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Functional analysis of subunits of mitochondrial RNA binding complex 1 in *T. brucei*

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Annotation:

In this study a functional analysis of four subunits of mitochondrial RNA binding complex 1 (MRB1) was performed using the tools of RNA interference. Examination of the generated knockdowns of GAP1, GAP2 and RNA helicase by RT-qPCR revealed that their downregulation results in a decline of edited transcripts as well as in a decrease of the steady state levels of gRNAs as shown by guanylyl transferase labelling assay. Thus, GAP1, GAP2 and RNA helicase play a role in maintaining the stability of gRNAs. In contrast to that, downregulation of Nudix hydrolase caused an overall reduction of both pre-edited and edited transcripts and had no effect on gRNA levels. Current data suggest that MRB1 participates in RNA metabolism in the mitochondrion of *Trypanosoma brucei*.

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I hereby declare that I did all work, summarized in this thesis, on my own or in collaboration with co-authors of the presented paper, and only using the cited literature.

České Budějovice, May 4, 2009 Zdeňka Čičová

Contents

Acknowledgements

Thankful

Thank you captain Jula for letting me go aboard your boat
me then fall into a stormy sea
You kept an eye on me, ready with help
how I can swim or how I sink
being eaten by the waves.

Thank you Hassan for being the wheelman for me
for five years on the same sea
captain and wheelman on the boat
me in the waters of no experience
gained and learned so much after all.

Thank you all the sailors from Jula`s boat
you never left ma as a lonely swimmer.
We have all been together through a lot
just now for me is the time to move on.

I have to leave your sailing boat but before that please let me
THANK YOU all.

Thank you all my friends for being the sun and the stars in the skies above the sea.

Děkuji Vám rodičové a Jožinku za vždy připravený záchranný kruh.

I am jumping to the OCEAN.

1. Introduction

1.1. *Trypanosoma brucei*

Trypanosoma brucei brucei is a subspecies of the *Trypanosoma brucei* complex and a member of the protozoan order Kinetoplastida. *T. brucei brucei* causes nagana, a wasting disease of livestock and cattle in sub-Saharan Africa, thus contributing to the malnutrition of the human population. During its lifecycle (Fig. 1.1), it alternates from the mammalian bloodstream to the midgut and salivary glands of its vector, the tsetse fly, a dipteran insect of the genus *Glossina.* The parasite's morphology, biochemistry and expression of major surface markers undergo rapid changes during the lifecycle.

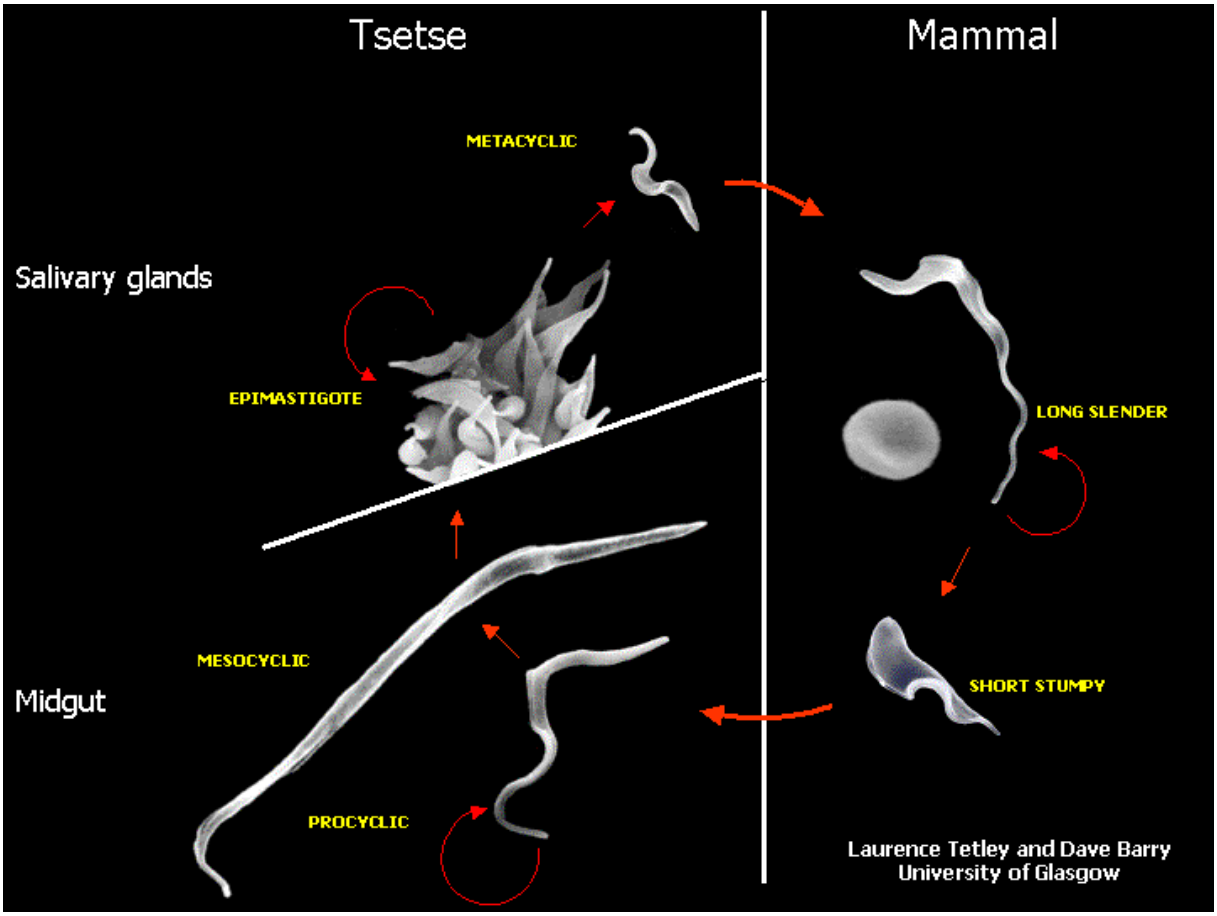

Figure 1.1: Lifecycle of *T. brucei*.
(Adapted from http://www.sanger.ac.uk/Projects/T_brucei/lifecycle.shtml)

