the mitochondrial genome that can contain up to 20% of the total DNA in this cell type (Hajduk & Ochsenreiter 2010). The term kinetoplastid DNA (kDNA) is used to refer to this structure. Transmission electron microscopy shows all kDNA organized into a disc-like structure. The kDNA is comprised of two classes of DNA that have been named minicircles and maxicircles based on their respective sizes (Shapiro & Englund 1995).

The complex life-cycle of *T. brucei* is divided between several distinct environments: the midgut and salivary glands of the tsetse fly insect vector as well as the bloodstream of mammalian hosts, such as humans, monkeys, buffaloes and cattle. There can be further division based on morphologically different appearance of cell into four major stages: two bloodstream forms, one procyclic and one metacyclic form. *T. brucei* proliferates in the midgut of tsetse fly as the procyclic stage, then it migrates to the salivary glands to transform into the metacyclic form, morphologically altered cells that are then introduced into the bloodstream of the mammalian host as the tsetse fly feeds (Vickerman 1985). The bloodform is further differentiated into two stages: the proliferating slender stage and the stumpy stage that is competent for completion of the lifecycle in the fly.

In mammals, *T. brucei* present in the bloodstream as the slender form, has the ability to cover its body with variant surface glycoproteins (VSG) in order to avoid the host immune response. When *T. brucei* is present in midgut of tsetse fly the cell surface is instead of glycoproteins covered with procyclin proteins (Dreesen et al. 2007). Other major physiological changes between the procyclic form and bloodstream can be distinguished.

Whereas the bloodstream environment is glucose rich supporting the parasite growth by glycolytic ATP production, the gut of tsetse fly is glucose poor. Thus the procyclic form relies on proline gathered from this environment as a primary source of energy.

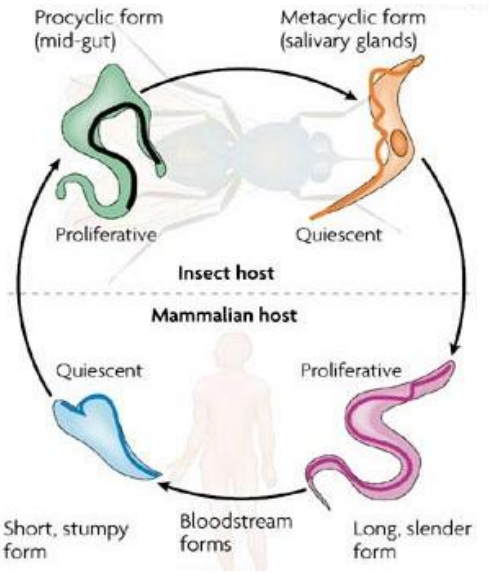

Fig. 1: Life cycle of *T. brucei*

The complex life cycle of *T. brucei* includes four major stages, that are distinguished into: two bloodstream forms (in mammalian hosts), one procyclic (in the midgut of the tsetse fly) and one metacyclic form (in the tsetse salivary gland). Bloodstream and metacyclic parasites are coated with a variant surface glycoprotein (VSG). While a procyclic parasites are coated with procyclins.

(Dreesen et al. 2007)

1.2 Maxicircles

Maxicircles, the less numerous components of kDNA, are the homologues of classical mitochondrial DNA observed in other eukaryotes, as it contains about 20 genes, most of which are typical protein coding genes, including two rRNA genes termed 9 and 12 S (Estévez & Simpson 1999). Surprisingly, 12 out of 18 mRNAs encoded by the maxicircle require post-transcriptional uridine insertion or deletion (U-insertion/deletion) in order to create appropriate open reading frames. This process is called RNA editing. Maxicircles encoded mRNAs can be classified as: 6 never-edited mRNA (requiring no U-insertion/deletion), 3 partially edited (requiring a few U-insertions) and 9 pan-edited transcripts (requiring a large amount of U-insertion/deletion).

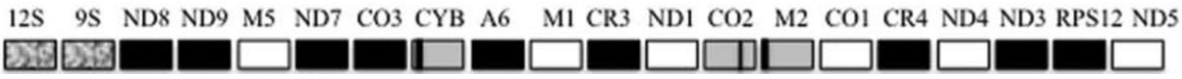

Fig. 2: Scheme of linearized maxicircle map of the mitochondrial genomes (kDNA) of *T. brucei.* On the top of each box the abbreviation for the particular genes can be found. The category of transcripts encoded by each

gene is indicated by colour: black - pan-edited RNA; grey - partially edited RNA, with the edited parts in black; white - never-edited RNA; textured grey - never-edited rRNAs.

The abbreviations denote: 12/9S - rRNAs; ND - NADH dehydrogenase subunits; M - maxicircle unidentified reading frame; CO - cytochrome oxidase subunits; CYB - apo-cytochrome b; A6 - ATPase subunit 6; CR - C-rich reading frame; RPS12 - ribosomal protein 12.
(Hajduk & Ochsenreiter 2010)

1.3 Minicircles

More than 95% of the total amount of the kDNA network is comprised of minicircles. Approximately 5000 minicircles per kinetoplast form a single large network, in which a minicircle is interlocked with three neighbouring molecules (Chen et al. 1995). The total minicircle population exhibits broad sequence heterogeneity (Onn et al. 2004). Each minicircle encodes 3 - 5 gRNA, which contains the genetic information needed for RNA editing as they are the template for editing of encrypted maxicircles transcripts. These relatively small gRNA molecules are in range of 50 - 70 nucleotides long and have a 3'-end containing post-transcriptionally added poly (U) tails (Blum et al. 1990).

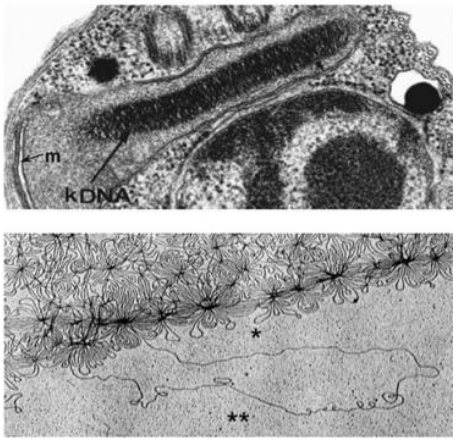

Fig. 3: Electron microscopy reveals the compact organisation of the mitochondrial DNA of *T. brucei* called kDNA. The close proximity to the mitochondrial membrane (m) is shown. Spreads of the kinetoplast DNA in the picture below show the concatenated network of mini- (*) and maxicircles (**)
(Hajduk & Ochsenreiter 2010)

1.4 The kRNA metabolism in *Trypanosoma brucei*

The kRNA metabolism in *T. brucei* consists of intertwined processes that require the cooperation of several complexes and enzymes. Although the mini- and maxicircle transcripts undergo different maturation pathways before gRNAs duplex with their cognate mRNAs, just a single mitochondrial RNA polymerase (mtRNAP) seems to be required for

their synthesis (Hashimi et al. 2009). Transcription of minicircles yields polycistronic gRNA transcripts. Endonucleolytic cleavage of these long molecules is needed in order to obtain individual gRNAs. For this purpose, minicircles are cleaved by a 19S protein complex into one or more gRNAs, followed by the process of polyuridylation of these transcripts by a TUTase dedicated for appending the 3'- ends of transcripts (Grams et al. 2000; Aphasizhev & Aphasizheva 2003).

In the case of maxicircles, large polycistronic transcripts have been detected, suggesting an endonucleolytic mechanism for the cleavage of these molecules into individual mRNAs and rRNAs. The identity of the enzymes performing this task remains unknown. RNA editing and the cleavage of the long precursor into monocistronic transcripts seem to be steps occurring independently of each other (Koslowsky & Yahampath 1997).

The kinetoplast poly(A) polymerase 1 (KPAP1) is the nucleotidyl transferase responsible for the addition a short(A) tail to the 3'-ends of individual mRNAs (Etheridge et al. 2008). The TUTase responsible of the short poly(U) tails also plays a role in 3'-tailing of mRNAs in addition to that of gRNAs (Stagno et al. 2007).

The process of mRNA 3'-tailing is very complex and the length of the poly(A/U) tail plays the major role for determination of the fate of the molecule (de Souza 2010). Short $poly(A)_{20-25}$ tails are present in pre-edited mRNA. In case of never- and fully-edited transcripts either short $poly(A)_{20-25}$ or long $poly(A/U)_{120-200}$ expansions are observed (Etheridge et al. 2008). The short 3' mRNA tails appear to affect transcript stability, while addition of long tails likely marks mRNAs as capable for translation (Aphasizheva & Aphasizhev 2010). However, the exact mechanism of all processing events has to be further investigated.

1.5 RNA Editing

U-insertion/deletion RNA editing is a process unique to kinetoplastids. Small gRNAs are composed of 3 domains. The 8 - 12 nucleotide anchor region positioned at the 5' end of the gRNA hybridizes to a complementary sequence on the pre-edited mRNA, via both normal and non-canonical guanine (G):U Watson-Crick base pairing (Hashimi et al. 2013). The middle part contains the information domain guiding the exact location and number of U-insertions/deletions. The editing sites (ES), where U-insertion/deletion occurs on the mRNA, is marked by a base-pair mismatch between gRNA and the to be edited mRNA (Kable et al. 1996), which is the target site for an endonuclease to cleave the mRNA into 3' and 5' fragments (Maslov & Simpson 1992). In dependence of the gRNA information domain

sequence downstream of the anchor domain, one or more Us are either inserted or deleted from the 5' cleavage product. During the U-insertion editing process, Us are added to the 3' hydroxyl of the 5' fragment by a terminal uridyltransferase (TUTase) (Aphasizhev et al. 2002). In the case of U-deletion editing, an enzyme termed an exonuclease removes U residues from the 3' end of the 5' cleavage product. Once the ES is edited, both fragments are joined back together by RNA ligase (Hashimi et al. 2013).

