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Ph.D. Thesis

Aspects of RNA editing in *Trypanosoma brucei*

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Annotation:
This work addresses various aspects of RNA editing, a unique processing event that is essential for mitochondrial (mt) RNA maturation in the model kinetoplastid *Trypanosoma brucei*. RNAi-mediated reverse genetics was used extensively throughout this work. TbRGG1 appears to have a role in this process or stabilizing edited RNAs and is associated with a putative complex we called mt RNA binding complex 1. Several subunits have been demonstrated to have various roles in RNA metabolism, guide RNA biogenesis being the most significant. The extensively edited ATP subunit 6 was shown to be incorporated into the F_OF_1 ATP synthase complex by indirect genetic means. This respiratory complex is essential in both insect and infectious bloodstream stages of the parasite. Work here supports the idea that *Trypanosoma evansi* and *Trypanosoma equiperdum*, pathogens of livestock that are RNA-editing incompetent, should be considered petite mutants of *T. brucei*.

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

České Budějovice, 20 January, 2009 Mir Mohamod Hassan Hashimi

Acknowledgements

When I heard the word trypanosome, just as I was entering the Laboratory of Molecular Biology of Protists in 2003, I pictured in my head  and was surprised to see  wriggling throughout the microscope field of my first culture. It's probably not an understatement to write that I have learned a lot since that time, which I hope is somehow reflected in the thesis you have in your hands.

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~hassan

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Abbreviations

ΔΨm	mitochondrial membrane potential
A	adenosine
A6	F_1/F_O-ATP synthase subunit 6
ak	akinetoplastic
base "J"	beta-D-glucosyl-hydroxymethyluracil
CBP	calmodulin binding protein
complex I	NADH dehydrogenase
complex III	cytochrome bc_1 complex
complex IV	cytochrome *c* oxidase
complex V	F_1/F_O-ATP synthase
coxX, coX	cytochrome *c* oxidase subunit X
cyB	cytochrome *b*
dk	dyskinetoplastic
dsRNA	double stranded RNA
G	guanosine
GAP	guide RNA associated protein
GAPDH	glyceraldehyde-3-phosphate
GRBC	guide RNA binding complex
gRNA	guide RNA
HA	hemagglutinin
HAT	human African trypanosomiasis
kDNA	kinetoplast DNA
KPAP	poly(A) polymerase
KREL	kinetoplastid RNA editing ligase
KREN	kinetoplastid RNA editing nuclease
KREP	kinetoplastid RNA editing proteins
KRET	kinetoplastid RNA editing 3' terminal uridylyltransferase
KREX	kinetoplastid RNA editing exonuclease
LSU	large subunit of risosome
mt	mitochondrial
MRB1	mitochondrial RNA binding complex 1

MRP	mitochondrial RNA binding protein
MURF	mitochondrial unidentified reading frame
NDx	NADH dehydrogenase subunit X
nt	nucleotide
Nudix	nucleoside diphosphate linked to some other moiety X
ORF	open reading frame
PARP	procyclic acidic repetitive protein
PPR	pentatricopeptide repeat
qPCR	quantitative real-time PCR
RNAi	RNA interference
RNAP	RNA polymerase
RPS12	ribosomal protein S12
SL RNA	spliced leader RNA
SSU	small subunit of ribosome
T	thymidine
TAO	trypanosome alternative oxidase
TAP	tandem affinity purification
tet	tetracycline
tetR	tetracycline repressor
U	uridine
UTR	untranslated region
VSG	variable surface glycoprotein

Summary

Members of the *Trypanosoma brucei* complex are the causative agents of several diseases, including human African trypanosomiasis and a wasting disease of ruminants called Nagana. This species has become a model species of its order Kinetoplastida, comprised of evolutionarily divergent flagellate protists, since it is amenable to RNA interference (RNAi). Several unique features of these cells include polycistronic nuclear transcription and *trans*-splicing, an extensive mitochondrial (mt) genome called kinetoplast (k) DNA and RNA editing. This thesis concerns aspects of RNA editing, an essential process in the maturation of transcripts encoded in kDNA maxicircles. It can be conceptually divided into two parts.

The first part (Chapters 2 and 3) deals with the functional analysis of proteins and protein complexes that are indirectly involved in RNA editing. RNAi-silencing of TbRGG1 showed that edited RNAs were downregulated. Steady state levels of guide (g) RNAs, vital genetic elements of this process encoded on kDNA minicircles, were not affected. Tandem affinity purification (TAP) of a TAP-tagged version of TbRGG1 over-expressed *in vivo* led to the discovery of a putative ~14 protein complex, provisionally named mitochondrial RNA binding complex 1 (MRB1). Three MRB1 subunits, named RNA helicase and gRNA associated proteins (GAPs) 1 and 2 have a role in some aspect of gRNA biogenesis or stability. The GAPs appear to be interacting partners, are essential in the bloodstream stage and have an interesting, punctuate localization in the mitochondrion of the procyclic stage. Downregulation of another MRB1 subunit, a predicted Nudix hydrolase, results in a general destability of maxicircle encoded RNAs without affecting gRNAs. A single mt RNA polymerase seems to transcribe both maxicircle and minicircle transcripts.

The second part (Chapters 4 and 5) deals with understanding the phenomena of dyskinetoplasty, in which the homogenization of minicircles leads to RNA editing incompetence and eventual loss of kDNA. *Trypanosoma evansi* and *Trypanosoma equiperdum*, important veterinarian pathogens, occur world-wide despite their aberrant kDNA. Analysis of these cells employing molecular and classical-parasitology approaches indicated that they are strains of *T. brucei* and should be given subspecies

status and considered petite mutants that are locked in the bloodstream stage. This state appears to arise spontaneously in perhaps a regular and/or autonomous fashion. However, their existence is not consistent with the finding that RNA editing is essential in the bloodstream stage of *T. brucei*, presumably for the extensive editing of a transcript provisionally assigned to be F_OF_1 ATP synthase subunit 6 (A6). This respiratory complex is essential in the bloodform to maintain membrane potential of the mitochondrion. A simple but unlikely explanation for dyskinetoplasty is that the mRNA in question does not encode A6 or that the protein product is not assembled into the complex. Resorting to an indirect, reverse genetics approach, since direct detection of almost all mt encoded proteins is not feasible, we provide evidence that A6 is indeed assembled into the F_OF_1 ATP synthase. Our results suggest that this complex may have an unusual structure as compared to those from other aerobic eukaryotes.

Chapter 1:
General Introduction

1. The Kinetoplastida

The order Kinetoplastida is one of the three clades that comprise the phylum Euglenozoa, along with Diplonemida and Euglendida. Several common features support the monophyly of this group of protozoan flagellates, including the addition of small spliced leader (SL) RNA to all cytoplasmic mRNAs (Liang et al., 2003) and the presence of the modified base "J"[1] in nuclear DNA (Doojiees et al., 2000). This phylum has been traditionally considered to represent one of the earliest diverging eukaryotes with typical mitochondria (Cavalier-Smith, 1997), although this notion has been challenged (Philippe et al., 2000). Regardless of whether it is an outcome of a long and independent evolutionary history, these organisms do possess remarkable biological properties, many of which are unique among eukaryotes.

The defining character that ultimately lends its name to the whole order Kinetoplastida is the kinetoplast, a dense structure within the single mitochondrion in proximity to the basal body from which the flagella arise (Vickerman, 1976). These protists typically are divided into two groups based on morphology, the biflagellate bodonids and the uniflagellate trypanosomatids[2] (Doležel et al., 2000; Simpson et al., 2002). While the bodonids inhabit a variety of ecological niches, ranging from free-living to parasitic lifestyles, the trypanosomatids are exclusively obligate parasites of invertebrates, vertebrates and plants. Arthropods serve as hosts for monoexones trypanosomatids, such as *Crithidia fasciculata*, while they are vectors transmitting dixenous trypanosomatids of the genera *Trypanosoma* and *Leishmania* to vertebrates and the genus *Phytomonas* to plants (Vickerman, 1976).

Trypanosomatids are by far the most studied group in the order Kinetoplastida or phylum Euglenozoa. One reason is that members of this group are amenable to genetic

[1] beta-D-glucosyl-hydroxymethyluracil

[2] The phylogeny of this order has been recently amended, establishing the subclass Prokinetoplastina to accommodate two kinetoplastids, *Ichthyobodo* and *Perkinsiella*, which are distantly related to the other members (Moreira et al., 2004; Simpson et al., 2006). Bodonids and trypanosomatids are contained in Metakinetoplastida.

manipulation, facilitating the dissection of molecular mechanisms underlying their biology (Beverly, 2003). However, an impetuous for establishing such a research platform is that a number of these parasites are pathogens causing important and yet often neglected human and veterinarian diseases. Twenty species of *Leishmania* are the causative agents of various forms of leishmaniasis, threatening 350 million people over a wide-range of countries in the inter-tropical and temperate regions throughout the old and new worlds (Herwaldt, 1999). *Trypanosoma cruzi* causes Chagas disease, a zoonosis that affects 8 to 11 million people mainly in poor, rural areas of Latin America (Barrett et al., 2003). This intracellular parasite is transmitted via the feces of its vector, triatomine bugs, and thus is classed into the Stercoraria group of trypanosomes. Although sharing a common ancestor and morphological characters of *T. cruzi* trypomastigotes, the subspecies comprising the *Trypanosoma brucei* complex are extracellular parasites with a mode of transmission through the saliva of a biting insect, and thus classed among the Salivarian trypanosomes.

2. The *Trypanosoma brucei* complex

Members of the *Trypanosoma brucei* complex are a significant source of human suffering in sub-Saharan Africa. Two subspecies, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, are pathogens that cause human African trypanosomiasis (HAT), the clinical name for sleeping sickness. The former is endemic in central and western Africa and is responsible for the chronic form of the disease, while the latter occurs in the eastern part of the continent and causes an acute infection (Barrett et al, 2003). The hallmark sign of the early stages of the disease is an undulating fever, corresponding to the rise and fall of parasitemia in the blood. Symptoms of the late stage, including a disrupted wake/sleep cycle, give the disease its common name and occur when the parasites cross the blood-brain barrier. If untreated, it is inevitably deadly. About half a million people are infected, but the disease is resurgent with 60 million people at risk in 36 of Africa's 52 countries (Barrett et al, 2003).

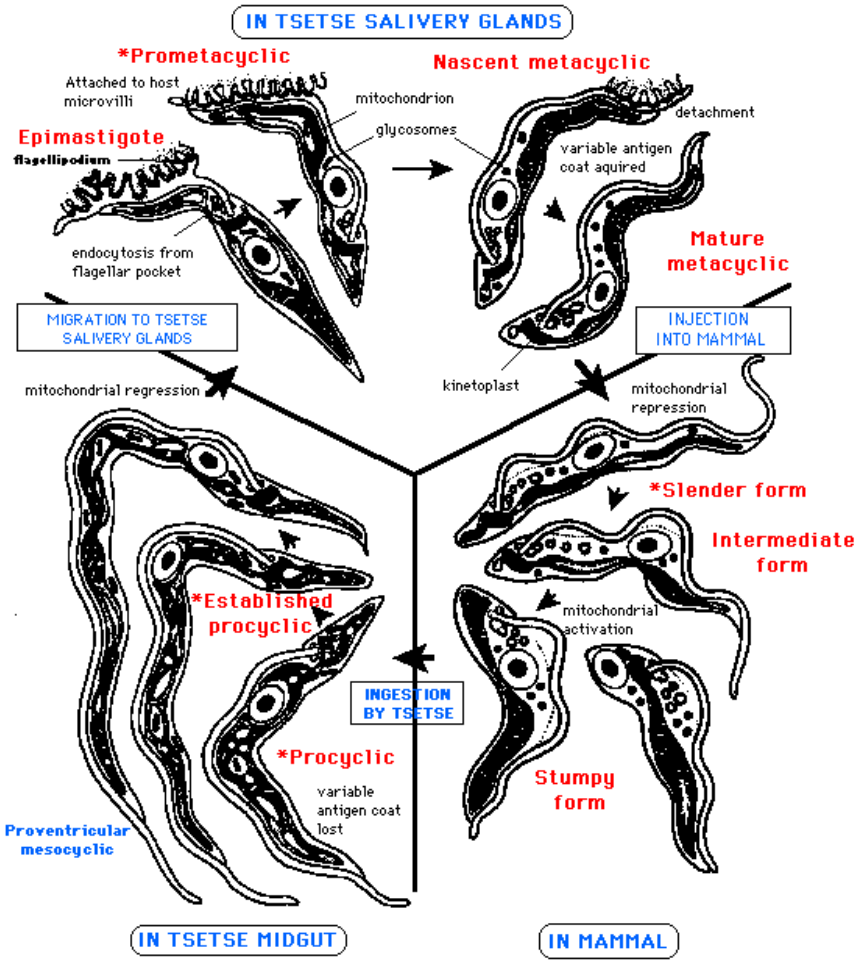


Figure 1. Life cycle of *T. brucei.* Compare elaborated, reticulated mitochondrion in procyclic stage to the reduced organelle in the slender bloodstream stage. From Vickerman (1985).

The third subspecies, *Trypanosoma brucei brucei*, causes nagana, a wasting disease of ruminant animals. Nagana contributes to the protein malnutrition of the populace by rendering ten million hectares of otherwise suitable land incapable of supporting cattle (Roberts and Janovy, 2004). Interestingly, humans are resistant to these trypanosomes because of a trypanolytic factor[3] in the sera conferring innate immunity (reviewed in Pays and Vanhollebeke, 2008). *T. b. gambiense* and *T. b. rhodesiense* have evolved resistance to this factor, and the so-called serum-resistance associated gene has been discovered in the latter subspecies (Xong et al., 1998; Perez-Morga et al., 2005).

[3] Although there is controversy of what is the tryanolytic factor and its mechanism of action, there is consensus that it is a component of high-density lipoprotein particles (Molina-Portela et al., 2005; Perez-Morga et al., 2005; Oli et al., 2006)

Because they are non-pathogenic to researchers, *T. b. brucei* has become the model organism in the laboratory and will be henceforth referred to as *T. brucei* in this thesis.

T. brucei has a complex, digenetic life cycle that alternates between the mammalian host and the *Glossina* vector, also called the tsetse fly (Vickerman, 1985; Matthews, 2005). As illustrated in Figure 1, the cells undergo three major developmental stages: the bloodstream stage in the host, procyclic stage in the vector midgut and the infectious metacylic stage in the vector salivary gland. Several physiological and morphological changes occur during the transition between these stages, the transformation of the mitochondrion being among them (Vickerman, 1985; Matthews, 2005). While the bloodstream stage has a reduced mitochondrion, because the parasite exclusively lives off of glycolytic catabolism of the bountiful sera glucose, the organelle is elaborated into a reticulated structure in the procyclic stage.

A significant portion of the life cycle takes place in the vector, where genetic exchange occurs (Gibson, 2001). As a consequence, the tsetse fly belt defines the epidemiological boundaries of the diseases caused by these trypanosomes, stretching north to south from the Sahel to the Kalahari Desert, respectively (Barrett et al., 2003; Roberts and Janovy, 2004). However, data in Chapter 4 of this thesis has proposed the inclusion of *Trypanosoma evansi*, and *Trypanosoma equiperdum*, the causative agents of surra and dourine, respectively, into the *T. brucei* complex as subspecies (Lai et al., 2008). Yet both are distinctive in that they are not confined to sub-Saharan Africa, as they are mechanically transmitted to their ungulate hosts within the mouth parts of bloodsucking insects or by coitus. The reason why they can circumvent the natural vectors of *T. brucei* will be discussed in section 8 of the introduction.

The control of the diseases caused by these trypanosomes is complicated by their fundamental biology, as well as the lack of will for costly drug development for an impoverished population without influence (Barrett et al., 2003). Chemotherapy for HAT relies on toxic, difficult to administer drugs that have been in use for decades, thus steadily succumbing to emerging resistance (Croft et al., 2005). Currently, only Elfornithine has been the only drug registered for fighting HAT in the last 50 years (Barrett et al., 2003), although a diamidine-based drug is undergoing clinical trials (Ansede et al., 2004). It is hoped that research into some of the unique, yet essential,

aspects of the biology of these organisms may uncover potential drug targets. A recent report shows the promise of such an approach (Amaro et al., 2008).

3. A brief discussion of some unusual biological properties of *Trypanosoma brucei*

Among the several biological peculiarities that set them apart from other eukaryotes is the compartmentalization of almost all of the enzymes of glycolysis into a peroxisome-derived organelle, called the glycosome (Opperdoes and Borst, 1977; reviewed in Hannaert et al., 2003). Another is the sophisticated system of antigenic variation that the bloodstream stage parasites use to evade host specific-immune response, relying on an extensive repertoire of variable surface glycoprotein (VSG) genes (reviewed in Pays, 2005). A periodic switch of VSG expression presents a new antigenic surface unfamiliar to the host, allowing another wave of parasitemia that underlies the characteristic cycling fever. The active VSG gene is transcribed by RNA polymerase (RNAP) I, typically restricted to rRNA expression, and a novel complex from a promoter near the telomere (Brandenburg et al., 2007).

Although other protein encoding genes are transcribed by RNAP II, it is also done so in an unusual fashion. The kinetoplastid genome is organized into long arrays of genes oriented in the same direction toward the telomeres and separated by only a few hundred base pairs (Palenchar and Bellofatto, 2006). They are transcribed as long, polycistronic units that initiate from strand-switch-regions of the chromosome, which do not contain canonical eukaryotic promoters or regulatory elements (Martinez-Calvillo et al., 2003). Individual mRNAs are subsequently split apart by *trans*-splicing, in which a 39 nt long, 5'-capped SL RNA is attached to the 5'-end of the precursor transcript (Liang et al., 2003). And while a ubiquitous feature of eukaryotic transcriptomes, only one *cis*-spliced intron has been discovered trypanosomes thus far (Mair et al., 2000). The overall consequence of this mode of transcription is that protein levels are predominantly regulated post-transcriptionally (Clayton, 2002; Mayho et al., 2006).

4. The extraordinary mitochondrion of *Trypanosoma brucei*

Because the main focus of thesis is mitochondrial (mt) RNA metabolism, I will briefly discuss some of the more unusual aspects of this organelle in *T. brucei* and other trypanosomatids.

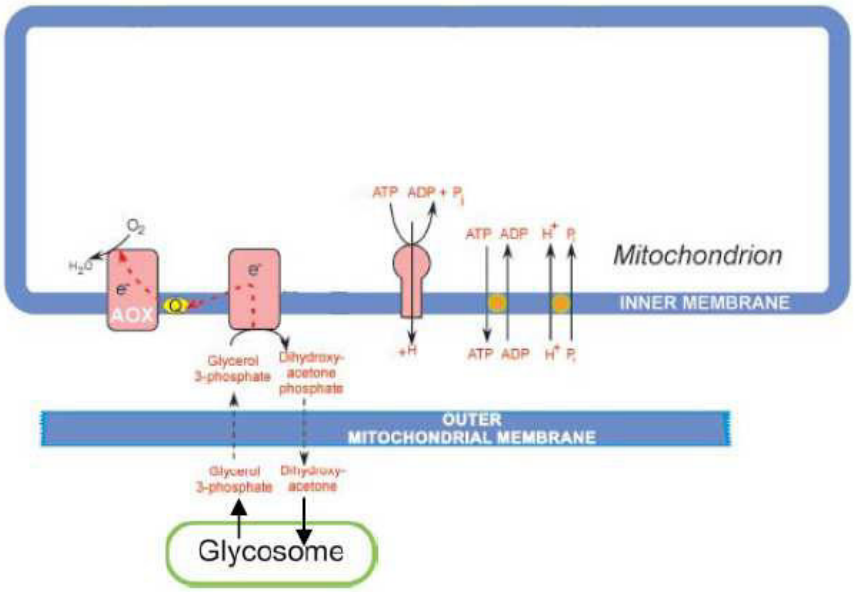
4.1 Atypical energy metabolism

As *T. brucei* cycles between its mammalian host and insect vector, the cell encounters very different environments to which it reacts by significant physiological changes. While in the glucose-rich confines of the host circulatory and lymphatic systems, the energy requirements of the bloodstream stage is sustained by fermentation of glucose to pyruvate, which is ultimately excreted (Hannaert et al., 2003). Consequently, the mitochondrion is drastically reduced. Citric acid cycle enzymes and two of the cytochrome-containing respiratory complexes, typically responsible for proton pumping out of the mt matrix to maintain membrane potential ($\Delta\Psi m$), are absent (Bienen et al., 1981) (Figure 2A).

However, the lack of energy catabolism pathways does not indicate that the mitochondrion is a sleeping organelle. A nuclear-encoded trypanosome alternative oxidase (TAO) is present in the inner membrane that is responsible for maintaining redox equilibrium in the glycosome, via a glycerol-3-phosphate/dihydroxyacetone-phosphate shuttle between the two organelles (Chaudhari et al., 1998; Hannaert et al., 2003) (Figure 2A). In addition, considering the presence of drastically reduced mitochondria in anaerobic parasites such as *Giardia intestinalis* or microsporidia, which retain them for iron-sulphur cluster assembly (Tovar et al., 2003; Goldberg et al., 2008), it is possible that this process occurs in the bloodstream stage organelle as well. Nevertheless, $\Delta\Psi m$ for protein import is still maintained in this organelle through the action of the F_1/F_O-ATP synthase (complex V), by hydrolyzing ATP to pump protons across the inner membrane (Schnaufer et al., 2005; Brown et al., 2006) (Figure 2A).