The impact of sleeping sickness on human population in Africa is severe as shown by numbers provided by the World Health Organization (WHO). Sleeping sickness occurs mostly in rural areas where the health systems are weak or nonexistent. More than 90% of the reported sleeping sickness cases are caused by *T. brucei gambiense*, resulting in chronic infection. After transmission of the trypanosome into

the host, it starts to multiply in subcutaneous tissues, blood and lymph, causing the first, haemo-lymphatic stage of the disease, with bouts of fever, headaches, joint pains and itching. The second stage begins when the parasite crosses the blood brain barrier and invades the central nervous system. Clear symptoms of the disease, such as confusion, sensory disturbances, poor coordination and disturbance of the sleep cycle begin. A person can be infected for months or even years with no symptoms. When the symptoms do emerge, the parasites have usually already crossed the blood brain barrier, a stage of the disease difficult to treat. Thus many infected individuals die before they can ever be diagnosed and treated. Up to date there are four drugs registered for the treatment of sleeping sickness: Suramin and Pentamidine for treatment of the first stage and Eflornithine and Melarsoprol for the second stage. In the case of Melarsoprol, resistance is increasing and moreover both Eflornithine and Melarsoprol are quite toxic and complicated to administer (Barrett et al., 2003).

Apart from its two relatives *T. brucei gambiense* and *T. brucei rhodesiense*, *T. brucei brucei* does not cause a sleeping sickness in humans and thanks to the striking similarity to its harmful brethren it has become a model system in the laboratory. *T. brucei* is an outstanding and interesting subject of research not only because of its clinical importance but also because it possesses evolutionary unique molecular and biochemical properties. Among the very unusual features of *T. brucei* belongs its ability to effectively evade the immune response of the infected mammals by continuously changing a Variant Surface Glycoprotein (VSG) coat (Berriman et al., 2002), known as antigenic variation. Other prominent molecular oddities are polycistronic transcription, trans-splicing and templated mitochondrial (mt) RNA editing to generate mature mRNAs. At the cellular level they contain novel organelle, the glycosome, a subcelullar organelle containing glycolytic enzymes and related to the peroxisome (Opperdoes et al., 1977).

A defining structure of the protozoan order Kinetoplastida is the kinetoplast. It is a peculiar DNA (kDNA) network comprising the mt genome (reviewed in Lukeš et al., 2002). The kDNA exists in the form of a complex network of both tightly concatenated minicircles and maxicircles (Fig 1.2.) The minicircles, present in about 5000 copies per network, are usually nearly identical in size (0.5-10 kb, depending on the species), but are heterogeneous in sequence, since they encode the small (50-70 nucleotide (nt)) guide RNAs (gRNAs), which are further discussed in section 1.2. An important exception from the concept of gRNA being encoded on minicircles is the gRNA for editing of cytochrome oxidase subunit

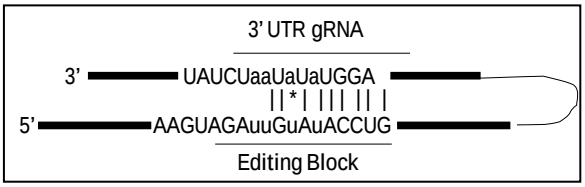

Figure 1.2: cox2 editing: *cis*- gRNA residing on its 3`end

2 (cox2) which uses its 3`-end sequence as gRNA (Golden and Hajduk, 2005). The maxicircles (~22 kb, 50 copies per cell) encode a subset of the subunits of mt respiratory complexes and two ribosomal RNA (rRNA) genes, with the coding capacity similar to the mtDNA of most eukaryotes. However, some of the protein-coding genes are encrypted. To generate functional mRNAs, the cryptic maxicircle transcripts undergo posttranscriptional modification via an intricate RNA editing process (Benne et al., 1986; for recent review Lukeš et al., 2005).

