The University of South Bohemia

Faculty of Science

Department of molecular biology and biochemistry

Bc.Thesis

Functional analysis of two subunits of the putative Mitochondrial RNA Binding complex 1 in *Trypanosoma brucei*

Lucie Hanzálková

Supervisor: Mgr. Mir Mohamod Hassan Hashimi, Ph.D.

Institute of Parasitology

Laboratory of molecular biology of protists

České Budějovice, 2010

Hanzáková, L., 2010 Functional analysis of two subunits of putative Mitochondrial RNA Binding complex 1 in *Trypanosoma brucei.* Bc. Thesis, in English -38 p., Faculty of Science, The University of South Bohemia, České Budějovice, Czech Republic

Anotation

The function of two subunits of the putative mitochondrial RNA binding complex (MRB1) found in parasitic protist *Trypanosoma brucei* was studied by creating single RNAi knockdowns of both genes as well as assaying the double knockdown cell line, previously obtained in our laboratory.

This work was supported by the Grant Agency of the Czech Republic 204/09/1667 and Ministry of Education of the Czech Republic (LC07032).

I hereby declare that I did all work, summarized in this thesis, on my own, and only using the cited literature.

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České Budějovice, April 21, 2010

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Lucie Hanzálková

Acknowledgements

I would like to thank to all the great people who were helping me to find my way around the whole new and a little confusing world of science. First of all I'd like to mention Hassan, who always tried to make me realize things on my own even if it sometimes took a bit longer, Lucka who was always ready to help me and Zdeňka, who taught me the basics when I came into the lab as a clumsy freshman. Every single person in lab was a target of my inquisitive questions over the two years and no one ever turned me down. For this my thanks goes to Pavel, Shao, big and small Zdeněk, Piya, Petr, Jirka, Milan, Eva, Zuzka, Tomáš, Esteban and DHL. At last but not least I feel grateful to Julius Lukeš for the opportunity he gave me, for his advice and guidance.

List of contents

1. Introduction

1.1 Several facts about trypanosomes

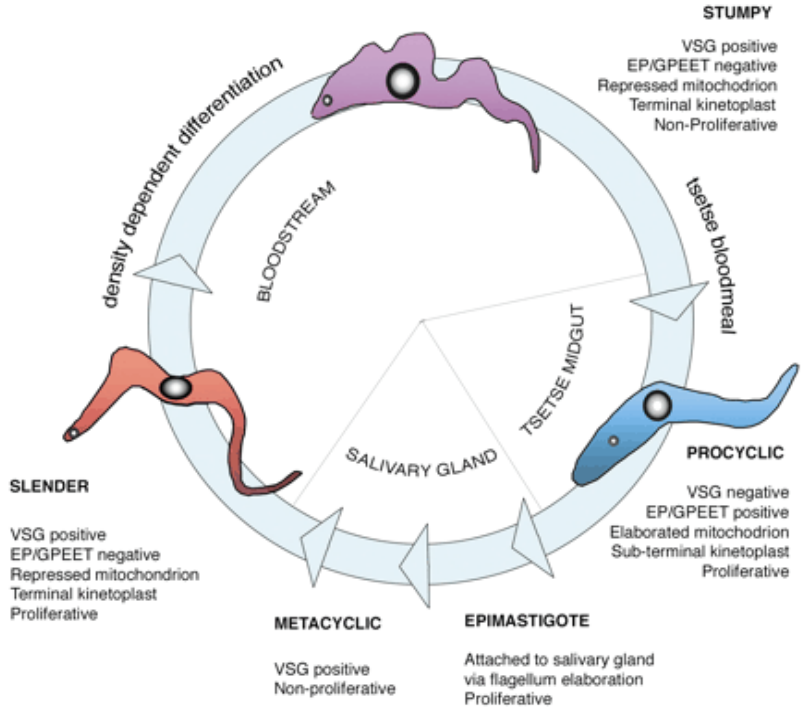
Trypanosoma brucei is a parasitic protist, belonging to the order Kinetoplastida, causing sleeping sickness in humans and Nagana in cattle on the continent of Africa. Of the sub-species, *T. brucei brucei* is responsible for disease prevalence in animals. Because it is harmless to humans, it became a model organism in laboratory research. *T. brucei gambiense* causes chronic infection in people living in western and central Africa and is responsible for around 90% of reported cases. Infection by *T. brucei rhodesiense* is acute and invades the central nervous system. Its prevalence is restricted to eastern and southern Africa (http://www.who.int/mediacentre/factsheets/fs259/en/).

Trypanosoma brucei undergoes dramatic physiological changes during its life cycle, in which we can distinguish three major forms: bloodstream, procyclic and metacyclic (Vickerman, 1985). In mammals, *T. brucei* thrives in its bloodstream stage, morphologically as the slender form. In order to avoid immune response, the trypanosome switches in this stage between variant surface glycoproteins (VSGs) covering its body. Its mitochondrial activity is repressed. As the cell count within the host bloodstream rises, the stumpy form starts to occur. These cannot proliferate, but are pre-adapted for transmission into the *T. brucei* vector *Glossina*, also knows as the tse-tse fly. The procyclic stage occurs in the midgut of *Glossina.* Instead of VSG, their cell surface is covered procyclin proteins. After establishment in the fly midgut, trypanosomes arrest in division and then migrate to the tsetse salivary gland, where they attach as epimastigote forms. These proliferative cells eventually generate non-proliferative metacyclic forms which have re-acquired VSG coat in preparation for transmission to new mammalian host (Matthews, 2005); see fig. 1.

In both procyclic and bloodstream stages, there can be found a single mitochondrion, which takes up significant portion of the cell cytoplasm. Although the physiology of this organelle is dramatically different between these two forms, one of the biological features of my interest, mitochondrial DNA, is contained in the same structure in both stages: the kinetoplast. Bizarre as it is, this feature of the mitochondrion is common to all members of the order Kinetoplastida, and is the source of its name. The kinetoplast, also called kDNA, comprises of a concatenated network of DNA circles (see fig.2). Two classes can be recognized. Maxicircles with its size of 20-40kb (depending on trypanosomatid species) encode subunits of mitochondrial

respiratory chain, mitochondrial ribosomal protein and some proteins of unknown function (MURFs). Minicircles, which mostly contribute to the mitochondrial DNA mass, are present in several thousands of copies that vary in sequence. Their size is about 1 kb in *Trypanosoma* species.

Fig. 1 *Trypanosoma brucei* life cycle



Trypanosomes proliferate in the bloodstream of mammals as the morphologically slender forms. In these cells, mitochondrial activity is repressed and the VSG coat allowing the parasite to avoid immune response is expressed. Stumpy forms occur as number of cells in host blood rises. These cannot divide but are pre-adapted for transmission to *Glossina*. In the vector midgut, cells proliferate in its procyclic form. Instead of VSG is their cell surface covered with procyclins. After establishment in the fly midgut, trypanosomes arrest in division and then migrate to the tsetse salivary gland, where they attach as epimastigote forms. These proliferative cells eventually generate non-proliferative metacyclic forms which have re-acquired VSG coat in preparation for transmission to a new mammalian host.

From Matthews, 2005

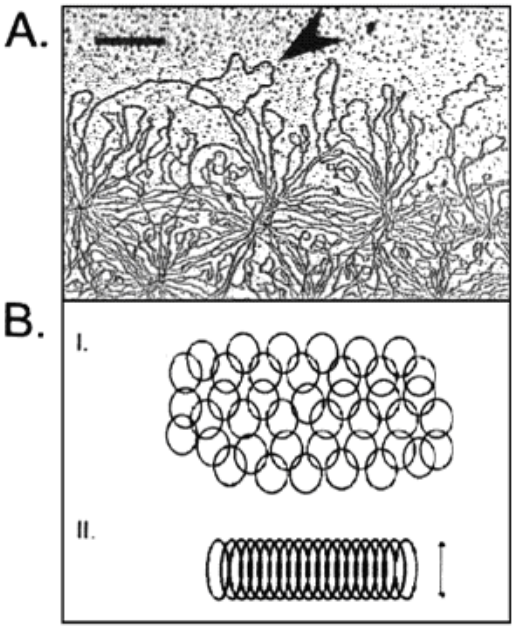


Fig. 2 kDNA network structure.

(A) Electron micrograph of the periphery of an isolated kDNA network fro *T. avium.* Loops represent interlocked minicircles. Bar, 500 nm.

(B) Diagrams showing the organization of minicircles.

(I) Segment of an isolated network showing interlocked minicircles in planar array.

(II) Section through a condensed network disk in vivo showing stretched-pit minicircles. Thickness of the disk is about half the circumference of a minicircle.

From Lukeš *et al*, 2002

1.2 RNA editing

RNA editing refers to an unusual form of RNA processing in which the sequence of the primary transcript is altered post-transcriptionally, as by insertion or deletion of Uridine (U) nucleic acids in the case of kinetoplastids. In *T. brucei*, the editing process is regulated during the life cycle of the parasite, often reflecting the differences in energy metabolism between bloodstream and procyclic stages (Feagin *et al*, 1986). This process was first discovered by Benne and colleagues in 1986 and has been the subject of intense study ever since.

1.2.1 Maxicircle and minicircle transcripts

As mentioned above, maxicircles encode proteins typically found on mitochondrial genomes. Some of these genes are encrypted to varying degrees and need to undergo editing process. There are basically three categories of genes (Fig. 3). So-called panedited genes require extensive editing in order to be functional; some genes undergo editing only in a small part of their sequence, those can be called partially edited or genes with limited editing. And finally there are genes that require no editing what so ever that can be called never edited or unedited.