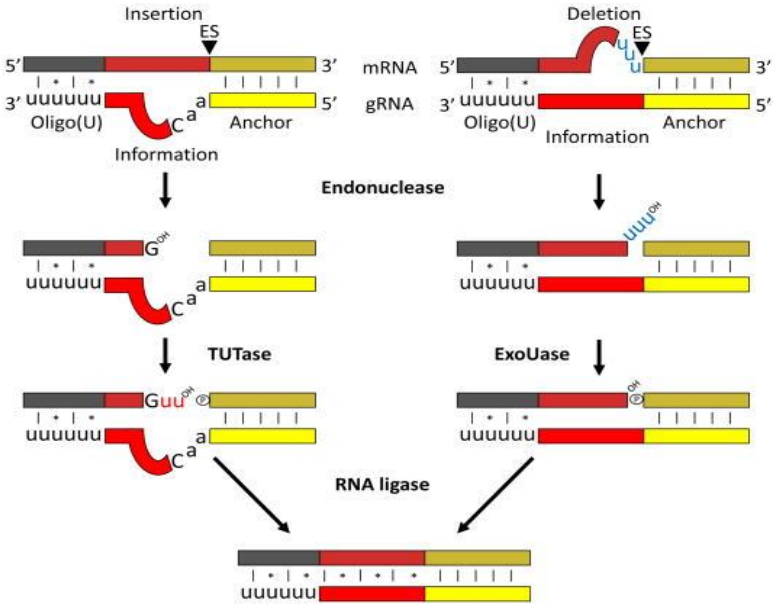

Fig. 4: Mechanism of RNA editing. The first base-pair mismatch in the duplex between gRNA and mRNA indicates the editing site ES, which is then cleaved by an endonuclease. In U-insertion, Us are added to the 3′-end of the 5′-mRNA fragment by a terminal uridylyltransferase (TUTase) as directed by the gRNA. In U-deletion, U-specific exonuclease cuts away extra Us on the 3'-end of the 5'-mRNA fragment. An RNA ligase then links the two mRNA cleavage products back together again after the appropriate editing event is completed (Hashimi et al. 2013).

1.6 The RNA editing core complex (RECC)

Whereas gRNAs serve as the informational element of RNA editing, a cascade of enzymatic activities is also required for this process. There are several macromolecular complexes whose proteins constitute the kRNA editing machinery. One of the best characterized complexes is the RNA editing core complex, known simply as RECC or the 20S editosome. RECC is comprised of 20 protein subunits that contains and coordinates the aforementioned enzymatic activities for U-insertion/deletion events (Carnes et al. 2008).

There are three major forms of functionally and compositionally different RECC, each bearing a defining endonuclease such as KREN1, KREN2 and KREN3 (Carnes et al. 2011). KREN2 cleaves the mRNA at the ES that requires U-insertion, while KREN1 does so for

ESs engaged to undergo U-deletion. The third KREN3 specifically cleaves the cytochrome oxidase subunit II (COII) mRNA, which does not need a traditional gRNA as its 3'-UTR has this element (Golden & Hajduk 2005). All three editing endonucleases are essential and have a single conserved RNase III motif required for the cleavage activity (MacRae & Doudna 2007).

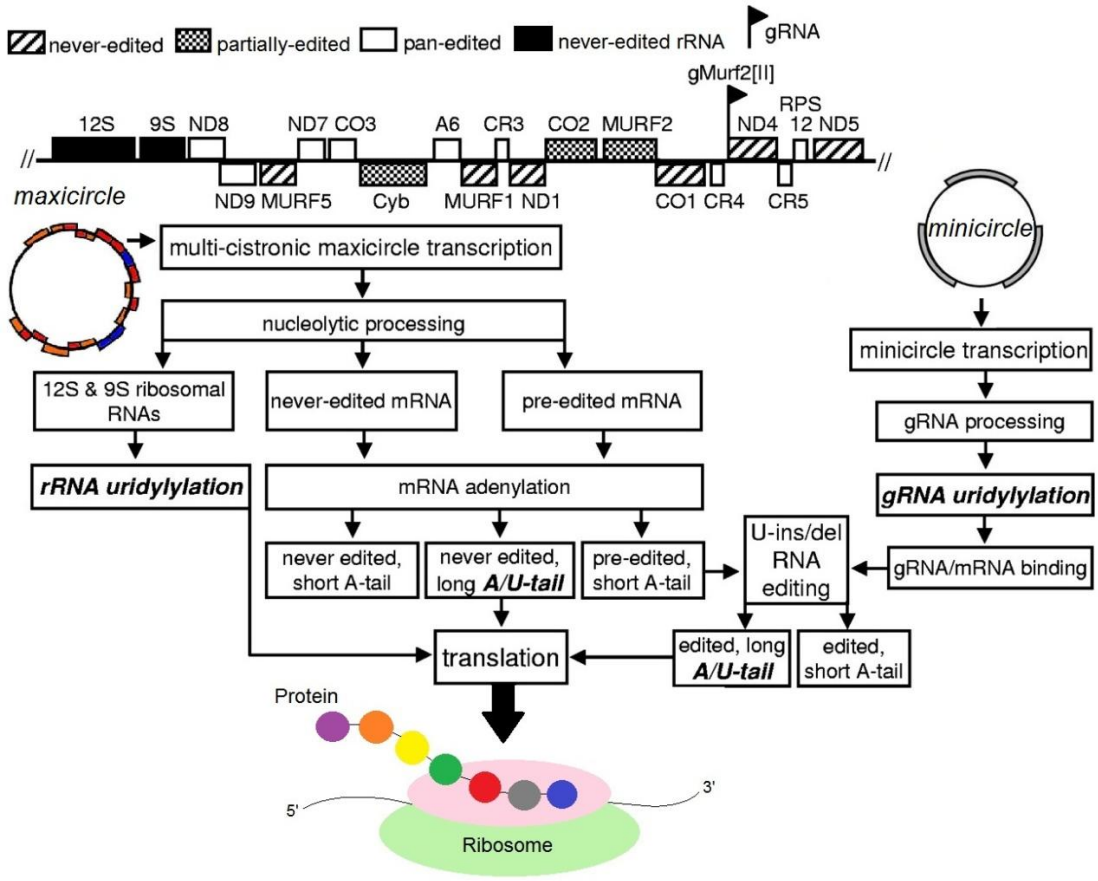

Fig. 5: Summary of kDNA metabolism of *T.brucei*. Maxi- and minicircle DNA leading to independent production of two types of transcripts - polycistronic mRNA - rRNA precursors and gRNA precursors, respectively. Schemes of linearized maxicircle map and non-linearized form are present. For linearized maxicircle: RNAs placed above the thick line are encoded from the same strand; RNAs shown below are transcribed in the opposite DNA strand. For non-linearized maxicircle: genes encoding pan-edited mRNAs are in red, minimally edited in blue, and never-edited in orange, whereas, the second circular structure represents minicircle with genes encoding gRNA in grey. RNA metabolism is described step by step by scheme with all the important events indicated in boldface type.

The polycistronic precursors are subsequently cleaved by an unknown endonuclease to yield monocistronic RNAs. The rRNAs attain short 3'(U) tails and are included into ribosomes; gRNAs preserve a triphosphate at the 5'-end but 3'-end goes through process and gets uridylated. Then, gRNA initiates the RNA editing process by U-insertion/deletion open reading frames are being generated. Whenever the mRNA editing is complete the existing 3'(A) tail is prolonged by the addition of an A/U heteropolymer.

Adapted from (Aphasizheva & Aphasizhev 2010; Hashimi et al. 2013).

1.7 Mitochondrial RNA-binding complex

Apart from RECC, RNA editing in the mitochondrion of *T. brucei* is promoted by another complex known as the Mitochondrial RNA-binding complex (MRB1) (Hashimi et al. 2013). MRB1 is composed of subunits which seem to have diverse functions, therefore the identification of their roles is crucial for understanding RNA editing as well as the integration of this process in other RNA maturation pathways. There are several proteins carrying signature motifs and domains signifying a role in RNA metabolism; others do not have such easily identifiable features and in addition are unique to the order Kinetoplastida (Hashimi et al. 2013; Hashimi et al. 2009). In general, it is thought MRB1 complex may play the pivotal role in coordination of RNA editing, stability, translation and polyadenylation. The idea of multifunctional processing of MRB1 can be supported by several results shown based on RNAi knockdown experiments of MRB1 subunits that results in a variety of different phenotypes.

The architecture of complex MRB1 was revealed, which has brought better understanding of the phenotypes resulting from the functional analyses of the MRB1 constituents. The complex is shown to consist of a core complex consisting of six proteins (GAP1, GAP2, MRB3010, MRB5390, MRB8620 and MRB11870), and interact with additional subcomplexes and proteins directly or in a manner enhanced by the RNA presence (Hashimi et al. 2013) To describe the architecture of the MRB1 composition, a comprehensive yeast two hybrid screen was used in order to map the interaction between 31 proteins present in various MRB1 isolations (Ammerman et al. 2012).

Among all core proteins, the functional analysis of MRB8620 protein has remained the last and therefore represents the main focus of the thesis as all the other proteins proved to be essential for the viability of *T. brucei* by affecting variety of aspects of the RNA editing process.