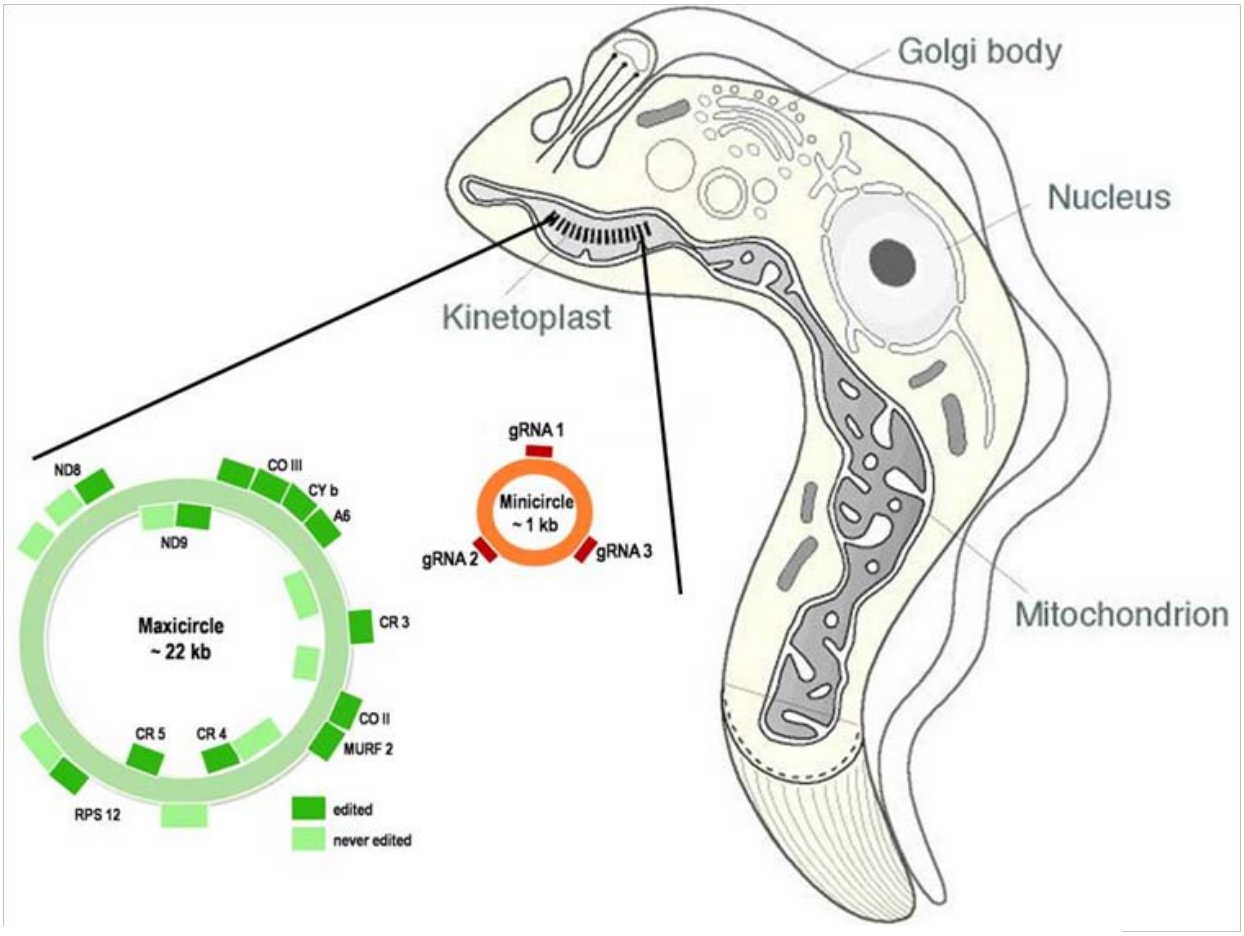

Figure 1.2: Structure of mitochondrial kDNA of *T. brucei* procyclic.
(Adapted from http://protist.i.hosei.ac.jp/protistology/Glossary/K/kinetoplast.jpg)

1.2. RNA editing

RNA editing is a posttranscriptional maturation of mtRNAs of kinetoplastids, which inserts and deletes numerous uridines (Us). It is a unique process and essential for kinetoplastids, therefore representing a drug target for the treatment of trypanosomiasis (Amaro et al., 2008). Editing can be extensive, with hundreds of Us inserted into and tens of Us deleted from a single mtRNA. Nevertheless, the predicted amino acid sequences are precise and have various degrees of homology to proteins encoded in the

mtDNA of a wide range of eukaryotes that do not edit mRNA. The edited mRNAs encode components of the mt oxidative phosphorylation system, including subunits of complex I (NADH dehydrogenase), complex III (cytochrome reductase), complex IV (cytochrome oxidase), and F_1F_0 ATPase synthase, a mitochondrial ribosomal protein S12 (RPS12), and a handful of proteins of unknown functions (Stuart et al., 2002).

Editing creates translatable ORFs by: i/ frameshift repairs, as originally discovered in cox2 subunit (Benne et al., 1986), ii/ generation of a start codon by insertion of the appropriate U, as found in cytochrome reductase subunit B (cyB) (Feagin et al., 1988a), iii/ reconstitution of a whole ORF by the insertion of hundreds of Us and also the deletion of tens of Us (Feagin et al., 1988b). The third variation, referred to as pan-editing, is typified by the edited mRNA of cox3, in which 547 Us are added and 41 Us are removed. Depending on the species, a varying number of mRNAs is produced without any RNA editing at all. Such transcripts are referred to as 'never-edited' (Lukeš et al., 2005).

The information for editing of specific transcripts is stored within the primary structure of gRNAs (Fig.1.3.), small RNA molecules of about 30 nucleotides in length, which can be divided into three regions. The 5' end is a so called anchor region and is complementary to the sequence of a pre-edited mRNA, whereas the central information region specifies how many Us will be inserted and/or deleted. The 3' end is comprised of a post-transcriptionally added oligo(U) tail, which is thought to be a stabilizing element for interaction between gRNA and its cognate mRNA (Blum and Simpson, 1990). The gRNAs provide the information for U-insertion and U-deletion at specific editing sites (ES) along the edited mRNA (Lukeš et al., 2005). Editing of the mRNAs is regulated during the lifecycle of trypanosomes and its pattern corresponds to the interstagial differences in energy metabolism (Feagin et al., 1986). It has been proposed that RNA editing is regulated at the level of gRNA utilization, but other factors such as stage-specific differential polyadenylation may play a role (Lukeš et al., 2005).

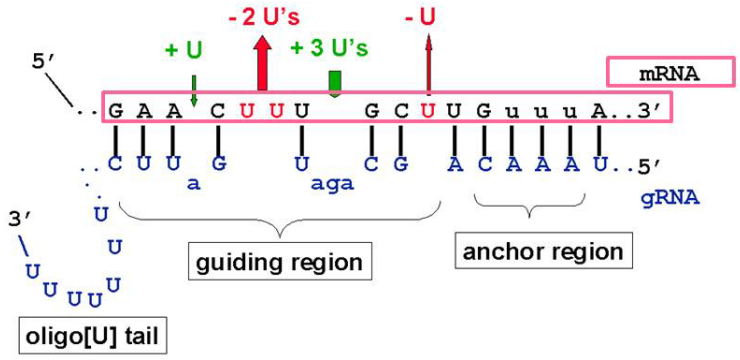

Figure 1.3: gRNA. (Adapted from L. Simpson)

Editing occurs by a series of coordinated catalytic steps in 3'→ 5' direction, in which enzymes such as 1) an endonuclease, that cleaves the mRNA at the specific ES, 2) an U-specific 3'→ 5' exonuclease

(exoUase) for deletion and a terminal uridylyl transferase (TUTase) for insertion editing and 3) a RNA ligase to rejoin the two fragments after processing, are involved (Fig. 1.4) (Stuart et al., 2005).