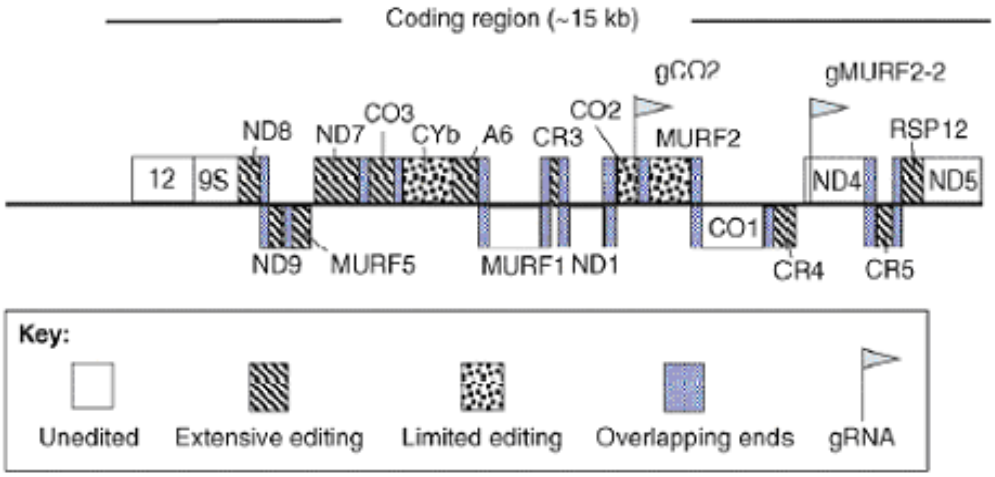


Fig. 3 Maxicircle map

Schematic of a linearized maxicircle map of the *Trypanosoma brucei* mitochondrial genome. You can notice a lack of t-RNA genes. Some genes overlap. Only the gene coding regions are shown. Gene abbrevations are as follows: 12S and 9S, rRNA subunits; ND, NADH dehydrogenase; CO, cytochrome oxidase; Cyb, cytochrome b; MURF, maxicircle unidentified reading frame; CR, C-rich reagion; RPS12, ribosomal protein subunit 12.

From Koslowsky, 2009

Minicircles encode small (about 60 nucleotides long) RNAs called guide (g) RNAs that serve as a template for editing of encrypted maxicircle transcripts (Sturm and Simpson, 1990). These nucleotide chains have a typical structure that contains three domains (Fig 4). The 5'-domain is called the anchor region and forms a duplex with pre-edited mRNA directly 3' to the block where the sequence is to be edited. The middle region contains the information domain, dictating specifically where and how many Us are inserted or deleted. The 3'-end contains an oligo (U) tail that is added post-transcriptionally (Blum & Simpson 1990). Also, two gRNAs are encoded on the maxicircles (Koslowsky, 2009). These two are: gRNA for CO2, which is located in the 3' UTR of said gene (Golden and Hajduk, 2005) and gRNA for MURF2 situated near ND4 gene. MURF2 gRNA can also be found on minicircles.

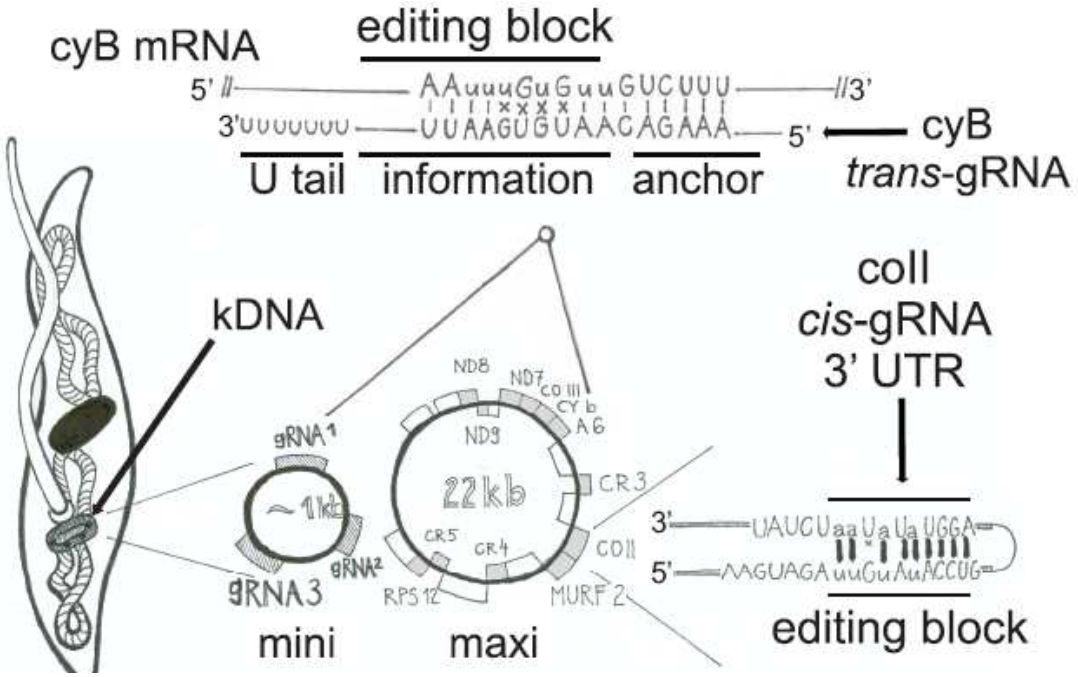


Fig. 4 Guide RNAs mediate RNA editing. A minicircle-encoded *trans*-acting gRNA forms a duplex with its cognate mRNA through hybridization of its anchor domain (**top**). The information domain guides U insertions into and deletions from the RNA until it complements the editing block. Non-canonical U:G pairings are depicted as crosses. The *cis*-gRNA residing of the 3' UTR of cox2 guides four U insertions (**right**).

From Hashimi, 2009

1.2.2 RNA editing mechanism and RNA editing core complex

Editing or mRNA proceeds from the 3' to 5' direction (Maslov and Simpson, 1992). At first the anchor region of gRNA hybridizes with its cognate mRNA. As the mRNA is being

altered, new sequences that are serve as anchor sites emerge, thus most pan-edited mRNAs contain multiple editing sites (ES) requiring multiple gRNAs. For all these events to take place, several catalytic steps must be coordinated (Kable *et al*, 1996, Seiwert *et al*, 1996, Igo *et al*, 2000). An endonuclease is responsible for cleaving mRNA at the ES, and then Us are either added to the 3'-end of the 5'-fragment by terminal uridynyl transferase (TUTase) or removed by a U-specific exonuclease (exoUase). The processed fragments are re-joined by a RNA ligase afterwards. These catalytic activities are contained in a macromolecular complex for which Larry Simpson *et al,* (2010) recently suggested name the RNA editing core complex (RECC), which will be used in this thesis. It is also refered to as 20S editosome, since it sediments at 20 Svedberg units in glycerol gradients (Pollard *et al,* 1992).

Recent studies made by Carnes and colleagues (2009) have revealed that there are actually three functionally and compositionally distinct RECCs. Kinetoplastid RNA editing endonucleases 1, 2 and 3 (REN1, REN2 and REN3) are three enzymes that are proposed to distinguish the function of the RECC depending on which one of these it contains. While REN1 cleaves RNA at U deletion sites, REN2 does so at U insertion sites. REN3 specifically cleaves the cytochrome oxidase subunit II (COII) mRNA, which is the only maxicircle transcript that contains its own gRNA on its 3' UTR (see figs.3 and 4).

Other proteins that are confirmed to have a role in RNA editing are the so-called mitochondrial RNA-binding proteins MRP1/2, which exist as a heterotetramer and facilitate hybridization between maxicircle transcript and gRNA (Schumacher *et al*, 2006; Zikova *et al*, 2008). RNAi knockdowns of MRP1/2 have virtually the same phenotype as those of another RNA binding protein, RBP16 (Vondruskova *et al*, 2005; Pelletier and Read, 2000). Their silencing has an adverse effect on Cyb editing and decreased stability of never-edited CoI and ND4 mRNAs. Simultaneous depletion of both factors seems to have a lethal effect in the procyclic stage of *T. brucei*, as opposed to an inhibition of growth, and downregulates several maxicircle mRNAs in a synergistic fashion (Fisk *et al*, 2009). Interestingly, RPB16 appears to enhance *in vitro* editing activities, perhaps suggesting that it facilitates gRNA/mRNA interactions (Ammerman *et al*, 2008).

1.2.3 Mitochondrial RNA binding complex

This thesis focuses on the so-called putative mitochondrial RNA binding complex (MRB1) (Hashimi *et al*, 2008; Panigrahi *et al*, 2008; Weng *et al*, 2008), the compositon of which is depicted in fig.5. MRB1 was described independently by three groups using the tandem

afffinity purification (TAP) approach. In this method, a fusion protein is created consisting of a protein of interest and the so-called TAP tag. This tag facilitates the isolation of the protein and potential binding partners using two purification steps (Puig *et al*, 2001). After the final elution, mass spectophotometry is used for identification of binding partners. The overlap of the proteins between the complexes identified by the three groups using the TAP-tag strategy, as well as overlaps with complexes involved in other aspects of mRNA metabolism (discussed below), suggest that there may be a dynamic interaction among these proteins, or that this collection of proteins is actually composed of smaller complexes and/or protein monomers. Furthermore, the data generated by RNAi knockdowns of these MRB1 subunits results in a variety of different phenotypes, which may mean they do not form a *bona fide* protein complex such as RECC.

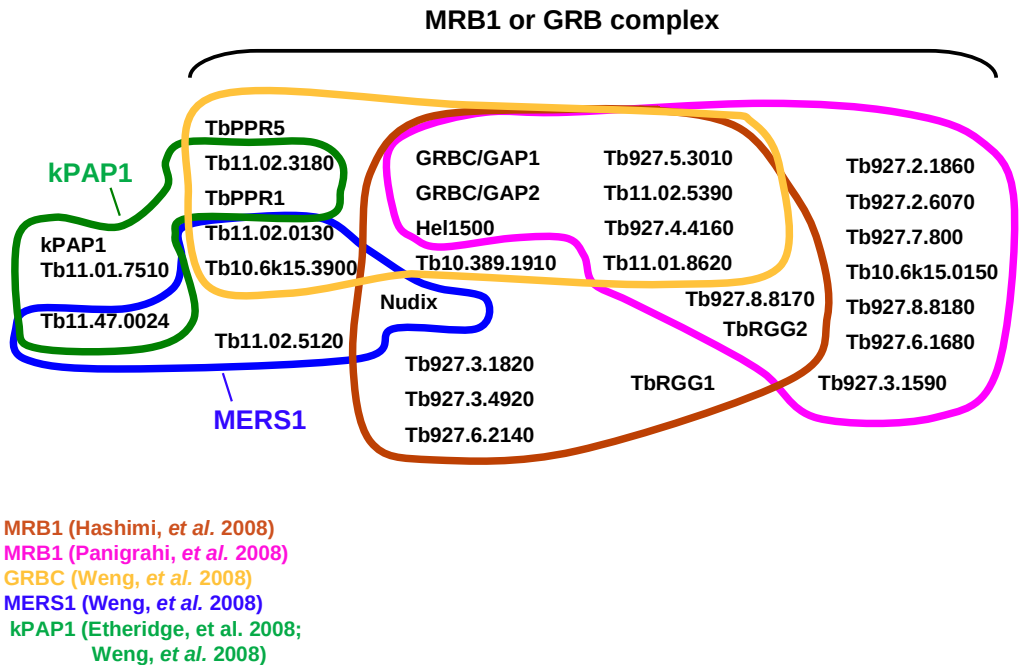


Fig. 5 Overlapping composition of putative MRB1 and GRB complex

Putative complexes isolated by different groups using TAP approach. Overlap occurs not only between MRB1 and GRBC but as well covers some complexes involved in mRNA metabolism. From Hashimi 2009