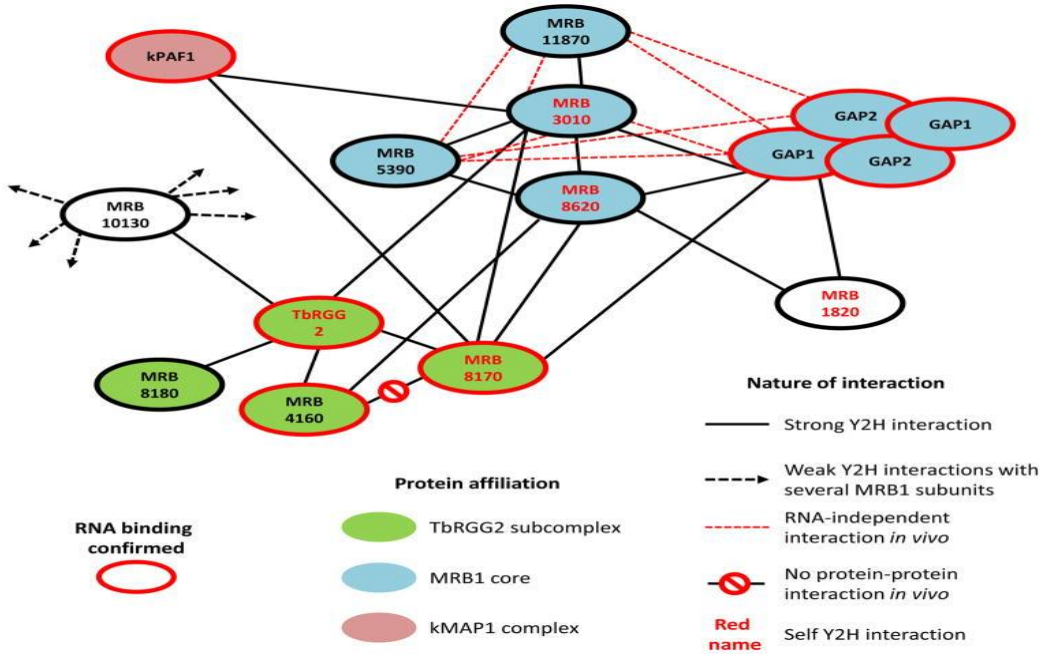

Fig. 6: MRB1 complex architecture. The resulting summary of the pair wise comprehensive yeast two hybrid (Y2H) screen that mapped MRB1 subunit interactions. Mutually exclusive interaction of MRB8170 and MRB4160 with TbRGG2 together with the heterotetrameric nature of the GAP1 and GAP2 interaction are shown. The legend to the figure describes different kinds of interactions.
(Hashimi et al. 2013)

2. MATERIALS AND METHODS

2.1 Knockout Construct Preparation

To create a knockout (KO) cell-line, two plasmids were generated to target each *MRB8620* allele: pLEW13-BLAS-sKO and pLEW13-HYGRO-dKO. These constructs were generated before my arrival in the lab, but I will briefly describe their construction. To generate pLEW13-BLAS-sKO, the 5' and 3' UTRs of MRB8620 were PCR-amplified from *T. brucei* 427 strain genomic DNA. The 380 bp MRB8620 5' UTR and 360 bp MRB8620 3' UTR PCR products were cloned into the Xho1/Sal1 and Xba1 sites of pLEW13, respectively creating the single knockout construct. The double knockout plasmid was created by replacing the Xba1 fragment containing Blast marker of pLEW13-BLAS-sKO with the Swa1/Stu1 fragment containing Hygromycin marker.

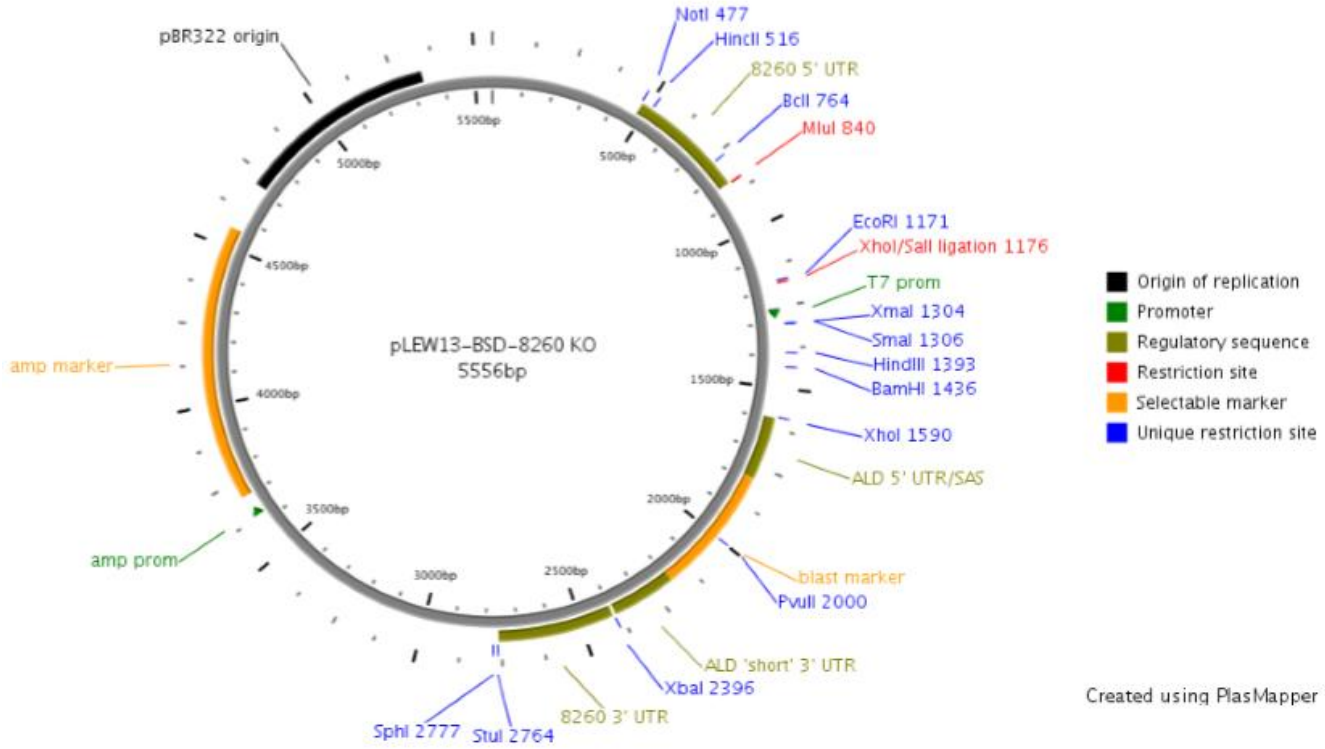

Fig. 7: pLEW13 plasmid created by using PlasMapper

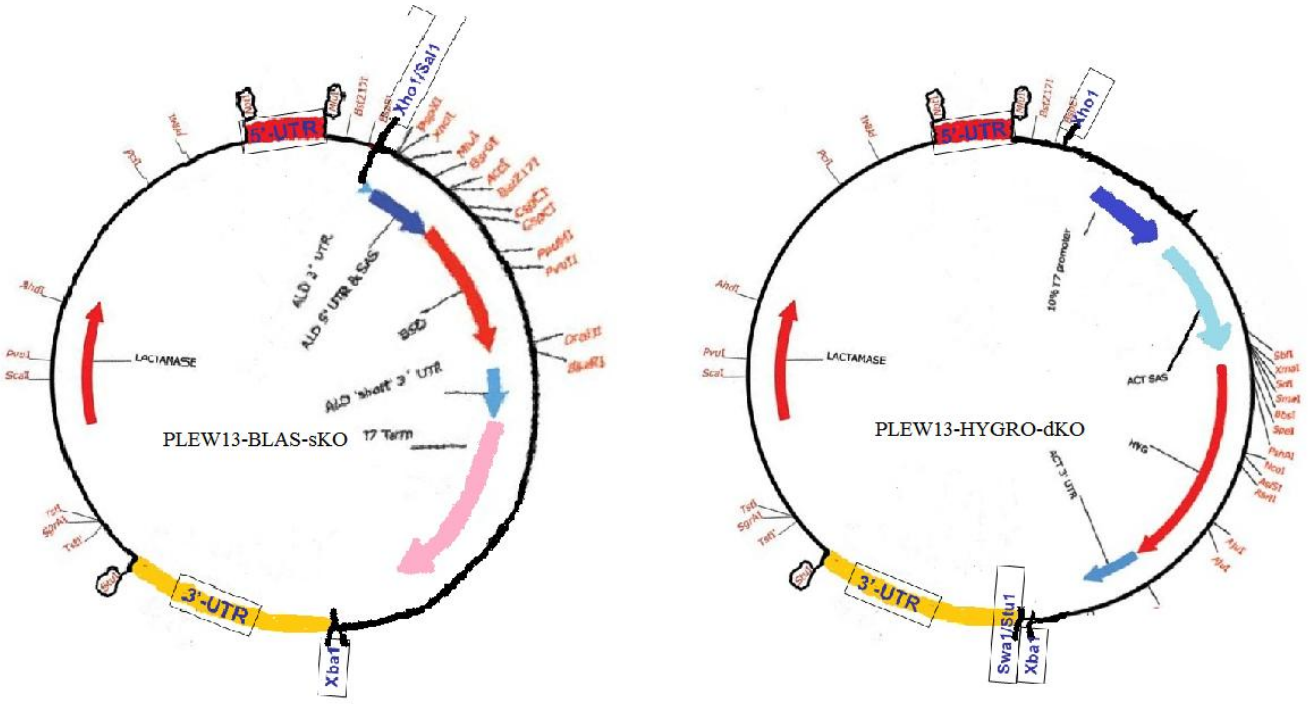

Fig. 8: Two plasmids that were generated to target each allele pLEW13-BLAS-sKO and pLEW13-HYGRO-dKO as the names suggest pLEW13-BLAS-sKO confers resistance to the drug blasticidin, while the pLEW13-HYGRO-dKO confers resistance to hygromycin.

2.1.1 Transformation to XL1 - Blue *Escherichia Coli* Cell-line and Cultivation

Note: Already pre - prepared in - 80 °C

Tab. 1: SOC media

Tryptone	20 g/L
Yeast extract	5 g/L
$MgSO_4$	4.8 g/L
Dextrose	3.603 g/L
NaCl	0.5 g/L
KCl	0.186 g/L

Tab. 2: Agar plate

Bactotryptone	1.0 g
Bacto yeast extract	0.5 g
NaCl	1.0 g
Bacto Agar	1.0 g
MiliQ	100 ml

Tab. 3: LB media

Bactotryptone	1.0 g
Bacto yeast extract	0.5 g
NaCl	1.0 g
MiliQ	100 ml
NaCl	0.5 g/L
KCl	0.186 g/L

As the constructs were made by another lab, the first step of my project was the transformation of the plasmids into competent cells. Competent *Escherichia coli* XL1-Blue cells were thawed out on ice. A volume 100 µl of cells were mixed with 5 µl of 30 ng/µl

plasmid stocks. Cells were kept on ice for 20 - 30 min, followed by a 60 sec heat shock for in a thermo block that was set to 42 °C. After the heat shock, cells were put back on ice for an additional 2 min. A volume of 300 µl of SOC media (**Tab. 1**) was added and cells were shaken for 30 - 60 min in 37 °C. After the shaking step, transformed *E. coli* were spread on an agar plate (**Tab. 2**) supplemented with 100 µg/ml ampicillin to select for *E. coli* containing the plasmid of interest, which all bear the ampicillin resistance gene, with a sterile loop. The plate was left in 37 °C incubator upside down. About 12 hours later, bacteria colonies appeared on the agar plate. A particular colony was picked with a pipette tip and seeded to grow in liquid LB media (**Tab. 3**), which was supplemented with 4 µl of 50 mg/ml ampicillin were added to the test tube. Cultures were grown overnight at 37 °C.