At the procyclic stage, the mitochondrion is a fully reticulated structure containing discoidal cristae (Vickerman, 1985). However, despite the presence of a functional respiratory chain and most of Krebs cycle enzymes, these mitochondria are

A. Bloodstream stage mitochondrion.



B. Procylic stage mitochondrion.

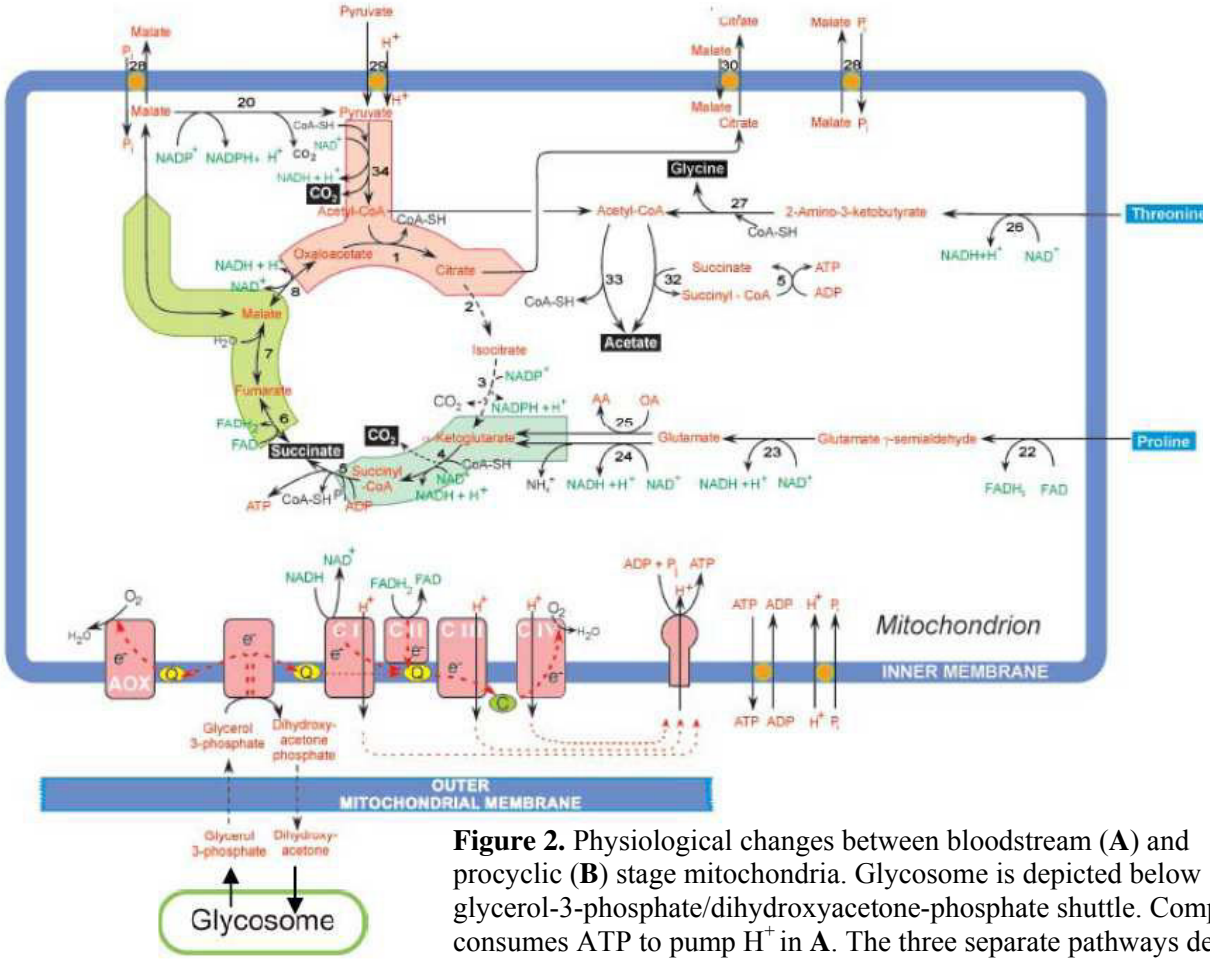


Figure 2. Physiological changes between bloodstream (**A**) and procyclic (**B**) stage mitochondria. Glycosome is depicted below glycerol-3-phosphate/dihydroxyacetone-phosphate shuttle. Complex V consumes ATP to pump H^+ in **A**. The three separate pathways derived from the Krebs cycle are shown as colored arrows in **B**. TAO labeled as AOX. From van Weelden et al. (2005), which includes glycolysis and fatty acid synthesis pathways in procyclic stage.

anything but typical. In the carbohydrate-poor environment of the tsetse midgut, different parts of the citric acid cycle are salvaged for proline-degradation to succinate, the production of malate for gluceogenesis or precursors for fatty acid synthesis (van Weelden, 2005) (Figure 2B). Thus, the citric acid cycle in these organisms does not proceed as a closed cycle to for the oxidation of pyruvate to CO_2. In terms of the electron transport chain, while having a unorthodox subunit composition (Maslov et al., 2002; Horvath et al., 2005), cytochrome bc_1 complex (complexes III) and cytochrome *c* oxidase (complex IV) are essential at this stage (Horvath et al., 2005). The presence and functionality of NADH dehydrogenase (complex I) has still not been resolved, and the paucity of identified nuclear-encoded subunits weakens the case for its existence (Tielens and Van Hellemond, 1998; Turrens, 1999; Čermáková et al., 2007). However, oxidative phosphorylation does not appear to be the main source of ATP production as in other eukaryotic cells with high energy requirements (Bochud-Allemann and Schneider, 2002).

4.2 Kinetoplast DNA

The kinetoplast (k) DNA network is a remarkable structure that can be seen by light microscopy after staining with dyes having affinity for DNA, such as the Giemsa stain. A unique feature of all kinetoplastids, it contains the mt genome on thousands concatenated circular DNAs that are condensed into a disk that is associated with the basal body of the flagella (Ogbadoyi et al., 2003). Two types of DNA circles exist in the network (reviewed in Lukeš et al., 2002; 2005). Minicircles make up the bulk of kDNA, are heterogenous in sequence and exist in a relaxed conformation (Rauch et al., 1993), which is an exception to the prevalence of supercoiled circular DNA in nature. The tens of identical maxicircles are significantly larger in size, ~23 Kb as compared to the ~1 Kb size of the minicircles in *T. brucei*.

The faithful replication of each of the thousands of minicircles is essential for preserving the genetic information contained by these molecules (Simpson et al., 2000; Lukeš et al., 2005). This process is performed by a careful orchestration of events, in which minicircles are first released into the so-called kinetoflagellar zone (Figure 3), in between the kDNA disc and the basal body, where they replicate (Drew and Englund,

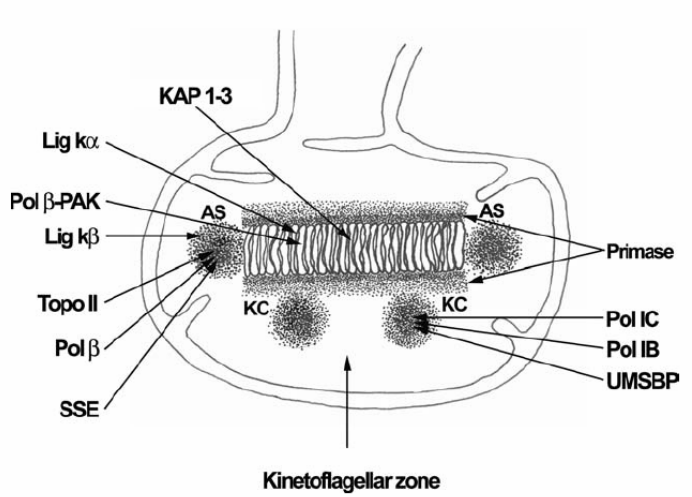


Figure 3. The complexity of the kDNA network. The localization of several representative proteins is shown, including four of the six DNA polymerases (Pol) and two DNA ligases (Lig). AS, antipodal sites; KC, kinetoflagellar centers. Taken from Lukeš et al. (2005).

2001). In *T. brucei*, the gapped progeny molecules are reattached to the network and accumulate at the antipodal sites, adjacent to the plane of the disk (Guilbride and Englund, 1998). Upon replication of all minicircles, the remaining gaps are repaired and the network is split into two (Lukeš et al., 2002; Liu et al., 2005).

An extensive number of proteins are imported into the mitochondrion for the maintenance and replication of kDNA (Liu et al., 2005). The positioning of these proteins around the disk contributes to the complexity of this structure (Figure 3). As a testament to this situation, *T. brucei* harbors six different DNA polymerase belonging to two families, while a single enzyme suffices for mt biogenesis in other eukaryotes (Klingbeil et al., 2002).

While the mechanism of maxicircle replication remains elusive, it has been known for a while that they contain recognizable mitochondrial genes, such as for subunits of the respiratory chain and two mt rRNAs (Simpson et al., 1987) (Figure 4). Twelve of the eighteen[4] protein-coding genes have been designated as cytochrome *b* (cyB), subunit 6 of the F_1/F_O-ATP synthase (A6), ribosomal protein S12 (RPS12), three cytochrome *c* oxidase subunits (cox1-3) and seven subunits of the NADH dehydrogenase[5] (ND1,3-5, 7-9). However, the sequences of many of these genes would not be recognizable as open reading frames (ORFs). The next section addresses the maturation of their transcripts into translatable mRNAs.

[4] The five remaining loci encode proteins of unknown function and have been labeled alternatively as mitochondrial unidentified reading frame (MURF) or CR.
[5] The presence of these subunits in the kDNA is supporting evidence for the presence of complex I in trypanosomes (Schnaufer et al., 2002), although many of these designations still require experimental support (Stuart et al., 1997).

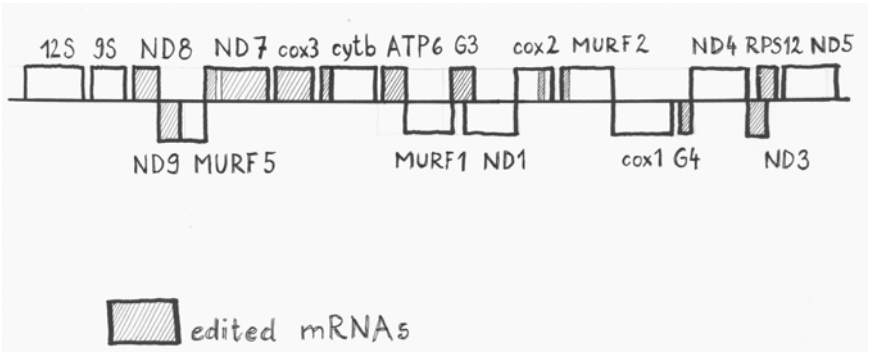


Figure 4. Organization of *T. brucei* maxicircle genes. The major and minor strands are shown on the top and bottom, respectively. Regions encoding polynucleotides undergoing RNA editing are shaded.

5. RNA editing in the mitochondrion of *Trypanosoma brucei*

In 1986, Benne and colleagues made the seminal discovery that four uridine (U) residues not encoded in the gene are inserted into specific locations the cox2 mRNA post-transcriptionally. This process was named RNA editing. A surge of reports followed indicating that RNAs from 12 of the 20 genes residing on *T. brucei* maxicircles required this process for their maturation (Figure 4). Translatable ORFs were created in three possible ways: 1) repairing frameshifts, as is the case for cox2 editing (Benne et al., 1986); 2) generation of start and/or adjacent codons at the 5' end of the mRNA, as in cyB (Feagin et al., 1987); 3) creation of entire ORFs through pan-editing, in which hundreds of Us are inserted and tens of Us are deleted, as shown by the famous Cell cover depicting cox3 editing (Feagin et al., 1988) (Figure 5). Molecules undergoing this kind of maturation are conceptually grouped as pre-, partially- and fully edited RNAs, depending on its current stage in the process, while those that bypass this route are referred to as never-edited.

5.1 Mechanism of RNA editing

Another breakthrough in the field was the discovery of small RNA molecules (50-70 nts long) almost entirely encoded on the minicircles of the kDNA network. These primary transcripts were called guide (g) RNAs because they provide the genetic information defining the editing sites (ES) on a given pre- and/or partially-edited mRNA (Blum et al.,

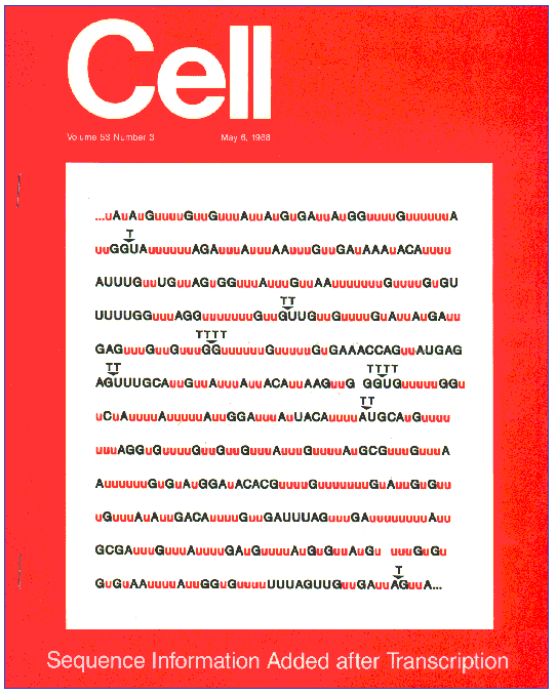


Figure 5. Pan-editing of cox3. Small red Us represent 547 insertions. Arrowheads with black Ts indicate 41 deletions. May 6, 1988 cover for article by Feagin et al. (1988).

1990; Sturm and Simpson, 1990). Examination of the three regions that make up the primary structure of a gRNA suggests how they act as blueprints for RNA editing events (Figure 6, *trans*-acting RNA, top). The 5'-positioned anchor domain is a small stretch of ~10 nts that hybridizes to a complementary sequence on the mRNA, just downstream of the ES (as oriented to mRNA). The information domain starts at the first base mismatch, providing a template for the appropriate U insertion/deletion. The transfer of information the gRNA to mRNA relies on both Watson-Crick and noncanonical G:U base pairing between the two molecules (Sturm and Simpson, 1990). After completion of editing, the edited part of the mRNA, called the editing block, is complementary to the information domain. The third part of the gRNA molecule, the 3'-oligo(U) tail, which is added to the molecule post-transcriptionally (Blum and Simpson, 1990) and demonstrated to interact with the purine-rich sequences upstream of the editing block (McManus et al., 2000). Almost all gRNAs act in *trans* in the described fashion. A notable exception is the editing of cox2, which utilizes a *cis*-acting gRNA in its 3'-UTR (Golden and Hajduk, 2005) (Figure 6, *cis*-acting gRNA, right).

While gRNAs represent the informational component of RNA editing, a cascade of enzymatic activities is also required (reviewed in Lukeš et al., 2005 and Stuart et al., 2005) (Figure 7). An endonucleolytic cleavage occurs at the ES, dividing the mRNA into 5' and 3' fragments that are bridged by the bound gRNA. What occurs next depends on whether a U insertion or deletion event is designated by the gRNA. In the case of the former, free UTP is added to the 3' hydroxyl group of the 5' fragment, the number of

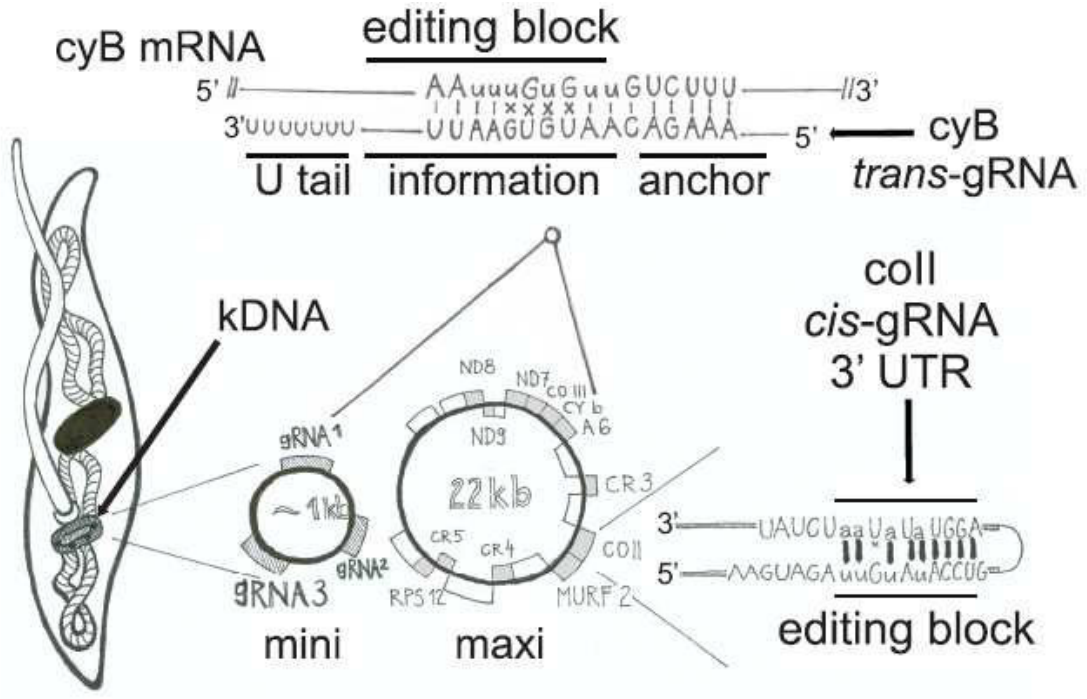


Figure 6. Guide RNAs mediate RNA editing. A minicircle-encoded *trans*-acting gRNA forms a duplex with its cognate mRNA through hybridization of its anchor domain (**top**). The information domain guides U insertions into and deletions from the RNA until it complements the editing block. Non-canonical U:G pairings are depicted as crosses. The *cis*-gRNA residing of the 3' UTR of cox2 guides four U insertions (**right**).

which dictated by A and G residues in the information domain. A 3'→5' exonuclease prunes away 3'-protruding U(s) from the 5' fragment in the case of deletion. Once the processing step results in a fully complementary duplex, the two mRNA fragments are rejoined by an RNA ligase. Once an editing block is completed, the current gRNA in unwound from the mRNA to allow the upstream hybridization of a subsequent gRNA for the next round. As a consequence, editing of pan-edited mRNAs proceeds with a 3' to 5' polarity (Maslov and Simpson, 1992).

5.2 The 20S editosome

The 20S editosome[6] macromolecular complex confers the core editing activities required for mt biogenesis (reviewed in Stuart et al., 2005). An inspection of the list of the ~20 protein subunits comprising the complex reveals that they often occur in sets or pairs

[6] The 20S modifier refers to the settling rate of 20 Svedberg units in glycerol gradient sedimentation experiments.

sharing motifs, domains and/or functions (Figure 8A). This arrangement reflects the complexes organization that facilitates the coordination of the required enzymatic steps.

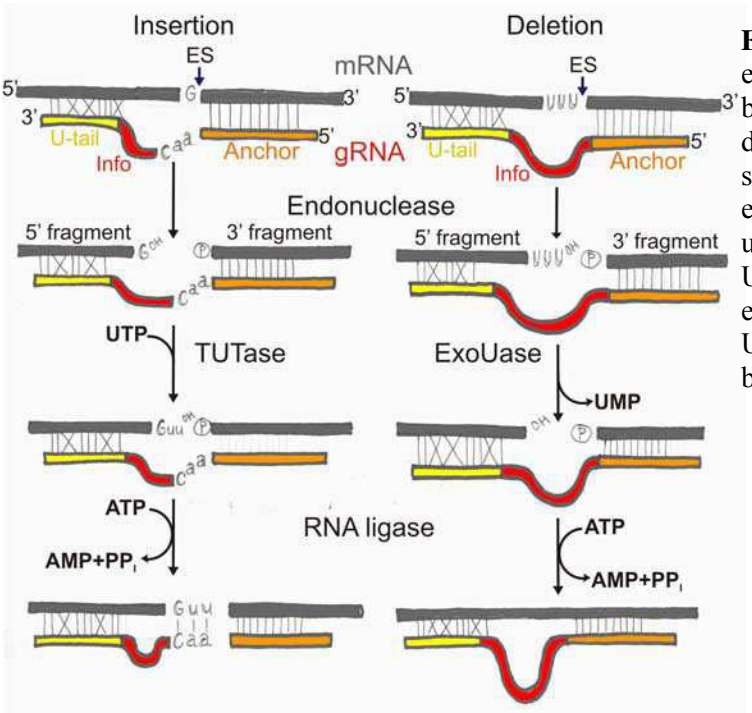


Figure 7. Mechanism of RNA editing. The first base pair mismatch between mRNA (top) and anchor domain (orange) defines the editing site (ES), which is cleaved by an endonuclease. A 3' terminal uridylyltransferase (TUTase) adds Us to the 5' fragment while a exonuclease (exoUase) deletes extra Us. The two fragments are joined back together by an RNA ligase.

Two subcomplexes encapsulate the proteins responsible for either U insertion or deletion editing events with a striking symmetry (Schnaufer et al., 2003) (Figure 8B). The U-insertion subcomplex contains the required 3' terminal uridylyltransferase, KRET2[7], bound to a protein called KREPA1, which in turn associates with the RNA ligase KREL2. On the other hand, the U-specific exonuclease, KREX2, is sequestered with KREL1 through mutual interactions with KREPA2 in the U-deletion subcomplex. It is hypothesized that the KREPA zinc-fingers facilitate their placement between their respective subcomplex partners. This composition allows the KREPA proteins to toggle substrate RNA bound to their OB-fold domains between the U addition or deletion enzymes and RNA ligase.

Three distinct types of 20S editosomes exist that differ in their incorporation of one of the three endonucleases that catalyze the initial mRNA cleavage step (Carnes et al., 2008). Those containing KREN1 or 2 have the capacity to cleave RNA editing substrates with U deletion or insertion editing sites, respectively (Carnes et al, 2005;

[7] Nomenclature introduced by Stuart et al. (2005) as follows: KRE= kinetoplastid RNA editing; L= ligase; N= endonuclease; P= protein; T= 3' terminal uridylyltransferase; X= exonuclease

Trotter et al., 2005). However, the editosomes with KREN3 specifically processes cox2 editing mediated by a *cis*-gRNA. Null-mutants of this endonuclease is lethal to the bloodstream trypanosomes, surprising given that respiratory complex to which it belongs is downregulated at this stage (Bienen et al., 1981).

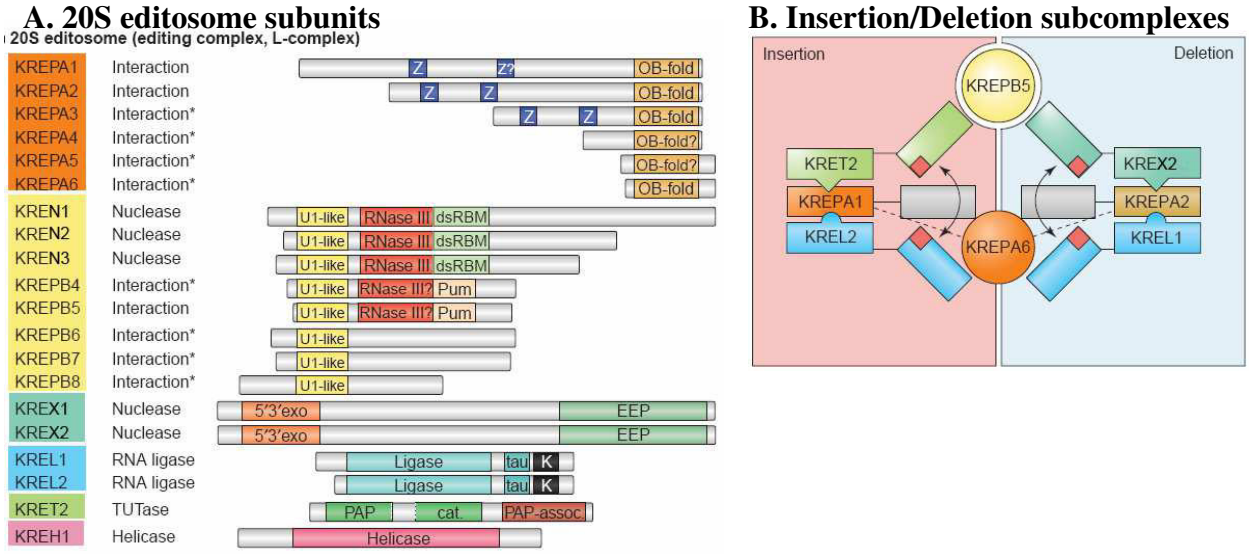


Figure 8. Composition of 20S editosome. **A.** Subunits of the 20S editosome, as organized according to sequence similarities. **B.** Toggle model proposed for how insertion/deletion subcomplexes orchestrate the subsequent steps mediated by either 3' terminal uridylyltransferase (KRET2) or exonuclease (KREX2) and RNA ligase. Notice the apparent symmetry of the two subcomplexes. Taken from Stuart et al., (2005).

At first, the lethality of the KREL1 gene knockouts in the bloodform was unexpected as well (Schnaufer et al., 2001). The existence of dyskinetoplastic (dk) representatives in the *Trypanosoma* genus locked in this stage, *e. g., T. b. evansi* and *T. b. equiperdum*, because they lack the gRNA repertoire for processing affected mRNAs, ostensibly implied that RNA editing is also not required during the infectious phase of the life cycle (Schnaufer et al., 2002) (discussed further in section 8). The finding that KREL1 is essential may have a practical outcome. The solved crystal structure of this protein (Deng et al., 2004) has primed a study for drug-like inhibitors of its function (Amaro et al., 2008), which may potentially be developed for drug treatment of the various diseases caused by trypanosomes.

5.3 Other proteins involved in RNA editing

Several other proteins and complexes have been described that have a role in RNA editing aside from imparting the core enzymatic activities this process. A DExD/H-box RNA helicase found unstably associated with the 20S editosome was proposed to have gRNA-unwinding role, although this hypothesis is not supported by the viability of null mutants (Missel et al., 1997). KRET1 is the 3' terminal uridylyltransferase responsible for the post-transcriptional addition of the 3'-oligo(U) tail to gRNA molecules, and is essential for RNA editing (Aphasizhev et al., 2003a).