In our laboratory, MRB1 was copurified with TbRGG1 protein in a RNase sensitive manner (Hashimi *et al*, 2009). This protein contains a RGG RNA binding domain and has an affinity towards poly(U) (Leegwater *et al*, 1995; Vanhamme *et al*, 1998). The same applies to TbRGG2, which is also present in this putative complex. RNAi knockdowns of both result in specific downregulation of edited-RNAs (Fisk *et al*, 2008; Hashimi *et al*, 2008; Acestor *et al*,

2009). Two gRNA associated proteins (GAP1/2) are mutually dependant (Weng *et al*, 2008; Hashimi *et al*, 2009), and their knockdown causes downregulation in those edited-RNAs requiring *trans*-acting gRNAs. This effect was shown to be due to a consequent decrease of the steady-state level of gRNAs upon GAP-RNAi induction (Weng *et al*, 2008; Hashimi *et al*, 2009). Homologs of these two proteins in *Leishmania tarantolae* were assayed by Weng *et al*, (2009) as a part of GRBC complex and were called GRBC1/2. Virtually the same phenotype as in GAP1/2 knockdowns was observed in in knockdowns of a predicted DExD/H-box RNA helicase (Hashimi *et al*, 2009; Hernandez *et al*, 2009). Another studied MRB1 protein is the predicted Nudix hydrolase (Nudix stands for NUcleoside DIphosphate linked to some other moiety X). RNAi-mediated depletion of this protein was found by two independent groups to either downregulate levels of both edited and pre-edited RNAs (Hashimi *et al*, 2009) or only the former molecules (Weng *et al*, 2008). No effect on gRNA stability was observed in either study. The latter group labelled this protein mitochondrial edited mRNA stability factor 1 (MERS1) to reflect such an apparent role.

In this thesis I will present my results regarding functional analysis of two other subunits of MRB1, provisionally named mitochondrial protein (MP) 100 and 102 (MP102), according to their predicted molecular weights. The corresponding GeneDB (www.genedb.org) accession numbers are Tb927.8.8170 for MP100 and Tb927.4.4160 for MP102.

1.3 The mitochondrial RNA metabolism in *Trypanosoma brucei*

The RNA metabolism in the *T.brucei* mitochondrion is a complex process requiring participation of several enzymes and complexes. While only a single mitochondrial RNA polymerase (mtRNAP) is required for transcription from maxi and minicircles (Grams *et al*, 2002; Hashimi *et al*, 2009), the maturation pathway of these transcripts dramatically differ afterwards.

Minicircles are thought to be transcribed polycistronically and then cleaved by a 19S complex into an induvidual gRNA molecule (Grams *et al*, 2000). Kinetoplastid RNA editing 3' terminal uridylyltransferase (KRET1) is responsible for polyuridylylation of these transcripts (Aphasizhev *et al*, 2003a). Maxicircles seem to undergo polycistronic transcripton as well. RNA editing occurs independently of cleavage of the long precursor into monocistronic transcripts (Koslowsky and Yahampath, 1997). Kinetoplast poly(A) polymerase (KPAP) then polyadenylates 3’ ends of the monocistronic transcripts with a short 3'-polyA tail (Etheridge *et al*, 2008). In general, mitochondrial transcripts in *T. brucei* have either short (20–25 nt) A-tail or

long (120–250 nt) poly(A/U) tail (Bhat *et al*, 1992; Etheridge *et al*, 2008; Militello and Read, 1999)., which seem to determine the fate of the modified transcript.

Pre-edited forms possess only short A-tails whereas neveredited and edited mRNAs have both short and long tails (Bhat *et al*, 1992; Militello and Read, 1999). While in pre-edited transcripts, short poly(A) tails lead to destabilization, on most edited and some never-edited RNAs it has the opposite effect (Kao and Read, 2005; Etheridge *et al*, 2008). Most mRNAs acquire long A/U tail, in case of RPS12 this event directly follows editing (Aphasizheva and Aphasizhev, 2010). It is possible that the 3' A/U tail marks that the mRNAs are prepared for translation, which would cover both never-edited and edited mRNA (Aphasizheva and Apasizhev, 2010). This model is in contrast with *in organello* studies made by Ryan and Read in 2005, which suggest that polyadenylated mRNAs are destabilized upon addition of UTPs.

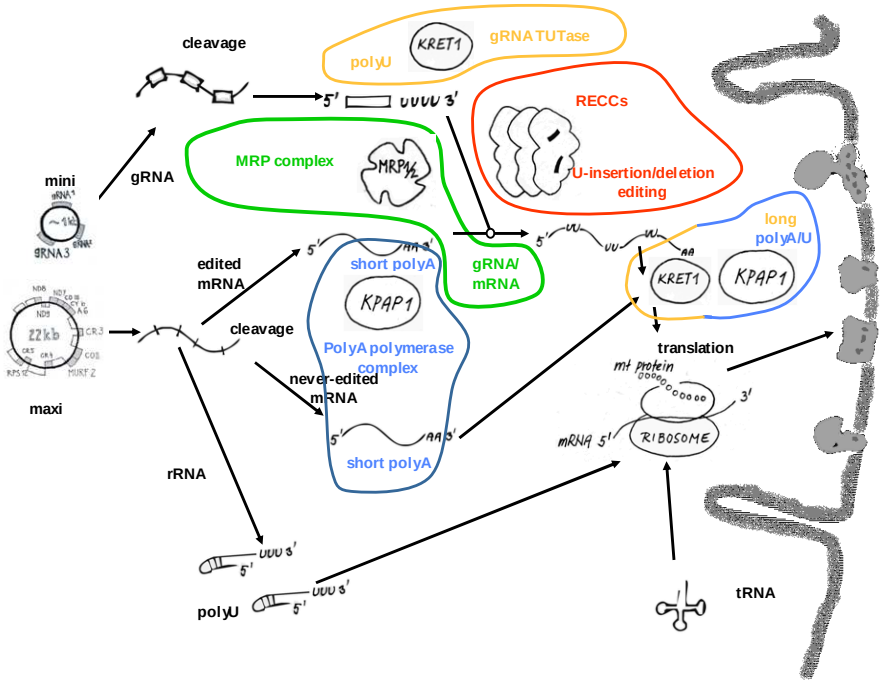


Fig. 5 Mitochondrial RNA metabolism in *T. brucei*

Several complexes play a role in T. brucei RNA metabolism. Both maxicircle and minicircle transcripts undergo polycistronic transcription and cleavage. KRET1 adds poly(U) tails to gRNAs, KPAP1 polyadenylates maxicircle encoded RNAs, apart from ribosomal RNAs.

2. Materials and methods

2.1 RNAi construct preparation

2.1.1 Primers

Primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome).

Primer name	**primer sequence**
MP100 Fw	CCG **CTC GAG** TTA CGC ACA CTG CTC ACA
MP100 Rv	GCC **GGA TCC** CTT CTT TTC GTC CCC ACA AG
MP102 Fw	GGA **CTC GAG** TTC ATA ACT TCA TTG AGC CCG
MP102 Rv	CGC **GGA TCC** ATG ACG GTG CTT CTT TTC GT
dKD Fw	**GGA TCC** CCT CCC GTT CTA CTG CTG AG
dKD Rv	**AAG CTT** AGC AGT TGC ATG AAG TGA CG

Restriction sites are in bold.

To each single knockdown forward primer's 5' end was added XhoI restriction site.

To each single knockdown reverse primer's 5' end was added BamHI restriction site.

Three extra bases added to 5' end of single knockdown primers, so the direct cloning into P2T7-177 vector would be possible.

In the case of double knockdown (dKD) XhoI restriction site was added to the 5' end of the reverse primer and HindIII site to the 5' end of the forward primer.

2.1.2 Polymerase chain reaction (PCR)

For each 50 µl reaction was used:

Genomic DNA	20 ng
Fw primer (10 µM)	2 µl
Rv primer (10 µM)	2 µl
Taq polymerase	10 units
Taq Pol buffer 10x	5 µl
dNTPs 2,5 µM	1,2 µl
$MgCl_2$	5 µl

Program:

1. denaturation	96°C 5 minutes	
2. denaturation	94°C 1 minute	25x
3. primers extension	58°C 1 minute	
4. polymerase	72°C 1,5 minutes	
5. polymerase	72°C 10 minutes	

2.1.3 Restriction

Both PCR product and P2T7-177 plasmid were digested with BamHI and XhoI enzymes (New England Biolabs) and recommended buffer for the double digest. Twenty units of each enzyme were used for each reaction. Incubated 1 hour in 37°C. Restriction of P2T7 was verified by electrophoresis.

2.1.4 Ligation into P2T7-177 plasmid

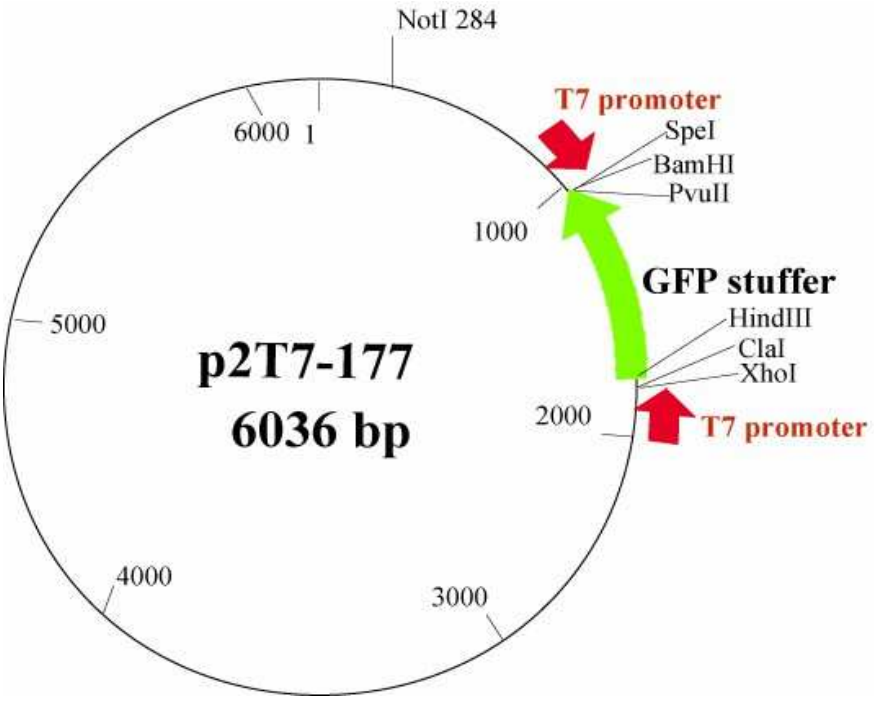


Fig. 6 P2T7-177 plasmid

Plasmid without the 800 bp GFP stuffer is about 6 Kb.