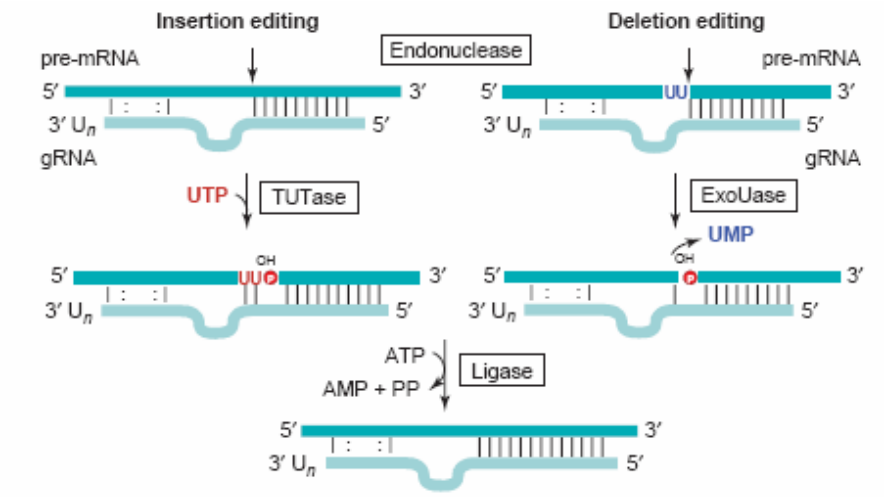

Figure 1.4: General mechanism of RNA editing (Stuart et al., 2005).

Enzymatic activities of RNA editing are contained within a multiprotein complex isolated from mitochondrial lysates and sedimenting at 20 S on glycerol gradients and thus named 20S editosome (Stuart et al., 2005). Most editosome proteins can be grouped up according to their sequence or domain (U1 like zinc finger, C2H2-type zinc-finger, 5`-3`exoribonuclease and endonuclease-exonuclease-phosphatase domains, RNase III domains, double stranded (ds) RNA binding motif, Pumilio motif etc.) similarities reflecting their function (Fig.1.5.). Recently, it has been shown that RNA editing is catalyzed by 3 different editosomes (Fig. 1.6.) (Panigrahi et al., 2006; Carnes et al., 2008; Ernst et al., 2009): editosome with kinetoplast RNA editing endonuclease (KREN) 1, which specifically cleaves deletion editing, KREN2 which specifically cleaves insertion editing site substrates. The third editosome was named KREN3 and it specifically cleaves cox2 pre-mRNA insertion editing site substrates *in vitro* (Carnes et al., 2008).

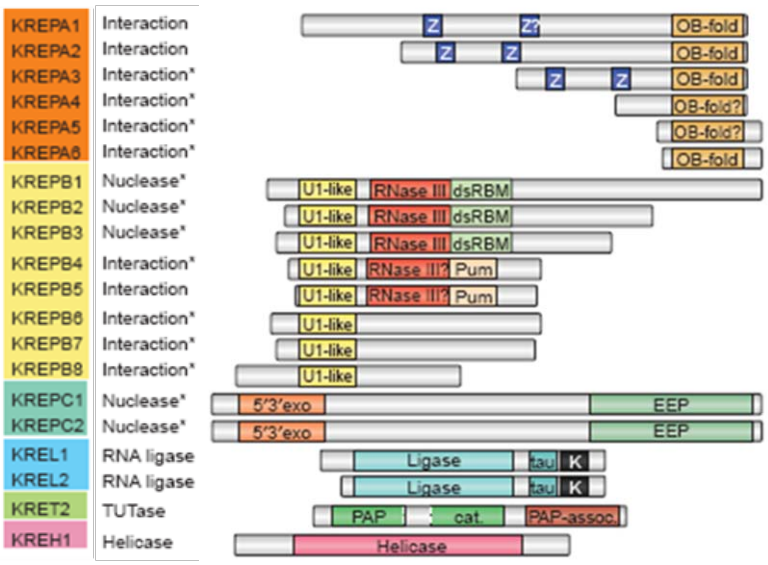

Figure 1.5: Components of 20S editosome (Stuart et al., 2005).

Proteins performing the catalytic steps of RNA editing have been characterized, such as the aforementioned KRENs. Kinetoplast RNA editing 3`- terminal uridylyl transferase (KRET) 2 adds U`s during insertion editing. Kinetoplast RNA editing U-specific exonuclease (KREX) 1 and 2 remove U`s at deletion sites. Either kinetoplast RNA editing ligase (KREL)1 or 2 ligate two mRNA fragments after U addition or removal. KREL1 plays a key role in the viability of both procyclic and insect stages of *T. brucei* (Schnaufer et al., 2001; Rusche et al., 2001; Huang et al., 2001; McMannus et al., 2001; Ernst et al., 2003; Kang et al., 2005; Trotter et al., 2005). There are no known close human homologs of KREL1 and thus it is a particularly attractive drug target. Drug-like inhibitors of this key enzyme have been reported (Amaro et al., 2008).

KREN2 editosome (insertion editing)	KREN1 editosome (deletion editing)	KREPB2 (=KREN3) editosome (COII editing)
KREPA1 to 6	KREPA1 to 6	KREPA1 to 6
KREPB4	KREPB4	KREPB4
KREPB5	KREPB5	KREPB5
KREPB7	KREPB8	KREPB6
KREX2	KREX1	KREX2
KREL1	KREX2	KREL1
KREL2	KREL1	KREL2
KRET2	KREL2	KRET2
	KRET2	

Figure 1.6: Composition of the three editosomes.