2.1.2 Plasmid Isolation

Tab. 4: Used devices:

Eppendorf minispin centrifuge
NanoDrop 1000 Spectrophotometer

Tab. 5: Used reagents:

Suspension Buffer	250 µl
Lysis Buffer	250 µl
Binding Buffer	370 µl
Wash Buffer 1	500 µl
Wash Buffer 2	750 µl
MiliQ	50 µl

The plasmid DNA was isolated and purified using the High Pure Plasmid Isolation Kit, according to manufacturer's protocol.

Plasmid purification follows a bind-wash-elute procedure. First, cultures of bacteria are lysed to free the plasmid DNA from the cell. After the solution is cleared of cell and chromosomal DNA, the supernatant is kept and transferred to the spin filter tube. With help of chaotropic salt (guanidine HCl) the nucleic acid binds specifically to the surface of glass fibres. Since the binding process is specific for nucleic acid the plasmid DNA is purified from salts, proteins and other cellular impurities by washing steps. After those washing steps plasmid is eluted in water.

Bacteria from overnight culture grown in test tube were first homogenized and 1 ml was transferred to 1.5 ml Eppendorf tube. Cells were spun down at 5 988 rcf for 30s. The supernatant was discarded, while the pellet was resuspended in 250 µl of Suspension Buffer in such way that no cell clumps were visible. Next 250 µl of Lysis Buffer was added and the solution was mixed gently. The tube was incubated at RT for 5 min. Then 370 µl of chilled Binding Buffer was added, the solution was mixed gently and centrifuged for 5 minutes at speed of 12 492 rcf. The supernatant with nucleic acid was transferred to High Pure Filter

Tube and centrifuged at 12 492 rcf for 30 - 60 s, while the pellet of lysate cells and chromosomal DNA was discarded. In the next step the flow-through was discarded. In order to purify the plasmid DNA bound on the surface of glass fibres from salts, proteins and other cellular impurities washing steps have to be done. A volume 500 µl of Wash Buffer I was added to High Pure Filter Tube and centrifuge at 12 492 rcf for 1 min. The flow-through was discarded again and 700 µl of Wash Buffer II was added. The centrifuge step was done as in previous part (and after discarding of flow-through), one addition minute of spinning was performed. The final flow-through together with the collection tube was discarded and the column part of the High Pure Filter Tube was transferred to the 1.5 µl Eppendorf tube. A volume of 50 µl of miliQ water was added and system was centrifuge for 1 min at 12 492 rcf. The column was discarded and purified plasmid was present in the Eppendorf tube as flow-through from the column. The final concentration of DNA was measured by NanoDrop 1000 Spectrophotometer.

2.1.3 Digestion

The purified plasmid was digested overnight at 37 °C by NotI High Fidelity (HF) and Stu I (**Tab. 7**) Digestion was verified by the electrophoresis on 1% Agarose gel (**Tab. 6**)

Tab. 6: Preparation of Agarose gel

Agarose	1 g
TAE Buffer	100 ml
EtBr	1.5 µl

Tab. 7: Recipe for digestion - 20 µl reaction volume

Plasmid	5 µl
NotI HF	0.5 µl
Stu I	0.5 µl
Buffer 4	2 µl
MiliQ water	12 µl

2.1.4 Preparation of Single Knockout and Double Knockout Constructs

Tab. 8: Used chemicals

96 % EtOH	250 µl
76 % EtOH	250 µl
3M NaCOOH	370 µl
Cytomix	500 µl

Tab. 9: Recipe for digestion before electroporation - 200 µl reaction volume

Plasmid	100 µl
Not HF	5 µl
Stu I	5 µl
Buffer 4	20 µl
MiliQ water	70 µl

Digestion of the described plasmids yielded two bands (**Tab. 9**).,While both bands were electroporated, only the fragment of the plasmid containing the homologous recombination sequence and drug resistance marker is assumed to be integrated into the genome (see **2.1.5**).

The digested construct was precipitated with 96% ethanol in a 2.5:1 ratio and sodium acetate at a final concentration of 0.3M in - 80 °C for at least 20 min. Pelleted DNA was washed with 70% ethanol. Then the pellet was air-dried and resuspended in 400 µl of sterile cytomix (**Tab.10)**.

Tab. 10: Cytomix composition

HEPES	25 mM
KCl	120 mM
$CaCl_2$	0.15 mM
K_2HPO_4/ KH_2PO_4	10 mM
EDTA	2 mM
Glucose	6 mM
$MgCl_2$	5 mM
BSA	100 µg/ ml
Hypoxanthine	1 mM

2.1.5 Electroporation of pLEW13-BLAS-sKO into Single Marker Strain

Tab. 11: Used devices

Eppendorf minispin centrifuge
Eppendorf centrifuge 5810 R for 50 ml falcons
AMAXA Nucleofector II

Tab. 12: Used chemicals

AMAXA cytomix
HMI - 9 Media

Around 50 million cells was harvested in two 50 µl Falcon tubes of single marker strain of bloodform *T. brucei* at 37 °C for 10 min with speed of 436 rcf. The supernatant was discarded with the exception of the last 500 µl that was used to resuspend the pellet of cells. The mixture was transferred to the 1.5 Eppendorf tube and centrifuge again. The supernatant was discarded and the pellet was resuspended with 50 µl of AMAXA cytomix. The 150 µl suspension of cells was supplemented with 50 µl of digested plasmid and transferred to cuvettes and electroporated with the AMAXA nucleofector II machine. Then, the contents of these cuvettes were transferred to tube A, containing 30 ml of media and gently inverted several times to mix well (cell density = 10 million cells/ ml). Additionally, 3 ml of the cells from tube A were transferred to pre-prepared tube B containing 30 ml of media and inverted well several times to mix well (cell density = 1 million cells/ml). The procedure was repeated one more time in the same way (3 ml from B tube to 30 ml of media in C tube) in order to get cell density = 0.1 million cells/ ml. In the last step, 1 ml aliquots of each of the three dilutions were distributed to three 24 well plates (A, B, C) and placed in a 37 °C incubator.

Tab. 13: Amaxa Nucleofector® cytomix

Human T-cell Nuceloefector Sol	25 mM
Supplement 1	120 mM

2.1.6 Selection and Cultivation of Single Knockout Transfectants

Tab. 14: Used chemicals

HMI-9 Media	10% fetal bovine serum (FBS) (Hirumi & Hirumi 1989)
Blasticidin	2.5 µg/ml

HMI-9 media with the final concentrations 2.5 µg/ml of blasticidin was added to the culture of transfectants about 12 hours after electroporation. The culture was cultivated and with density of 1 x 10^6 cell/ml and further used for electroporation.

2.1.7 Electroporation of pLEW13-HYGRO-dKO into Single Knockout Cell-line

In order to perform the double – knockout, the digested pLEW13-HYGRO-dKO construct that targets the second allele of MRB8620 was electroporated into single-knockout cell-line. The construct was electroporated using the same protocol for electroporation of pLEW13-BLAS-sKO into Single Marker Strain (see 2.1.5)

2.1.8 Selection and Cultivation of Double Knockout Transfectants

Tab. 15: Used chemicals

HMI-9 Media	10% fetal bovine serum (FBS)
Blasticidin	2.5 µg/ml
Hygromycin	50 µg/ml

HMI-9 media with the final concentrations 2.5 µg/ml of blasticidin and 50 µg/ml of hygromycin was added into the culture of transfectants about 12 hours after electroporation.

2.1.9 Identification of Transfectants

Note: The following list of set of primers (Tab. 16 and 17) was kindly provided by my Co-Supervisor Zhenqiu Huang, because the primers to identify the single- and double-knockout were already generated when I came to the laboratory (More in detail in result part 3.3).

Tab. 16: pLEW13-BLAS-sKO for identification of 8620 single knockout

pLEW13-BLAS-sKO for identification of 8620 single knockout	
5' UTR upstream Fw	tcaactatacgcccacagatcagcg
3' Rv Blasticidin	gccctcccacacataaccagagg
5' Fw Blasticidin	ATG CCT TTG TCT CAA GAA GAA TCC ACC
3' UTR downstream Rv	gtgaggcgcgattgctccgtcttac
3' UTR for sequence for KO2	ATGGTAAAGACG GCACCGACA GG

Tab. 17: pLEW13-HYGRO-dKO for identification of double knockout

pLEW13-HYGRO-dKO for identification of 8620 double knockout	
5' UTR upstream Fw	tcaactatacgcccacagatcagcg
3' Rv Hygromycin	acgctgtcgaacttttcgatcagaa
5' Fw Hygromycin	gcggccgtctggaccgatggctgt
3' UTR downstream Rv	gtgaggcgcgattgctccgtcttac

All the primers listed above were constructed in order to determine by PCR whether the knockout constructs were properly integrated into the *MRB8620* loci (see 2.2.1).

2.2.0 Extraction of *T. brucei* genomic DNA

The isolation of genomic (g) DNA from the transfected *T. brucei* was done according to manufacturer's protocol for the illustra Tissue and Cells genomicPrep Mini Spin Kit (GE Healthcare). A simple genomic DNA purification protocol uses chaotropic agents to extract DNA from cells and denature protein components.

Around 4 x 10^7 cells were centrifuge at 753 rcf for 10 min. The supernatant was discarded and the pellet was resuspended in 600 µl of lysis buffer so that no clumps of cells were visible and incubated at RT until the pellet was entirely dissolved. Three µl of RNaseA solution was added and the reaction was incubated at 37 °C for 1 hour. RNaseA is an endonuclease used to remove single-stranded RNA. Afterward, 200 µl of protein precipitation solution was added and vortexed in order to separate proteins present in the solution, which were centrifuged at 31 799 rcf for 5 min. The supernatant containing genomic DNA was transferred into a new tube, where 500 µl of 100% isopropanol was added. The DNA pellet was washed with 70% ethanol and air-dried. Then 50 µl of DNA Hydration Solution was used to resuspend the pellet. The gDNA was used in the subsequent polymerase chain reaction.