Ligation was done using T4 ligase in a 10 µl reaction volume according to manufacturer's protocol except for the incubation, which was done at room temperature

overnight. The PCR product insert was at least three of four times more abundant than the cut plasmid to ensure that the plasmid was the limiting factor in the reaction.

2.1.5 Transformation to XL1- Blue *E. Coli* cells

Competent *E. coli* XL1-Blue cells were thawed out on ice, and 50 µl of cells was mixed with 5 µl of the ligation reaction. The cells were incubated 20 min on ice, then 40 sec in a 42°C water bath, then 2 min on ice again. A volume of 100 µl of SOC was added and the cells were shaken for 30 mins in 37°C. The cells were spread on ampiciline lates that were left at 37°C overnight.

2.1.6 Cultivation of *E. coli* and plasmid isolation

E. coli colonies were picked from the plate (3 per gene) and seeded into 3 ml of LB media with ampicilin (0.1 mg/ml). Cultures were grown overnight at 37°C. The plasmid was isolated using the QIAGEN mini-prep kit, according to manufacturer's protocol.

2.1.7 P2T7-177 linearisation

Plasmid was linearised by the Not1 enzyme (New England Biolabs), using 20 units for 5 µg of DNA at 37°C overnight. The digest was tested by running an aliquot of the reaction on a gel in parallel with an uncut plasmid. The linearized construct was precipitated with 96% ethanol in a 2:1 ratio and sodium acetate at a final concentration of 0,3M in -80°C for 20 minutes. Pelleted DNA was washed with 70% ethanol. The pellet was air-dried and resuspended in 400 µl of sterile cytomix.

Cytomix composition	25 mM HEPES
	120 mM KCl
	0,15 mM $CaCl_2$
	10 mM K_2HPO_4 / KH_2PO_4
	2 mM EDTA
	6 mM glucose
	5 mM $MgCl_2$

2.1.8 Electroporation into 29-13 strain of *T. brucei*

Mid-log culture of wild type 29-13 strain of *T. brucei* was harvested by careful centrifugation (1300 g). The pellet was washed with 10 ml of cold cytomix and resuspended in 400 µl of cytomix with 10 µg of linearised plasmid. The mix of cells with plasmid was loaded into cuvettes and electroporated by ECM650 (BTX) machine with two pulses using following setting: 1500 V, 25 Ω, 50 µF, 10s, 1700 V, 25 Ω, 50 µF. Afterwards, cells were resuspended in 10 ml of medium supplemented with hygromycin (H) final concentration 50 mg/ml and G418 (G) to final 15 mg/ml, and incubated at 27°C overnight.

2.1.9 Drug-based selection of transfectants

2,5 µg/ml of phleomycin (P) was added into the culture of transformants. Cells were distributed on 24-well plate for semi-cloning by limiting dilution. First row was loaded with 1,5 ml of culture, second and third with 1 ml of SDM-79 medium and fourth with 0,5 ml of SDM-79 medium. Then 0,5 ml from the first row was transferred to the second, 0,5 ml from second to the third and so on (see fig. 7) Plates were kept in 27°C until the drug selection was done and the cell lines stabilized.

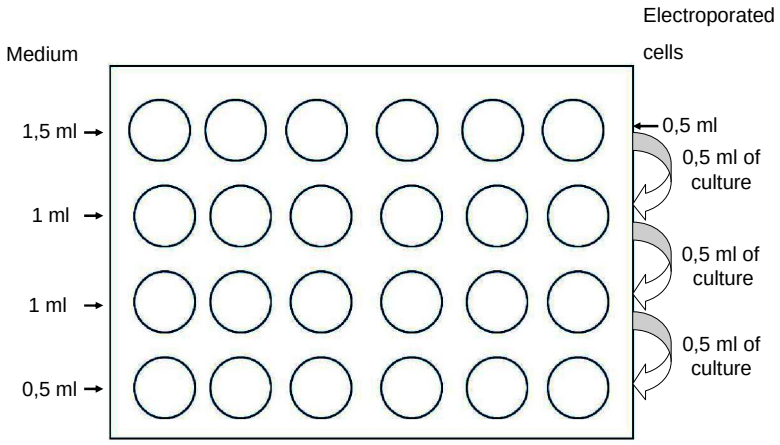


Fig. 7 Dilution of electroporated cells

2.2 Cultivation of *T. brucei*

The procyclic stage of *T. brucei* was cultivated at 27°C in SDM-79 medium (Brun and Schonenberger, 1979). Hygromycin (50 µg/ml) and neomycin (G418) (15 µg/ml) were added

into the wild type cell culture (H,G), stable non-induced cell line culture contained phleomycin (2,5 µg/ml) in addition to those (H,G,P). Tetracycline (1 µg/ml) was used to induce RNAi.

2.3 Northern analysis

Northern analysis was used for knockdown verification using a radioactively labelled probe that hybridized both the target gene and dsRNA gerated by tetracylin induction. Compared to real-time quantitative (q) PCR, it provides limited information about gene abundance upon interference induction, but the result also indicates whether the dsRNA is transcribed even in the absence of tetracycline. This phenomenon is called leakage.

2.3.1 Induction of *T. brucei*

Two cell cultures were grown with a starting concentration of $2x10^6$ cells/ml and a 10 ml volume. One of them was supplemented with tetracycline (1 µg/ml) to induce RNAi. Cells were harvested by centrifugation (1300 g) after 48 hours. The cell pellet was resuspended in 1 ml of RNA Blue (TopBio, Czech Republic).

2.3.2 RNA isolation

To each sample in RNA Blue was added 200 µl of chloroform. The emulsion was vortexed for 15 s, kept 2 mins to settle down and spun down in 4°C for 15 min, 9750 g. The upper aqueous layer was transferred to a new tube and mixed with 500µl of isopropanol. The RNA was precipitated at room temperature for 10 min, and then centrifuged as described before. The supernatant was discarded, pellet washed with 70% ethanol and air dried. The dry pellet was resuspended in 30 µl of water and heated for 10 min at 60°C. The concentration of RNA was measured using the NanoDrop (Thermo Scientific) spectrophotometer.

2.3.3 RNA gel electrophoresis

A 1% agarose gel containing 6,7% of formaldehyde and 1xMOPS buffer was made. Samples and RNA marker were mixed with appropriate volume of 1,5x sample buffer and incubated 10 min in 65°C before loading. The gel was run for 3 hours at 60 V. The running buffer was shaken every 30 min.

RNA gel 1% 100 ml	
10x MOPS	10 ml
Agarose	1g
milliQ water	72 ml
after cooling down add 18 ml of formaldehyde 37%	

1.5 x Sample Buffer	
Formamide	600 ul
Formaldehyde 37%	210 ul
10x MOPS	156 ul
Ethidium Bromide	5 ul

10x MOPS 100 ml	
0,5 M MOPS	40 ml
3 M NaO Acetate	1,67 ml
0,5 M EDTA pH 8	2 ml
milliQ water	56,33 ml

Running Buffer (500 ml)	
10x MOPS	50 ml
milliQ water	450 ml

2.3.4 Blotting

The Northern blot apparatus consisted of filter paper, the RNA gel, membrane and more filter paper in this order from bottom to top. 5x SSC was poured on blot during assembly, all layers were smoothened to avoid air bubbles. The current was created by dry paper towels on the top of the blot. Pressure was applied and the apparatus was secured against evaporation and let to stand overnight. The transferred RNA was then immobilized to the membrane by UV crosslinking (UV Stratalinker, Stratagene).

2.3.5 Membrane pre-hybridization

Membrane was pre-incubated in Na-Pi (sodium phosphate buffer) in a cylinder. It was let to rotate for two hours at 60°C.

2.3.6 Radioactive labelling

HexaLabel DNA labelling Kit (Fermentas) was used according to manufacturer's protocol using 100ng of purified PCR product as a template. The probe was purified in a spin column (MicroSpin G-50 Sephadex-GE Healthcare) and then heated at 100°C for three minutes,

cooled down on ice, put into approximately 5 ml of NaPi and poured into the cylinder with pre-hybridized membrane. The cylinder was then rotated at 55°C over night.

2.3.7 Membrane washing

Hybridizaton solution was poured out of the cylinder. This was replaced by 5 mls of the 2x SSC + 0,1% SDS solution and rotated for 20 min at room temperature. The liquid was discarded and 5 ml of 0,2x SSC + 0,1% SDS was poured into the cylinder and let to rotate for another 20 min at 55°C. The membrane was then wrapped into foil and stored in a phosphoimager cassette over night. The Northern data was captured on a Typhoon Phosphoimager (Amersham).

2.4 Growth curve

This method is used to follow the growth of the cell line where RNAi has been induced and determine by comparison with non-induced cells whether the RNA-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 starting cultures contained $2x10^6$ cells/ml. Their concentrations were measured with Beckman Coulter Z2 Particle Counter every 24 hours for fourteen days, with dilution back to $2x10^6$ cells/ml every second day. Four cultures were grown for each gene knockdown; two with the tetracycline in media (tet+) and two without (tet-). The graph was then acquired using the average of the two values.

2.5 Western blot

Western blot analysis is a technique used to detect specific proteins in whole cell or subcellular lysates using an antibody against a protein of interest. Four major steps were used. Gel electrophoresis, wet blot transfer to a PVDF membrane, antibody detection and analysis using a luminoimager.

2.5.1 Gel electrophoresis

Cells were harvested at day five after induction and resuspended in sample buffer consisting of 100 mM Tris, 200 mM DTT, 4% SDS, 0,2% Bromophenol Blue and 20% glycerol. A volume of lysate cooresponding to approximately $5x10^6$ cells was loaded into each well of a 12% polyacrylamide gel (PAGE). The gel was run for 3 hours at 80 V to separate proteins around 100 kDa.