Given the presence of RNA substrates, the presence of accessory factors transiently associating with the editosomes and mediating RNA-RNA interactions, RNA-protein interactions and RNA stability has been predicted. One such accessory factor is RBP16 (Pelletier et al., 2003). It was shown to be an essential protein in *T. brucei* procyclic, which mediates the stability of a specific subset of edited and never-edited mRNAs (Pelletier et al., 2003) and stimulates RNA editing *in vitro* (Miller et al., 2006). Two other accessory factors, mitochondrial RNA binding protein (MRP) 1 and 2 are essential for the growth of procyclic *T. brucei* and the RNAi-phenotype of both is similar to that of RBP16 KD in the destabilization of never-edited NADH dehydrogenase (ND) subunit 4 and cox1 mRNAs and a massive decrease in cyB mRNA editing (Vondrušková et al., 2005). MRP1 and MRP2 form a heteromeric complex that acts as a RNA matchmaker by stabilizing the RNA molecule in the unfolded conformation, which is suitable for pre-mRNA-gRNA hybridization in the initial stage of RNA editing (Aphasizhev et al., 2003a; Schumacher et al., 2006). Another protein belonging among the accessory factors is TbRGG1 (Vanhamme et al., 1998). It possesses the arginine-glycine-glycine (RGG) trieptide which is repeated within close proximity with each other and interspersed with aromatic residues, constituting a motif present in some RNA binding proteins (Burd and Dreyfuss, 1994). RNA binding by TbRGG1 is thought to be facilitated by electrostatic interactions between the positively charged arginines and negatively charged nucleic acid. TbRGG1 is an essential protein for cell growth, its depletion results in an overall decline of edited mRNAs whereas the levels of never-edited RNAs remain unchanged. TAP-tag purification of TbRGG1 revealed its RNAse-sensitive association with a novel complex named the

Mitochondrial RNA binding complex 1 (MRB1) (Hashimi et al., 2008, Hashimi et al., 2009), the functional analysis of which is the subject of this thesis.

1.3. The MRB complex 1

MRB1 is a complex of ~14 proteins (Table 1.1.) co-purified upon the tandem affinity purification of the protein TbRGG1 (Hashimi et al., 2008). Most of the obtained proteins possessed known domains or motifs involved in RNA binding or protein-protein interactions such as DExD-box helicase, RNA recognition motif (RRM), glycine rich protein (GRP) and ankyrin repeat. Among them were two exceptions, Tb927.2.3800 and Tb927.7.2570, with no known motifs but having 31% sequence identity and 48% similarity over 432 amino acids between them. They were identified in the TbRGG1 TAP-tag output with high protein coverage and thus selected for reciprocal TAP-tag analyses. Data from all three TAP tags were compiled and 14 proteins found in all three TAP tag pull-downs with a probability of ≥ 0.9 and at least by two unique peptide matches in one of them, were assigned as the components of the complex (Hashimi et al., 2008).

Protein name	GeneDB	TbRGG1	Tb927.2.3800	Tb927.2.2570	Homology (*E*-value)	Motif/domain (*E*-value)
Tb927.6.2230^{tag}	TbRGG1	√	√	√	*Caenorhabditis* hypothetical protein (4e-13)	—
Tb927.7.2570^{tag}	HP*	√	√	√	Tb927.2.3800^{1}	—
Tb927.2.3800^{tag}	HP*	√	√	√	Tb927.7.2570^{1}	—
Tb927.5.3010	HP	√	√	√	*Babesia* hypothetical protein (9e-09); bacterial putative RNA-binding protein (6e-06)	Ribosomal S2 protein signature
Tb927.4.1500	HP	√	√	√	*Oryza* ATP-dependent RNA helicase (1e-121)	dsrm (9e-07); Helicase C (2e-13)
Tb11.02.5390	HP	√	√	√	—	Ankyrin repeat (0.83)
Tb11.01.8620	HP	√	√	1	—	—
Tb10.406.0050	RBP$^{\#}$	√	√	√	*Caenorhabditis* RNA helicase (1e-09)	RRM (6.3e-11)
Tb927.8.8170	HP	√	√	1	Tb927.4.4160^{2}	—
Tb927.4.4160	HP	√	1	√	Tb927.8.8170^{2}	—
Tb927.3.1820	HP	√	√	√	—	GRP (0.028)
Tb11.01.7290	Nudix hydr*	√	√	1	—	NUDIX (4e-05)
Tb927.3.4920	HP	1	√	√	*Arabidopsis* calcium binding mt protein (9e-06)	LETM1 (4e-08)
Tb927.6.2140	HP	1	√	1	Bacterial hydratase (6e-05)	2-keto-4 pentenoate hydratase (5e-07)

(√) Protein was identified with more than two unique tryptic peptides; (1) protein was identified with 1 unique tryptic peptide; (*) proteins also known as LtAP1-3 identified in *L. tarentolae* MRP1/2 complex (3); (superscript "tag") tagged proteins in this study; (superscript "#") protein also known as TbRGGm (20); (superscript "1") proteins have 31% sequence identity and 48% similarity over 432 amino acids between them; and (superscript "2") proteins have 77% sequence identity and 85% similarity over 904 amino acids between them.

Table 1.1: Composition of the Mitochondrial RNA binding complex 1 in *T. brucei* (taken from Hashimi et al., 2008).

The function of two constituent MRB1 proteins, Tb927.2.3800 and Tb927.7.2570, was analyzed by the RNAi approach in both procyclic and bloodstream stages. The proteins turned out to be essential and their stability was mutually dependent on each other. Downregulation of each resulted in strikingly similar phenotypes on RNA editing, in which edited transcripts are downregulated and corresponding pre-edited transcripts upregulated. Importantly, editing employing *cis*-acting gRNA, cox2, remained unaffected. Silencing of both negatively affected steady state levels of gRNA in both procyclic and bloodstream forms. Taken together, these results indicate that they are likely involved in gRNA stability or transcription and thus they were named guide RNA associated proteins (GAP) 1 and 2.

Another component of this complex that is a part of this study is Tb927.4.1500 a predicted DexD/H box RNA helicase. The protein family of DexD/H box RNA helicases is characterized by the presence of conserved motifs that bind and hydrolyse ATP and using the the resulting energy to unwind dsRNA. It has been shown that this family is associated with virtually all biological processes employing RNA molecules. RNA helicases are regarded to be RNA chaperones, maturases, unwindases and even RNPases, that disrupt RNA-protein interactions (Tanner and Linder, 2001; Lorsch 2002; Fuller-Pace, 2006). RNA helicase had a RNAi-phenotype resembling that of GAP1 and GAP2 and based on that we concluded that RNA helicase from MRB1 has a role in gRNA biogenesis.