The participation of RNA binding proteins has always been expected feature of RNA editing. A common strategy for isolation of these factors is the screening for proteins binding to synthetic gRNAs (Köller et al., 1994) or poly(U) polymers (Leegwater et al., 1995; Bringaud et al., 1995). RBP16 is an example of a protein discovered in this fashion (Hayman and Read, 1999). Containing hallmark features of Y-box nucleic acid-binding proteins (Miller and Read, 2003) and modification by arginine methylation (Pelletier et al., 2001), its silencing by RNA interference (RNAi) had a pleimorphic phenotype, affecting never-edited transcripts as well as the editing of cyB (Pelletier and Read, 2003). Furthermore, RBP16 stimulates *in vitro* RNA editing activity (Miller et al., 2006), which is consistent with its demonstrated gRNA/mRNA annealing activity (Ammerman et al., 2008). The mitochondrial RNA binding proteins MRP1 and 2 were identified in a similar fashion and have been a focus of intense research. They associate in a heterotetrameric complex that was shown to have *in vitro* RNA matchmaking activity (Müller et al., 2002; Schumacher et al., 2006; Zíková et al., 2008a). Although RNAi-knockdowns of these proteins also affected a subset of both never-edited and edited mRNAs (Vondrušková et al., 2005) (as summarized in Table 1 of Chapter 5), the crystal structure of this complex, in which positively-charged amino acids on its surface bind gRNAs, is consistent with a role in gRNA:mRNA duplex formation (Schumacher et al., 2006; Zíková et al., 2008a).

TbRGG1 was found during screening a *T. brucei* cDNA library for a homolog to nucleolin by Vanhamme and co-workers (1998), a cross-hybridization that occurred probably because both proteins contain the so-called RGG domain, an established RNA binding motif (Burd and Dreyfuss, 1994). This serendipitous event was ultimately a

rediscovery of a mt protein that was previously discovered by virtue of its affinity for poly(U) (Leegwater et al., 1995; Vanhamme et al., 1998). Circumstantial evidence based on glycerol gradient sedimentation studies suggested a potential role in RNA editing and association with a large macromolecular complex. This study prompted us to investigate these possibilities, as detailed in Chapter 2, using RNAi to silence expression of TbRGG1. While RNA editing was affected, the steady state level of gRNA was not (Hashimi et al., 2008). However, tandem affinity purification (TAP) of this protein did show RNA-mediated association with what we named the putative mitochondrial RNA binding complex 1 (MRB1).

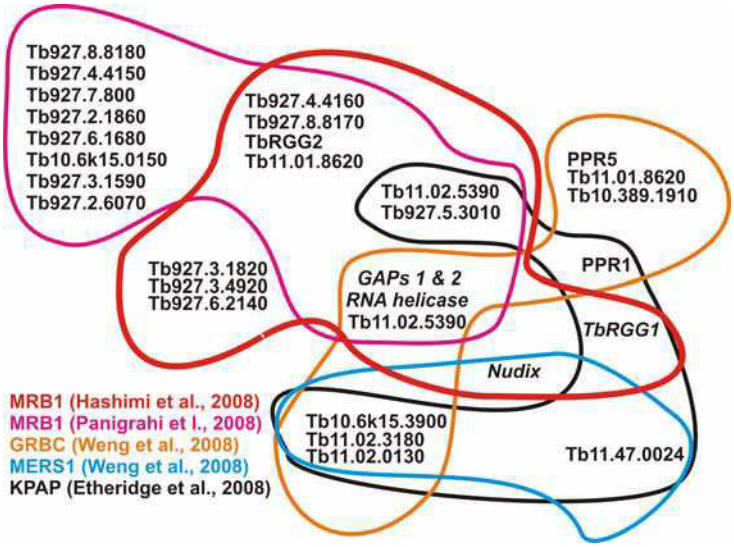


Figure 9. The varying compositions of the putative mitochondrial RNA complex isolated by different groups. GeneDB accession numbers from the *T. brucei* genome sequence are given to unnamed proteins. Groupings are color coded according to reports describing their MRB1 preparations, all based on the TAP approach. Italicized names are given to proteins discussed in this thesis (Chapters 1 and 2). Included are subunits of the KPAP complex, named after a mt poly(A) polymerase, that also co-purify with MRB1 in the other studies. GAP, guide RNA associated protein; PPR, pentatricopeptide repeat protein.

5.4 The mitochondrial RNA complex 1

MRB1 appears to be a complex or collection of smaller complexes and/or monomers that was described independently by three groups using the TAP approach (Hashimi et al., 2008; Panigrahi et al., 2008; Weng et al., 2008). Weng and colleagues (2008) further divide this set of proteins into the guide RNA binding (GRBC) and MERS complexes,

the latter containing a predicted Nudix hydrolase. Furthermore, several putative MRB1 subunits were also found to associate with a recently discovered mt poly(A) polymerase (Etheridge et al., 2008). As illustrated in Figure 9, while these preparations did not yield identical compositions, they do share a tremendous degree of overlap[8].

What is clear from an overview of this collection of proteins is that there are several with apparent motifs and domains indicating a role in some aspect of RNA metabolism. The aforementioned Nudix hydrolase belongs to a superfamily of ubiquitous proteins that process organic pyrophosphates, such as those of nucleosides (McLennan, 2006). Several proteins have pentatricopeptide (PPR) repeats, which are prevalent in plant organelles and are involved in post-transcriptional events (Delannoy et al., 2007). Six such proteins have been shown to have a role in mt rRNA biogenesis in *T. brucei* (Pusnik et al., 2007). Both TbRGG1 and 2 have been described in the MRB1 protein mix (Hashimi et al., 2008; Panigrahi et al., 2008), sharing the RGG motif as well as an affinity for poly(U) sequences (Vanhamme et al., 1998; Fisk et al., 2008). A putative RNA helicase has been identified in several of the several MRB1 isolations.

The data indicate that MRB1 is more a collection of complexes and/or monomers than a *bona fide* protein complex held together by direct interaction between subunits, as are the 20S editosome and MRP complex. First of all, several putative subunits have been shown to associate with macromolecular structures in an RNase-sensitive manner (Fisk et al., 2008; Hashimi et al., 2008; Weng et al., 2008; Acestor et al., 2009), suggesting they may assemble around RNA, while others do not appear to have this property (Acestor et al., 2009).

RNAi knockdowns of several subunits result in different phenotypes. While depletion of both TbRGGs results in specific downregulation of edited-RNAs (Fisk et al., 2008; Hashimi et al., 2008; Acestor et al., 2009), silencing of other subunits results in a decrease in steady state gRNA levels (Weng et al., 2008; Hashimi et al., in press). Furthermore, our Nudix hydrolase knockdowns exhibited a general destabilization of maxicircle-encoded RNAs, perhaps reflecting a potential role in repairing oxidative

[8] Three subunits of MRB1, namely guide RNA associated proteins (GAPs) 1 and 2 and Nudix hydrolase, were initially reported to interact with the MRP complex (Aphasizhev et al., 2003b). However, this observation was not confirmed by later reports (Zíková et al., 2008a; Hashimi et al., 2008; Panigrahi et al., 2008).

damage to ribonucleosides (McLennan, 2006), whereas this phenotype was not observed by Weng and coworkers (2008).

For now, the nature of MRB1 remains an enigma. However, the data thus far indicate that the comprising proteins play a central role in mt RNA metabolism. Chapter 3 discusses our contribution to research on MRB1.

5.5 The *raison d'etre* of RNA editing

Shortly after its discovery, it was proposed that kinetoplastid RNA editing may be a relic of an ancient "RNA world", when only these molecules existed (Benne 1990). The lack of catalytic activity of the substrate RNAs and the participation of a sophisticated protein complex has negated this idea. In addition, a possible link of seemingly cumbersome process to parasitism has been invalidated by its existence in free living Bodonids (Blom et al., 1998). Several hypotheses have suggested the evolutionary advantages bestowed by RNA editing, including: 1) extra level of regulation of mt gene expression (Stuart et al., 1997); 2) fixing mutations that have accumulated in a non-functional mitochondrion (Cavalier-Smith, 1997); 3) accelerated evolution by creating more genetic variation (Landweber and Gilbert, 1993); 4) multiple proteins coded by one gene (Read et al., 1994).

The persistent editing of cox3 in the bloodstream stage (Feagin et al., 1988; Stuart et al., 1997; Schnaufer et al., 2002), despite the downregulation of its encompassing complex, has spurred the exploration of the idea that RNA editing contributes protein diversity. An interesting study has provided evidence for a protein product of an alternatively edited cox3 mRNA that has a role in kDNA maintenance (Ochsenreiter and Hajduk, 2006; Ochsenreiter et al., 2008a). However, the viability of dk trypanosomes (discussed in section 7), in which RNA editing is not functional and its protein apparatus not essential (Schnaufer et al., 2005), tempers this finding. Although more alternatively edited RNAs of other mt gene transcripts have been described (Ochsenreiter et al., 2008b), hard evidence of their translation is needed to confirm this exciting theory.

6. The mitochondrial RNA metabolism of *Trypanosoma brucei*

RNA editing is process that is integrated into what is emerging as a byzantine RNA metabolism. While only a single mt RNAP appears to be required for mini- and maxicircle RNA synthesis (Grams et al., 2002; Hashimi et al., in press), their transcripts undergo different maturation pathways before the gRNAs are duplexed with their cognate pre-edited mRNAs. Minicircles are thought to be transcribed polycistronically and cleaved by a 19S protein complex into one or more gRNAs (Grams et al., 2000)[9], before being polyuridylylated by KRET1 (Aphasizhev et al., 2003a). These molecules are believed to assume a secondary structure with two hairpin loops, perhaps as a way of being recognized by the protein machinery of editing (Schmid et al., 1995). The two mt rRNAs also undergo post-transcriptional modification, forming their short 3' oligo(U) tails (Adler et al., 1991).

The dense gene structure of the *T. brucei* maxicircle (Figure 4) indicates that it is transcribed polycistronically, although this is not the case in other trypanosomatids (Blum et al., 1990; van der Spek et al., 1991). Editing mediated by *trans*-gRNAs occurs independently of cleavage of these precursors into monocistronic transcripts, sometimes even preceding this event (Koslowsky and Yahampath, 1997). An ortholog of the KRETs polyadenylates the resulting mRNAs, and has been named kinetoplast poly(A) polymerase (KPAP) (Etheridge et al., 2008). Interestingly, KPAP appears to be in a complex with some of the subunits of the MRB1 complex (Figure 9), although the nature of this association remains uncertain.

In mitochondria throughout eukaryotes, polyadenylation either stabilizes mRNAs, as occurs in humans, or marks them for degradation, as in plants (Carpousis et al., 1999). Its role is more complex in *T. brucei*, in which the length of the poly(A) tail is a key determinant. Pre-edited mRNAs have short $poly(A)_{20\text{-}25}$ tails, while never- and fully-edited transcripts have either short $poly(A)_{20\text{-}25}$ or long $poly(A/U)_{120\text{-}200}$ extensions, in

[9] There are few problems with this idea. One is that this processing event would yield only one 5'-triphophorylated gRNA, which is a defining feature of their being primary transcripts (Sturm and Simpson., 1990). Another is that the mt endonucleolytic activity attributed to cleave polycistronic gRNAs *in vitro* may be an artifact of the endogenous activity of the 20S editosome, which has the same sedimentation properties.

which oligo(U) tracts are interspersed among the poly(A)[10] (Read et al., 1994; Militello and Read, 1999; 2000; Ryan and Read, 2006; Etheridge et al., 2008). While the short tail destabilizes pre-edited molecules, it has the opposite effect in edited RNAs (Kao and Read, 2005). There is disagreement on whether partially-edited RNAs contain both types of tails or exclusively the long form (Militello and Read, 1999; Etheridge et al., 2008). Proponents of the former propose that UTP and the 3' polyuridylylation activity of KRET1 stimulates mRNA turnover (Militello and Read, 2000; Ryan and Read, 2005), while those of the latter suggest it signifies a translatable mRNA (Etheridge et al., 2008). Another mt mRNA degradation pathway exists that is independent of the poly(A) tail or UTP (Militello and Read, 2000).

The 12S rRNA and ND3 genes represent the 5' ends of major and minor strands of the maxicircle, respectively, as they are adjacent to the variable sequence domain (Figure 4). TbDSS-1, which is a homolog to the eponymous yeast mt degradosome exonuclease (Penschow et al., 2004), targets aberrant byproducts of these loci which still contain their unprocessed 5'-ends (Mattiacio and Read, 2008). Thus, this enzyme has a role in surveillance of the mt transcriptome for improperly processed RNAs.

7. Translation of mitochondrial RNA

It took almost 15 years from the discovery of RNA editing to find direct evidence of a protein product of an edited RNA, cyB, in *Leishmania tarentolae* (Horvath et al., 2000a). Reports of the translation of cox1 and cox2 from never-edited and edited transcripts from the same organism soon followed (Horvath et al., 2000b; 2002). As the generation of antibodies against mt proteins has proven to be unfruitful, perhaps due to their high hydrophobicity, these studies had to resort to more difficult techniques. This situation has ultimately been a barrier in detecting the other 15 mt proteins.

The synthesis of the characterized respiratory chain subunits was resistant to chloramphenicol, which typically inhibits the action of mt and bacterial ribosomes (Horvath et al., 2000a; 2000b; 2002), and may reflect an unusual aspect to these structures in trypanosmatids. Indeed, the 9S and 12S mt rRNAs from these flagellates are

[10] This feature was initially observed in edited RNAs of *C. fasciculata* (Van der Spek et al., 1988; 1990). It has not been determined poly(A/U) is a feature of never-edited RNAs, although it is possible given the propensity of mt RNA to be undergo some degree of oligouridylylation (Madej et al., 2007).

among the shortest homologs of the *E. coli* 16S and 23S rRNAs in eukaryotes (de la Cruz et al., 1985a; 1985b; Sloof et al., 1985). Recent studies examining the protein composition of mt ribosomes in *T. brucei* (Zíková et al., 2008) and *L. tarentolae* (Maslov et al., 2006; 2007), have identified several proteins with varying degrees of homology to eukaryotic and bacterial subunits. Not surprisingly, most of the identified peptides were unique to kinetoplastids. Still, the large subunit (LSU) and small subunit (SSU) particles form ribosomes with a shape reminiscent of those from bacteria and bovine (Maslov et al., 2006). A novel ribonucleoprotein complex that is comprised of the SSU and an uncharacterized protein mass, and without a known function, is present in these organelles as well (Maslov et al., 2006; 2007).

The unique composition of the mt ribosomes may be a result of selective pressure for the utilization of eukaryotic-type tRNAs (Schneider and Marechal-Drouard, 2000). Because no tRNA genes reside within kDNA, the whole set of these molecules are imported into the mitochondrion (Hancock and Hajduk, 1990) There is some controversy about how tRNA import is facilitated, with one group claiming that it is performed by a novel complex containing nuclear encoded subunits of the respiratory chain and even mt encoded proteins (Mukherjee et al., 2007). However, there is consensus that protein entities do mediate this process in conjunction with ATP hydrolysis (Rubio et al., 2000). Once inside the organelle, some tRNAs are modified in order to conform to mt codon usage, as illustrated by the C to U editing event occurring in the imported $tRNA^{Trp}$ anticodon (Alfonso et al., 1999).

8. Dyskinetoplastic *Trypanosoma brucei*

The plasticity of *Trypanosoma* is evident in the in the existence of diskinetoplatsic (dk) and akinetoplastic (ak) cells, which survive despite substantial loss to their mt genomes (Schnaufer et al., 2002). The latter exhibit a complete loss of kDNA, whereas dk cells appear to have a morphologically intact kinetoplast (Stuart, 1971; Lai et al., 2008). However, under the ultrastructural surface lurks the homogenization of minicircle classes, diminishing the repertoire of gRNA genes to a handful of sequences, and a consequent incapacity to express edited maxicircle genes. The larger component of kDNA in turn

accumulates deletions, because of an absence of selection pressure for their maintenance (Speijer, 2006), presumably another step on a slippery slope to the ak state.

These types of cells can be induced in the laboratory by a rigorous course of mutagenic drug treatment targeting kDNA replication in *T. brucei* (Stuart, 1971). However, it is quite significant that dk *T. b. equiperdum* and ak *T. b. evansi* occur in nature, where they have an economic impact as causative agents of veterinarian diseases of horses, water buffalos and camels. Unlike other members of the *T. brucei* species complex, they have spread outside of the tsetse fly belt of sub-Saharan Africa (Lun and Desser, 1995). This advantage is due to the reduction of their heteroxenous life cycle, requiring the *Glossina* vector, to a monoexonous one strictly as a bloodstream trypanosome transmitted by mechanical means. Although the textbook mode of transmission between horses for *T. b. equiperdum* is by coitus, *T. b. evansi* is spread to various mammalian hosts by the mouthparts of a wide range of bloodsucking insects, and even vampire bats in South America (Brun et al., 1998; Roberts and Janovy, 2004).

How dk/ak trypanosomes are able to survive in the bloodstream stage, and not have the capacity to transform to the procyclic stage, is apparent upon a comparison of the energy metabolisms of these two phases (Section 4.1). The elaborated mitochondrion of the insect stage requires the complete expression of the maxicircle for biogenesis of the cytochrome containing respiratory complexes (Figure 2B). In addition, recycled parts of the Krebs cycle are present for other pathways, such as proline catabolism. These features are missing in the reduced mitochondrion of the bloodstream stage, which derives energy only from glucose fermentation (Figure 2A). *Petite* (ρ) mutants of yeast, named for the diminutive size, thrive under similar conditions (Chen and Clark-Walker, 2000). These cells, which either completely lack (ρ^0) or have aberrant (ρ^-) mt DNA, are able to grow in fermentable media in the absence of complete mt biogenesis.

However, the above discussion does not take into account that complex V still present during the bloodstream stage of all *T. brucei* subspecies, consuming ATP to preserve $\Delta\Psi m$, allowing protein import for organellar maintenance (Schnaufer et al., 2005; Brown et al., 2006). This complex requires the A6 subunit, which is part of the proton channel of the membrane-associated F_O moiety (Velours and Arselin, 2000) (Figure 10), and retained in virtually all mt genomes (Funes et al., 2002). In *T. brucei*, a

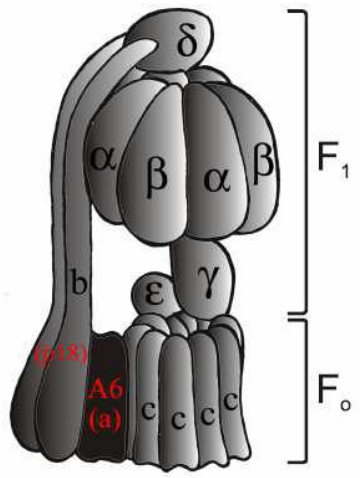


Figure 10. Structure of complex V. The soluble F_1 and membrane bound F_O moieties are depicted. The position of A6, which with the subunit c oligomer forms the proton channel, is indicated in black. Subunit b, spanning the two particles, is also referred to as p18 in Chapter 4.

pan-edited transcript has been assigned to encode this protein, based on its extreme hydrophobicity and low sequence similarity of its C-terminus those of other A6 homologs (Bhat et al., 1990). Experimental evidence of the incorporation of A6 into complex V, albeit indirect, is provided in this thesis (Chapter 5).

This situation raises the question as to why RNA editing is needed when a complete mt genome is present, but superfluous in a ρ-like background (Schnaufer et al., 2001; 2002)? It seems that the answer is that compensatory mutations have evolved in other subunits of the F_1 moiety of complex V to offset the missing A6 and F_O proton channel (Schnaufer et al., 2005; Lai et al., 2008). An introduced γ subunit containing such a compensatory mutation from an induced dk cell line converted a ρ^- *Kluyveromyces lactis* yeast into a ρ^+ form, which can grow on non-fermentable media, providing compelling experimental support for this theory (Schnaufer et al., 2005). An elegant model was proposed as to how ΔΨm is generated in dk/ak cells, in which ATP^{4-} is converted to ADP^{3-} by the F_1 moiety; the antipodal exchange of the former into and latter out of the matrix by the ATP/ADP carrier maintains a net positive charge on the outside face of the inner membrane (Schnaufer et al., 2005).

9. Tools for functional analysis of *Trypanosoma brucei*

A majority of the work presented in this thesis was made possible by the elaborate system of inducible expression established in *T. brucei*. This platform was used for two reasons. The first was to ascertain the function of certain genes by RNAi-mediated reverse genetics. The second was for the over-expression of tagged proteins, in order to determine potential interacting proteins or sub-cellular localization. The system established procyclic cells will be discussed briefly here, keeping in mind that the same principles are the basis of the equivalent bloodstream stage cell lines.

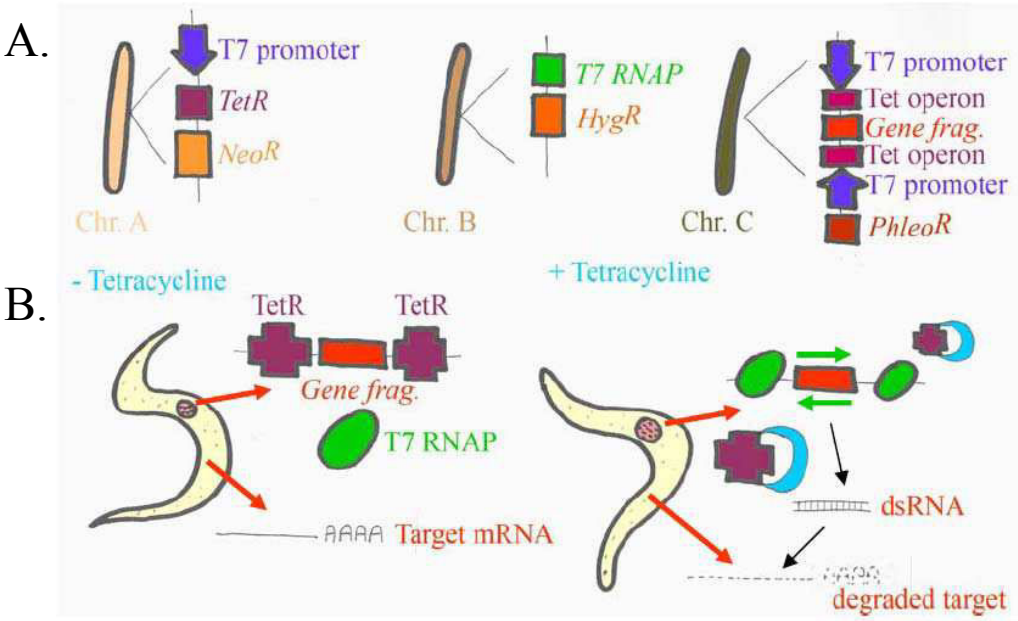


Figure 11. (**A**) Genetic background of 29-13 cell line. Chromosomes containing *TetR* and neomycin resistance genes under control of T7 promoter (Chr. A) and constitutively expressed *T7 RNAP* and hygromycin genes (Chr. B) are shown. A third cassette for mediating RNAi is shown of Chr. C, with studied gene fragment in between opposed T7 promoter and Tet operons. (**B**) Mechanism of tetracycline induction. In absence of tetracyline (left) T7 RNAP is blocked from T7 promoters by TetR binding to Tet operon. Induction by tetracyline (right, light blue crescents) occurs when it binds TetR, allowing T7 RNAP access to its promoters. dsRNA is transcribed, which degrades target mRNA in cytoplasm. Gene names are italicized.