2.2.1 Polymerase Chain Reaction

Used device: Biometra T3000 Thermocycler.

This experiment was designed in order to confirm whether the electroporated two KO constructs were incorporated into the *MRB8620* loci of *T. brucei,* leading to knockout of both alleles of the gene of interest. Therefore a polymerase chain reaction (PCR) was run using previously described designed primers (2.1.9 Tab. 16 and 17) according to recipe Tab. 18 and setting of machine Tab. 19.

Tab. 18: Recipe for PCR - 50 µl reaction volume

genomic DNA	15 µl
Fw primers	1 µl
Rv primers	1 µl
Phusion Buffer 5x	10 µl
dNTPs (10 mM)	1 µl
Phusion polymerase	0.5 µl
MiliQ water	22.5 µl

Tab. 19: The PCR machine program

1. step 94 °C	2 min
2. step 94 °C	30 sec
3. step 55 °C	1 min
4. step 72 °C	1 min (back to 1.step 25x)
5. step 72 °C	5 min
6. step 14 °C	0.5 µl

Tab. 20: Supermix combination of primers were done according Tab. 16, 17

1. supermix	**5' UTR upstream Fw** **3' Rv Blasticidin**
2. supermix	**5' UTR upstream Fw** **3' Rv Hygromycin**
3. supermix	**3' UTR downstream Rv** **5' Fw Blasticidin**
4. supermix	**3' UTR downstream Rv** **5' Fw Hygromycin**

2. 3 Growth Curve of Procyclic RNAi Cell-line Cultured in Glucose-Free Media

A previously generated procyclic cell-line, where RNA interference (RNAi) targeting MRB8620 can be induced, was used follow the impact of the knockdown of this protein on growth by growth curve analysis. A growth phenotype is determined by comparison of non-induced cells with cells where RNAi was induced by tetracycline to determine RNAi-silencing of the target gene has an effect on the growth rate of *T. brucei*. If growth is inhibited, the gene is considered to be essential.

The preliminary result of RNAi-silencing of the target gene did not show any effect on the growth rate of *T. brucei* when cultured in a medium containing 6 mM glucose. Based on this result and the knowledge that the energy metabolism of procyclic *T. brucei* differs in the presence and absence of glucose, the growth curve determination was repeated in glucose

free media. The previously generated inducible MRB8620 RNAi cell-line was stored as a glycerol stock in liquid nitrogen.

2.3.1 Cultivation of MRB8620 RNAi Cell-line

Tab. 21: Used chemicals

SDM-79 media	(Brun & Schönenberger 1979)
Hygromycin	50 µg/ ml
Phleomycin	2.5 µg/ ml
G418	15 µg/ ml

The glycerol stock of the MRB8620 RNAi cell-line was thawed and transferred from the vial to a culture flask containing SDM-79 medium and left to recover in a 27 °C incubator. This medium also contained a final concentration of 50 µg/ml Hygromycin (H), 2.5 µg/ml Phleomycin (P) and 15 µg/ml G418 (G). The density of cells was checked every day until they reached the appropriate value of 5 x 10^7 cells/ml. The density was measured with the following steps: 50 µl of cells were transferred to a cuvette containing 10 ml of Hemasol solution and measured with the Beckman Coulter Z2 Particle Counter.

Cells were kept growing for several days to get better adapted to normal conditions again. Dilution with glucose and drug containing media was done regularly so that, cells were able to reach the same density every second day after each dilution.

When cells started to grow well in SDM79, they were slowly introduced into glucose-free media. As the next step, cells were divided into 2 flasks. The first flask was used for culturing cells in glucose free media whereas the second one was grown in SDM79 as a back-up.

2.3.2 Adaptation of MRB8620 RNAi Cell-line to Glucose-Free Media

To get the cell adapted to glucose-free media successfully, the dilution was done step by step and started with $\frac{1}{2}$ dilution of cells grown in SDM79 to glucose-free media. In two days, these cells were diluted $\frac{2}{3}$ into glucose-free media. After two more days, a $\frac{3}{4}$ dilution was performed. As the step of adaptation to glucose free media was crucial, cells were cultured for one week more with following dilutions every second day:

a) 1 ml of cells in 3 ml of glucose-free media with HGP

b) 1 ml of cells in 5 ml of glucose-free media with HGP

c) 1 ml of cells in 10 ml of glucose-free media with HGP

2.3.3 Induction of MRB8620 RNAi Cell-line

Cells in glucose-free media were taken from the 27 °C incubator and checked under the microscope. The density of cells was measured. Procyclic culture with density of 2 x 10^6 cells/ml was divided into 3 wells (each well 1 ml of culture provided) of two different 24 well cultivation plates. While one plate named T- had three wells filled with procyclic culture, wells of the second plate labelled T+ were additionally supplemented with 1 μg/ml Tetracycline to induce RNAi.

2.3.4 Growth Curve of MRB8620 RNAi Cell-line in Glucose Free Media

The induced and non-induced cultures were monitored for 14 days by growth curve analysis. The density of the cells was measured with Beckman Coulter Z2 Particle Counter every 24 hours with dilution back to 2 x 10^6 cells/ml every second day. The experiment was done in triplicate; three wells with the tetracycline in media T+ and three without T-. The graph was performed by using the average of the three values.

2.4 Real-Time Quantitative PCR

PCR technology is a very helpful tool in quantifying DNA amount. Ideally, the target sequence quantity should double during every amplification cycle. During the method of real-time quantitative PCR (qPCR) the amount of amplified product is linked to fluorescent intensity using a fluorescent dye, in this particular case SYBR green, which fluoresces only when bound to double-stranded DNA. The level of florescent data are reported whenever the amplification reaction is complete (about 30-40 cycles) and based on the final fluorescence the amount of a PCR product over the reaction course can be measured. However the PCR reaction efficiency can decrease over the amplification process as the reagents are getting consumed. The result of the experiment is analysed by relative quantification analysis.

Principle of relative quantification analysis is based on the comparison of two ratios, the ratio of a target DNA sequence to reference DNA sequence for an unknown sample and the ratio of the same two sequences in a standard sample. The nucleic acid of interest is set as the target, whereas the nucleic acid that is found at constant copy number in case of all samples serves as reference. The reference is used for purpose to normalize sample-to-sample differences.

The analysis uses the sample Ct value that stands for threshold cycle value, which is the number of the cycle where the transcript abundance reaches a set fluorescent value. The

relative abundance of maxicircle transcripts and target gene transcrips are calculated by Pfaffl method. The method uses the sample's Ct value, the efficiency of the reaction, the number of cycles completed to determine how much the DNA concentration must have increased for each sample by the end of amplification. In such way the ratios are generated. Therefore the final ratio from normalized Relative Quantification is only function of PCR efficiency and of determined threshold cycle values (Pfaffl 2001).

$$\text{Relative Quantification} = \frac{\text{PCR efficiency (target)}^{\text{Ct value (target)}}}{\text{PCR efficiency (reference)}^{\text{Ct value (reference)}}}$$

Fig. 9: Pfaffl method formula using PCR efficiency to the power of Ct value for target to reference ratio

The qPCR was used in order to verify the RNAi knockdown of MRB8620. Primers used were designed for a selection of mitochondrial genes to see whether the knockdown affects the edited and pre-edited transcripts or not. By this method, one can determine the impact of MRB8620 knockdown on RNA metabolism.

2.4.1 RNAi-induction and harvesting of *T. brucei*

Induction with 1µg/ml tetracycline was done according to the procedure described in section 2.3.3. Cells were induced, further cultivated in glucose-free media and then harvested just before the start of the growth phenotype on the 4th day after induction. Both cultures were harvested in Falcon tubes by the Eppendorf 5810 R centrifuge using a swing-out rotor for 5 min at 1 744 rcf at 4 °C. The supernatant was discarded and the cell pellet was further used for RNA isolation.

2.4.2 RNA Isolation

Tab. 22: Used chemicals to each tube with pellet

RNA Blue	1 ml
Chloroform	1 ml
Isopropanol	circa 1 ml
75% EtOH	1 ml
MiliQ water	100 µl

The pellets of harvested induced and non-induced MRB8620 RNAi cell-line were resuspended in 1 ml of RNA Blue in such way that no clumps of cells were visible. The system was incubated for 5 minutes at room temperature (RT). RNA Blue has the ability to

dissociate proteins and helps to avoid the degradation of RNA by inactivating enzymes that can degrade RNA and also maintaining a suitable pH for RNA isolation. The technique works on the principle of liquid - liquid extraction therefore the next step required addition of 1 ml of chloroform in order to separate the aqueous and organic phases. The solution was vortexed vigorously for 15 sec and left stand for 2 min. Then the solution was centrifuged for 15 min at 4 °C with the speed of 27 901 rcf. RNA stays exclusively in the top aqueous phase while DNA and proteins are present in the interphase and organic phase. The aqueous phase was transferred into a new 15 ml falcon tube and the same volume of isopropanol added. The solution was spun down for 10 min at 4 °C with speed of 27 901 rcf. Isopropanol was aspirated as much as possible and 1 ml of 75% ethanol (EtOH) was added to wash the RNA pellet. The solution was spun down for 5 min at 4 °C with the speed of 27 901 rcf. EtOH was aspirated out of the tube as much as possible. The pellet was air dried until the moment when it started to get transparent around its periphery and then resuspended in 100 µl of 18.2Ω MiliQ water. The solution was heated for 10 min at 60 °C to dissolve the pellet completely. The isolated RNA was transferred to 1.5 ml Eppendorf tube and its concentration was measured by NanoDrop 1000 spectrophotometer.

2.4.3 RNA gel electrophoresis

The state of isolated RNA was verified in order to prove that the RNA did not degrade during the procedure. 1% agarose gel (**Tab. 24**) containing 37% formaldehyde and 10 x MOPs buffer (**Tab. 23**) was prepared. Each RNA sample was mixed with 1.5 X sample buffer (**Tab. 25**), so that the latter was diluted to 1 x and incubated for 10 min at 65 °C before loading. The gel was run for 1 hour at 80 V (**Tab. 26**).