2.5.2 Wet blot transfer

The PVDF membrane was prepared by washing with methanol for 10 min and transfer buffer (20% methanol by volume, 38,6 mM glycine, 48 mM Tris, 1,3 mM SDS) for another 10 min. The gel was also soaked in the transfer buffer before the blot assembly. The wet blot was assembled in the following order from anode to cathode: sponge, filter paper, membrane, gel, filter paper, sponge. The blot was run for two hours to make sure that the proteins around 100 kDa, which was the size of the target protein, will transfer. The membrane was blocked in 5% milk dissolved in PBS-Tween over night at 4°C.

2.5.3 Protein detection

The membrane was incubated with primary antibody against MP100 (supplied by L. Read), which was diluted 1:2000 in 5% milk. The membrane was then washed in PBS-Tween 5 x 5 min and incubated with secondary antibody, in this case α-rabbit antibody, which was diluted 1:2000 in 5% milk. Washing in PBS-Tween was repeated as before.

2.5.4 Membrane analysis

The Pierce ECL Western Blotting Substrate was applied on the membrane for 2 min. The immunodecorated protein was visualized using the LAS-3000 Luminoimage analyser set to high sensitivity and an appropriate exposure time.

2.6 Real time quantitative PCR (qPCR)

This method follows the general PCR principle, but allows the reaction to be followed in real time and thus quantify the abundance of a transcript. This procedure is facilitated by a dsDNA binding fluorescent dye, SYBR green in our case, which allows the measurement of concentration of a PCR product over the course of the reaction. The qPCR method was used in this project to verify RNAi knockdowns as well as assaying RNA editing *in vivo*. The latter assay was done using primers designed for selected mitochondrial genes, some of which undergo full editing, some just editing in a small region and some that are not edited at all (Carnes *et al*, 2005). Primers were designed to be specific for edited and pre-edited versions of genes. This approach allows us to see whether the knockdown affects these transcripts and even draw some conclusions about the part of the RNA metabolism a given protein may participate, based on the effect of their downregulation.

2.6.1 RNA isolation

RNA was isolated according to the same protocol that was used for RNA isolation in the preceding section on Northern analysis (see 2.2.2)

2.6.2 DNAse treatment of RNA

Since this assay focuses on quantification of transcript in a sample, it is important to get rid of any DNA that may potentially be isolated together with the RNA prior to using it a template for cDNA synthesis (described in section 2.6.3).

10-15 μg of RNA mixed with 1U of TURBO DNAse (Ambion) and 10x supplied buffer in 50μl reaction, and then incubated 30 min in 37°C. One more unit of TURBO DNAse was added to each reaction and incubated for another 30 min. Then the sample was incubated 2 min in RT with 10μl of DNAse inhibition mixture to stop the reaction, spun down for 2 min at 5850 g and the same volume of supernatant from each sample was transferred to new tubes. A volume of 300 μl of 96% ethanol, 5 μl of 3M acetate and 1 μl of glycogen were added to each tube and left over night to precipitate in -80°C.

Samples were centrifuged at 4°C at 13200rpm for 20 min, supernatant was discarded, RNA was washed with 70% ethanol and spun at the same conditions for another 10 min. The

ethanol was then discarded and the RNA air dried. The volume of water required for resuspension was chosen according to the starting amount of RNA, so the final concentration would be approximately 1 µg/µl. The quality of RNA was checked on a denaturing formaldehyde gel.

2.6.3 Creation of cDNA

Complementary DNA (cDNA) for qPCR was created in duplicate for each sample. In addition, a negative control was created for each RNA sample where reverse transcriptase was missing from the master mix. In total, we therefore obtained A and B RT+ samples (reverse transcriptase present) and one RT- cDNA for each RNA sample. Since accuracy is critical in this method, every reagent including RNA was vortexed and spun before using.

At start each tube contained 5 µg of RNA, 250 ng of random hexamer primers and 4 µl of 2.5 mM dNTPs in a total volume of 10 µl. Tubes were put into a PCR cycler and after 5 min at 65°C 10 µl of master mix containing 40 U of recombinant ribonuclease inhibitor (RNaseOUT^{TM}, Invitrogen), 200 U of reverse transcriptase (SuperScript® III, Invitrogen), 1 µl of 0,1 M dithiothreitol (DTT) and 4 µl of 5x SuperScript III buffer was added to RT+ samples. All of above apart from the SuperScript enzyme was added to RT- samples. The cycler program was resumed using the following: 5 min at 25°C, 60 min 50°C, 15 min at 70°C. The volume of all samples was then brought to 200 µl.

2.6.4 Real-time quantitative PCR

Each reaction was done in triplicate and contained 2 µl of cDNA, 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.3 µl of each primer (c= 100µM) in a total volume of 20 µl. For quantification of 18S and β-tubulin housekeeping genes the cDNA was further diluted 50x. Samples were put into the Rotor-Gene RG 3000 (Corbett Research) thermocycler and the following program was run: 2 min at 50°C, 10 min at 95°C, (15s at 95°C, 60s at 60°C) repeated 40 times, melt: 60°C-> 95°C with 1 degree/step increment, first step took 45s, following ones 5s each. In the first run abundancy of housekeeping genes was measured in all; A and B, tet+ and tet- samples, then the levels in induced and non-induced cDNA were compared and the pair with the closest Ct value was chosen for further experiments.

For the primers used for the quantification of various mitochondrial proteins see Carnes *et al* (2005). Primers used for knockdown verification were designed using NCBI primer blast,

numbers in brackets determine the position in the gene sequence; MP100 Fw: TACGCTCGATTCGCGACGCC (112-131), MP100 Rv: GGGTGTGTGAGCAGTGTGCGT (187-207), MP102 Fw: CGGAAAATTGGCGAGAAGTA (22-41), MP102 Rv: CCGGGGGAGAAGACTAAGAC (65-84). All the primers used for qPCR were HPLC purified.

The Relative abundance of maxicircle transcripts as well as target gene transcripts were calculated using the Pfaffl method (Pfaffl, 2001). For the used formula, see Fig. 7. Error bars were calculated using the data obtained for the triplicates in which each reaction was done.

$$\text{Relative abundancy} = \frac{\text{PCR efficiency (target gene)} \wedge \text{Ct value (target gene)}}{\text{PCR efficiency (reference gene)} \wedge \text{Ct value (reference gene)}}$$

Fig. 7 Pfaffel method formula

Ct value stands for threshold cycle value, which is the number of the cycle where the transcript abundance reached a set flouresence value.

3. Results

3.1 Orthologs of MP100 and MP102 in Kinetoplastids

We have found orthologs of Tb927.8.8170 (MP100) and Tb927.4.4160 (MP102) in various organisms belonging to order Kinetoplastida. Two proteins similar to those mentioned above are found only in two organisms: *T. brucei gambiense* and *Trypanosoma congolense*. Other kinetoplastida, which have only one gene with significant sequence identity with our query, are *Trypanosoma cruzi*, *Trypanosoma vivax*, *Leishmania braziliens*, *Leishmania infantum* and *Leishmania major*.

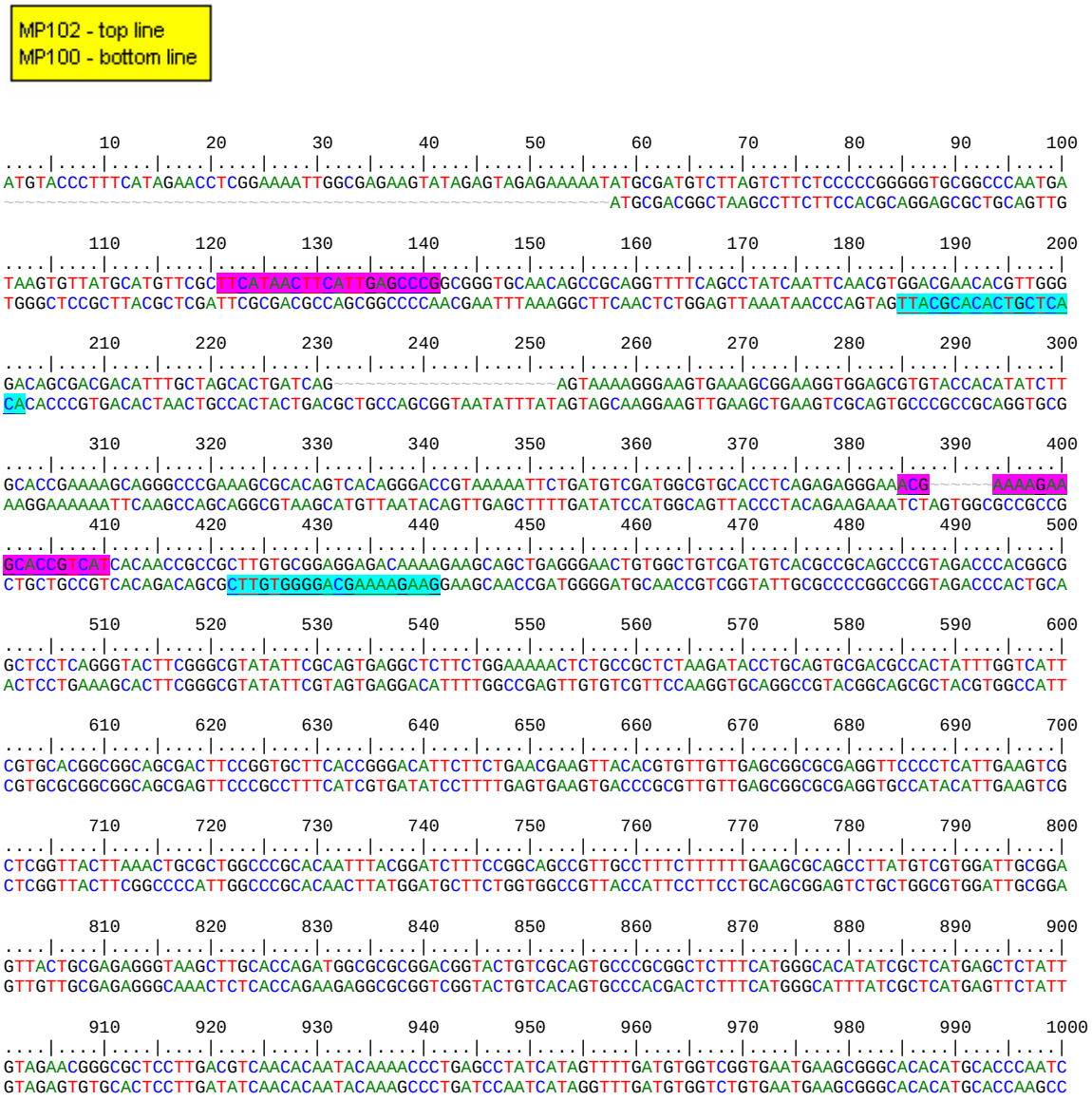
Organism	**Accession number (GeneDB)**
T. brucei brucei	Tb927.8.8170
T. brucei brucei	Tb927.4.4160
T. brucei gambiense	Tbg927.8.8500
T. brucei gambiense	Tbg927.4.4290
T. congolense	TcIL3000.4.3570
T. congolense	TcIL3000.8.833
T. cruzi	Tc00.1047053509895.20
T. vivax	TvY486_0807570
Leishmania braziliens	LbrM31_V2.0810
Leishmania infantum	LinJ31_V3.0670
Leishmania major strain *Fridelin*	LmjF31.0640

3.2 Single knockdowns of MP100 and MP102

Both single knockdowns were done only in the procyclic form of *T. brucei*. This task was not as straightforward as anticipated because of the high DNA sequence identity of the two genes. I chose two ways to present the DNA sequence similarity between MP102 and MP100. First is a standard alignment, created using ClustalW application, second is a simplified scheme which offers clear picture of position of primers used for generation of single and double knockdown in the context of sequence similarity.