The procyclic cell line named 29-13 contains two cassettes that contain the *T7 RNAP* and *tetracycline repressor* (*tetR*), each with an adjoining *neomycin* or *hygromycin* resistance gene, respectively (Wirtz and Clayton, 1995; Wirtz et al., 1998) (Figure 11A). To ensure these transgenes are stably integrated into the genome, this cell line is grown constantly in the presence of these two antibiotics. *T7 RNAP* is constitutively transcribed from the *β-tubulin* locus, whereas the *tetR* cassette is targeted to a weakly transcribed locus. This transgene is under the control of a T7 promoter containing a point mutation that reduces its activity by 90% (Wirtz and Clayton, 1995; Wirtz et al., 1998). In this genetic background, both T7 RNAP and the tetR are expressed, setting the stage for inducible expression, a useful tool for expression of a product that may be detrimental to the cell.

RNAi is a pathway in which introduced double stranded RNA instigates the degradation of cytoplasmic RNAs with homologous sequence. This process was discovered in *T. brucei* (Ngo et al., 1998) independently of the Nobel Prize winning

discovery in *Caenorhabditis elegans* (Fire et al., 1998). There have not been any reports of its identification in any other trypanosomatids, making *T. brucei* the workhorse for functional gene analysis. In our studies, the inducible expression of dsRNA is facilitated by the electroporation of a third construct into the 29-13 cell line, in which a gene fragment is flanked by T7 promoters. These elements are under control of the tet operator, to which tetR binds and blocks T7 RNAP access to its promoters (Figure 11B). After selection of successful transformants by growth in the presence of phleomycin, RNAi is induced by introduction of tetracyline (tet), which competitively binds tetR, freeing it from the operator and allowing T7 transcription to proceed.

We used the p2T7-177 construct, which integrates into the transcriptionally-silent 177 locus present on minichromosomes, with the intention of preventing the leaky expression of dsRNA by endogenous read-through transcription activities (Wickstead et al., 2002). The first plasmid using opposed T7 promoters is pZJM, which gets its name from the first initials of the researchers who generated it for creating an RNAi-library to facilitate forward genetics (Wang et al., 2000; Morris et al., 2002). This cassette is targeted to the rDNA locus, which is transcriptionally active and more prone to leaky transcription of dsRNA in the absence of tet. It is widely believed in the community that the generation of a hairpin dsRNA from one T7 promoter provides tighter regulation, although they do not provide the straightforward cloning of other approaches. Examples of such constructs include the pQuadra plasmids (Inoue et al., 2005).

Constructs under the control of a single promoter were also used for over-expression of proteins containing in frame C-terminal tags. The utilized vectors are based on the pLew79 vector (Wirtz et al., 1999), with a tet-inducible PARP[11] promoter that is transcribed by RNAP I (Liang et al., 2003). The T7 RNAP transcribes the phleomycin resistance marker. The pJH54 plasmid allows cloning of an ORF upstream of a sequence coding for three tandem hemagglutinin (HA) epitopes. The tagged proteins are subsequently detected with the α-HA antibody in immunolocalization experiments.

Originally developed to study protein interactions in yeast (Riggaut et al., 1999), tandem affinity purification (TAP) has been ported into trypanosomatids to purify native protein complexes, often for subsequent mass spectroscopy analysis to identify

[11] Acronym for procyclic acidic repetitive protein

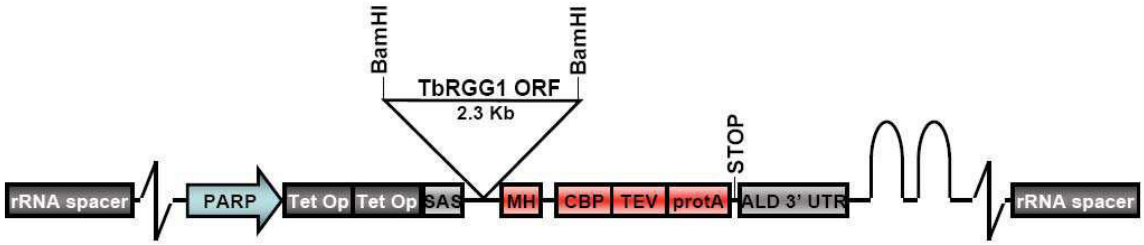


Figure 12. pLew79-MHTAP cassette map, after linearization for integration into *rDNA* locus. PARP, promoter; Tet Op, tet operon; SAS, actin splicing signal; MH, myc/his epitopes; CBP, calmodulin binding protein; TEV, protease cleavage site; protA, Protein A.

comprising peptides (*e.g.*, Schnaufer et al., 2003; Brandenburg et al., 2008; Panigrahi et al., 2008; Weng et al., 2008; Zíková et al., 2008a; 2008b). The TAP tag of the utilized pLew79-MHTAP vector contains (listed in order from the myc/his epitopes to the C-terminus), Protein A region, TEV protease cleavage site and calmodulin binding protein (CBP) (Jensen et al., 2006) (Figure 12). This scheme allows the use of a two-step affinity purification through a IgG column, binding Protein A, followed by a calmodulin column, which CBP binds in the presence of Ca^{2+} (Riggaut et al., 1999); the bound material is freed from the columns for subsequent steps by TEV protease cleavage in the first and EGTA-mediated chelation of Ca^{2+} in the second. Another variation on the TAP-tag replaces CBP with a so-called Protein C epitope, which binds a specific monoclonal antibody in the presence of Ca^{2+} (Schimanski et al., 2005).

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Chapter 2:

TbRGG1, an essential protein involved in kinetoplastid RNA metabolism that is associated with a novel multiprotein complex. (reprint of Hashimi *et al*., 2008, RNA *14*, 970-980)

Abstract:

The uridine insertion / deletion RNA editing of kinetoplastid mitochondrial transcripts is performed by complex machinery involving a number of proteins and multiple protein complexes. Here we describe the effect of silencing of TbRGG1 gene by RNA interference on RNA editing in procyclic stage of *Trypanosoma brucei*. TbRGG1 is an essential protein for cell growth, the absence of which results in an overall decline of edited mRNAs, while the levels of never-edited RNAs remain unaltered. Repression of TbRGG1 expression has no effect on the 20S editosome and MRP1/2 complex. TAP-tag purification of TbRGG1 co-isolated a novel multi-protein complex and its association was further verified by TAP-tag analyses of two other components of the complex. TbRGG1 interaction with this complex appears to be mediated by RNA. Our results suggest that the TbRGG1 protein functions in stabilizing edited RNAs or editing efficiency and that the associated novel complex may have a role in mitochondrial RNA metabolism. We provisionally name it putative mitochondrial RNA binding complex 1 (put-MRB complex 1).

Chapter 3:
Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex 1 and mitochondrial RNA polymerase.
(manuscript of RNA article *in press*)

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 in *Trypanosoma brucei*. Based on the phenotype caused by downregulation 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 downregulation 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

proximity of the kinetoplast. Finally, we demonstrate that the same mt RNA 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

Introduction

Kinetoplastid flagellates, the most studied of which are causative agents of African sleeping sickness, Chagas disease and leishmaniases in humans, have a strikingly complex mitochondrial (mt) DNA, also termed kinetoplast (k) DNA. It is composed of two types of molecules – maxicircles and minicircles. In the single mitochondrion, maxicircles are present in about a dozen of copies and represent the equivalents of classical mt DNA, bearing genes for subunits of respiratory complexes, mitoribosomal RNAs and one mitoribosomal subunit (for recent review see Simpson et al., 2004; Lukeš et al., 2005; Stuart et al., 2005). A unique feature of these flagellates is that the transcripts of some of the maxicircle-coded genes are post-transcriptionally altered by additions and/or deletions of uridylate (U) residues by a process termed RNA editing (Benne et al., 1986).

The function of kDNA minicircles had remained mysterious for decades. These molecules are concatenated in a network, number in the thousands, and their heterogenous sequence make up the bulk of the mt genome (Liu et al., 2005). The seminal discovery that they encode genes for small RNA molecules called guide (g) RNAs revealed their biological role as a genetic reservoir complementing the maxicircles (Sturm and Simpson, 1990). It is these gRNAs that provide information for multiple and specific insertions and deletions of uridines into pre-edited maxicircle mRNAs. Each gRNA is composed of three regions, differing in their function and content. The 5' anchor region hybridizes to a complementary sequence of the pre-edited mRNA, while the downstream information region of the RNA molecule provides the template for U insertions/deletions in the cognate mRNA via both Watson-Crick and non-canonical G:U base pairing. The 3' end of the gRNA is comprised of the post-transcriptionally added oligo(U) tail (Blum and Simpson, 1990). While the identity of the RNA components of editing was established almost 20 years ago, the identification of the myriad of proteins involved in mt RNA metabolism remains an ongoing endeavor.

The core RNA editing process has been ascribed to the 20S editosome, a multiprotein complex composed of 20 proteins (Simpson et al., 2004; Lukeš et al., 2005; Stuart et al., 2005). It has been firmly established by *in vitro* and *in vivo*

experiments that editing is carried out by this complex, which contains all catalytic activities necessary for the cleavage of the pre-mRNA, addition or deletion of the U(s), and ligation of the processed RNA molecules. The 20S editosome appears to be represented by a population of at least three dynamic protein complexes slightly differing in their composition and specific role in this process (Panigrahi et al., 2006; Carnes et al., 2008).

Mitochondrial RNA binding proteins have been a focus of intense research. The MRP1/2 complex is a heterotetrameric structure, composed of the MRP1 and MRP2 proteins, with positively charged residues concentrated at one face, to which the gRNAs anneal (Schumacher et al., 2006) Data obtained with the *in vivo* purified as well as *in vitro* reconstituted MRP1/2 complexes are consistent with a role in matchmaking the gRNAs with cognate pre-edited mRNAs (Muller et al., 2001; Zikova et al., 2008). RBP16 is another RNA binding protein that appears to have an *in vivo* role in editing (Pelletier and Read, 2003), perhaps via its demonstrated ability to stimulate insertion editing *in vitro* (Muller et al., 2006).

The initial characterization of TbRGG1, so-called because it possesses the RGG RNA binding motif, suggested the existence of another protein complex with a role in RNA editing (Vanhamme et al., 1998). The downregulation of TbRGG1 in the procyclic (=insect) stage of *T. brucei* caused an overall decline of edited mRNAs, but the never-edited transcripts were unaffected (Hashimi et al., 2008). This protein is stably associated with a putative protein complex composed of about 14 different subunits (Hashimi et al., 2008; Panigrahi et al., 2008), provisionally named the mitochondrial RNA binding complex 1 (MRB1 complex). Several of its components have features that point to RNA processing activities associated in an RNA mediated manner (Hashimi et al., 2008; Panigrahi et al., 2008). Another component of this complex, TbRGG2 (also known as TbRGGm), has affinity for poly(U) and its depletion leads to dramatic decrease of edited mRNAs (Fisk et al., 2008). Interestingly, a recent report on the newly discovered KPAP1 protein, poly(A) polymerase that polyadenylates maxicircle transcripts, shows that it is associated with a complex of proteins of overlapping composition with the MRB1 complex (Etheridge et al., 2008; Weng et al., 2008).

From these data, the role of the MRB1 complex is not obvious. However, its importance is underscored by the fact that orthologs of its subunits are found in all trypanosomatid genomes sequences thus far. Here, we attempt to ascertain the function of the MRB1 complex by functional analysis of four subunits of this complex by RNAi-silencing in the procyclic stage. The examined subunits are summarized in Table 1, including a nomenclature introduced in a similar study performed by Weng et al. (2008). Two of these subunits, Tb927.2.3800 and Tb927.7.2570, are paralogs that have no known protein motifs or domains (Hashimi et al., 2008). They will be referred to herein as guide RNA associated

proteins (GAP) 1 and 2, respectively, since we provide evidence for their participation in the biogenesis of these molecules. The third subunit, Tb927.4.1500, is predicted to be a ~240 kDa protein with DExD/H-box RNA helicase domains (Hashimi et al., 2008), and is referred to as such in this paper. The last studied subunit, Tb927.11.7290, is annotated as a Nudix hydrolase due to its conservation to other such proteins. Moreover, we show that mt RNA polymerase (mtRNAP), known to transcribe the maxicircle-encoded protein-coding genes (Grams et al., 2002), appears to have a similar role for minicircle-encoded gRNAs.

Materials and Methods

Generation of RNAi-knockdown and transgenic cell lines – Primers for generation of 400-600 bp gene fragments for cloning into the p2T7-177 vector were designed using the RNAit online tool available on the TrypanoFAN website (http://trypanofan.path.cam.ac.uk/software/RNAit.html). The following primer pairs (with restriction site underlined and indicated in parenthesis) were used: GAP1 gene – GAP1-F (5'-CTCCTCGAGCCTTTCAGC-3') (XhoI) and GAP1-R (5'-GGCAAGCTTCTGCGAATGTAG-3') (HindIII), amplified a 533 bp- long fragment; GAP2 gene – GAP2-F (5'-CGAGGATCCACAACGGCATT -3') (BamHI) and GAP2-R (5'-CATCATCAGCAAGCTTTATGATG-3') (HindIII), amplified a 580 bp-long fragment; Hel1-F (5'-GGATCCGTAGGAACTGGCAGAGACGC-3') (BamHI) and He1-R (5'-CTCGAGGCTACTGATTGCACGCAAAA -3') (XhoI), amplified a 453 bp-long fragment; Hyd1-F (5'-GGATCCCGTAAGGTGTCAGGACCGAT-3') (BamHI) and Hyd1-R (5'-CTCGAGTCTACGGTAATGCCCGTTTC-3') (XhoI) amplified a 467 bp-long fragment. All amplified fragments were cloned into the p2T7-177 vector creating constructs, which were upon linearization electroporated into the procyclic *T. brucei* strain 29-13, and the clones were obtained by limiting dilution at 27^{o}C as described elsewhere (Vondrušková et al., 2005). Synthesis of dsRNA was induced by the addition of 1 μg/ml tetracycline to six clonal cell lines for each knockdown. For further experiments clones were selected based on the tightness of tetracycline expression of dsRNA and the robust elimination of target mRNA, as determined by Northern blot analysis using the appropriate gene fragment as a probe. The procyclic knockdown generated by cloning a fragment of the mt RNA polymerase gene in the pZJM vector (Grams et al., 2002) was kindly provided by P.T. Englund (Johns Hopkins University, Baltimore).

Linearized p2T7-177 vectors containing the GAP1 and 2 gene fragments were electroporated using the Amaxa Nucleofector II electroporator into the 427 bloodstream *T. brucei* strain. About 3 x 10^7 cells of exponentially growing culture were used for transfection, following the protocol of Vassella et al.(2001) with

minor modifications. Transfectants were selected with 1.25 μg/ml phleomycin and cloned by limiting dilution. RNAi was triggered and assessed in six clonal cell lines by the addition of 1 μg/ml tetracycline using the same strategy as that described for the procyclics.

The full-length open reading frames of the GAP1 and 2 genes were cloned into the pJH54 plasmid containing the C-terminal HA_3-tag (a gift from Christine Clayton, University of Heidelberg). The PCR amplified insert was digested with either HindIII and XbaI (GAP1) or just XbaI (GAP2). The orientation of the latter insert was verified by diagnostic restriction digests and sequencing. The utilized PCR primers are (with restriction site underlined): GAP1-HA-F (5'-AAGCTTATGCTGCGCGCGCCTG-3') and GAP1-HA-R (5'-TCTAGAGTATGCCGAAACGGCAGT-3'); GAP2-HA-F (5'-TCTAGAATGCTTCGCTTATTGCGG-3') and GAP2-HA-R (5'-TCTAGACAACTTCGCCTCACAGCC-3').

Generation of antibodies – Affinity purified polyclonal rabbit antibodies against GAPs 1 and 2 were raised against synthetic oligopetides by the GenScript corporation (New Jersey, USA). The GAP1 synthetic oligopeptide (EYGDWGAEPGFEDRC) corresponds to the 137-150 amino acid region of the *T. brucei* protein while the GAP2 oligopeptide (TIKRSKDQLHVDLDC) is derived from the 297-310 amino acid region.

Western and Northern blot analyses – For Western blot analysis, cell lysates corresponding to 5×10^6 procyclics/lane or 1×10^7 bloodstreams/lane were separated on a 12% SDS-PAGE gel. The polyclonal rabbit antibodies against the MRP2 protein (Vondrušková et al., 2005), enolase (provided by P.A.M. Michels) and frataxin (Long et al., 2008) were used at 1:2000, 1:150,000 and 1:1000 dilutions, respectively. The antibodies against GAP1 and GAP2 were used at the respective dilutions 1:2000 and 1:500. Monoclonal antibodies against KREPA2, KREL1 and KREPA3 and Tbmp45 were used as described elsewhere (Panigrahi et al., 2001; Madison-Antenucci et al., 1998). Commercial antibodies against the HA_3 tag were used according to manufacturer's protocol (Sigma).

For Northern blot analysis, ~ 10 μg per lane of total RNA from procyclic or bloodstream cells was loaded on a 1% formaldehyde agarose gel, blotted and cross-linked as published elsewhere (Vondrušková et al., 2005). After pre-hybridization in NaPi solution (0.25M Na_2HPO_4 and 0.25M NaH_2PO_4, pH 7.2, 1 mM EDTA, 7% SDS) for 2 hrs at 55 ^oC, hybridization was performed overnight in the same solution at 55 ^oC. A wash in 2x SSC + 0.1% SDS at room temperature (RT) for 20 min was followed by two washes in 0.2x SSC + 0.1% SDS for 20 min each at 55 ^oC.

Growth curves – Following Northern blot analysis of several clones for each knockdown, one clone was selected for further experiments. Growth curves obtained over a period of 14 days in the presence or absence of the RNAi-induction agent tetracycline for transfected procyclics or 8 days for transfected bloodstreams. Cell density was measured every 24 hours using the Beckman Z2 Cell Counter.

Cell fractionation, glycerol gradients, digitonin fractionation – For separation on glycerol gradients, mitochondria from 10^9 procyclic cells were purified following the protocol detailed in Hashimi et al (2008). Digitonin fractionation and glycerol gradient sedimentation were performed as described elsewhere (Smid et al., 2006).

Quantitative real-time PCR and guanylyltransferase labeling – RNA for quantitative real-time (q) PCR and guanylyltransferase labeling were collected and DNased as described in Hashimi et al. (2008). After checking the integrity by running an aliquot on a formaldehyde gel, 4.5 μg of RNA was used as a template for cDNA synthesis for subsequent qPCR analysis (see Hashimi et al., 2008 for details). A parallel cDNA synthesis reaction was set up with the same amount of RNA but without the reverse transcriptase to serve as a control for effective DNase treatment. Primers for maxicircle mRNAs, as well as the housekeeping β-tubulin and 18S rRNA transcripts serving as reference genes, are listed in Carnes et al. (2005) and Hashimi et al. (2008). New primer pairs amplifying cDNAs corresponding to transcripts of the subunits of the MRB1 complex are: GAP1-qPCR-Fw (5'-AACGTATTGCGGATGCTTAC-3') and Rv (5'-GAACCACACGCTCACAACAG-3'); GAP2-qPCR-Fw (5'-CGGCTCATATTCCTGCCAATG-3') and Rv (5'-CGAAGTCCTCAGCAACCAACC-3'); Hel-qPCR-Fw (5'-ATCGCGTTAGGTGAAGCAGT-3') and Rv (5'-AAATGGGGATCCCTAAGGTG-3'); Hyd-qPCR-Fw (5'-ATTTTTCACCCTTGCACGTC-3') and Rv (5'-TCGATGGATTTGTTGTCACC-3'). Relative abundances of maxicircle transcripts were calculated using the Pfaffl method (Pfaffl, 2001).

Guanylyltransferase reactions were performed on 2.5-5 μg of total RNA to cap gRNAs with [α-^{32}P]GTP as previously described (Hashimi et al., 2008). The recombinant enzyme was either purchased from Ambion or provided by Ruslan Aphasizhev (University of California, Irvine). The reactions were separated on a denaturing 12% acrylamide-8M urea gel.

Immunolocalization – In order to visualize mitochondria, 5 x 10^6 to 1 x 10^7 cells were incubated in SDM-79 media supplemented with 1 mM of MitoTracker Green

FM (Molecular Probes) for 20 min at 27°C. Cells were subsequently washed 3x in phosphate-buffered saline (PBS) and then spread on slides, fixed and permeabilized as described in Klingbeil et al. (2002). All subsequent steps were carried out in a humid chamber. The slides were first blocked at RT for 1 hr with 10% goat serum diluted in PBS. Afterwards, the primary rabbit antibody was applied to the slides at the appropriate dilution in 10% goat serum + PBS for 90 min. After washing 3 x 10 min in PBS, the slides were incubated with secondary goat α-rabbit antibody conjugated to the cy3 fluorophore (Jackson Immunoresearch) at a 1:800 dilution for 1 hr. The slides were then washed 3 x 10 min in PBS, and mounted in Vectashield containing DAPI. The α-GAP1 antibody was used at dilution 1:100 and the α-HA_3 antibody was used according to the manufacturer's protocol (Sigma). Samples probed with the latter antibody were not pretreated with MitoTracker. Cells were examined on the Olympus Fluoview FV1000 confocal microscope using the accompanying Fluoview v1.7 software. A composite of the Z-stack of the fluorescent images was rendered using the ImageJ software and processed using Adobe Photoshop v6.0.

Results

Subunits of the MRB1 complex are essential for the procyclic stage – Comparison of the growth of non-induced and RNAi induced GAP1, GAP2, RNA helicase and Nudix hydrolase cell lines revealed that these proteins are essential for the growth of *T. brucei* procyclics. In these cell lines, growth inhibition is apparent 3 to 4 days after addition of the RNAi-induction agent tetracyline (Fig. 1). In the case of the GAP knockdowns, the cells became increasingly resistant to the dsRNA and recovered around day 12 (Figs. 1A and 1B), as previously reported in the *T. brucei* system (Pelletier and Read, 2003). However, the RNA helicase and Nudix hydrolase knockdowns did not recover their wild-type growth over the 14-day time-course. Based on these data, the timepoints after 3 to 4 days of RNAi induction were selected for all subsequent experiments with the knockdowns.

Stability of GAPs1 and 2 are mutually dependent – To confirm the mutual association of the GAP proteins as predicted by the mass spectrometry analysis (Hashimi et al., 2008), the mt lysates of cells with a single down-regulated GAP were probed with polyclonal antibodies against both proteins. The specificity of each α-GAP antibody for the appropriate target protein was verified by probing recombinant GAPs overexpressed in *E. coli* (data not shown). Indeed, elimination of the target protein by RNAi was followed by the disappearance of the other GAP protein (Fig. 2A), testifying to their mutual dependence. Furthermore, we checked

whether the GAPs are also destabilized upon silencing of either the RNA helicase or Nudix hydrolase. In either of these backgrounds, both proteins persisted (Fig. 2B).

RNAi-silencing of MRB1 complex subunits affects maxicircle transcripts – Since TbRGG1 and TbRGG2 from the MRB1 complex were shown to be essential for RNA editing (Hashimi et al., 2008; Fisk et al., 2008), we decided to investigate in detail the effect of down-regulation of the studied subunits in procyclic cells on the stability and editing of mt-encoded mRNAs. The levels of several pre-edited, edited and never-edited RNAs were measured by a quantitative real time (q) PCR based assay, using cDNA from RNAi-induced and non-induced cells. Primers amplifying the pre-edited and edited mt mRNAs of ATPase subunit 6 (A6), cytochrome oxidase subunits 2 and 3 (cox2 and cox3), cytochrome reductase subunit B (cyB), maxicircle unknown reading frame 2 (MURF2), NADH dehydrogenase subunit 7 (ND7) and mitoribosomal protein S12 (RPS12) were used. The levels of two never-edited mRNAs (ND4 and cox1) and, in some cases, the 9S and 12S mitoribosomal RNAs were also measured, as were the mRNAs targeted by RNAi. The obtained values were normalized to the measured levels of the cytoplasmic β-tubulin and 18S rRNA transcripts, since they are unaffected by RNAi. All qPCR reactions with a given primer pair were done in triplicate, and the average and median standard deviations of the measured cycle threshold values are given in Fig. 3.