Used Solutions:

Tab. 23: 10 x MOPs Buffer

Formamid	600 µl
37% Formaldehyde	210 µl
10 x MOPS	156 µl
EtBr	5 µl

Tab. 24: 1% Agarose gel

10x MOPs	55 ml
37% Formaldehyde	50 ml
MiliQ water	445 ml

Tab. 25: 1.5 M Sample Buffer

0.5 M MOPs	40 ml
3 M NaCOOH	1.67 ml
0.5 M EDTA pH 8.00	2 ml
MiliQ water	56.33 ml

Tab. 26: Running Buffer

10 x MOPs	12 ml
Agarose	1.2 g
MiliQ water	104 ml
37% Formaldehyde	4 ml

2.4.4 Reverse Transcription - creation of cDNA

Tab. 27: Used reagents and volumes required per tube

gDNA Wipeout Buffer, 7x	2 µl
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer, 5x	4 µl
RT Primer Mix	1 µl
18.2Ω MiliQ water	variable

The complementary DNA (cDNA) was generated from the RNA template using the QuantiTect Reverse Transcription Kit from QIAGEN according to manufacturer's protocol.

Note: cDNA for qPCR was created in duplicate for each sample. In addition, a negative control was created for every RNA sample where the reverse transcriptase was absent in order to detect potential gRNA contamination, which could influence the results. Thus, it was obtained A and B RT+ samples and one RT- cDNA for each RNA sample.

Template RNA previously stored at - 80 °C was thawed on ice together with gDNA Wipeout Buffer, Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase free 18.2Ω MiliQ water. Every solution mentioned has a specific function during the procedure of cDNA creation from the RNA template. The solution termed gDNA Wipeout Buffer plays the role in effective and rapid removal of gDNA from RNA samples, which otherwise could influence the downstream PCR step. Quantiscript Reverse Transcriptase and Quantiscript RT Buffer enable high yields of cDNA from any RNA template. RT Primer Mix contains specially optimized mix of oligo-dT and random hexamer primers allowing cDNA synthesis from all regions of RNA in the sample. Each solution was mixed by flicking the tube and centrifuge briefly to collect residual liquid from the sides of the tubes and then kept on ice. The gDNA elimination reaction (Tab. 28) was prepared on ice and mixed well.

Test tubes were incubated for 2 min at 42 °C and immediately placed on ice. The reverse-transcription master mix was prepared on ice (Tab. 29). The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA. RNA template from DNA elimination step was added to each tube containing reverse-transcription master mix. The solution was mixed and stored on ice. Then reaction was incubated for 15 min at 42 °C to promote the annealing of random primers to the RNA and for 3 min at 95 °C in order to inactivate Quantiscript Reverse Transcriptase. The reverse-transcription reaction was placed on ice and each 20 µl of reaction was diluted to 100 µl with MiliQ water. Out of this dilution, a further 50 x fold dilution was performed (2 µl of cDNA to 98 µl water) to serve as a template for the very abundant housekeeping cDNA.

Tab. 28: Recipe for Genomic DNA elimination - 14 µl reaction volume

gDNA Wipeout Buffer, 7x	2 µl
Template RNA, 2 µg/ml	5 µl
18.2Ω MiliQ water	7 µl

Tab. 29: Recipe for Reverse-transcription - 20 µl reaction volume

Template RNA (entire genomic DNA elimination reaction **Tab. 28**)	14 µl
Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer, 5x	4 µl
RT Primer Mix	1 µl

2.4.5 Real-Time PCR

Every reaction was done in triplicates and contained the components described in Tab. 30 while all the used primers were diluted and mixed according to Tab. 31.

Tab. 30: SYBR Green PCR Master Mix

Power SYBR Green Master Mix	10 µl
Primer Mix (Tab. 30)	8 µl
cDNA Template	2 µl

Tab. 31: Primer Mix

Primer:	**Stock Solution**	**Amount**	**MiliQ**	**Final concentration**
Reverse	100 µM	7.5 µl	492.5 µl	1.5 µM
Forward	100 µM	7.5 µl	492.5 µl	1.5 µM

The final concentration of every diluted primer was 300 nM. The primer pair master mix was done for each set of primers used. Tubes were put into Rotor-Gene RG 3000 (QIAGEN) thermocycler and the following program was started: 2 min at 50 °C, 10 min at 95 °C and then a 2 step cycle (15s at 95 °C, 60s at 60 °C) repeated 40 times. In order to determine whether the reaction yielded one or more products, a melting curve analysis was performed on the reaction: 60 °C to 95 °C with 1 degree / step increment, first step lasted 45s, following ones took 5s each. In the first run, the house-keeping genes 18 S rRNA and β-tubulin were measured in each of the T+ and T- cDNAs which were made in duplicate (see section 2.3.4). The appropriate T+ and T- cDNA pair was chosen based on which cDNAs exhibited the most similar levels.

3. RESULTS AND DISCUSSION

Note: During my laboratory work I was focused on the MRB8620 protein that is known to be part of MRB1 complex, and thus may play a role in RNA editing in *T. brucei*. The idea to try the knockout experiment of MRB8620 was based on preliminary results generated by other colleagues before I came to the laboratory indicating that MRB8620 is not essential for the *T. brucei* viability.

3.1 MRB8620 RNAi Cell-line preliminary results

Tetracycline-inducible RNAi construct was prepared and electroporated into 29-13 strain of procyclic *T. brucei*. Transfectants were selected for the appropriate drug resistance marker and cultivated in glucose-containing SDM79. After several days RNAi was induced by tetracycline and growth was measured over a time course. By comparison with non-induced cells, this experiment was to determine whether the RNA-silencing of the target gene has an effect on the growth rate of *T.brucei*. Whenever growth is inhibited, the targeted protein is considered to be essential.

The starting density of cultures was 2 x 10^6 cells/ml and the growth of the RNAi cell-line was followed for ten days. Cells were diluted back to their starting density every second day. However, the resulting growth curve analysis shows no effect on growth of RNAi-silencing of MRB8620 (Fig. 10), suggesting it is not essential for the cells. Therefore the next KO experiments were undertaken to test the essentiality of the protein.

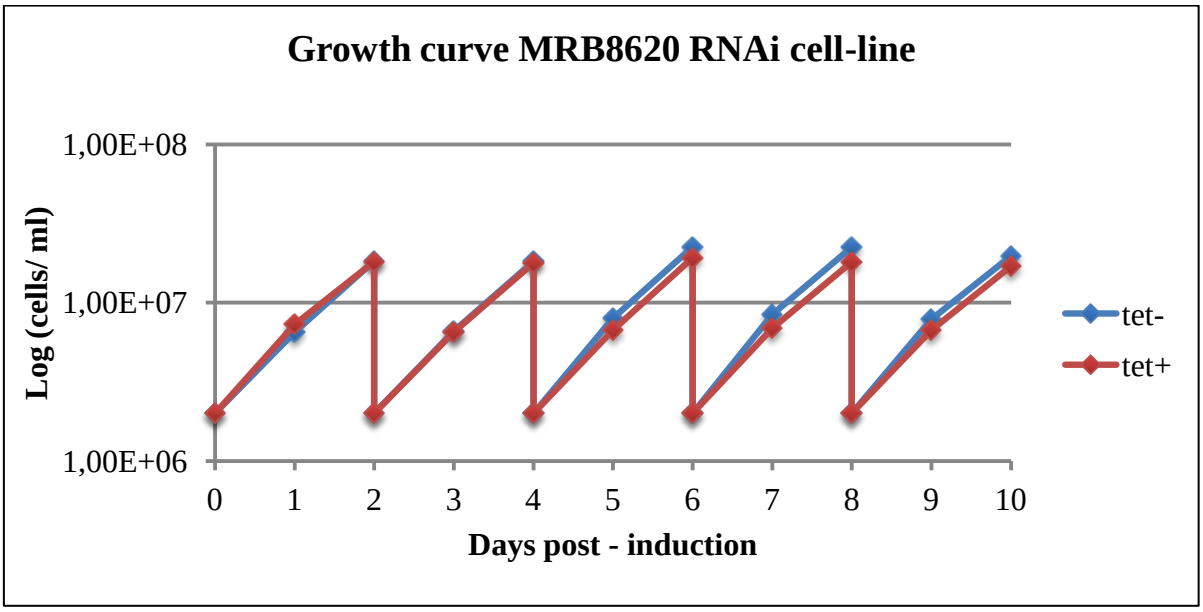

Fig. 10: Growth curve of RNAi knockdown of MRB8620 in SDM79 medium.

The growth inhibition did not occur.

3.2 Double Knockout Experiment

The double knockout of *MRB8620* was performed as described in 2.1. In the first step, the pLEW13-BLAS-sKO construct conferring resistance to blasticidin and targeting 1st allele of *MRB8620* was electroporated to single marker strain of *T. brucei*. Knockout of first allele was done by homologous recombination (Fig. 11) after selection using 2.5 µg/ml blasticidin. When the cells reached a density of 1 x 10^6 cells/ml, the electroporation of pLEW13-HYGRO-dKO conferring resistance to hygromycin, into single knockout cell-line was performed. Homologous recombination should allow the knockout construct to integrate into the second MRB8620 allele.

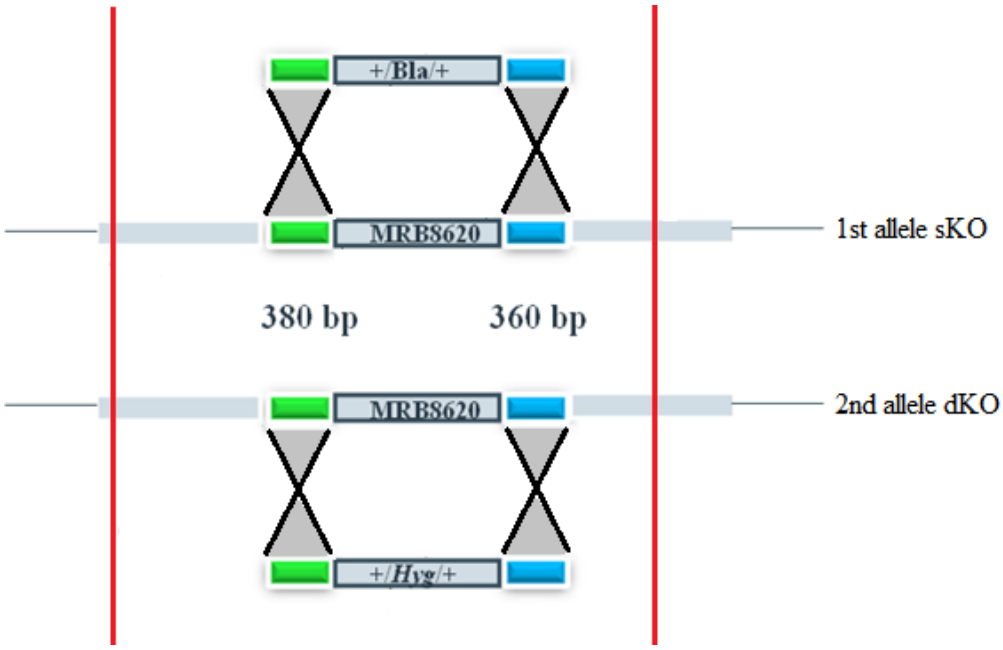

Fig. 11: Homologous recombination scheme between fragment and MRB8620 loci leading to knockout of both alleles. The first *MRB8620* allele supposed to be replaced by the construct conferring resistance to blasticidin while the second allele is replaced by the construct conferring resistance to hygromycin.