The last MRB1 component examined in this study was Tb11.01.7290, a protein belonging to the family of nucleoside diphosphate linked to some other moiety x (Nudix) hydrolases and named as such here. The Nudix hydrolase family functions in an array of biological processes, such as hydrolysis of intracellular nucleotides and elimination of potentially toxic derivatives. It is also essential protein for *T. brucei* procyclic but in contrast to the three above mentioned MRB1 components its downregulation resulted in overall destabilization of maxicircle encoded RNAs and it had no effect on the steady state levels of gRNA. The particular function of Nudix hydrolase in the MRB1 has yet to be determined.

A role of the MRB1 in RNA metabolism is further underscored by data on the associated protein TbRGG2 (formerly known as TbRGGm) (Fisk et al., 2008). TbRGG2 is essential for the growth of both procyclic and bloodstream stages of *T. brucei*. TbRGG2 binds RNA due to the presence of N-terminal RGG boxes and a C-terminal RRM, which facilitate binding of gRNAs *in vitro* and generally prefers poly (U). It appears to be multifunctional, strongly facilitating the editing of pan-edited RNAs and modestly destabilizing minimally edited and never edited RNAs (Fisk et al., 2008).

1.4. Mitochondrial RNA metabolism

Many unique aspects of the mt RNA metabolism are found in *T. brucei*, such as RNA editing and promiscuous polyuridylylation. RNA species encoded by the mt genome include rRNAs, edited transcripts, never-edited transcripts, pan-edited transcripts and gRNAs. Interestingly, even though translation of mRNAs occurs, there are no tRNAs of mt origin and thus must all be imported (for recent review Salinas et al., 2008).

Following the general flow of events going from gene to the protein, DNA should be mentioned before discussing RNA. In *T. brucei* mt DNA is comprised of a tightly concatenated network of mini- and maxicircles (see section 1.1.). Transcription of gRNAs from minicircles may be processed from polycistrons (Grams et al., 2000) and the single mt RNA polymerase appears be involved, based on the fact that its downregulation decreases gRNA abundance (Hashimi et al., 2009). In order to create mature gRNA, 3`-oligo(U) tail is added by KRET1 3`-terminal uridylyl transferase which is essential for RNA editing (Aphasizhev et al., 2003b). In case of maxicircles, transcription is performed by the mt RNA polymerase as well, producing polycistronic transcripts (Grams et al., 2002), which are further processed into rRNA and pre-mRNA. Never-edited maxicircle transcripts, such as the 12S, 9S rRNAs as well as ND4 and cox1, are directly subjected to polyadenylation.

Polyadenylation has diverse roles in the mitochondrion throughout the eukaryotes. While the poly (A) tail is a signal for degradation (Gagliardi et al., 2004), polyadenylation stabilizes human mt mRNA, whereas de-adenylation by polynucleotide phosphorylase has the opposite effect (Nagaike et al., 2005). Yeast mt mRNAs are not polyadenylated at all. In *T. brucei*, current data suggest that polyadenylation has a key role in the regulation of mt genome expression by altering mRNA stability. Polyadenylation is carried out by a complex of proteins associated with the kinetoplast poly (A) polymerase 1 (KPAP1) (Etheridge et al., 2008). KPAP1 contains several proteins which have been previously linked to mt RNA processing and translation, such as TbRGG1 (Vanhamme et al., 1998; Hashimi et al., 2008), the pentatricopeptide repeat (PPR) proteins (Mingler et al., 2006; Pusnik et al., 2007) and a putative EF-Tu elongation factor. KPAP1 has been shown to be essential for parasite viability and mt function and directly involved in the synthesis of short A-tails required for the stability of never-, partially and fully edited mt RNAs. This enzyme was localized to two punctate antipodal regions adjacent to the kinetoplast disc. Co-immunoprecipitation experiments revealed RNA independent association of KPAP1 with KRET1. However, its depletion had no effect on gRNA abundance (Etheridge et al., 2008).

Besides mt transcripts containing a short A- tail (~20-25 nt), transcripts having both short and long A/U heteropolymers (~100-200 nt), previously thought to be long A-tails, are present (Bhat et al., 1992;

Etheridge et al., 2008). The length of the poly (A) tail seems to correlate with the editing status of the mRNA, since short A-tails are present on pre-edited mRNAs whereas both short and long A/U extensions are added to never-edited and only fully edited mRNAs. It has been suggested that the A/U tail represents a signal of a translationally competent mRNAs (Etheridge et al., 2008). However, the exact mechanism of all of these events has to be further explored since some reports claim A/U-tails are destabilizing elements, marking the RNA for decay (Miliitelo and Read, 2000; Ryan and Read, 2005).

As *T. brucei* cycles between the mammalian and insect stages, adjustments to its energy metabolism occur. An obvious question of regulation of protein expression arises since some proteins are needed only transiently. The order of Kinetoplastida is exceptional in this aspect because there is no regulation on the level of nuclear transcription, with expression being regulated post-transcriptionaly (Torri and Hajduk, 1988). There is no evident system of regulation of mt gene expression either, although it has been proposed that differential editing in the two lifecycle stages may play such a role (Stuart et al., 1997). In the steady state RNA pool within the organelle, there is a large amount of improperly edited mRNAs as well as nonfunctional by-products of the many processing reactions (Decker and Sollner-Webb 1990). A RNA surveillance system which would recognize and subsequently degrade those RNAs is needed. In other eukaryotes, different mechanisms removing aberrant or improperly processed RNAs evolved. In yeast, the degradosome complex composed of DSS-1 exoribonuclease and SUV3 RNA helicase, degrades aberrant RNAs in mitochondria (Dziembowski et al., 1998; Dziembowski et al. 2003). In *Arabidopsis*, a mt polynucleotide phosphorylase degrades rRNAs and tRNAs maturation by-products (Holec et al., 2006). In *T. brucei* a homolog of the yeast DSS1 exoribonuclease and member of the RNR family was found and named TbDSS1 (Penschow et al., 2004). TbDSS1 is an essential protein that is localized to the mitochondrion, although not associated with ribosome as it is in yeast. Downregulation of TbDSS1 results in mixed phenotype, implicating its participation in multiple aspects of mtRNA metabolism. Further study using 12S/9S rRNA locus as a model system provided evidence for the role of TbDSS1 in the RNA surveillance pathway and degradation of 12S rRNA maturation by-products and indicated a wider role in decay of similar products from other regions in the mt genome (Mattiacio and Read, 2007). SUV3 RNA helicase was found in the *T. brucei* database but its association with TbDSS1 is in question (Penschow et al., 2004).