The qPCR assay revealed a virtually identical phenotype for both GAP1 and 2 knockdowns (Figs. 3A and 3B). With a single but important exception (see below), all examined pre-edited mRNAs (A6, cox3, cyB, MURF2, ND7 and RPS12) were up-regulated between 1.5 and up to 20-fold, as compared to RNA from the non-induced cells. Importantly, this up-regulation was correlated to the decrease of the respective edited transcripts. No effect was observed on the never-edited mRNAs (cox1 and ND4) and rRNAs (9S and 12S) (Figs. 3A and 3B). Significantly, pre-edited and edited cox2 mRNAs were also not affected by RNAi-mediated elimination of either GAP. This transcript is unique in that its single gRNA is contained in its 3'-untranslated region, acting as a guide for insertion editing in *cis* (Golden and Hajduk, 2005). The assay also reveals that dsRNAs specifically target only the intended GAP RNA (Figs. 3A and 3B).

This assay revealed a similar effect on maxicircle transcripts in cells with silenced RNA helicase: the combined increase and decrease of pre-edited and edited transcripts, respectively, with the exception of cox2 transcripts (Fig. 3C). However, it should be noted that while never-edited transcripts were unaffected, so were the levels of pre-edited and edited ND7. In contrast, silencing of Nudix hydrolase showed a general effect on almost all maxicircle-encoded transcripts (Fig. 3D). Most never-edited, pre-edited and edited mRNAs and rRNAs are

downregulated upon depletion of this protein. This phenotype represents a highly significant departure from the otherwise relatively uniform phenotype of the other examined knockdowns.

The MRB1 complex has a role in gRNA expression– The disruption of RNA editing, observed in cells in which either GAP1, GAP2 or RNA helicase are downregulated, appears to be independent of the core editing activities of the 20S editosome since cox2 mRNA editing proceeds normally. Another component of this process are the small 30-60 nt gRNAs. To see whether these primary transcripts are affected, they were capped with guanylyltransferase and [α-^{32}P]GTP and visualized on a high-resolution denaturing acrylamide gel. An upper band corresponding to the cytoplasmic 5S rRNA is used as a loading control. Indeed, repression of GAP1 and 2 as well as RNA helicase in the procyclic cells leads to a decrease of the steady-state level of gRNAs (Figs. 4A, 4B and 4C). In contrast, neither the abundance nor 3'-oligo(U) tail of gRNAs is not affected upon depletion of Nudix hydrolase (Fig. 4D).

GAP1 assembly into macromolecular complexes is affected in the knockdowns for subunits of the MRB1 complex– To further study the sedimentation properties of GAP1 in cells with down-regulated MRB1 subunits, lysates from hypotonically isolated mitochondria from non-induced and RNAi-induced cultures were loaded onto 10 to 30% glycerol gradients. Gradient fractions were first probed with monoclonal antibodies against three of the core editosome proteins, KREPA2, REL1 and KREPA3 (Panigrahi et al., 2001), used as markers for 20S. As an additional control, the fractions were immunodecorated with an antibody binding Tbmp45, a protein previously labeled REAP1, as a marker for 40S sedimentation (Madison-Antenucci et al., 1998; Fisk et al., 2008). A representative immunoblot performed on GAP1 induced and non-induced samples is in agreement with previous studies, as all proteins recognized by these antibodies localized to fractions 13 thru 17, the peak of 20S editosome sedimentation (Fig. 5A, top panel). Furthermore, it has been demonstrated that this complex is not disrupted when substrate RNAs are absent since it is held together by protein-protein interactions (Domingo et al., 2003; Fisk et al., 2008; Hashimi et al., 2008). The Tbmp45 protein was found to be localized in the lowest fractions, as expected (Fig. 5A, bottom panel). The other gradients depicted in Fig. 5B were also validated in this fashion (data not shown).

In the separated mt lysates from the non-induced cell lines, GAP1 is distributed throughout the gradient, although with some variation in the pattern as revealed by Western analysis (Figs. 5B and 7B). A broad distribution of GAP2 has been previously reported (Panigrahi et al., 2008). As expected, GAP1 is reduced

upon interference of expression of either GAP subunits (Fig. 5B). A dramatic shift of the S value of GAP1 is observed in the RNA helicase-silenced cells, in which it is concentrated in the lighter fractions (Fig. 5B). Such a striking difference in GAP1 sedimentation is not observed in the induced and non-induced Nudix hydrolase knockdowns (Fig. 5B). In the hydrolase-silenced sample, a reduction of GAP1 immunopositive signal in the dense fractions is apparent (Fig. 5B).

mtRNA polymerase appears to transcribe minicircles – The participation of a multiprotein complex in the transcription of minicircle gRNA genes, independent of mtRNAP, has been hypothesized (Grams et al., 2002). In the inducible mtRNAP knockdowns (kindly provided by P.T. Englund), we have first verified the RNAi effect by Northern analysis, as antibodies against the *T. brucei* mtRNA polymerase are not available. As shown in Fig. 6A, the mtRNA polymerase-encoding mRNA is undetectable 3 days after tetracycline induction, the time-point in which growth inhibition is first observed (Grams et al., 2002). Next, we have hybridized blots containing mt RNA with a probe against cox1 mRNA. In agreement with the previous study (Grams et al., 2002), this never-edited transcript is undetectable 3 days after RNAi is triggered (Fig. 6B). To test whether this polymerase is responsible for the transcription of minicircles, the initial step in expression of gRNA molecules, we have performed the guanylyltransferase labeling assay on total RNA isolated from non-induced and induced knockdowns for mtRNA polymerase. Clearly, the gRNA population is decreased in cells lacking the enzyme (Fig. 6C).

MRB1 complex is disrupted upon downregulation of mtRNA polymerase– The similarity in the effect on gRNA expression of the knockdowns for mtRNAP and MRB1 subunits GAP1, GAP2 and RNA helicase inspired us to investigate the integrity of this complex in the absence of the polymerase. For this purpose, total lysates prepared from non-induced cells as well as from cells in which mtRNAP was targeted by RNAi for 3 days were loaded on glycerol gradients and the obtained fractions were screened with the α-GAP1 antibody. The quality of the gradient was tested by Western blot analysis using antibodies against the editosome KREL1 and Tbmp45 (Fig. 7A). GAP1 is broadly distributed in the non-induced cells in fractions 7 to 17 (Fig. 7B). Indeed, an apparent shift towards the lighter density fractions (with a marked peak in fractions 7 and 9) of the gradient in the GAP1 distribution occurs when mtRNAP is downregulated. This result indicates that in the absence of mtRNAP, the MRB1 complex is disrupted in the same manner as in the absence of RNA helicase (Fig. 5B).

GAP1 and 2 and gRNAs are essential for the bloodstream stage – Since RNA editing is essential also in the bloodstream stage (Schnaufer et al., 2001), we decided to test whether the absence of GAP1 and 2, and consequently gRNA expression, is also lethal in this stage. The constructs containing the same GAP1 or 2 gene fragments as those used for RNAi in the procyclics were transfected into the bloodstream stage. Western blot analysis revealed that in the GAP1 and 2 knockdowns, the targeted protein was undetectable after 2 days of induction (Figs. 8A). Moreover, as in the procyclic stage, the ablation of GAP1 leads to the reduction of GAP2, and vice versa, testifying to their mutual dependence.

Downregulation of either GAP1 or 2 results in growth inhibition, indicating that these proteins are also essential in this life cycle stage. When compared with the non-induced cells, the growth of the induced clones decreases at day 3 and stops at day 4 (Fig. 8B). Both induced cell lines recovered from the effect of RNAi at day 7 post-induction. Labeling of total RNA collected at days 2 and 3 post-induction with guanylyltransferase revealed that steady state levels of gRNAs are decreased when either GAP1 or 2 are silenced in the bloodstream stage (Fig. 8C). This result indicates that the function of these proteins is conserved between the two major life stages of *T. brucei,* and that gRNAs are also required for survival of the infective bloodforms.

GAP1 and GAP2 have a novel localization within the mitochondrion – Previous data provided evidence for localization of the MRB1 complex in the mitochondrion (Hashimi et al., 2008; Panigrahi et al., 2008). We have therefore resorted to indirect immunofluorescence experiments to determine where these proteins are located in the organelle. Using the α-GAP1 antibody, we show that in the procyclic cells, the protein is confined to several discrete foci, distributed throughout the reticulated mitochondrion (Fig. 9A, top row), often in the proximity of the kDNA disk. Specificity of the used antibody was confirmed by its failure to detect target protein in RNAi-silenced GAP1 cells (Fig. 9A, bottom row). The polyclonal α-GAP2 antibodies proved to be less useful in the immunolocalization studies.

To corroborate this unexpected observation, cells were transfected with a construct for tetracycline-inducible expression of either GAP1 or 2 proteins bearing an HA_3-epitope tag. Immunocytochemistry and DAPI staining performed on cells grown in the presence of the antibiotic for 2 days confirmed that both epitope-tagged proteins are confined to multiple discrete punctuate loci unevenly distributed throughout the organelle (Fig. 9B). As observed with the α-GAP1 antibody, an immunopositive signal is also often observed near the kinetoplast.

Although attachment of the HA_3 tag to the C-terminus of the protein should not interfere with the mt import signal located at the N-terminus, possible extra-mitochondrial localization had to be ruled out. Therefore, the 29-13 procyclics

containing the HA_3-tagged GAP1 or 2 were fractionated by extraction with increasing concentrations of digitonin. Indeed, probing the obtained cytosolic and mt fractions, as well as the total cell lysate, with antibodies against the HA_3 tag showed that both tagged GAP proteins are properly imported into the organelle (Fig. 9C).

Discussion

Since the discovery of RNA editing in 1986 (Benne et al., 1986), considerable efforts have been directed at understanding the molecular mechanisms underlying what was initially an enigmatic biological process. A major breakthrough was the discovery of gRNAs, which revealed that the information for U insertion/deletion events reside in these molecules and a function of the thousands of minicircles is to encode them (Blum and Simpson, 1990). The establishment of an *in vitro* editing assay led to the validation that proteins provided the enzymatic machinery for RNA editing (Seiwert et al., 1996). The isolation and characterization of the 20S editosome have dissected how a multi-protein complex can orchestrate the catalytic steps required for the maturation of most mt RNAs in trypanosomes (Simpson et al., 2004; Lukeš et al., 2005; Stuart et al., 2005).

Studies focusing on identifying proteins involved in other aspects of mt RNA metabolism have uncovered the MRB1 complex in three different instances (Etheridge et al., 2008, Hashimi et al., 2008, Panigrahi et al., 2008). Although the subunit composition in these studies is not identical, the degree of overlap is quite significant. The MRB1 complex contains up to 14 proteins, some of which possess known motifs and domains involved in RNA processing or protein interactions (Hashimi et al., 2008; Panigrahi et al., 2008). Two components of the MRB1 complex have been already characterized to some extent, namely TbRGG1 and TbRGG2, the latter of which was suggested to be a transiently associated editing factor required for the processivity of RNA editing (Fisk et al., 2008). RNAi-silencing of both proteins leads to a downregulation of edited transcripts, suggesting some kind of a role in RNA editing (Fisk et al., 2008; Hashimi et al., 2008). However, these data do not adequately address a possible role of the MRB1 complex.

Here we addressed the question of function by examining the phenotype resulting from RNAi-silencing of four subunits of this complex: GAP1, GAP2, RNA helicase and Nudix hydrolase. We have shown that a subset of the MRB1 complex, GAP1, GAP2 and RNA helicase, is involved in gRNA biogenesis. This notion is supported by the absence of gRNAs when any of these proteins is depleted as a consequence of RNAi. Furthermore, editing of the maxicircle-

encoded RNAs is subsequently reduced, with the notable exception of cox2. This transcript contains its own *cis*-gRNA in the 3'-untranslated region, thus circumventing the requirement for minicircle-encoded gRNAs acting in *trans* (Golden and Hajduk, 2005). The independence of cox2 mRNA editing, in the *trans*-acting gRNA-deficient background, on these MRB1 proteins clearly indicates that they are not involved in core RNA editing activities.

The question is specifically what role do these proteins play in the expression of gRNAs? It was hypothesized that a multiprotein complex may be involved in the transcription of minicircle-encoded genes, independent of the canonical mt RNA polymerase (mtRNAP) involved in maxicircle transcription (Grams et al., 2002). Here we provide evidence that mtRNAP is the most likely candidate for the transcription of gRNA genes as well as maxicircle genes. This observation indicates that the MRB1 complex may have a distinct role in gRNA synthesis, since mtRNAP was not demonstrated to be associated with this complex in any of the published reports (Etheridge et al., 2008, Hashimi et al., 2008, Panigrahi et al., 2008). Furthermore, the possibility that the mtRNAP transcribes both minicircle-encoded gRNAs and maxicircle-encoded mRNAs is surprising given the requirement of the multiple DNA ligases and DNA polymerases for kDNA maintenance and replication (Klingbeil et al., 2002; Liu et al., 2005).

The GAPs and RNA helicase do appear to be involved in some aspect of gRNA processing and/or stability. The most established processing event in the biogenesis of a gRNA is its post-transcriptional addition of the 3'-oligo(U) tail (Blum et al., 1991) by the KRET1 enzyme (Aphasizhev et al., 2003b). It was also proposed that functional gRNAs are spliced from the 5'-ends of polycistronic minicircle transcripts (Grams et al., 2000). However, a corresponding accumulation of these large precursor RNAs was not detected in the GAP knockdowns (data not shown), suggesting that if this processing step does exist, it is not mediated by these proteins.

Another proposed feature of gRNA is the presence of two intramolecular hairpin structures with single stranded ends (Schmid et al., 1995; Hermann et al., 1997). The presence of a predicted DExD/H-box RNA helicase in the gRNA-expression component of the MRB1 complex raises the possibility that these proteins may play a role in facilitating the formation of this secondary structure in these small transcripts. Perhaps these thermodynamically unfavorable loops (Schmid et al., 1995) are not formed in the absence of the GAPs or RNA helicase, leading to the apparent destabilization of the gRNAs. However, this speculative model is tempered by the involvement of the DExD/H box RNA helicases in diverse roles in RNA metabolism not restricted just to processing, such as transcription regulation and RNA turnover (reviewed by Fuller-Pace, 2006).

We show that the GAP proteins are essential for the bloodstream stage, where they appear to have the same role in gRNA processing/stability as they do in the procyclic stage. Although an active RNA metabolism has been established as a requirement for survival of this infective stage (Schnaufer et al., 2001; Fisk et al., 2008), to our knowledge this result is the first to indicate that the gRNA biogenesis is required in this part of the life cycle as well. This result is significant in light of the existence of naturally occurring dyskinetoplastic and akinetoplastic trypanosomes (Lai et al., 2008), which must circumvent the lack of the requisite repertoire of gRNAs for the expression of normally essential mt-encoded genes, such as the ATPase subunit 6 (Schnaufer et al., 2005). Apparently, in both stages all kDNA is transcribed, and any regulation occurs at the post-transcriptional level, a situation strikingly similar to what is known for the expression of nuclear genes in trypanosomes and other kinetoplastid flagellates (Haile and Papadopoulou, 2007).

We also establish here that the group of proteins that we designate to the MRB1 complex do not share an overall function. While GAP1, GAP2 and RNA helicase are involved in gRNA expression, TbRGG1 and 2 are clearly not (Hashimi et al., 2008; Fisk et al., 2008). Furthermore, we show here that the Nudix hydrolase is also not involved in gRNA expression, since these molecules persist in a background deficient of this protein. The family of Nudix hydrolases has a diverse array of functions in the cell, such as decapping mRNAs (Cohen et al., 2005) and clearance of oxidized nucleosides (reviewed by McClennan, 2006). Designation of a specific role of this hydrolase is still not feasible based on the observed destabilization of most of the maxicircle transcripts upon its depletion. However, these results counter those of Weng and colleagues (2008) indicating a specific decrease in edited RNAs.

The array of phenotypes affecting different aspects of mt RNA metabolism upon downregulation of various putative subunits of the MRB1 complex, along with the assortment of associating proteins, is quite striking (Etheridge et al., 2008; Fisk et al., 2008; Hashimi et al., 2008; Panigrahi et al., 2008; Weng et al., 2008). These observations raise the question whether it represents a single *bona fide* complex, held together by protein-protein interactions, or a collection of monomers and/or smaller complexes connected by substrate RNAs. Indeed, the latter situation has been proposed (Weng et al., 2008) and incorporation of both TbRGG proteins into large complex(es) appears to be RNase sensitive, while the 20S editosome complex remains intact (Fisk et al., 2008; Hashimi et al., 2008). Interestingly, RNAi-silencing of either RNA helicase or mtRNAP clearly disrupts the GAP1 assembly into macromolecular complexes. This result may reflect a genuine interaction of these proteins with GAP1 or be a result of the consequent decrease of RNA molecules via which these proteins seem to associate.

We conclude by discussing the unique localization of the GAP proteins to discrete points along the reticulated mitochondrion, often proximal to the kDNA network. To our knowledge, such a pattern has not been reported for other proteins involved in mt nucleic acid metabolism (Klingbeil et al., 2002; Liu et al., 2005; Vanhamme et al., 1998; Etheridge et al., 2008). Panigrahi and co-workers (2008) have previously reported a more uniform distribution of different subunits of the MRB1 complex throughout the mitochondrion. However, given the aforementioned diversity of RNAi-silencing phenotypes for the MRB1 complex proteins, it is possible that they may also exhibit diverse yet overlapping sites in the organelle as well. Nevertheless, as the GAP proteins have a demonstrated role in gRNA biogenesis, we imagine these loci may represent centers, where gRNAs are processed and distributed for their participation in RNA editing as catalyzed by the 20S editosome.

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Figure legends

Fig. 1. Subunits of the MRB1 complex are essential for growth of procyclic stage trypanosomes. Cell densities (cells/ml) that were measured every 24 hours are plotted on a logarithmic scale on the y-axis over 14 days. Cells were diluted to 2 x 10^6 cells/ml every 2 days. Cells grown in the presence or absence of the RNAi-induction agent 1 μg/ml tetracycline are indicated by grey (tet+) or black (tet-) lines, respectively. Growth curves of knockdowns of GAP1(**A**), GAP2 (**B**), RNA helicase (**C**) and Nudix hydrolase (**D**) are shown.

Fig. 2. The stability of the GAP proteins is dependent on their mutual association. (**A**) Crude mt lysates from 10^8 cells were loaded per lane on a SDS-PAGE gel and transferred onto a PVDF membrane for immunodecoration with either α-GAP1 or α-GAP2 polyclonal antibody, as indicated on the left. Antibody against the mitochondrial protein frataxin was used as a loading control. Lysates from cells grown for 3 days in the presence or absence of 1 μg/ml tetracycline are indicated at

the top ("+" or "-") for either the GAP1 or GAP2 RNAi knockdown. 50 and 15 kDa protein markers are indicated on the right.
(**B**) Samples from the RNA helicase and Nudix hydrolase RNAi knockdown cells grown 4 days in the presence and absence of 1 μg/ml tetracycline were prepared and labeled as described above.

Fig. 3. Effects of RNAi-silencing of the MRB1 complex subunits on maxicircle transcripts. Quantitative real time PCR analysis of pre-edited, edited and never-edited mRNAs and rRNAs was performed on knockdowns of GAP1 (**A**), GAP2 (**B**), RNA helicase (**C**) and Nudix hydrolase (**D**). The reaction for each amplicon was done in triplicate on cDNA generated from cells grown in the presence or absence of the RNAi-inducer tetracycline. The relative abundance of each of the examined RNAs upon the synthesis of interfering dsRNA was plotted on a logarithmic scale: 1 represents the wild-type levels, while a value above or below this number signifies an increase or decrease of a given RNA, respectively. These values were normalized to the cytosolic transcripts β-tubulin or 18S rRNA, whose levels are not affected by RNAi. The average (AVE $\sigma(C_T)$) and median (MED $\sigma(C_T)$) of the measured cycle threshold (C_T) values for all the reactions performed for each knockdown are indicated under each bar graph. The following pre-edited (P) and edited (E) RNAs were assayed: ATPase subunit 6 (A6), cytochrome oxidase subunits 2 (cox2) and 3 (cox3), cytochrome reductase subunit b (cyB), maxicircle unknown reading frame 2 (MURF2), NADH dehydrogenase subunit 7 (ND7) and ribosomal protein S12 (RPS12). The following never-edited RNAs were assayed: 9S RNA, 12S RNA, cox1, and ND4. The appropriate cytoplasmic mRNA targeted by RNAi were assayed for each knockdown, and are indicated on the right.

Fig. 4. Effects of RNAi-silencing of the MRB1 complex subunits on minicircle transcripts. The total population of minicircle encoded gRNAs were labeled in knockdowns of GAP1 (**A**), GAP2 (**B**), RNA helicase (**C**) and Nudix hydrolase (**D**). Labeling reactions were performed with guanylyltransferase, [α-^{32}P] GTP and either 2.5 (**A** and **B**) or 5 μg RNA (**C** and **D**). The gRNAs, appearing as a ladder mainly because of their heterogeneous 3'-oligo(U) tails, are indicated on the left. The top band marked with the "*" is a cytosolic RNA that is also labeled in this reaction and is used as a loading control.

Fig. 5. GAP1 assembly into macromolecular complexes is affected in the knockdowns for subunits of the MRB1 complex. Cleared lysates from hypotonically isolated mitochondria are separated by sedimentation in glycerol gradients. Odd fractions from the gradients were run on SDS-PAGE gels and transferred onto PVDF membranes.

(**A**) All gradients were verified by immunodecoration with monoclonal antibodies against editosome subunits KREPA2, KREL1 and KREPA3 (top panel), as well as Tbmp45 (bottom panel). A representative blot for GAP1 samples is shown here in which the appropriate antibody signals are indicated on the left. The presence (+) or absence (-) of tetracycline is specified on the right.
(**B**) α-GAP1 antibody was used to probe the glycerol gradient fractions from knockdown cell lines grown in the absence (-) or presence (+) of tetracycline as indicated on the right. The GAP1, GAP2, RNA helicase and Nudix hydrolase cell lines are indicated on the left.

Fig. 6. Evidence that mt RNA polymerase transcribes kDNA minicircles.
(**A**) Effect of RNAi induction on the mtRNAP mRNA was followed by Northern blot analysis. Its levels were analyzed by blotting 10 μg of total RNA extracted from non-induced cells (-) and cells 3 days after RNAi induction (+) with a probe hybridizing to the gene fragment used for generation of dsRNA. The position of the targeted mRNA and the inducibly synthesized dsRNA are indicated by black and grey arrowheads, respectively. To visualize characteristic rRNA bands, used here as a loading control, the gel was stained with ethidium bromide.
(**B**) The same RNA was hybridized with a labeled probe for never-edited mRNA encoding cytochrome oxidase subunit 1 (cox1).
(**C**) Guanylyltransferase labeling of gRNAs using same RNA samples as used in (**A**) and (**B**) are performed and marked as in Fig. 4. 5 μg of total RNA was used per lane.

Fig. 7. GAP1 assembly into large macromolecular complexes is reduced in cells with RNAi-silenced mt RNA polymerase. Western blot analysis of glycerol gradients was performed and marked as described in Fig. 5. Western blots of fractions were verified using the α-KREL1 (top panel) or α-Tbmp45 (bottom panel) monoclonal antibodies (**A**) or probed using the α-GAP1 polyclonal antibody (**B**).