Transfectants were drug-based selected by using HMI-9 medium with the final concentrations 2.5 µg/ml blasticidin and 50 µg/ml hygromycin.

3.3 Knockout verification

In order to verify proper integration into the *MRB8620* gene loci, genomic DNA was isolated from bloodform *T. brucei* cells electroporated with the MRB8620 KO constructs. Specific PCR primers were constructed to characterize integration of knockout constructs into the *MRB8620* loci. The knockout scheme how this verification PCR was designed is shown in Fig. 12). It presents both the alleles with the *MRB8620* and drug resistance marker genes and

the position of the used primers together with approximated sizes of all the fragments that should get amplified (Tab. 16, 17 and 21).

Additionally, the primers for amplification of MRB8620 itself were used to assure that the MRB8620 was knocked out and therefore not detectable by amplification.

Tab. 33: Sequences of primers that were used to amplify MRB8620 ORF

MRB8620 Fw:	AAGCTTATG TTA CTG AGC ACC CGT GCC A
MRB8620 Rv:	GGATCCCTT ACC ACC ATC TTG CGA CAA CCG C

The specific primers used for this experiment are also described in Materials and Methods.

Tab. 34: Supermixes of primers used for knockout verification and expected sizes of PCR products

1. supermix	**5' UTR upstream Fw** **3' Rv Blasticidin**	**Expected fragment size** **1 900 bp**
2. supermix	**5' UTR upstream Fw** **3' Rv Hygromycin**	**Expected fragment size** **2 500 bp**
3. supermix	**3' UTR downstream Rv** **5' Fw Blasticidin**	**Expected fragment size** **900 bp**
4. supermix	**3' UTR downstream Rv** **5' Fw Hygromycin**	**Expected fragment size** **1 800bp**

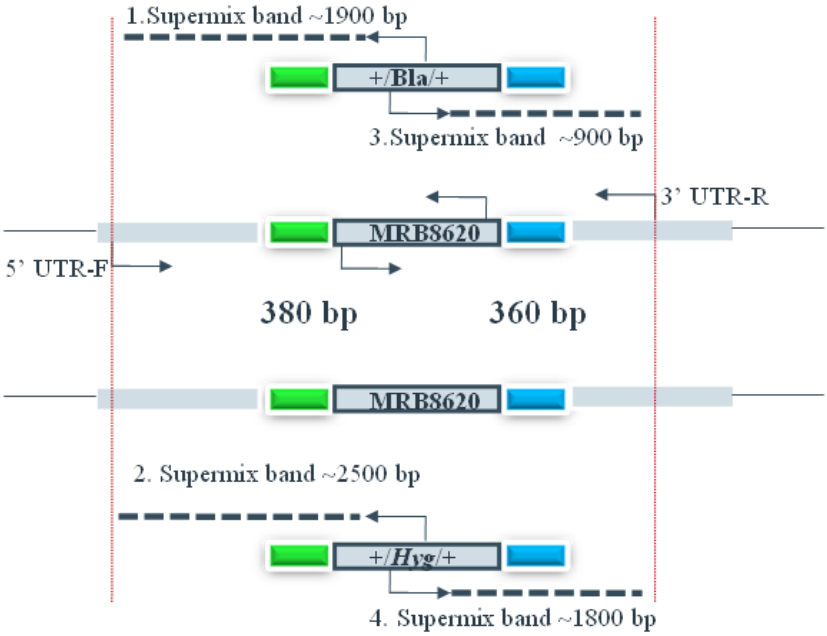

Fig. 12: Scheme depicting PCR primers used to characterize integration of knockout constructs into MRB8620 loci.

PCR products were checked by agarose gel electrophoresis.

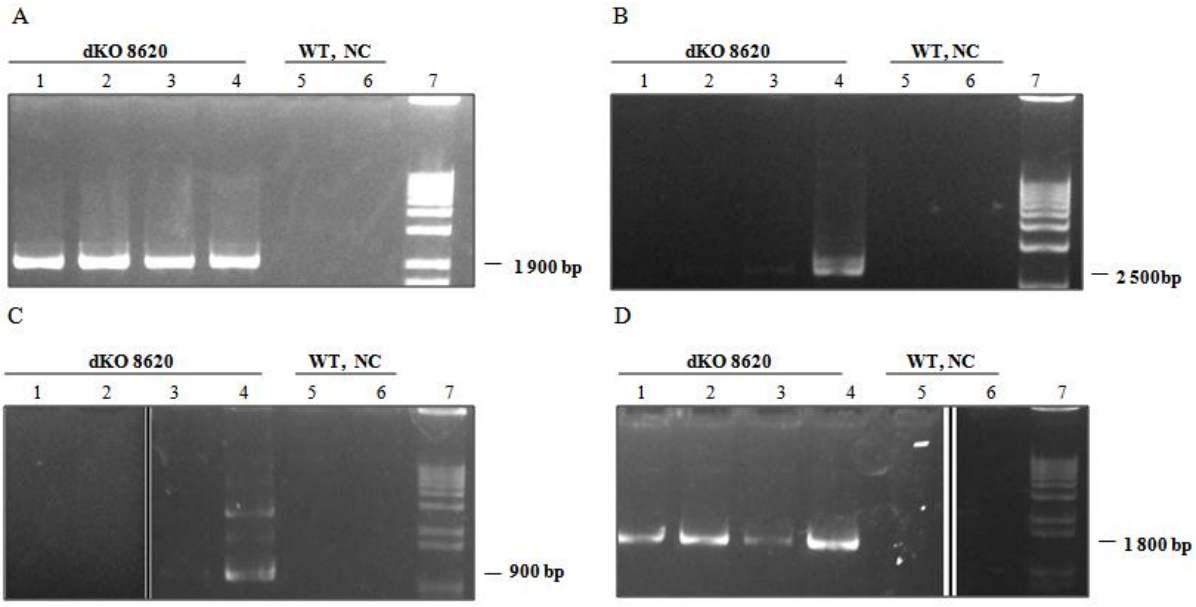

Fig. 13: Gel electrophoresis of gene fragments (to test each fragment amplification quadruplicates of genomic DNA were used); A – fragment amplified by 1. Supermix, which tests for integration of the blasticidin selection marker into the 5'-part of the *MRB8620* gene and whose expected size is 1 900 bp; B – fragment amplified by 2. Supermix, which tests for integration of the blasticidin selection marker into the 3'-part of the *MRB8620* gene and whose expected size is 2 500 bp; C – fragment amplified by 3. Supermix, which tests for integration of the hygromycin selection marker into the 5'-part of the *MRB8620* gene and whose expected size is 900 bp; D – fragment amplified by 4.supermix, which tests for integration of the hygromycin selection marker into the 5'-part of the *MRB8620* gene and whose expected size is 1 800 bp. WT stands for wild type cell line (well 5), containing no double – knockout of *MRB8620* gene, NC stands for negative control, containing no template (well 6). The well labelled by number 7 represents 5 kb DNA ladder

The result showed the expected size bands of 1 900 bp Fig. 13 A 1 – 4 wells, whereas in case of WT and NC any band was present. This example confirmed the successful integration of the blasticidin selection marker into the 5'-part of the *MRB8620* gene. Fig. 13 B 1 – 4 wells showed the expected size bands of 2 500 bp confirming the integration of the blasticidin selection marker into the 3'-part of the *MRB8620* gene, while there is no band present in NC and WT. Fig. 13 C wells 1 - 3 seemed to be slightly problematic, probably due to low concentration of isolated genomic DNA. The band of expected size showed in case of well 4, while NC and WT did not show any specific band. The integration of the hygromycin selection marker into the 5'-part of the *MRB8620* gene was successful. Fig. 13 D 1 – 4 wells showed the expected size bands of 1 800 bp, while there was no band in case of WT well neither in case of NC. The integration of the hygromycin selection marker into the 5'-part of the *MRB8620* gene was confirmed. Out of these results the conclusion was done indicating

that the two resistance markers were integrated into the two *MRB8620* loci, replacing the 8620 ORF.

However, to see whether the MRB8620 ORF has thus been eliminated from the genome, a further PCR amplifying the MRB8620 ORF was checked by agarose gel electrophoresis. If the KO experiment worked, there should be no amplified MRB8620. As shown (Fig 14.), this PCR amplified the MRB8620 ORF even in the double knockdowns, indicating that this element is still present in the genome despite the integration of the knockout constructs into the *MRB8620* loci.

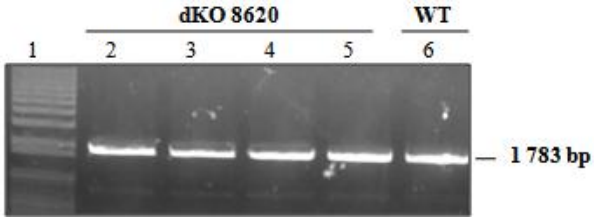

Fig. 14: Gel electrophoresis of gene fragment MRB8620 showed bands confirming that the knockout of *MRB8620* was unsuccessful

The procedure to perform the double knockout of MRB8620 was repeated three times (but only one attempt is shown here in the thesis) all the trials proved that the knockout was not successful.