3.2.1 Alignment of MP100 and MP102

Alignment was done in BioEdit using the ClustalW application. It shows 90% sequence identity in approximately 85% of the sequence. The remaining 15% with no significant sequence homolgy is located in the 5'-region. PCR primers for amplifying the fragment for dsRNA generation for single knockdowns were therefore designed in this 5'-region in order to avoid off target RNAi. In contrast, the RNAi fragment used for double knockdown generation is longer and targeted near 3'-end, where there are nucleotide sequences with 100% similarity for sequence stretches longer than 24 nts, the minimal length of interfering dsRNAs (Djikeng *et al*, 2001). The parts of the sequence where the primers anneal are marked in the alignment according to the fig. 8 description.



```
        2710      2720      2730      2740      2750      2760      2770
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAACTGGCGCGCCGATTTGGTGGGGAGCAGGAGCAGCAAATGGCACAGGAAATGTTAGCGAAGGTGCTTTATTAG
GAACTGGCGCGCCGATTTGGTGGGGGACAGGAGCAGCAAATGGCGCAGAAGATGTTAGCGAAGGTATTTTGTTAG
```

Fig. 8 Alignment of MP100 and MP102

The alignment was done using ClustaW application. Top line is the gene sequence of MP102, bottom line the sequence of MP100. The sites where the primers anneal are highlighted in the following manner: single MP102 KD primers, single MP100 KD primers, double KD primers.

3.2.2 Schematic drawing of MP 100 and MP 102

The scheme in Fig. 9 presents the genes and the position of primers used for RNAi fragment creation. Double knockdown primer design was based on the sequence of MP102. Please note that the scheme is not drawn to scale.

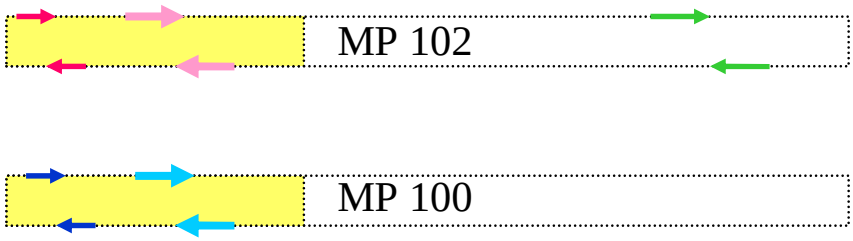


Fig. 9 A scheme of MP 100 and MP102 gene sequence identity

represent primers used for real-time quantitative PCR in MP102
represent RNAi primers used to generate MP100 KD dsRNA fragment
represent RNAi primers used to generate MP102 KD dsRNA fragment
represent RNAi primers used to generate MP100/102 double KD dsRNA fragment
represent primers used for real-time quantitative PCR in MP100
The yellow background marks the drawn region where the two genes differ in sequence. The white background represents the area where the two genes have 90% sequence identity. The scheme is not drawn to scale.

3.2.3 RNAi fragment creation

PCR products were checked by agarose gel electrophoresis before and after digestion with BamHI and XhoI restriction enzymes. The P2T7-177 plasmid was also controlled after restriction with these enzymes.

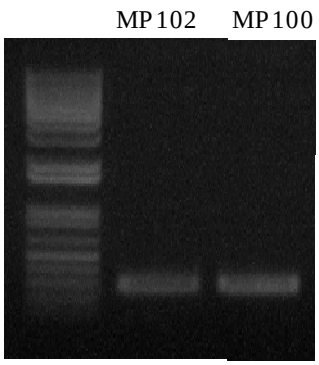


Fig. 10 Gel elecrophoresis of gene fragments used to generate dsRNA obtained by PCR

· The sizes of both fragments are about 280 bp

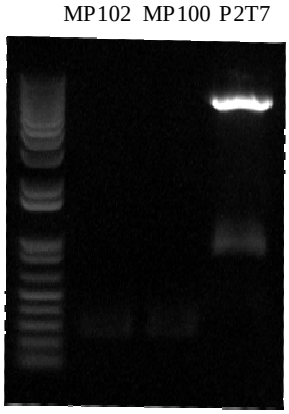


Fig. 11 Gel electrophoresis of plasmid and inserts after restriction digest.

P2T7-177 plasmid and inserts after restriction by XhoI and BaMHI. The plasmid size is about 6000 bp while the freed GFP stuffer 800 bp in size.

After ligation of the digested PCR amplicons into p2T7-177, subsequent transformation into competent *Escherichia coli* bacteria, inoculation of *E. coli* transformants into LB media cultures and isolation with the QIAGEN miniprep kit, the plasmid was again digested with XhoI and BamHI to diagnose the presence of inserts.

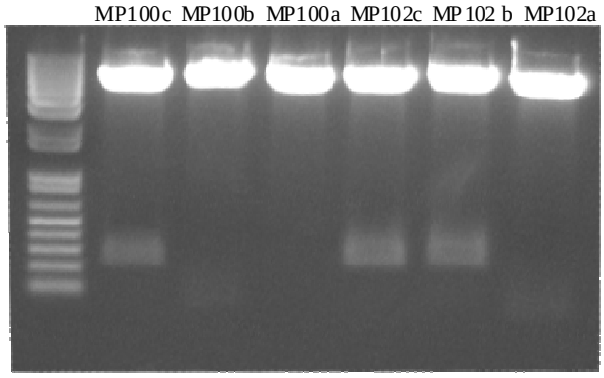


Fig. 12 Plasmid isolated from *E. coli* after digest

Minipreps of plasmids isolated from the *E. coli* cultures after restriction with BamHI and XhoI. Plasmid size is about 6000bp, insert size about 300bp. One successful ligation for MP100 and two for MP102 were obtained.

One successful ligation of MP100 and two of MP102 inserts into the p2T7-177 vector were obtained. These three plasmids were also sequenced as a further control of their identity.

3.2.4 Knockdown verification

Two clones of MP102 and four of MP100 knockdown cell lines survived after electroporation of one of the previously described constructs into the 29-13 strain of *T.brucei* and subsequent selection by 2,5 µg/ml of phleomycin. Knockdowns were confirmed by real-time quantitative (q) PCR (see Fig 10). Clones MP100 D1 and MP102 D5 were chosen for subsequent experiments.

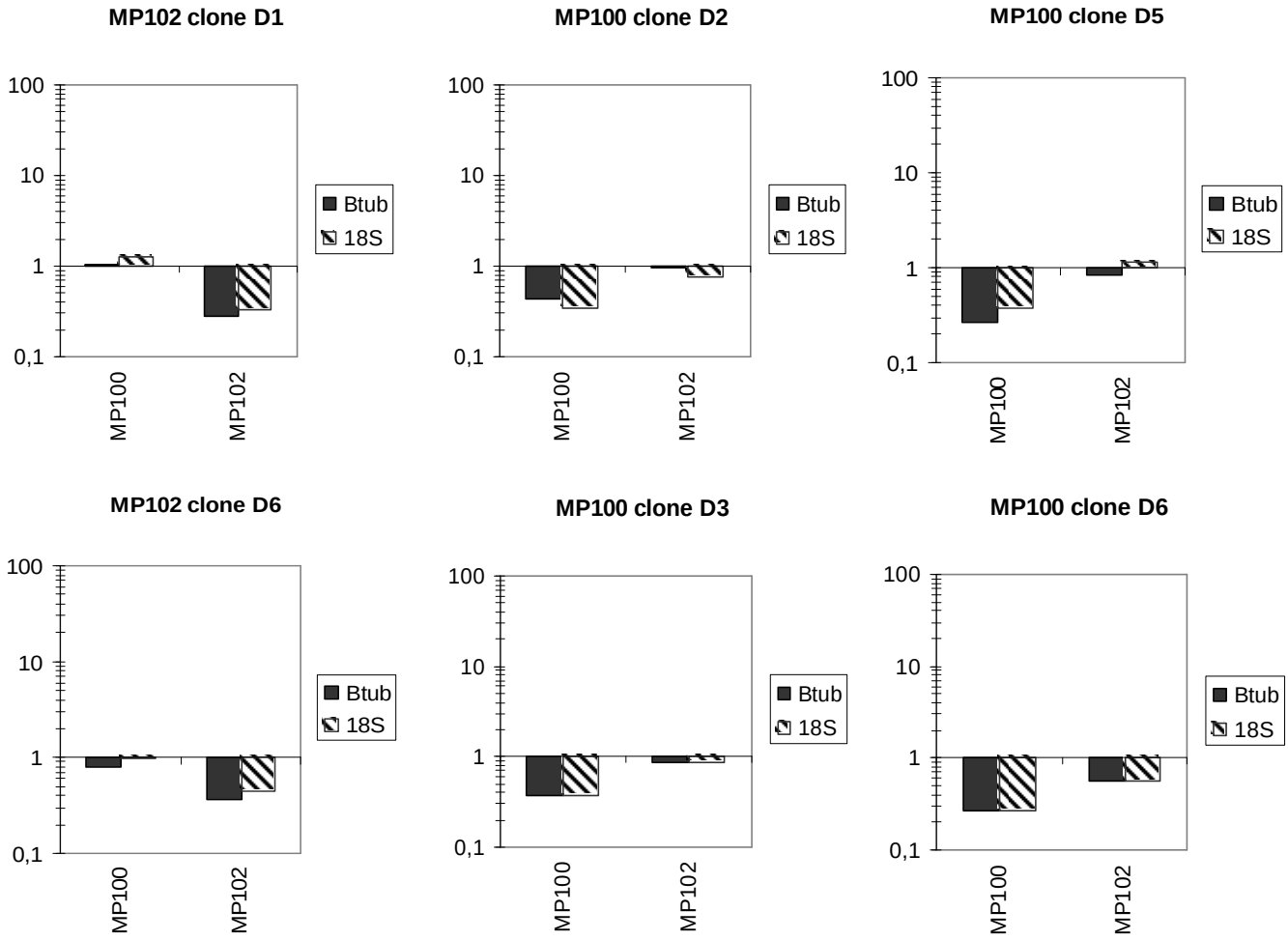


Fig. 13 Single knockdown verification by qPCR

Single knockdowns were verified by qPCR. The graph shows downregulation of transcript in induced cells compared to non-induced ones. Two different housekeeping genes were used for normalization of the data: 18S rRNA and β-tubulin. Based on this analysis MP102 clone D1 and MP100 clone D5 were chosen for subsequent experiments.