1.5. Mitochondrial translation

Translation in *T. brucei* mt is carried on mitoribosomes, ribosomal particles of bacterial origin, sticking to the inner mt membrane (Maslov et al., 2006). They are composed of two particles: the small (SSU) and large subunit (LSU), as reported in other eukaryotes. However events such as reduction of RNA and

acquisition of proteins happened during evolution The reduced sizes of 9S SSU rRNA and 12S LSU rRNA are the smallest found in nature (de la Cruz et al., 1985a; de la Cruz et al., 1985b). In mammals it has been proposed that a higher protein content compensated for the reduction of rRNAs (Sharma et al., 2003). A comprehensive analysis of *T. brucei* mt proteome (Panigrahi et al., 2009) together with affinity purification and mass spectrometry analysis of some components of mitoribosomes (Zíková et al., 2008) identified compositional similarities to bacterial ribosomal proteins and to those of higher eukaryote but also proteins that have no significant homology outside Kinetoplastida. Moreover proteins with various functions were found. Among those were eight PPR motif proteins of which three proteins were shown to be required for the stabilization of 12S and 9S rRNAs (Pusnik et al., 2007). The PPR is a degenerate 35 amino acid motif that defines a wide eukaryotic protein family, having 400-500 members in the plant kingdom but usually fewer than 5 members in other eukaryotes. In *T. brucei*, the 28 annotated PPR proteins is extraordinarily high for a nonplant organism. Eight of these were randomly chosen, named TbPPR1 to TbPPR8, and were studied by tools of functional analysis such as RNAi. With the exception of TbPPR8, all selected PPR proteins are exclusively localized in the mitochondrion and are associated with the mt rRNAs (Pusnik et al., 2007). Only TbPPR1, which appears in the KPAP1 complex as well as in the guide-RNA binding complex (GRBC) (Weng et al., 2008), is not linked to mt rRNAs but to cox1 mRNA (Pusnik et al., 2007). TbPPR5 is associated with 12S RNA and GRBC.

2. Results

Hashimi H, Čičová Z, Novotná L, Wen YZ and Lukeš J (2009). Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex and mitochondrial RNA polymerase. RNA 15: 588-599

Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex 1 and mitochondrial RNA polymerase

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ABSTRACT

The mitochondrial RNA binding complex 1 (MRB1) is a recently discovered complex of proteins associated with the TbRGG1 and TbRGG2 proteins in *Trypanosoma brucei*. Based on the phenotype caused by down-regulation of these two proteins, it was proposed to play an unspecified role in RNA editing. RNAi silencing of three newly characterized protein subunits, guide RNA associated proteins (GAPs) 1 and 2 as well as a predicted DExD/H-box RNA helicase, show they are essential for cell growth in the procyclic stage. Furthermore, their down-regulation leads to inhibition of editing in only those mRNAs for which minicircle-encoded guide (g) RNAs are required. However, editing remains unaffected when the maxicircle-encoded *cis*-acting gRNA is employed. Interestingly, all three proteins are necessary for the expression of the minicircle-encoded gRNAs. Moreover, down-regulation of a fourth assayed putative MRB1 subunit, Nudix hydrolase, does not appear to destabilize gRNAs, and down-regulation of this protein has a general impact on the stability of maxicircle-encoded RNAs. GAP1 and 2 are also essential for the survival of the bloodstream stage, in which the gRNAs become eliminated upon depletion of either protein. Immunolocalization revealed that GAP1 and 2 are concentrated into discrete spots along the mitochondrion, usually localized in the proximity of the kinetoplast. Finally, we demonstrate that the same mtRNA polymerase known to transcribe the maxicircle mRNAs may also have a role in expression of the minicircle-encoded gRNAs.

Keywords: RNA editing; guide RNA; mitochondrion; trypanosome

3. My contributions to the data published

- generation of RNA helicase and Nudix hydrolase RNAi knocked down (KDs) cell lines in *T. brucei* procyclic stage
- verification of these KDs by Northern analysis
- measurement of growth of KDs
- RT-qPCR to test effect of RNA helicase and Nudix hydrolase downregulation on RNA editing
- assisted in the Western analysis of glycerol gradients
- assisted in the guanylyltransferase experiments

4. Conclusions and future prospectives

In this study, initial data about the function of MRB1 is provided that may be used as a springboard for future experiments. Examination of the generated RNAi KDs of its four subunits, GAP1, GAP2, RNA helicase and Nudix hydrolase, has shown that all four proteins are essential for the growth of procyclic stage *T. brucei*. Moreover both GAP1 and GAP2 were shown to be essential for the bloodstream stage of this parasite. GAP1, GAP2 and RNA helicase downregulation generally results in the decline of the steady state abundance of gRNAs and a consequent reduction of editing as well. In all of the KDs editing of cox2, which employs *cis*-gRNA, remained unaffected. In contrast, Nudix hydrolase RNAi-silencing causes an overall reduction of pre-edited and edited transcripts and has no effect on the steady state gRNA levels. This finding leads us to the conclusion that GAP1, GAP2 and RNA helicase somehow play a role in maintaining the stability and/or transcription of gRNAs from minicircles.

To sum up, a clear evidence for participation of the MRB1 in RNA metabolism has been provided. However, based on the diversity of phenotypes obtained by RNAi based downregulation of subunits of this complex, it is likely that some of its subunits may associate with MRB1 only transiently or that this complex is composed of several smaller complexes and/or monomers acting together in different processes. This hypothesis may be further corroborated by the fact that some of the MRB1 components were detected in tandem affinity purifications of other complexes involved in mt RNA metabolism (Fig. 1.7), such as KPAP1 (Etheridge et al., 2008), mitochondrial edited RNA stability factor 1 (MERS1) (Weng et al., 2008) and GRBC (Weng et al., 2008). The overlapping but still different compositions of the complexes are probably due to the fact that some components posses RNA binding domains and thus their association with the complexes is mediated by RNA. This was confirmed by application of RNAse A treatment on TAP tagged and purified GAP1 and GAP2 (unpublished data).