3.4 Growth curves in Glucose – free Media

Because the failure to create *MRB8620* double knockout suggests that this gene is essential, we decided to assay the effect of MRB8620-RNAi in procyclic *T. brucei* under glucose-free conditions. After the MRB8620 RNAi cell-line was adapted to glucose-free media, growth of the knockdown was followed for ten days in the presence and absence of tetracycline. At the beginning the cultures had a starting density of 2×10^6 cells/ml. Cell concentration was measured every 24 hours with dilution back to the starting concentration every second day. The inhibition effect on growth of RNAi – silencing of MRB8620 was observed on the fifth day after the induction (Fig. 15). Thus, we conclude that MRB8620 is essential for growth of *T. brucei*.

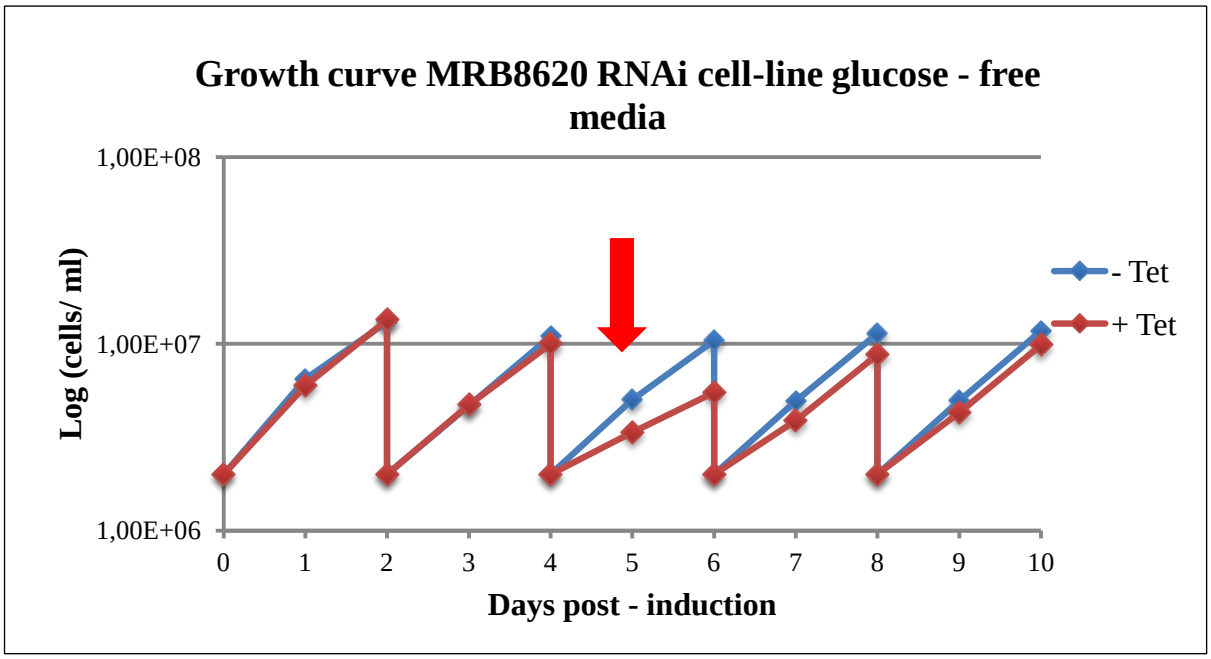

Fig. 15: Growth curve of RNAi knockdown of MRB8620 in glucose-free media.
The growth inhibition occurred during the fifth day post – induction (red arrow)

3.5 Quantitative real – time PCR

The level of maxicircle RNAs was assayed by qPCR on cells whose RNA was harvested four days post-induction, which is the time point preceding the manifestation of growth inhibition. The nuclear-encoded housekeeping genes 18S rRNA and β-tubulin were used in order to normalize the data between tetracycline-induced and non-induced samples.

The result qPCR showed down-regulation of MRB8620 transcript. Never-edited transcripts CO1 and ND4 do not seem to be affected by the down-regulation of MRB8620. Whereas CO2 pre-edited one does not show any significant effect the edited CO2 seem to be slightly down-regulated. In case of cyB pre-edited transcripts present up-regulation, while the edited one is down-regulated. Pre-edited and edited transcripts MURF2 do not seem to be affected. Edited form of pan-edited transcripts A6, CO3, ND7 and RPS12 show significant down-regulation, whereas the pre-edited form of A6, CO3 and RPS12 describes accumulation of transcript in induced cells. In case of ND7 there is no accumulation of pre-edited mRNA. From this experiment, we conclude that like other MRB1 subunits, MRB8620 plays a role in RNA editing.

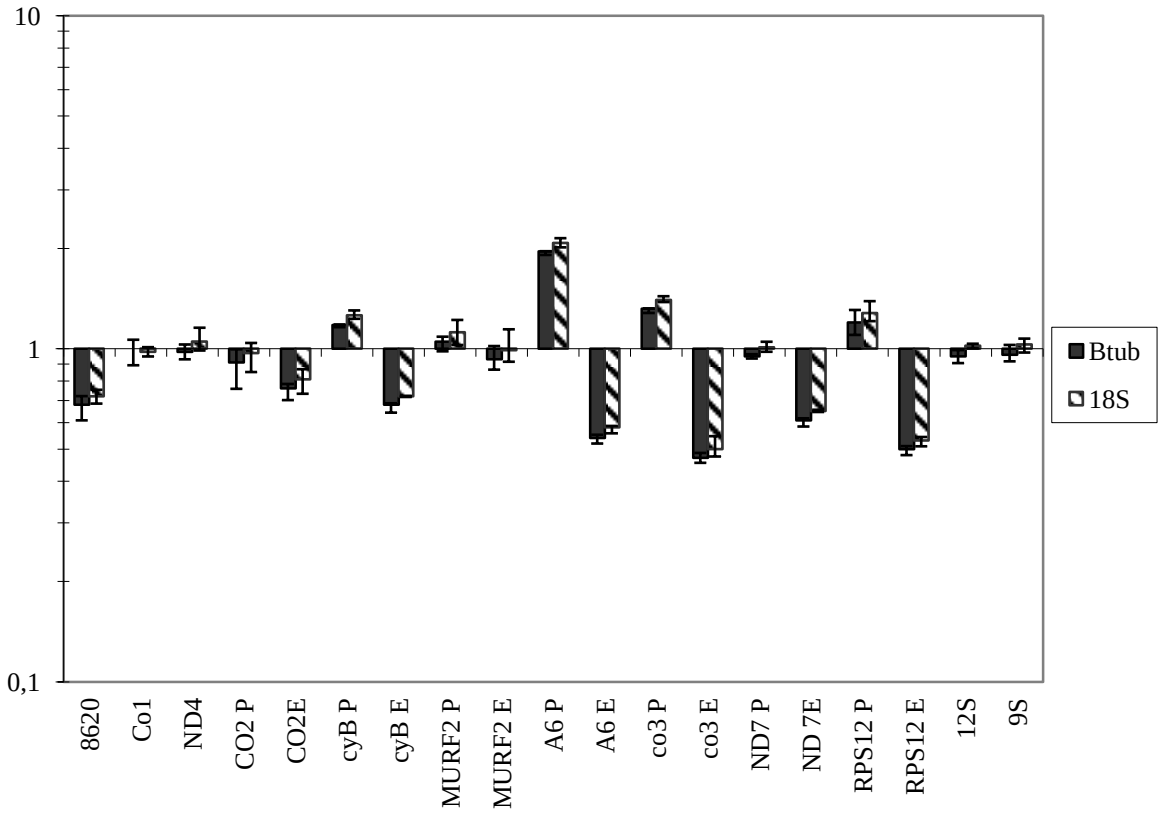

Fig. 16: qPCR analysis of the relative abundance by selection of maxicircle-encoded mRNAs in knockdown after RNAi inhibition.

Abbreviations of RNA names are present on the X axis, whereas logarithmic scale of Y axis shows relative abundance of those RNAs in RNAi silenced MRB8620 knockdowns compared to non-induced cells. Whenever the abundance in induced cells is above 1 (the midline of the graph), it indicates accumulation of the transcript upon RNAi induction. In the case of down-regulation of a transcript in induced cells, the bar extends below the value 1. Error bars represent standard deviation based on 3 replicates. RNA levels normalized to either 18S and β – tubulin RNA levels are depicted as striped and black bars, respectively.

4. CONCLUSION

One of the main goals of this thesis, to perform functional analysis of protein MRB8620 of *Trypanosoma brucei* was partially achieved. Future studies will likely refine our current understanding of MRB8620 function, however based on the qPCR result, it is clear that MRB8620 is essential player in RNA editing.

We focussed on the use of homologous recombination to knockout MRB8620 to test its essentiality in *T. brucei*. This experiment was repeated three times, however, the result always confirmed that the gene is essential since the knockouts were not achieved. While it appears that we were able to target the MRB8620 loci with the knockout constructs that replace the ORF with drug resistance markers, it appears that the MRB8620 shifted its location and therefore remained in the genome.

To probe the essentiality of MRB8620 we further examined RNAi knock down cell-line by monitoring its growth rate in glucose-free media. The result showed the negative growth phenotype on 5^{th} day onwards, indicating towards the essentiality of MRB8620 in mt RNA editing.

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6. LIST OF USED ABBREVIATIONS

A/As - Adenine/s

A6 - ATPase subunit 6

ATP - Adenosine triphosphate

CO - Cytochrome oxidase subunits

CR - C - rich reading frame

CYB - Apo - cytochrome b

ES - editing site

EtBr - Ethidium Bromide

EtOH- Ethanol

G - G418 drug

GAP - guide RNA associated protein

gRNA - guide RNA

H - Hygromycin

kDNA - kinetoplastid DNA

KO - knockout

KPAP - kinetoplastid poly(A) polymerase

KREN - kinetoplastid RNA editing endonuclease

M/MURF - maxicircle unidentified reading frame

mRNA - messenger RNA

MRB1 - mitochondrial RNA - binding complex

ND - NADH dehydrogenase subunits

nts - nucleotides

P - Phleomycin

RECC - RNA editing core complex

RET1 - RNA editing TUTase

RNAi - RNA interference

RPS12 - ribosomal protein 12

rRNA ribosomal RNA

T - Tetracycline

TUTase - terminal uridyltransferase

U/Us - Uridine/s

UTR - untranslated region

VSG - variant surface glycoprotein

Y2H - yeast two hybrid screen