Fig. 8. GAP RNAi are essential for gRNA biogenesis in bloodstream stage.
(**A**) Whole cell lysates from 1.5 x 10^7 cells were immunoblotted with either the α-GAP1 or α-GAP2 antibody for each of the GAP1 (top) and GAP2 (middle) cell lines. Cells grown in the absence (-) or presence (+) of 1 μg/ml of tetracycline are indicated, as well as the days post-induction in the latter samples. An antibody decorating the cytosolic enolase was used as a loading control (bottom). All antibodies are indicated on the left.
(**B**) RNAi-silencing of the GAP proteins in the bloodstream stage results in growth inhibition. Growth curves over 7 days are shown for the GAP1 (top) and GAP2

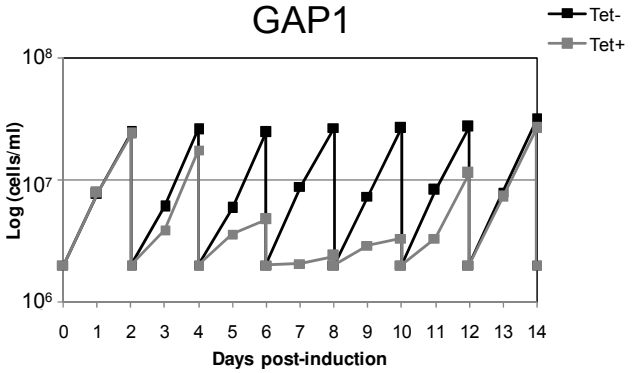
(bottom) knockdown cell lines and are labeled as in Fig. 1. Cells were diluted every 24 hours to a density of 10^5 cells/ml.
(**C**) Guide RNA levels are diminished in the GAP-silenced bloodstream stage. The guanylyltransferase assay was performed on 5 μg of total RNA as described in Fig. 4. The gels shown for GAP1 (left) and GAP2 (right) are also labeled as in Fig. 4., with the days post-induction by tetracycline indicated below the "+".

Fig. 9. The GAP proteins are localized in punctuate loci along the reticulated mitochondrion of procyclic *T. brucei*.
(**A**) Immunolocalization using rabbit α-GAP1 antibody in the GAP1 non-induced (top row, labeled Tet-) and RNAi-induced cells (bottom row, Tet+). Signal from cy3-conjugated α-rabbit antibody (Cy3), MitoTracker Green FM (Mito), the two images merged with DAPI staining of nucleus and kDNA (Merge-DAPI) and a composite image with a differential interference contrast image (Merge-DIC) are indicated along the top. White arrowheads indicate position of kDNA.
(**B**) Immunolocalization of the HA_3-tagged versions GAP1 (top row) and GAP2 (bottom row), which is immunodecorated with rabbit polyclonal antibody against the epitope. Signal from cy3-conjugated α-rabbit antibody (Cy3), DAPI staining of nucleus and kDNA (DAPI), a merged image of the two (Merge) and a composite image with a differential interference contrast image (Merge-DIC) are indicated along the top. White arrowheads indicate position of kDNA.
(**C**) Immunoblot analysis of the localization of overexpressed HA_3-tagged GAP1 (left) and 2 (right) proteins. Whole cell lysates (W) and digitonin extracted cytosolic (C) and mitochondrial (M) fractions of these cell lines grown for 2 days in the presence of 1 μg/ml tetracycline. Antibody against the HA_3-tag was used as well as those against the MRP2 and enolase proteins, serving as mitochondrial and cytosolic markers, respectively. 10 μg of protein of each digitonin fraction was loaded per lane.

Table 1. Nomenclature of examined MRB1 complex proteins.

Nomenclature introduced in this study	Nomenclature introduced by Weng et al. (2008)	GeneDB accession number
GAP1	GRBC1	Tb927.2.3800
GAP2	GRBC2	Tb927.7.2570
Nudix hydrolase	MERS1	Tb927.11.7290
RNA helicase	--	Tb927.4.1500

A


GAP1
Tet-
Tet+
10^8
10^7
10^6
Log (cells/ml)
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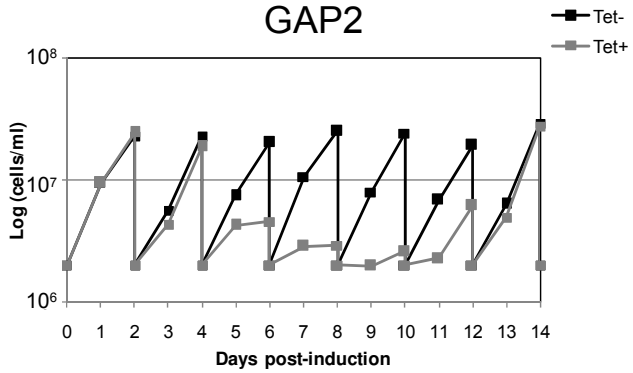
Days post-induction

B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10^6
Log (cells/ml)
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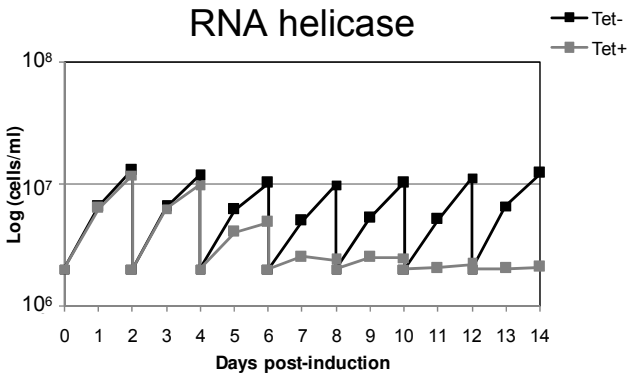
Days post-induction

C


RNA helicase
Tet-
Tet+
10^8
10^7
10^6
Log (cells/ml)
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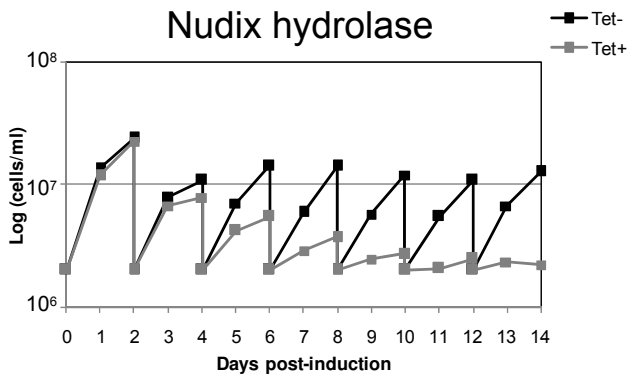
Days post-induction

D


Nudix hydrolase
Tet-
Tet+
10^8
10^7
10^6
Log (cells/ml)
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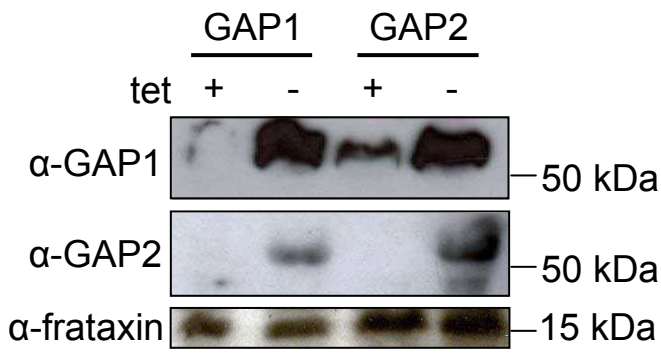
Days post-induction

A


GAP1
GAP2
tet + - + -
α-GAP1
50 kDa
α-GAP2
50 kDa
α-frataxin
15 kDa

B

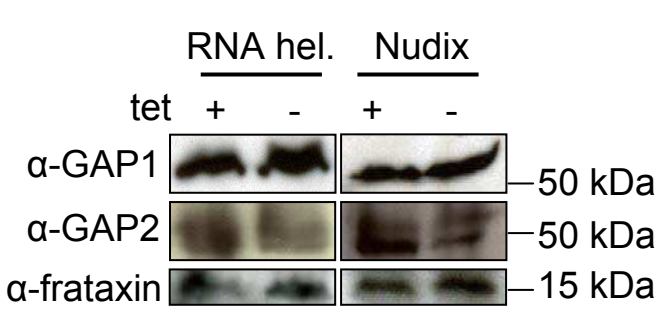
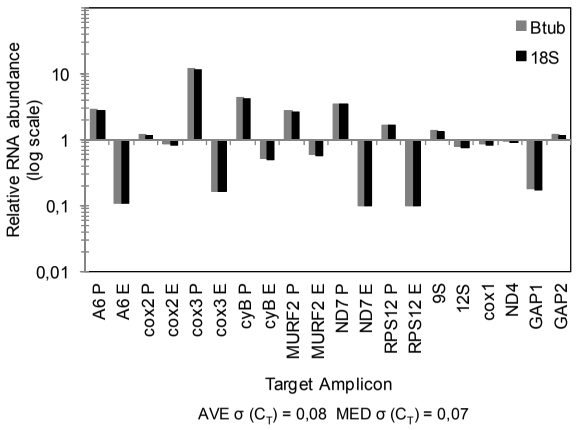

RNA hel.
Nudix
tet + - + -
α-GAP1
50 kDa
α-GAP2
50 kDa
α-frataxin
15 kDa

Fig. 3

A



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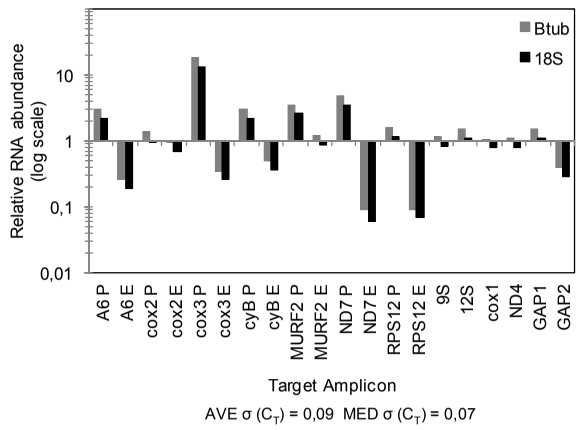

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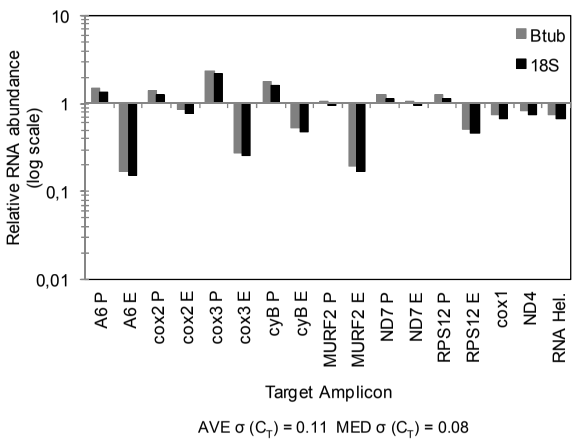

AVE σ (C_T) = 0.11 MED σ (C_T) = 0.08

D

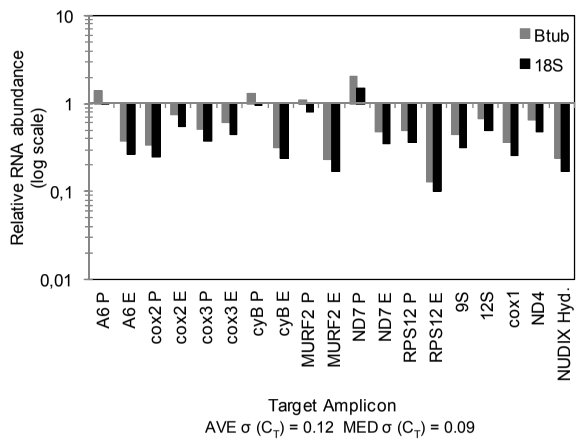

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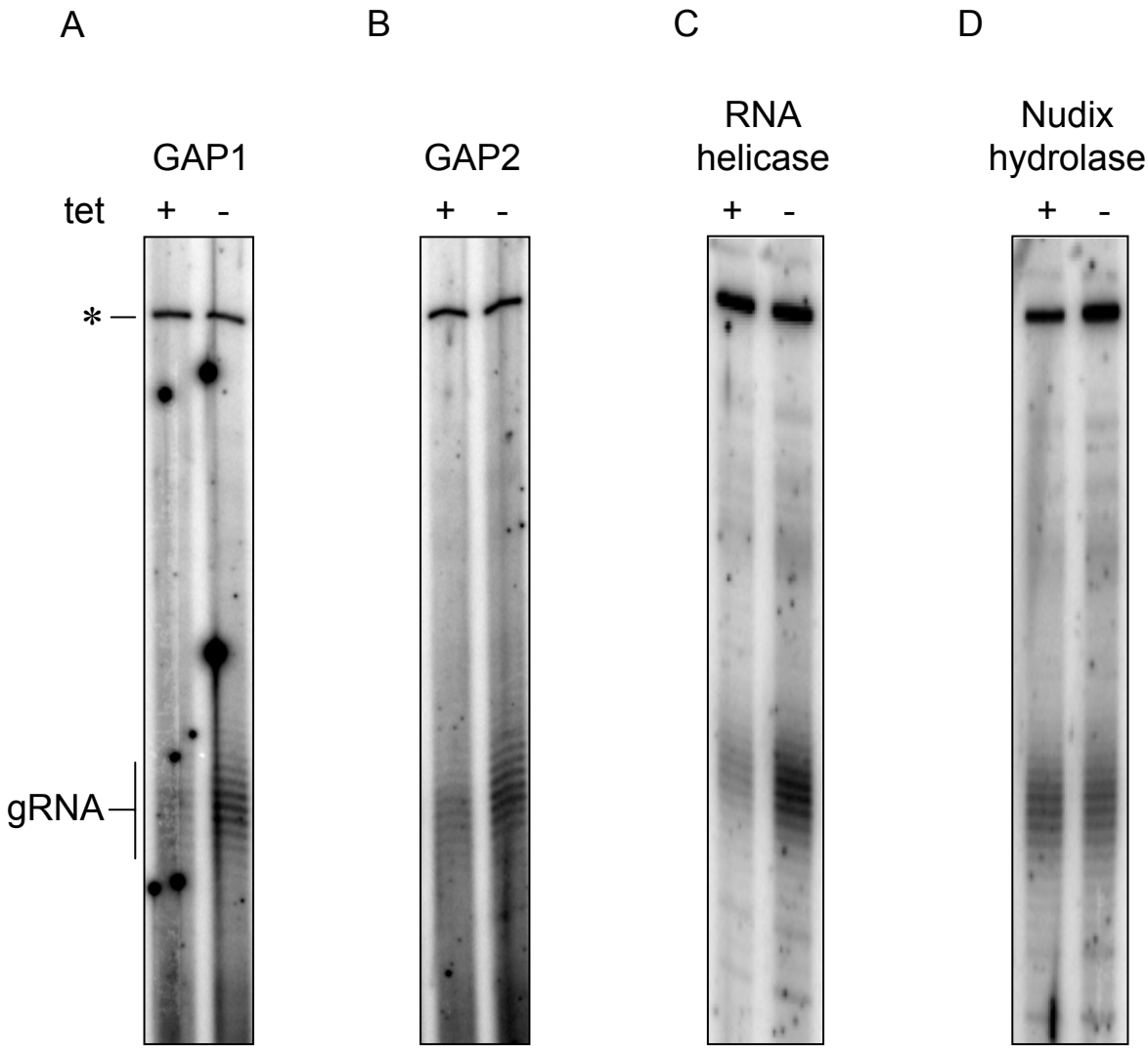
Fig. 4



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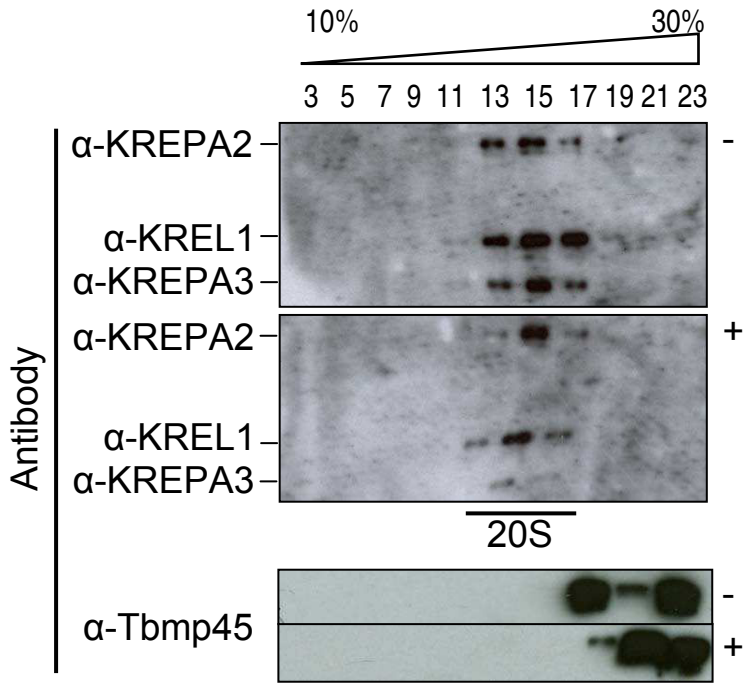
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α-KREL1
α-KREPA3
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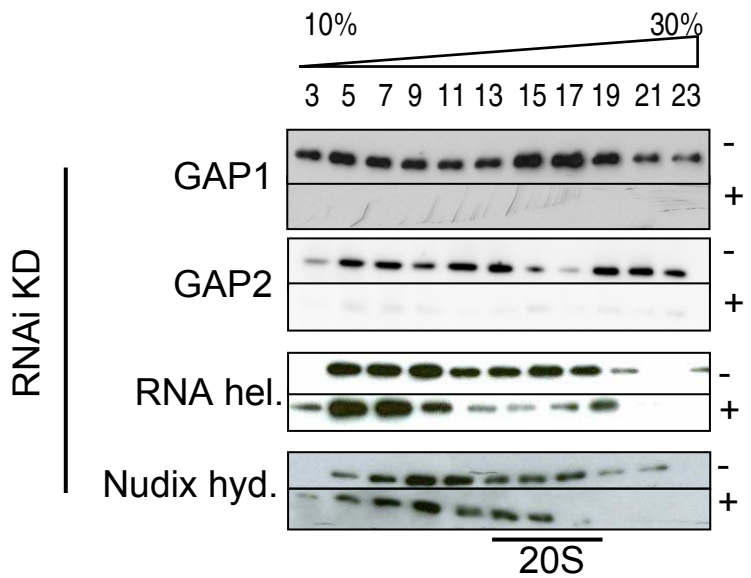
-
+
20S
α-Tbmp45
-
+

B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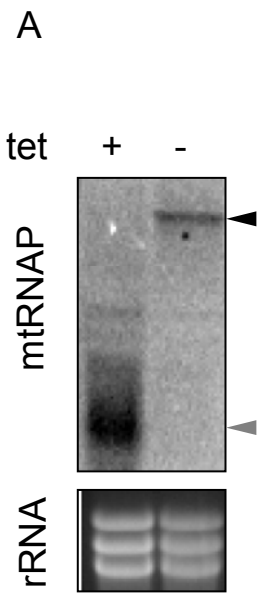
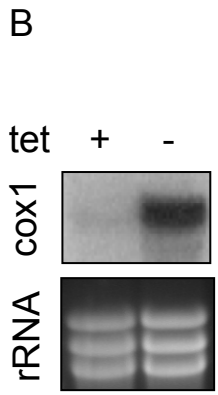
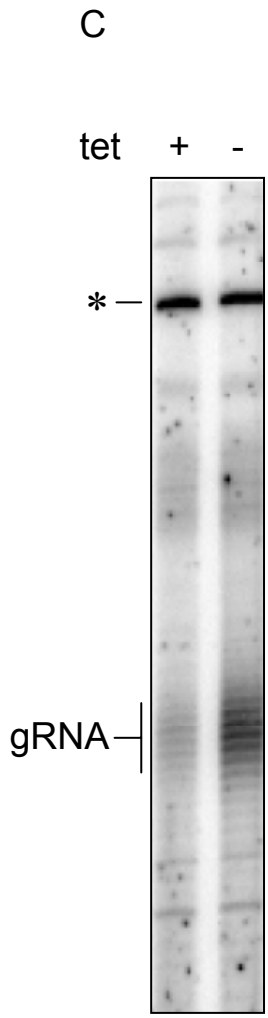
RNAi KD
GAP1
GAP2
RNA hel.
Nudix hyd.
-
+
20S

Fig. 6







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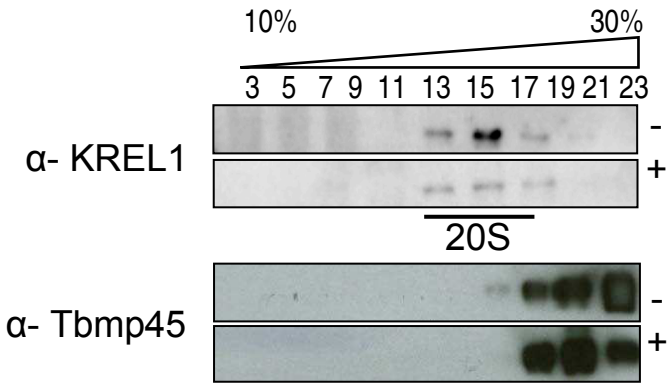
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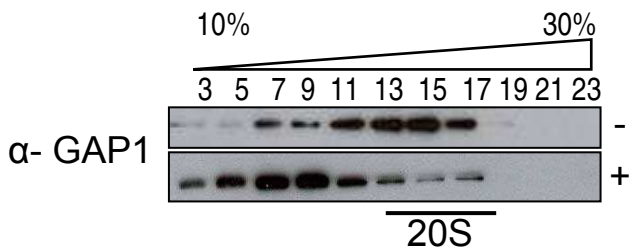
-
+
20S
α- Tbmp45
-
+

B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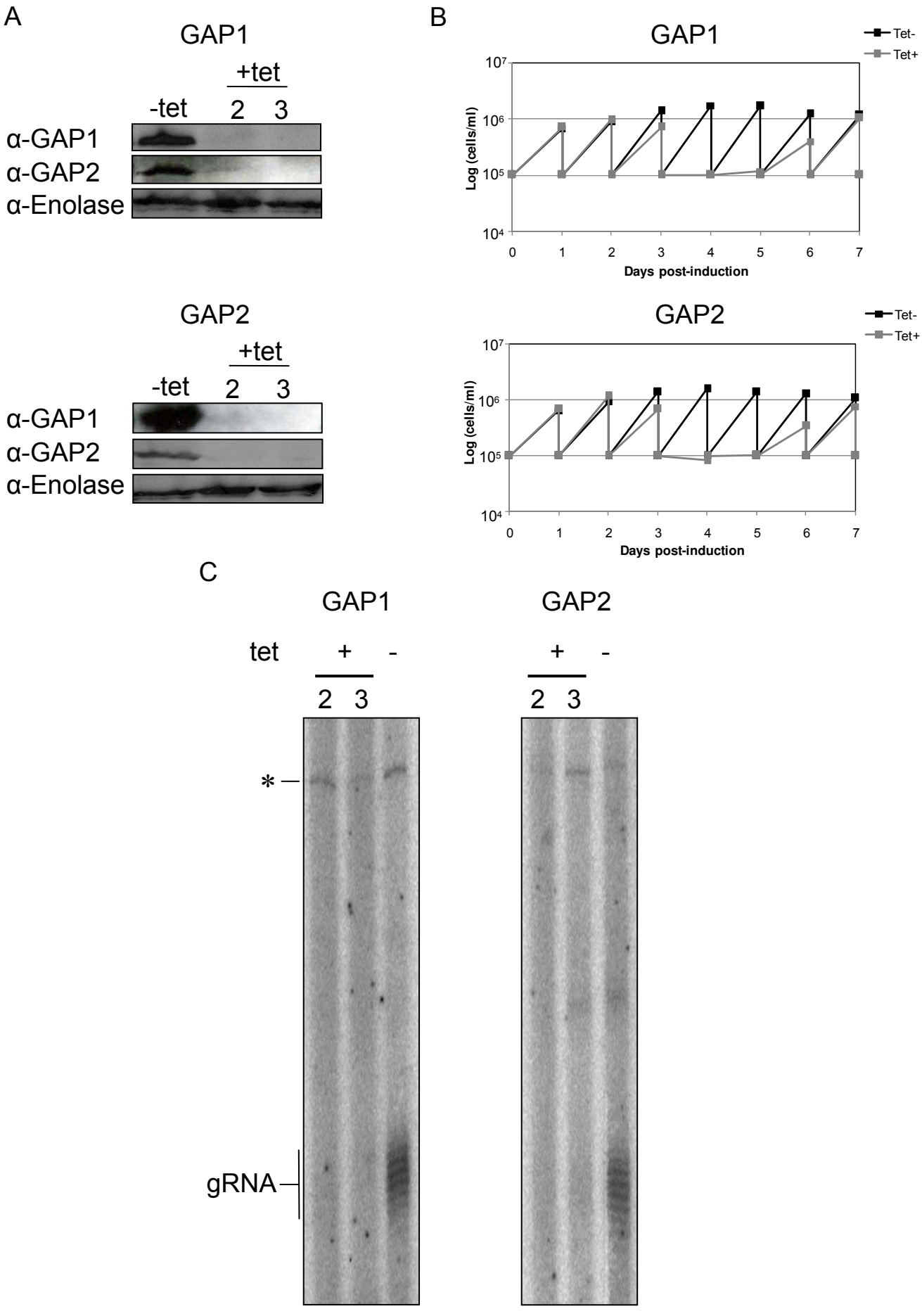
α- GAP1
-
+
20S