Characterization of the rest of components in order to complete the whole set and state the role more clearly is being intensively pursuit using the tools of RNAi for knocking down the MRB1 subunits one by one in procyclic and bloodstream stages of *T. brucei.* These KDs are being subjected to various assays such as growth measurement, RT-qPCR, guanylyltransferase labeling of gRNAs and examining if their silencing affects the glycerol gradient sedimentation of other subunits of MRB1. To establish a map of protein-protein interactions within this complex, a yeast-two hybrid screening is being done.

Having the whole dataset at hand in the future could enable us to elucidate the actual composition of MRB1 as well as the function of the individual parts in order to incorporate it into the overall mtRNA metabolism, a brief review of which was also attempted in this work.

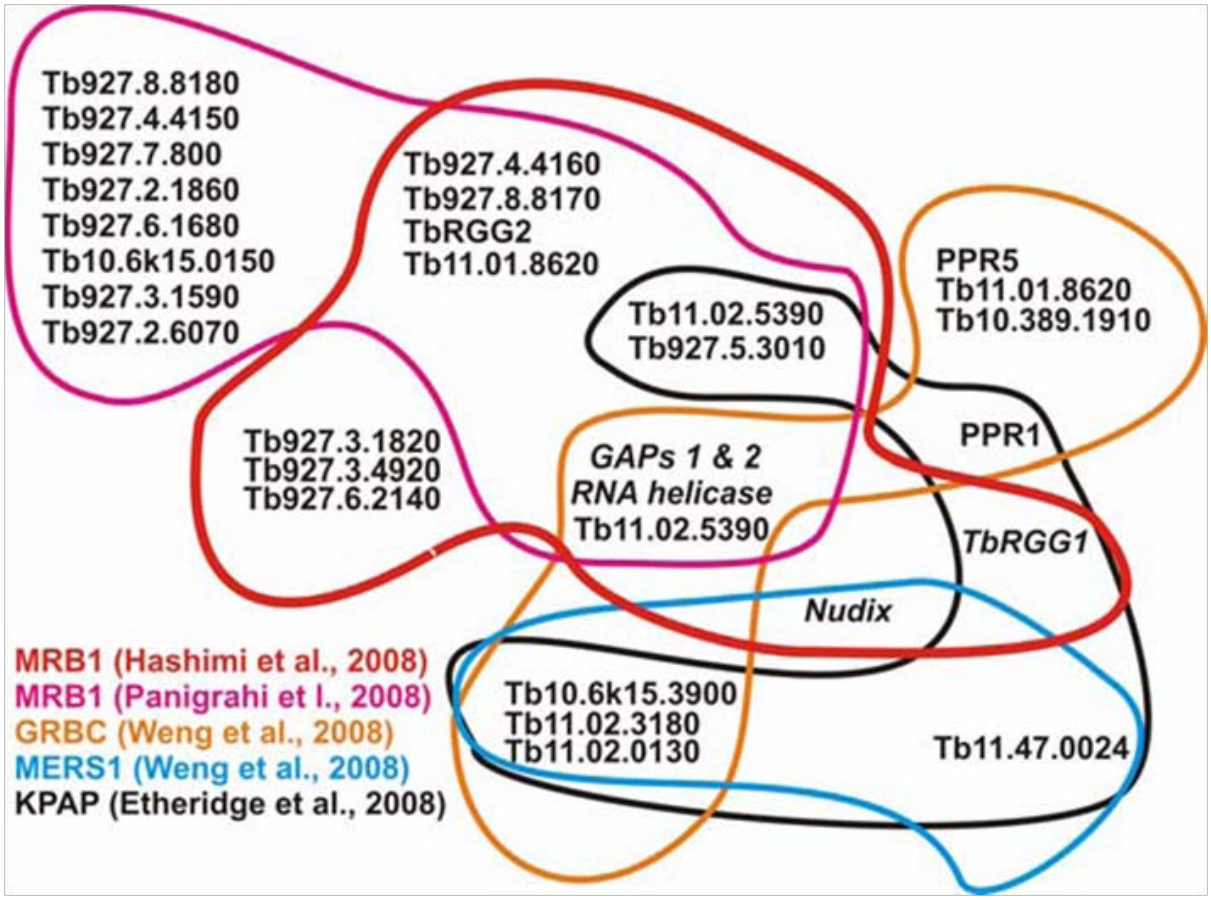

Figure 1.7: Compositions of complexes overlapping with MRB complex 1 in *T. brucei* (taken from Hashimi 2009).

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6. List of abbreviations

cox	cytochrome oxidase
cyB	cytochrome reductase subunit B
dsRNA	double-stranded RNA
ES	editing site
exoUase	U-specific 3'→5` exonuclease
GAP1	guide RNA associated protein 1
GAP2	guide RNA associated protein 2
GRBC	guide RNA binding complex
gRNA	guide RNA
GRP	glycine rich protein
KD	knock-down
kDNA	kinetoplast DNA
KPAP1	kinetoplast poly (A) polymerase 1 complex
KREN	kinetoplast RNA editing endonuclease
KRET	RNA editing 3`- terminal uridylyl transferase
KREX	kinetoplast RNA editing U-specific exonuclease
MERS1	mitochondrial edited mRNA stability factor 1
MRB1	mitochondrial RNA binding complex 1
MRP	mitochondrial RNA binding protein
mt	mitochondrial
ND	NADH dehydrogenase
nt	nucleotide

Nudix	nucleoside diphosphate linked to some other moiety x
PPR	pentatricopeptide repeat
RGG	arginine-glycine-glycine
RNAi	RNA interference
RPS12	ribosomal protein S12
RRM	RNA recognition motif
rRNA	ribosomal RNA
TAP	tandem affinity purification
TUTase	terminal uridylyl transferase
Us	uridines
VSG	variant surface glycoprotein
WHO	World Health Organization