3.2.5 Growth curves

Clones MP102 D1 and MP100 D5 were chosen for the growth curve analysis (Fig. 14). The starting cultures contained $2x10^6$ cells/ml. Their concentrations were measured every day for fourteen days, with dilution back to the starting concentration every second day. No effect on growth of RNAi-silecning of either MP100 or MP102 was observed over the two weeks period.

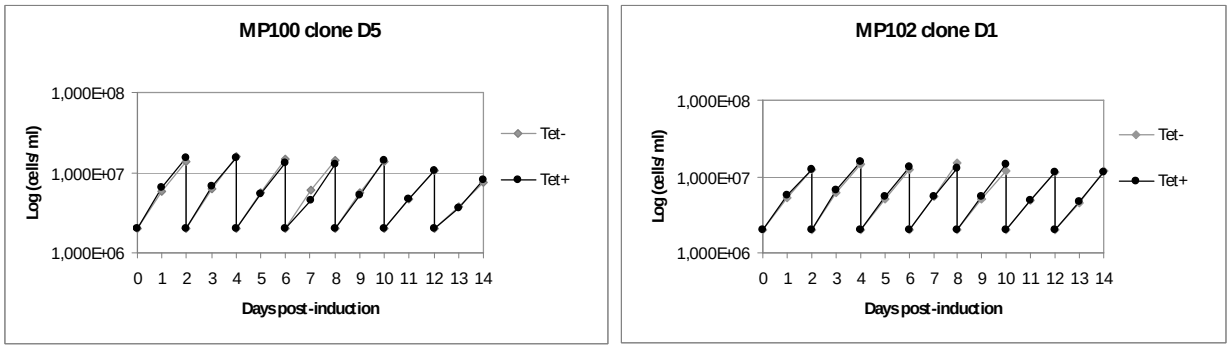


Fig. 14 Growth curves of single knockdowns of MP100 and MP102

Growth of both MP100 and MP102 KDs was measured for fourteen days. Tet+ lines are cells in which RNAi was induced by tetracycline; Tet- are non-induced cells. No effect on growth was observed over the two weeks period for either knockdown.

3.3 Double knockdown of MP100 and MP102

A double knockdown in the procyclic form of *T. brucei* was obtained by Lucie Novotná by electroporation of the RNAi fragment that was set into the region with high similarity between the two genes in a manner described above.

3.3.1 Northern analysis

Northern analysis was performed on clones that survived selection with phleomycin. The probe, which was a radioactively marked PCR product created using the same primers as for the amplification of RNAi fragment, should detect dsRNA used for RNA silencing and target mRNA. It confirmed that the dsRNA is transcribed only in induced cells, but the transcript itself is not clearly visible. A possible explanation could be that the abundance of target mRNA

is below the threshold of Northern analysis detection or that the band is obscured by ribosomal RNA running around the same size.

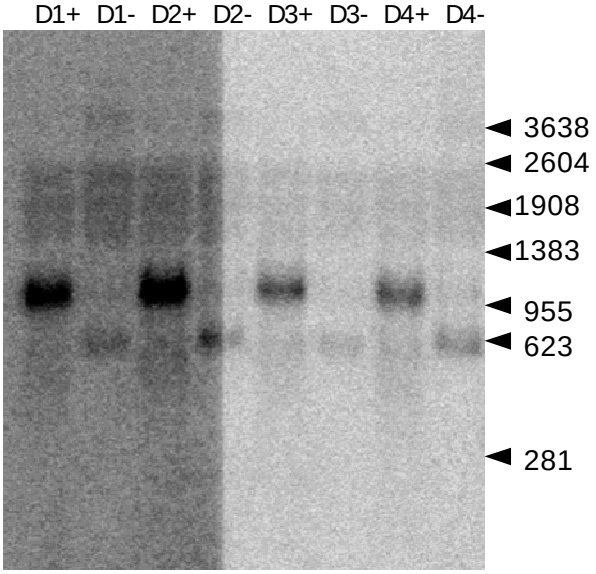


Fig. 15 Northern blot of double knockdown of MP100 and MP102

Transcription of dsRNA in induced cells was verified. Transcript (2700 nts) is not clearly visible. Clone D3 was chosen for subsequent experiments.

3.3.2 Growth curve

The growth of the double knockdown was followed for fourteen days in the presence and absence of tetracycline, starting at a concentration of $2x10^6$ cells/ml and diluted every second day. The growth inhibition of cells cultured in the presence of tetracycline was observed around the fifth day after the induction (see fig. 16)

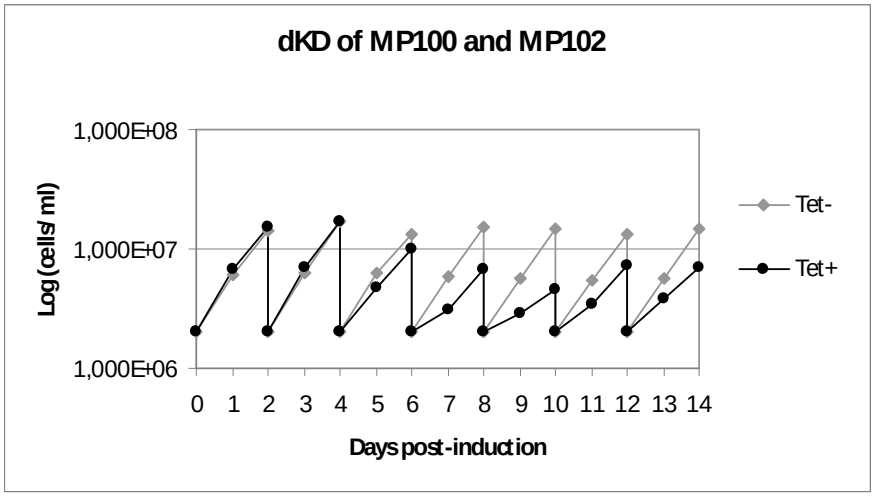


Fig. 16 Growth curve for double KD of both MPs

Performed as described in fig. 14. Growth inhibition is observed starting at day five post-induction.

3.3.3 Western analysis

Western analysis was performed on both single knockdowns as well as double knockdown using a MP100 antibody, generated against a synthetic peptide of this protein and supplied by Laurie Read (State University of New York at Buffalo). An antibody against the cytoplasmic enolase protein was used as a loading control. The predicted size of MP100 is 100 kDa. However, the signal can be observed at the top of the membrane, which would indicate the size of the protein above 250 kDa. Furthermore, the band intensity was quite low, making interpretation of the western blot difficult. Nevertheless, the signal intensity follows the expected pattern, which is downregulation in induced MP100 KD and double KD while the band intensity not affected in the MP102 KDs as compared to the non-induced cells.

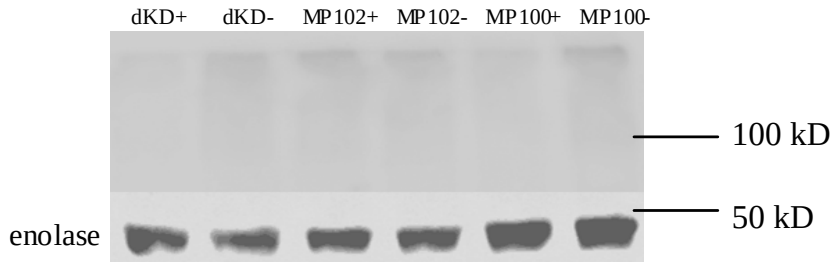


Fig. 17 Western analysis of MP100

Western analysis was performed on single knockdowns of both MP100 and MP102, as well as the double knockdown, using anti-MP100 antibody. An anti-enolase antibody was used as loading control. The protein size was expected to be 100 kDa. The "+" marks lysates of tetracycline induced cells;'–' lysates from non-induced cells.

3.3.4 Quantitative real-time PCR

The qPCR-based assay of maxicircle RNAs was performed on cells four days post induction, which is the time point directly preceding the manifestation of growth inhibition. Two housekeeping genes were used to normalize the data between tetracycline induced and non-induced samples: 18S rRNA and β-tubulin. The qPCR shows downregulation of both MP100 and MP102 transcripts. As for the mitochondrial genes, we observe decrease in abundance of edited transcripts with the exception of MURF2, which still appears to accumulate in the double KD in its pre-edited form despite no significant change in the abundance of the edited form.

Another gene with outstanding reaction to the depletion of both MP100 and MP102 is CO3, which shows a decrease in both edited and pre-edited transcript abundance.