Fig. 8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GAP1
+tet
-tet
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α-Enolase
GAP2
+tet
-tet
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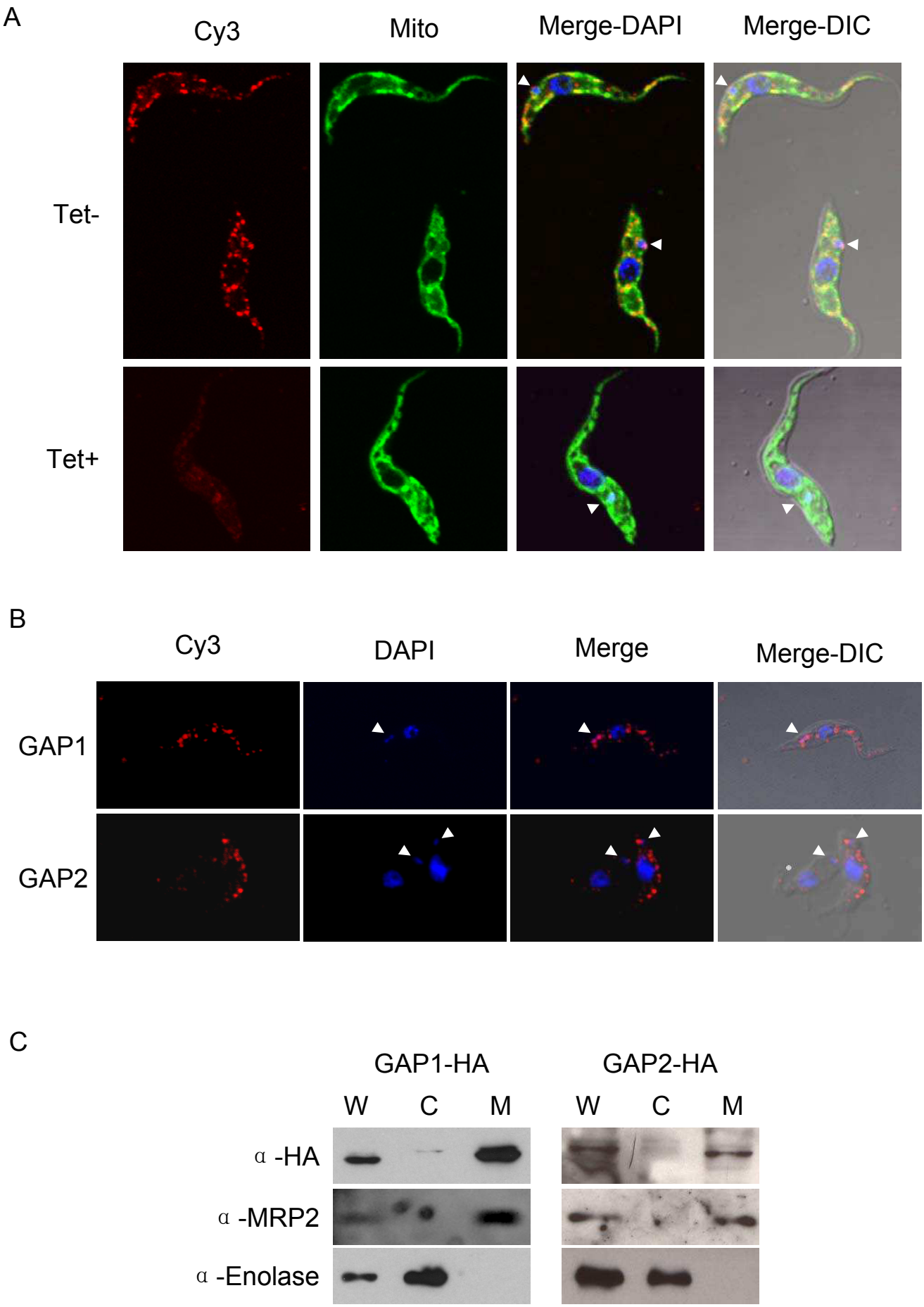
α-GAP2
α-Enolase
B
GAP1
Tet-
Tet+
Log (cells/ml)
Days post-induction
GAP2
Tet-
Tet+
Log (cells/ml)
Days post-induction
C
GAP1
GAP2
tet
+
-
+
-
2
3
2
3
*
gRNA

Fig. 9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Cy3
Mito
Merge-DAPI
Merge-DIC
Tet-
Tet+
B
Cy3
DAPI
Merge
Merge-DIC
GAP1
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C
GAP1-HA
GAP2-HA
W
C
M
W
C
M
α -HA
α -MRP2
α -Enolase

Chapter 4:

Adaptations of Trypanosoma brucei to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of *T. brucei.*
(reprint of Lai *et al.*, 2008, Proc. Natl. Acad. Sci. USA 105, 1999-2004)

Abstract:

Trypanosoma brucei is a kinetoplastid flagellate, the agent of human sleeping sickness and ruminant nagana in Africa. Kinetoplastid flagellates contain their eponym kinetoplastDNA(kDNA), consisting of two types of interlocked circular DNA molecules: scores of maxicircles and thousands of minicircles. Maxicircles have typical mitochondrial genes, most of which are translatable only after RNA editing. Minicircles encode guide RNAs, required for decrypting the maxicircle transcripts. The life cycle of *T. brucei* involves a bloodstream stage (BS) in vertebrates and a procyclic stage (PS) in the tsetse fly vector. Partial [dyskinetoplastidy (Dk)] or total [akinetoplastidy (Ak)] loss of kDNA locks the trypanosome in the BS form. Transmission between vertebrates becomes mechanical without PS and tsetse mediation, allowing the parasite to spread outside the African tsetse belt. *Trypanosoma equiperdum* and *Trypanosoma evansi* are agents of dourine and surra, diseases of horses, camels, and water buffaloes. We have characterized representative strains of *T. equiperdum* and *T. evansi* by numerous molecular and classical parasitological approaches. We show that both species are actually strains of *T. brucei*, which lost part (Dk) or all (Ak) of their kDNA. These trypanosomes are not monophyletic clades and do not qualify for species status. They should be considered two subspecies, respectively *T. brucei equiperdum* and *T. brucei evansi*, which spontaneously arose recently. Dk/Ak trypanosomes may potentially emerge repeatedly from T. brucei.

Chapter 5:
The protein product of pan-edited ATP synthase subunit 6 is incorporated into a non-canonical Complex V in *Trypanosoma* species.
(submitted to Eukaryotic Cell)

The protein product of pan-edited ATP synthase subunit 6 is incorporated into a non-canonical complex V in *Trypanosoma* species

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Throughout eukaryotes, the gene encoding subunit 6 (ATP6) of the F_1F_0 ATP synthase (complex V) is maintained in mitochondrial genomes, presumably because of its high hydrophobicity due to its incorporation into the membrane-bound F_0 moiety. In *Trypanosoma* species, a mitochondrial (mt) transcript that undergoes extensive processing by RNA editing has a very low sequence similarity to ATP6 from other organisms. The notion that the putative ATP6 subunit is assembled into the F_0 sub-complex is ostensibly challenged by the existence of naturally occurring dyskinetoplastic (Dk) and akinetoplastid (Ak) trypanosomes, which are viable despite lacking the mt DNA required for its expression. Taking advantage of the differential phenotype between RNAi knockdown cell lines in which the expression of proteins involved in mitochondrial RNA metabolism and editing can be silenced, we provide several lines of support that ATP6 is encoded in the mt genome of *Trypanosoma* species and that it is incorporated into complex V. The reduction of the F_1F_0 oligomer of complex V coincides with the accumulation of the F_1 moiety in ATP6-lacking cells, which also appear to lack the F_0 ATP9 multimeric ring. The oligomycin sensitivity of ATPase activity of complex V in ATP6-lacking cells is reduced, reflecting the insensitivity of the Dk and Ak cells to this drug. In addition, the F_1 moiety of complex V appears to exist as a dimer in steady state conditions and contains the ATP4 subunit traditionally assigned to the F_0 sub-complex.

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INTRODUCTION

Trypanosoma brucei is a pathogen with a broad impact on the inhabitants of sub-Saharan Africa, subspecies of which are the causative agents of human sleeping sickness and ruminant nagana. The haemoflagellate switches between the tsetse fly vector and mammalian host, where it exists in the procyclic (PS) and bloodstream (BS) stages, respectively, and has to adapt to the different environments of the insect midgut and mammalian bloodstream. During its life cycle the parasite

undergoes substantial physiological and morphological changes [1], the transformation of its single mitochondrion belonging to the most dramatic of them [2]. The mitochondrion in the BS is considerably reduced and appears to lack cristae [reviewed in 3,4]. This morphological difference, in addition to the absence of cytochrome-containing respiratory complexes III and IV, reflects the exclusivity of glycolysis in the energy metabolism of *T. brucei* in the glucose-rich bloodstream of the mammalian host. In contrast, the PS contains a fully developed mitochondrion, appearing as a reticulated structure meandering throughout the cytoplasm, with the participation of complexes III and IV in the generation of ATP via oxidative phosphorylation [5].

Although the BS has a reduced mitochondrion, it is hardly a dormant organelle, as it still requires the expression of the mitochondrial (kinetoplast) DNA (kDNA), consisting of thousands of interlocked minicircles and dozens of maxicircles [reviewed in 6,7]. The latter molecules are the equivalents of classical mitochondrial (mt) DNA, containing genes required for the organelle's biogenesis, most of which encode subunits of the respiratory complexes. The expression of majority of these genes is not as straightforward as in other eukaryotes however, as their maturation requires extensive RNA editing in the form of post-transcriptional insertion and/or deletion of uridines (U) in preordained positions of the mRNA. A diverse population of minicircles encode small guide (g) RNAs, which direct the enzymatic machinery encompassed by the editosome protein complex to properly edit the mRNAs [reviewed in 8,9].

Trypanosomes maintain the mt membrane potential ($\Delta\Psi m$) requisite for the import of essential proteins [10,11] and Ca^{2+} [12]. Typically, eukaryotes rely on respiratory complexes I, III and IV to pump protons outside of the mt matrix to generate $\Delta\Psi m$, and the latter two complexes indeed have such a role in the PS [13]. However, noteworthy experiments have shown that in the BS it is the F_0F_1-ATP synthase (complex V) that adopts the role of sustaining $\Delta\Psi m$ [12,14,15].

In a typical mitochondrion, complex V acts as a dynamo driven by the flux of protons down the electrochemical gradient inherent in $\Delta\Psi m$, which is coupled to the production of ATP. Its mechanism of action can be explained by exploring the remarkably conserved structure of complex V, the basic architecture of which is present in bacteria, archea and eukaryotes [reviewed in 16,17]. Complex V is composed of two parts: the hydrophobic F_0 moiety embeds the complex into the mt inner membrane, while the F_1 sub-complex extends into the mt matrix. The F_0 part is composed of one ATP6 subunit (subunit *a* in the mammalian nomenclature), one or two ATP4 (*b*) subunits and a ring of 10-15 ATP9 (*c*) subunits. It is believed that this moiety mediates proton flux through the inner membrane via the interface between ATP6 and the ATP9-multimer ring, powering its rotary motion [17]. The resulting energy is used for ATP synthesis at the F_1 moiety, which is composed of a ring of three alternating α and β subunit pairs, a central stalk made up of the γ and ε subunits. The central stalk connects the spinning ATP9-composed ring to the stationary $\alpha_3\beta_3$ hexamer, facilitating conformational changes to the three catalytic sites present in the latter, which in turn allows ATP synthesis. Interaction between the F_0 ATP4 and F_1 OSCP subunits forms the peripheral stalk, which is believed to comprise part of a stator running parallel to the central stalk [18]. The action of complex V is reversed in the BS, in which ATP is consumed in order to pump protons out of the matrix [14,15].

The essential ATP6 subunit is retained in virtually all mt genomes, presumably due to its extreme hydrophobicity [19]. In *T. brucei*, a pan-edited mt transcript, requiring information from an estimated 21 gRNAs for the insertion of 448 Us and deletion of 28 Us to render a translatable ORF, has been hypothesized to encode ATP6 [20,21]. Such an assignment was supported by

analysis of the *in silico* translated product, revealing a similar hydropathy profile and low sequence similarity of the C-terminus to ATP6 orthologs from other species [20]. However, conclusive evidence for this speculative designation is missing [22].

The notion that this pan-edited mRNA encodes the ATP6 subunit, and that it is assembled into the F_0 moiety is, however, further complicated by the existence of natural and laboratory-induced dyskinetoplastic (Dk) and akinetoplastic (Ak) trypanosomes, which are viable despite a partial and complete loss of kDNA, respectively [reviewed in 23]. These cells are locked in the BS because they lack the mitochondrial-encoded subunits of the cytochrome-containing complexes III and IV, and are viable despite the absence of the ATP6 gene [23]. Yet RNA editing is essential in the BS of *T. brucei* [24], suggesting that the product of ATP6 is not entirely dispensable. Furthermore, the consumption of ATP by the F_1 moiety of complex V is required for maintaining $\Delta\Psi m$, which is considered indispensable for the survival of any trypanosome, including the Dk and Ak strains [14]. One interpretation of these findings is that the protein product of the edited mRNA putatively assigned as ATP6 in the mt genome of *Trypanosoma* species is not incorporated into the F_0 moiety, perhaps because it does not encode this subunit. Another explanation postulates that in the Ak cells mutations have evolved in the nuclear-encoded subunits of the complex to offset the absence of ATP6, in which the ATPase activity of the F_1 is decoupled from the incomplete F_0 that lacks proton pumping capacity. A single amino acid substitution in the C-terminus of the γ subunit from a laboratory-induced Dk strain was elegantly demonstrated to be such a mutation [14], while additional mutations in the same region with a putative compensatory role were identified in several natural Ak strains [25]. Consistent with such a scenario is the insensitivity of *T. b. evansi* to oligomycin, a specific inhibitor of F_0, to which the BS *T. brucei* remains susceptible [14,26]. Furthermore, the putative ATP6 mRNA is edited in both PS and BS trypanosomes, while other transcripts are preferentially edited only in one of these stages [22,23].

Despite significant efforts in the last two decades, proteins translated from edited mRNAs remain elusive. So far, apocytochrome B (cyB) and cytochrome *c* oxidase subunit 2 (cox2) from the model trypanosomatid *Leishmania tarentolae* remain the only detected protein products of a moderately edited mRNA [27,28], whereas a protein translated from a pan-edited mRNA has yet to be found [28,29]. Recently, an important claim has been made that pan-editing generates alternative transcripts, leading to the production of several different proteins from one gene, a function analogous to alternative splicing [30]. Interestingly, only very few molecules were found to be fully edited and thus translatable into predicted subunits of respiratory complexes [31]. Such an inefficient production of these subunits, mostly deemed essential for a eukaryotic cell, further questions the presupposition that pan-editing creates translatable transcripts.

Unfortunately, direct recombinant manipulation of kDNA is still not feasible despite valiant efforts [32,33]. Thus, we have decided to address the translation and incorporation of the predicted ATP6 protein by taking advantage of the differential phenotypes resulting from RNA interference (RNAi)-mediated silencing of two nuclear-encoded proteins involved in mt RNA metabolism: mitochondrial RNA binding protein 2 (MRP2) and kinetoplastid RNA editing proteins KREPA6 (summarized in Table 1). MRP2 with its partner MRP1 forms a heterotetrameric MRP1/2 complex, which facilitates annealing of gRNAs to their cognate mRNAs [34]. RNAi knockdown (KDs) of MRP2 in the PS resulted in the downregulation of a subset of never-edited and edited mRNAs, with the important exception of ATP6 [35], which consequently caused the disruption of complexes III and IV (Table 1) [36]. Since KREPA6 is a subunit of the editosome [9], its depletion in the PS results in a general reduction of RNA

editing, affecting mRNAs for subunits of complexes III and IV, as well as ATP6 (Table 1) [37]. Although the secondary effect of its silencing on the respiratory complexes was not directly investigated, their disruption is considered inevitable, since this outcome is observed in the RNAi KD of the terminal uridylyl transferase required for gRNA maturation, which also affected all edited mRNAs in a similar fashion [38,39]. Here we present several lines of evidence, albeit indirect, for the existence of a genuine ATP6 subunit of an apparently non-canonical complex V in the mitochondrion of procyclic *T. brucei*.

EXPERIMENTAL PROCEDURES

Strains, cultivation and isolation of mitochondria - The MRP2 and KREPA6 RNAi cell lines were described elsewhere [35,37]. The PCR-generated fragment (forward primer with *BamH*I restriction site: 5' - TGGGATCCAACACTGCACCATGGATTG – 3'; reverse primer with *Xho*I site: 5' – AAGCTCGAGTGGATGTTCTTTCCCCTC -3'; both restriction sites underlined) of ATP4 (p18) was cloned into the p2T7-177 vector. The ATP4 knockdown cell line was established by transformation of the parental 29-13 cell line with resulting cassette, as described elsewhere [40]. All transgenic cells, as well as the 29-13 cell line, were cultivated in SDM-79 medium under conditions described elsewhere [35]. The *T. brucei* strain 920, *T. b. equiperdum* strain 818 and *T. b. evansi* strain 810 were described previously [25]. The kinetoplast-mitochondrial vesicles from 5 x 10^8 non-induced and induced procyclic cells were isolated by hypotonic lysis as described elsewhere [13]. Pelleted mitochondrial vesicles were stored at -70 ºC upon further use.

Quantitative real-time PCR - Quantitative real-time PCR reactions were performed as in Hashimi et al. [40]. Primers for amplification of pre-edited and edited mt mRNAs are as described elsewhere [41], as are those for KREPA6 [37]. The primer pair for detection of MRP2 cDNA is MRP2-qPCR-Fw (5'- GAAGCTTGGCTGTGTCCTTC-3') and MRP2-qPCR-Rv (5'-TGCGTCCGAATACGATTACA-3'). Relative RNA abundance between RNAi-induced and non-induced samples were calculated as described previously [25,37,40,41].

Measurement of ATPase activity and inhibition experiments- 1 mg of mt proteins was resuspended in 1 ml of the TC buffer (0,2 M KCl; 10 mM Tris-HCl, pH 8.2; 2 mM $MgCl_2$). The reaction was started at room temperature by adding ATP to the final concentration 5 mM and after 5 min was stopped by mixing 95 µl aliquots with 5 µl of 3 M $CHCl_3$-COOH, incubated for 30 min on ice, and spun (16 000 g for 10 min at 4°C). Ninety µl of the supernatant were added to 1 ml of the Sumner reagent (8,8% [w/v] $FeSO_4$; 375 mM H_2SO_4; 6,6% [w/v] $(NH_4)Mo_7O_{24}$). After 15 min incubation at room temperature, absorbance of free P_i was measured at 610 nm. Parallel experiments were performed with untreated samples and those in the presence of 10 µg/ml oligomycin or 1 mM azide. Inhibition experiments were reproduced in 10 independent measurements from four RNAi inductions with parallel non-induced controls. Statistical significance of observed differences in inhibition data were determined by Student's t-test.

In-gel activity staining and two-dimensional gel electrophoresis **–** For activity staining, 100 µg of mitochondrial lysate in 0.5 M aminocaproic acid and 2 % dodecylmaltoside was loaded per lane and analyzed on a 4-15% gradient blue native polyacrylamide (BN) PAGE gel as described

elsewhere [13]. Immediately after the run, the gel was transferred into either ATPase reaction buffer (35 mM Tris; 270 mM glycine; 19 mM $MgSO_4$; 0,3% [w/v] $Pb(NO_3)_2$; 11 mM ATP) for overnight incubation, or into cytochrome oxidase buffer (50 mM sodium phosphate, pH 7.4; 1 mg/ml 3,3'-diaminobenzidine; 24 U/ml catalase; 1 mg/ml cytochrome *c*; 75 mg/ml sucrose) for 3 hours staining, both by slow agitation. The ATPase activity appears as a white and cytochrome *c* oxidase activity as brown precipitate. The resulting bands were quantified with the scanning densitometry ImageQuant program (Molecular Dynamics). The BN PAGE gels for the first dimension of two-dimensional gel electrophoresis were prepared in the aforementioned fashion. The second dimension was resolved on 10% Tricine-SDS PAGE gels as in [13, 36].

Western blot analysis – After electrophoresis, the BN PAGE gel was blotted on a nitrocelulose membrane, and probed with polyclonal rabbit antibodies raised against complex V subunits (designation in parenthesis) of *Crithidia fasciculata* (F_1 moiety) [42], *Leishmania tarentolae* (ATP4) [43] and *T. brucei* (ATP9) [15] were used at 1:1,000, 1:3,000 and 1:500 dilutions, respectively. Secondary α-rabbit antibodies (1:2,000) (Sevapharma) coupled to horseradish peroxidase were visualized according to the manufacturer's protocol using the ECL kit (Pierce).

Isolation of F_1 moiety of complex V by chloroform extraction- In a protocol adapted from Linnet et al. [44], mitochondrial vesicles from 4 x 10^8 cells were resuspended in 300 µl STE buffer (0.25 M sucrose; 20 mM Tris.HCl, pH 7.9; 2 mM EDTA), and sonicated 4 x 10 s with 30 s intervals in between. The sonicate was centrifuged at 100,000 g for 30 min at 4 °C, and the pellet was then resuspended in 300 µl STE. A 150 µl volume of chloroform was added and the two phases were emulsified by vortexing for 10 s and spun in a microcentrifuge at maximum speed for 5 min at room temperature. The aqueous phase was transferred into a new centrifuge tube and ultracentrifuged at 100,000 g for 30 min at 25 ^oC. The proteins present in the supernatant were concentrated to 0.4 µg /µl using Microcon YM-30 centrifugation filters (Millipore), in which the solvent buffer was replaced with the resuspension solution (0.5 M aminocaproic acid; 0.25 % dodecylmaltoside). This sample was divided and stored overnight at either 0^oC or room temperature to assay for the cold lability of the isolated F_1 moiety. About 5 µg of extracted proteins were loaded per lane of the BN PAGE gel.