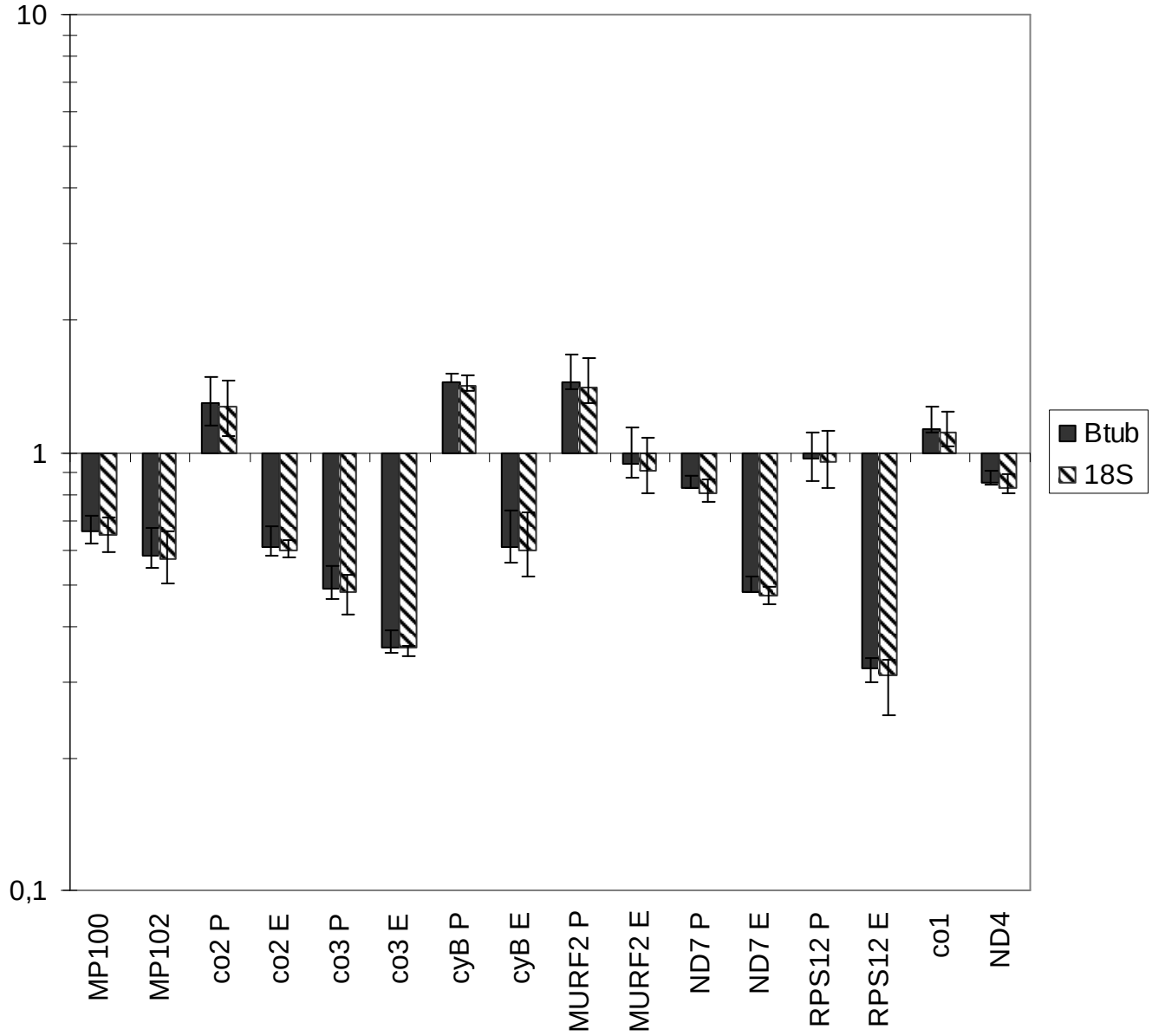


Fig. 18 qPCR analysis of the relative abundance of selected maxicircle-encoded mRNAs in double knockdown after RNAi induction

On the X-axis are the names of mRNAs and on the Y-axis is relative abundance of those mRNAs in the RNAi silenced double KD as compared to non-induced cells on a logarithmic scale. If the abundance in induced cells surpasses 1, it indicates accumulation of the transcript upon RNAi induction. In the other case of the reduction of the transcript in induced cells, the column is drawn below the X-axis. 18S and β-tubulin (Btub) are two housekeeping genes used for normalization of the acquired data. The error bars indicate the standard deviation.

Gene abbrevations are as follows: ND, NADH dehydrogenase; CO, cytochrome oxidase; CyB, cytochrome B; MURF, maxicircle unidentified reading frame; RPS12, ribosomal protein subunit 12.

4. Discussion

In my work I have focused on two proteins introduced by two different groups as a part of putative MRB1 complex (Hashimi *et al*, 2008, Panigrahi *et al*, 2008) and believed to play some role in RNA editing or metabolism in *T. brucei*. Tb927.8.8170 was given a working name MP100, which stands for a mitochondrial protein of 100 kDa mass. The other, Tb927.4.4160 has an atomic mass of 102 kDa, therefore we call it MP102. These two proteins, which do not contain any known domains or motifs, seem to be a result of gene duplication in *T. brucei*, which is supported by the level of their sequence identity as well as the fact that in other Kinetoplastids, with the exception of *T. congolense* and *T. brucei gambiense*, only one protein homologous to our proteins of interest can be found. This situation made it particularly complicated to create single knockdowns, because the stretch that unique between the two genes only takes up about 15% of the total coding sequence on the 5'-end. Within this region, I had to design primers for qPCR determination of the downregulation of individual MP100 and MP102 mRNAs, as well as for amplifying fragments for generation of dsRNAs specific against either of these transcripts. On the other hand, the double knockdown was quite easy to obtain. The RNAi fragment designed near 3'-end of MP102 gene sequence downregulates the abundance of the mRNA of both genes.

Since Northern analysis that was performed on the double knockdown did not really visualise target mRNA, perhaps because the mRNAs run at around the same size as rRNAs during gel electrophoresis or transcript abundance was below the sensitivity threshold of this method, we decided to use qPCR as a way of verifying the knockdowns. This method proved that the attempt to downregulate only one of the gene transcripts at a time was successful, as well as creation of the double knockdown.

The growth curves revealed that although the downregulation of both proteins at once slows the growth of the tetracycline induced cells to approximately half the growth rate of their non-induced counterparts, the lack of only one of the two proteins has no effect on growth as evidenced by the lack of growth inhibition in the single knockdowns. This result would suggest that the MP100 and MP102 proteins serve redundant function.

Quantitative real-time PCR measuring maxicircle RNAs was done only for the double knockdown so far. The result is quite surprising and not exactly easy to interpret. The downregulation of both MP100 and MP102 does not seem to have any significant effect on never-edited genes, which are represented by NADH dehydrogenase 4 (ND4) and cytochrome oxidase 1 (Co1). Cytochrome oxidase 2 (Co2) and cytochrome B (CyB) transcripts are both

upregulated in its pre-edited form and downregulated in edited form. Both of these mRNAs are only edited in a small region. However, another mRNA which undergoes editing only in small region, mitochondrial unknown reading frame 2 (MURF2), does not seem to be downregulated in its edited form, although the pre-edited transcript accumulates in induced cells. When we look at the genes which are fully edited, represented by NADH dehydrogenase 7 (ND7), ribosomal protein subunit 12 (RPS12) and cytochrome oxidase 3 (Co3), we can observe significant downregulation of the edited form of their mRNA. In the case of RPS12 and ND7 there is no accumulation of the pre-edited mRNA. However the level of pre-edited Co3 mRNA goes down upon induction.

My hypothesis about the possible functions of MP100 and MP102 in the mitochondrion of *T. brucei* is mostly based on the qPCR. Since the double knockdown influences the levels of Co2 mRNA, which has its gRNA encoded within its 3'-UTR and acts in *cis* (Golden and Hajduk, 2005), a role in *trans*-acting gRNA stability is unlikely. When the GAP protein are knocked down, the level of *trans*-acting gRNAs is decreased while Co2 editing is unaffected (Hashimi *et al*, 2009) A good way how to address whether MP100 and MP102 affects gRNAs is to do the guanylyltransferase labelling assay, which can directly visualise these molecules. Another possible role in RNA metabolism could be investigated by determining which RNA binds these proteins, if any. There are various methods which can be performed to find out. One is an EMSA assay, also called gel shift assay. The general principle of this method lies in the fact that nucleic acid migrates in gel slower when it has a protein or protein complex bound to it (Pelletier *et al*, 2000). Another possibility is the so-called Cross-Linking and ImmunoPrecipitation (CLIP) method, which can identify direct interaction sites between RNA-binding proteins and RNAs *in vivo* (Ule *et al*, 2005). In this method, a cross-linking agent is used to fixate the nucleic to the protein it interacts with, and then a specific antibody against the protein of interest is used for immunoprecipitation. The nucleic acid can then be identified.

The downregulation of the pre-edited as well as edited Co3 mRNA upon RNAi-silencing of both proteins could indicate a role in cleavage of the maxicircle transcript, which is transcribed polycistronically. Co3 is an extensively edited gene overlapping with pan-edited ND7 on its 5’-end and partially-edited Cyb on its 3’-end. If the function of MP100 and MP102 was to make sure that editing takes place on sites that are then recognized as the cleavage sites, the lack of these proteins may lead to the downregulation of this RNA even in its pre-edited form. This hypothesis may be confirmed by Northern analysis with a probe hybridizing pre-edited and edited Co3. In case this hypothesis is correct, we would be able to see a larger size of these mRNAs, albeit with a lower intensity.

The last hypothesis I would suggest would be, that MP100 and MP102 are simply increasing the efficiency of editing and therefore their knockdown results in increased abundance of transcripts where editing is in progress. Since primers targeting pre-edited transcripts are designed near 3'-end of target mRNA, which is where editing starts, and primers targeting edited transcripts are designed near 5'-end, therefore targeting only transcripts where editing is complete, transcripts where intensive editing is still in progress are not detected by either pair. This situation could explain why we do not see any upregulation in abundance of pre-edited transcripts of panedited genes or even, in the case of Co3, we can observe a downregulation, since an accumulation may occur in the "invisible" edited intermediates. Perhaps it may be interesting to look at these molecules to see if there are any editing stall sights. However, transcripts that require only one or a few gRNA to correct their sequence have a considerably less complex mix of editing intermediates. Thus, if MP100 and MP102 would be involved in the progress of editing, I would expect a slight upregulation in pre-edited and slight downreguation in edited mRNA abundance upon their RNA-silencing. While the partially edited MURF2 adheres to this scenario, partially edited CyB and Co2 are significantly affected by the double knockdown.

5. Abbreviations

complementary DNA	cDNA
cytochrome b	Cyb
cytochrome oxidase	Co
double knockdown	dKD
double stranded RNA	dsRNA
editing site	ES
guide RNA	gRNA
knockdown	KD
messenger RNA	mRNA
mitochondrial edited mRNA stability factor	MERS
mitochondrial protein	MP
mitochondrial RNA	mtRNA
mitochondrial unidentified reading frame	MURF
NADH hedydrogenase	ND
polymerase chain reaction	PCR
real time quantitative PCR	qPCR
ribosomal protein subunit 12	RPS12
RNA binding protein	RBP
RNA editing core complex	RECC
RNA editing endonuclease	REN
RNA interference	RNAi
terminal uridynyl transferase	TUTase
tetracycline	tet
untranslated region	UTR
uridine	U
variable surface glycoproteins	VSGs

6. References

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