Electron microscopy – Cells were washed in 0.1 M phosphate-buffered saline (PBS) and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h at 4 ^oC, then washed in 0.1 M PBS and post-fixed with 2% (w/v) OsO_4 in the same buffer for 1 h at room temperature. After dehydration in ethanol, the cells were embedded in PolyBed (Polysciences) and sectioned. Thin sections were stained with lead citrate and uranyl acetate, and examined in a JEOL JEM 1010 microscope. In order to estimate the number of cristae in mitochondria of the PS and BS of *T. brucei*, as well as KREPA6-silenced cells, the cristae were counted in over 200 carefully examined cross-sections of mitochondria from each cell line.

RESULTS

In-gel ATPase activity reveals oligomerization of complex V and dimerization of F_1 moiety in PS - The lysates from hypotonically isolated mitochondria from the parental PS 29-13 cell line, from which the MRP2 and KREPA6 RNAi KD cells are derived, were resolved on a BN-PAGE gel.

Complex V was visualized by subsequent staining for in-gel ATPase activity (Fig. 1A), revealing two major upper ~2700 kDa and lower ~ 900 kDa bands. Both appear to be comprised of doublets or multiple smaller bands, with the bottom signal being the most intense. A smear is present between the upper and lower major bands, which are often resolved as multiple bands of less intensity than the upper and lower bands (see below). In order to confirm that these ATPase activity bands represent genuine complex V, Western blots of lysates separated by BN-PAGE were probed with two antibodies immunodecorating subunits of complex V. The α-subunit β and α-ATP4 antibodies, used in previous studies of *T. brucei* [14, 25], were originally raised against the F_1 moiety of *Crithidia fasciculata* [42], and the F_0-subunit of *Leishmania tarentolae* [39,43] (Figs. 1B and C). Both antibodies positively labeled all the bands with the same relative intensities as the in-gel activity stain. Polyclonal antibodies raised against the *Saccharomyces cerevisiae* ATP4, 6 and 9 subunits did not cross react with antigens present in *T. brucei* (data not shown).

Depending on the conditions used, multiple bands are resolved by native gel electrophoresis that correspond to different forms of complex V in yeast, such as oligomers, dimers and monomers of F_1F_0, as well as free F_1 particles [45]. We attempted to identify the form of complex V corresponding to the upper and lower bands, as well as the smear in between, by liquid chromatography-tandem mass spectroscopy (LC-MS/MS). However, only F_1 subunits were identified in all three samples, plus ATP4, possibly because the technique is not amenable to identification of the hydrophobic peptides comprising F_0 (data not shown). The presence of the canonical F_0 ATP4 in all bands also complicated this task. We resorted to the assignment of these bands by how their estimated molecular weights match the canonical stoichiometry [16, 17, 18] of the currently annotated complex V subunits in the GeneDB *T. brucei* genome database (http://www.genedb.org/genedb/tryp/). The top ~2700 kDa band has been provisionally assigned as an oligomer of complex V. The identity of the lower ~900 kDa doublet was more difficult to ascertain, since its migration could correspond to either a monomer of complex V or a dimer of the F_1 sub-complex. The isolated F_1 moiety from *L. tarentolae* has been reported to have a similar ~ 900 kDa size that we observe in *T. brucei*, and was also proposed to be a dimer [46].

To address this issue, the soluble F_1 moiety was prepared by chloroform extraction from hypotonically isolated mitochondria from the 29-13 cells following the protocol of Linnett et al. [44]. The F_1 moiety purified by this method from the membrane-bound hydrophobic subunits was electrophoresed on a BN gel along with the whole mitochondrial lysate. Upon staining by the in-gel ATPase activity assay, the lower band from the mt lysate migrates the same distance as the isolated F_1 moiety (Fig. 1A). The size implies the catalytic portion of complex V exists as a dimer, whose activity is cold labile (Fig. 1A), a typical characteristic of the isolated F_1 sector. Western analyses using antibodies against the F_1 β subunit and F_0 subunit ATP4 showed that both are present in the chloroform extracted F_1 sub-complex (Figs. 1B and C). ATP4 was also reported to be present in the chloroform extracted F_1 moiety of *C. fasciculata* [42].

ATP4 is required for the integrity of the F_1 moiety – The presence of ATP4, traditionally assigned to the F_0 moiety [16,17], in the chloroform-extracted F_1 sub-complex prompted us to generate RNAi KD of this subunit to investigate its position within the complex. Silencing of ATP4 resulted in slower growth of the procyclic cells (Fig. 2A). After 90 hours of RNAi-induction, the steady-state level ATP4 protein was virtually eliminated (Fig. 2A; inset), which was the time point chosen for subsequent experiments. In-gel ATPase activity and Western blot analysis using antibody against the β subunit showed that the catalytic portion of complex is

destabilized upon downregulation of ATP4 (Figs. 2B and C). This result is reminiscent of destabilization of the β subunit of F_1 upon silencing of its partner, the α subunit, and *vice versa* [14, 15], which may be an indication that ATP4 interacts directly with this moiety as well. This interpretation is consistent with its apparent association with isolated F_1 particle, as well as its interaction with the F_1 OSCP subunit of complex V. In any case, it is clear that ATP4 plays a role in maintaining the integrity of this moiety.

Differential effect of KREPA6 and MRP2 RNAi silencing on ATP6 editing - Inhibition of cell growth was re-evaluated in the PS cells, in which the KREPA6 or MRP2 transcripts were downregulated by RNAi (Figs. 3A and C). In agreement with previous reports [35,37], tetracycline induction of double stranded RNA targeting of these transcripts resulted in slower growth. Based on these results, subsequent experiments were performed on cells grown for six days in the presence and absence of tetracycline. This time point was chosen since our study concerns a secondary phenotype affecting the respiratory complexes, a strategy already employed in similar studies [36,39], taking advantage of the consequential results of the already direct effect of these RNAi KDs on mt RNAs.

In the original report, mt RNAs were assayed in the MRP2-silenced cells by poisoned primer extension [35], showing reduction of edited and never-edited subunits of complexes III and IV, without an apparent effect on the ATP6 mRNA (Table 1). We have verified the differential effect on ATP6 editing in the KREPA6 and MRP2 KDs using a more sensitive method based on quantitative real-time (q) PCR (Figs. 3B and D). Using previously described primers against pre-edited and edited ATP6 mRNAs [40], editing of this transcript is virtually abolished in the KREPA6 KD six days after tetracycline induction compared to its non-induced counterpart (Fig. 3B). In contrast, MRP2-silencing only slightly reduces the levels of pre-edited and edited ATP6 mRNAs (Fig. 3D), perhaps reflecting a general role in RNA stability and/or processing suggested for its eponymous complex [34,35]. Nonetheless, the level of the translatable ATP6 transcript is considerably closer to the wild-type level upon the depletion of MRP2 as compared to KREPA6.

A subset of the mt-encoded RNAs for subunits of respiratory complexes III and IV, plus the edited maxicircle unidentified reading frame 2 (MURF2), were also assayed by reverse transcription-qPCR to confirm that they are affected as originally reported in the KREPA6 and MRP2 KDs [35,37] (summarized in Table 1). As expected, the never-edited transcript of complex IV cytochrome *c* oxidase subunit 1 (co1) and the edited RNA of the complex III cytochrome reductase subunit b (cyB) were downregulated upon MRP2 silencing (Fig. 3D). Editing of co3 and cyB was also reduced in the cells with downregulated KREPA6 (Fig. 3B). Moreover, editing of the MURF2 transcript, encoding a protein of unknown function, was equally affected in both KDs (Figs. 3B and D).

The accumulation of the F_1 moiety and reduction of the F_1F_O oligomer of complex V in ATP6-depleted cells - Kinetoplasts from the non-induced and tetracycline-induced KREPA6 and MRP2 KDs were subjected to the aforementioned BN-PAGE/in-gel ATPase activity experiments. Complex V was visualized by running these lysates on the BN gels, followed by staining for the in-gel ATPase activity (Fig. 4A). In all the samples besides the KREPA6-silenced one, three bands between the upper and lower bands are present. The cells interfered against KREPA6, in which editing of ATP6 is virtually abolished (Fig. 3B), exhibit a reduction in oligomerization of complex V and a corresponding accumulation of free F_1 dimers (Fig. 4A). Semiquantification of

the ATPase bands reveal that the lowest doublet represents 96% of the sum density of the upper and lower bands in the lane containing the RNAi-silenced sample, while it comprises 57% and 58% in-gel activity from the untreated and parental cell lines (Fig. 4A). A similar enrichment of the free F_1 dimers was observed in KREN1 KD cells, in which one of the endonucleases essential for RNA editing has been ablated (data not shown) [47]. Such a disruption of complex V is not observed in the MRP2 KDs, in which editing of ATP6 persists, while the amount of never-edited, pre-edited and edited mRNAs of the other mitochondrial-encoded subunits of respiratory complexes is decreased [35,36]. The in-gel activity of free F_1 represents 32% and 67% of the sum density of the upper and lower bands observed in the non-induced and RNAi-induced samples, respectively (Fig. 4A). This pattern is apparent in western blot analyses with antibodies against the β and ATP4 subunits of the F_1 and F_0 sub-complexes, respectively (Figs. 4B and C). Interestingly, a signal corresponding to the uppermost of the three middle bands is observed in KREPA6-silenced lane of both immunodecorated blots. As expected, the in-gel activity of complex IV was dramatically reduced by about 70% when either MRP2 or KREPA6 are downregulated (Fig. 4D).

Formation of the F_0 ATP9 multimeric ring is disrupted in the absence of ATP6 – Two-dimensional gel electrophoresis was employed to further examine complex V in the KREPA6 KDs, since a polyclonal antibody against ATP9, a subunit forming the multimeric ring of the F_0 sub-complex, appears to recognize proteins resolved under denaturing conditions. Native complexes of RNAi cell lines grown in the presence and absence of tetracycline were separated on BN gels in the first dimension followed by SDS-PAGE in the second dimension to break apart individual subunits. As seen in Western blot in Fig. 5A, the β subunit and ATP4 have been detected in the upper and lower bands (as visualized by in-gel ATPase activity), as well as in the middle bands. However, ATP9 is confined to the region corresponding to the upper bands, and is also present to a lesser degree in the middle bands (Fig. 5A). In the KREPA6-silenced samples, ATP4 and F_1 are concentrated in the lowest bands, while ATP9 is undetectable, presumably because in the absence of ATP6, it cannot be assembled into the complex and is degraded (Fig. 5B). This finding also further supports the notion that the lower band represents a dimer of the F_1 moiety.

Sensitivity to oligomycin is decreased in ATP6-depleted kinetoplasts - The Ak trypanosome *T. b. evansi* is insensitive to oligomycin, a specific inhibitor of the F_0 subunit [14,26]. We investigated the possibility that the KREPA6-depleted cells may exhibit a decrease in sensitivity to oligomycin that would be reminiscent of the behavior of the ATP6-lacking Ak cells. The ATPase activity of hypotonically-isolated mitochondria from the non-induced and RNAi-induced KREPA6 and MRP2 KDs, as well as the parental 29-13 cells, were assayed for the release of P_i in the presence of 10 μg/ml oligomycin and 1 mM azide, which inhibit the F_1 sub-complex (Fig. 6), as compared to samples without inhibitors. The observed ~40% reduction of ATPase activity by either inhibitor in mitochondria from the 29-13 cell is similar to what has been previously reported for the BS trypanosomes [14]. Downregulation of KREPA6 results in more than 50% lower sensitivity to oligomycin, as compared to the non-induced cells, while both cell lines do not differ in their sensitivity to azide (Fig. 6). The observed changes in oligomycin sensitivity are statistically significant ($p= 0.0288$), while fluctuations in azide inhibition are not ($p= 0.4907$), as determined by the Student's t-test. The MRP2 KD cells also exhibit somewhat reduced sensitivity to oligomycin, although they remain more susceptible to the drug than the KREPA6-

silenced cells. This relatively minor decrease in sensitivity of the MRP2-silenced cells was not statistically significant (oligomycin p= 0.1810; azide p= 0.5199), and may be due to the slight decrease of edited ATP6 mRNA, as revealed by qPCR (Fig. 3B).

Prevalence of the uncoupled F_1 moiety in Dk and Ak trypanosomes - In the induced KREPA6 KD cells, the accumulation of the F_1moiety is apparent in the absence of the edited ATP6 mRNA (Fig. 4A). Therefore, in the BN-PAGE gels we have assayed the in-gel ATPase activity of lyzed mitochondria purified from the Ak and Dk strains of *T. b. evansi* and *T. b. equiperdum*, respectively, as well as from the PS and BS of *T. brucei* (Fig. 7A). Two prominent bands are apparent in the BS that correspond to the top ~2700 kDa F_1F_O-oligomer and the bottom ~900 kDa F_1-dimer present in the PS cells. In the Dk and Ak strains only the lower ATPase activity band is apparent, however, with a higher intensity than that found in the BS cells (Fig. 7A). The BS and the Dk trypanosomes show considerably lower in-gel ATPase activity than the PS cells, which is in agreement with previous observations that complex V is downregulated in the stages infecting mammals [49]. The native forms of complex V were also visualized by Western blot analysis using an antibody directed against the β subunit, revealing the predominance of the F_1 dimer in the Dk and Ak trypanosomes (Fig. 7B). This finding was confirmed by Western blot analysis of two-dimensional gels resolving complex V, as in Fig. 5, showing that in the Ak *T. b. evansi* strain, the ATP4 subunit is restricted to the lower ATPase activity band.

Ultrastructure of PS mitochondrion is not altered in the absence of ATP6 - Oligomerization of complex V has been implicated in generating the inward folds of the mt inner membrane to form cristae [48]. Since multimers of complex V are reduced in the ATP6-depleted cells, we investigated this possibility by examining mitochondrial ultrastructure of the non-induced and induced KREPA6 and MRP2 KD cells by transmission electron microscopy. By this method, typical discoidal cristae of kinetoplastid flagellates were present in both KDs (Fig. 8A and data not shown), and their abundance was similar to that found in the PS parental 29-13 cells (Fig. 8B and Table 2). It is worth noting that cristae of very similar morphology were also observed in the BS of *T. brucei* (data not shown) and the Ak *T. b. evansi* (Fig. 8C), yet their abundance was about ten times lower in the mammalian stages (Table 2).

DISCUSSION

Since its seminal discovery [50], RNA editing in the mitochondrion of kinetoplastid flagellates is an intensely studied process [for review see 8,9]. There was very little doubt that the edited transcripts are translated into mt proteins. Still, so far only two proteins produced from a moderately edited and never-edited mt mRNA were shown to exist [27,28], while all attempts to detect predicted protein products of pan-edited mRNAs failed. An extensive analysis of mt transcripts in *T. brucei* and *L. tarentolae*, however, detected a widespread occurrence of partially edited molecules, with fully edited ones representing only a tiny fraction of the mt RNA population [51,52]. Yet evidence has recently been put forward that these are alternatively edited molecules translatable into different proteins [30,31]. In the particular case of pan-edited ATP6, only 10% of analyzed molecules could serve as blueprints for the synthesis of canonical ATP6 subunit of complex V [31].

Although perhaps inefficiently produced, the product of pan-edited ATP6 mRNA appears to be incorporated into the complex V of *Trypanosoma* species, as supported by several lines of evidence presented in this study. The PS cells, in which the 20S editosome subunit KREPA6 was RNAi-silenced, are deficient for ATP6, as compared to cells with downregulated MRP2. Indeed, RNA editing is generally inhibited by silencing proteins directly involved in the process, invariably affecting mt mRNAs encoding subunits of respiratory complexes, whereas complex V escaped such interference in the latter KD (Table 1 and Figs. 1B and D). The absence of the ATP6 protein caused by the disruption of RNA editing drastically reduces the level of F_1F_0 oligomers of complex V, while an accumulation of the free F_1 moiety, the soluble portion of the ATP synthase, takes place. The KREPA6 KD represents an ATP6-lacking background as compared to the MRP2-silenced cells, in which oligomerization occurs at approximately the wild type level. This situation is also apparent in Dk and Ak trypanosomes, which naturally lack the capacity to express ATP6, since they also exhibit an accumulation of the uncoupled F_1 moiety as compared to BS *T. brucei* containing the F_1F_0 oligomers. Furthermore, we show that ATP9, the subunit responsible for the formation of the multimeric ring of the F_0 moiety, is destabilized in the absence of ATP6. Finally, the sensitivity of complex V to oligomycin is reduced in cells lacking the capacity to edit ATP6 mRNAs, which correlates with the situation documented for the Dk trypanosomes naturally lacking ATP6 [14].

The deletion of ATP6 in *S. cerevisiae* resulted in the loss of F_1F_0 dimers and monomers and the appearance of free F_1, as revealed by in-gel ATPase activity staining [45]. Our findings in *T. brucei* are consistent with these results. However, ATP9 appears to be still assembled into complex V in the yeast null mutants [45]. In trypanosomes, the absence of ATP6 appears to destabilize the F_0 moiety, as implied by the diminished ATP9 ring, leading to the apparent deficiency of F_1F_0 formation. This observation indicates that ATP6 may not be the last subunit incorporated into complex V as has been suggested for the yeast ortholog, as a measure to prevent a premature leakage of protons by the incomplete sub-complex [45,53]. Furthermore, while complex V oligomerization has been demonstrated to participate in the formation of cristae in yeast [48], this does not appear to be the case in the ATP6-lacking *T. brucei*. Moreover, few cristae were even observed in the BS *T. brucei* and the Ak *T. b. evansi*, a surprising result given the paradigm that mitochondria of the mammalian stage do not contain these structures [2,3].

Several other differences between kinetoplastid and canonical complex V were underscored by this study. It appears that ATP4, a subunit traditionally assigned to F_0, is firmly associated with F_1 in these flagellates. This conclusion is viable given its role in the formation of the peripheral stalk by direct interaction with the F_1 OSCP subunit and is supported by several lines of evidence presented in this study. First, ATP4 is a constant presence within the soluble moiety, even after separation from the membrane-bound subunits by chloroform extraction [44], as also reported in *C. fasciculata* [42]. This localization is in contrast to ATP9, which is absent in the free F_1 moiety. Second, RNAi silencing of ATP4 leads to the destabilization of F_1, an indication of the mutual interdependence of interaction partners [13, 35] and reminiscent of studies showing that silencing of one of the subunits forming the F_1 αβ heterodimer results in the degradation of the other [14, 15]. In addition, silencing of the α subunit leads to degradation of ATP4 [15]. This phenotype is in contrast with the one resulting from the disruption of the ATP4 gene in *S. cerevisiae*, triggering the decoupling of F_0 from F_1 without destabilizing proteins of the latter moiety [54].

This study also suggests that the F_1 subunit exists as a dimer across kinetoplastid flagellates, as originally reported for *L. tarentolae* [46]. Dimerization of this moiety has been

reported in other eukaryotes [55]. However, in these organisms it is promoted by an inhibitor protein in response to conditions that lead to diminished ΔΨm, such as oxygen deprivation, in order to conserve ATP that would otherwise be spent by reversal of complex V [55]. The chloroform-extracted F_1 moiety of *T. brucei*, whose migration in native gels also suggests a dimer conformation, is apparently not inhibited, since they maintain in-gel ATPase activity. Thus, its apparent dimerization is formed by another mechanism. Homodimers of the ATP4 subunit, assigned to the membrane-bound F_0 sub-complex, have been demonstrated to form between two adjacent complexes in yeast [56]. The presence of this subunit in the isolated F_1 sector in trypanosomes may mediate the dimerization of this moiety. Nonetheless, the occurrence of F_1 as a dimer appears to be unique to these organisms.

Although the presented data suggest for the first time that a protein product of a pan-edited RNA is indeed translated, and in this case assembled into complex V, due to the limitations of our system, we were unable to directly address the question whether ATP6 is essential for ATP synthesis. A logical experiment would be to test *in vitro* ATP synthase activity [57] from mitochondria isolated from the analyzed KDs. However, this assay requires that the capacity to generate ΔΨm is preserved, which both KDs lack due to the inevitable disruption of proton pumping complexes III and IV. Nevertheless, the notion that ATP6 is indispensable is further supported by reports that RNA editing is essential in the BS *T. brucei*, which uses complex V to sustain ΔΨm [23], while its maturation by this process is redundant in the Dk and Ak trypanosomes because of compensatory mutations in some of the F_1 subunits [14,25].

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FIGURE LEGENDS AND TABLES

Fig. 1. Oligomerization of complex V and dimerization of free F_1 moiety in procyclic *T. brucei*. 100 μg of protein from lysates of hypotonically isolated mitochondria and the equivalent of 5 μg of the F_1 moiety after chloroform extraction were elecrophoresed on a 4-15% blue native gel. The position of the ferritin dimer (Sigma) used as molecular weight marker are indicated on the left. 0°C, F_1 moiety isolated by chloroform extraction and incubated overnight at 0°C; Chl, F_1 moiety isolated by chloroform extraction and incubated overnight at room temperature; Mt, lysates from hypotonically isolated mitochondria; u – upper activity bands (~2700 kDa); l – lower activity bands (~900 kDa).
After the run**,** the gel was either stained for in-gel ATPase activity (**A**), or transferred onto a Nitrocellulose membrane and immunodecorated with polyclonal antibodies against either the F_1 β subunit (**B**) or the F_O subunit ATP4 (**C**).

Fig. 2. Downregulation of ATP4 results in slower growth and disassembly of the F_1 moiety.
(**A**) The growth curves for non-induced (grey open circles) and RNAi-induced cells (black filled squares) are shown for a period of 14 days. The y axis is depicted on a log scale and represents the cell densities of the cultures measured every 24 hours, as specified on the x axis. Inset displays the downregulation of the ATP4 protein after 90 hours of RNAi-induction (+) as compared to the non-induced cells (-), as determined by Western blot analysis (Ab). Ponceau staining of the membrane is shown as a loading control (Pon).
(**B**) Complex V from mitochondrial lysates of the 29-13 parental cell line, as well as the ATP4 RNAi knockdown cell line grown for 90 hours in the absence (-) and presence (+) of tetracyline,

was resolved on a 4-15% blue native gel and visualized by the in-gel ATPase activity assay, as in Fig 1A. The upper (u, ~2700 kDa) and lower (l, ~900 kDa) bands, in addition to the position of the ferritin dimer marker (880 kDa), are indicated on the left and right, respectively. 100 μg of protein was loaded per lane.
(**C**) Visualization of F_1 β subunit after the blue native gel was transferred to nitrocellulose and probed with specific antibody. Labeling as in Fig. 2B.

Fig. 3. Effect of RNAi-silencing of KREPA6 and MRP2 on cell growth and editing of ATP6 mRNA.
The growth curves for non-induced (grey open circles) and induced KREPA6 (**A**) and MRP2 (**C**) RNAi knockdowns (black filled squares) are shown for a period of 10 days and depicted as in Fig. 2A. Real time quantitative PCR analysis of a subset of mitochondrial-encoded RNAs, including pre-edited and edited ATP6 mRNAs, as well as the levels of the particular nuclear-encoded and RNAi-targeted mRNA (KREPA6 – **B**; MRP2 – **D**) was performed in triplicate on cDNAs generated from cells grown for 6 days in the presence or absence of tetracycline. For each target amplicon, the relative change in RNA abundance due to induction of RNAi-silencing of the particular transcript was determined by using cytosolic transcripts of β-tubulin (striped bar) and 18S rRNA (grey bar) as internal references, since their transcription was not affected by treatment. The following pre-edited (P) and edited (E) mRNAs were assayed: ATPase subunit 6 (A6), cytochrome oxidase subunits 1 (co1) and 3 (co3), cytochrome reductase subunit b (cyB) and maxicircle unknown reading frame 2 (MURF2). The average and median standard deviation of the measured triplicate cycle threshold (C_t) values are 0.13 and 0.10, respectively.

Fig. 4. Decrease of complex V F_1F_0 oligomers and accumulation of free F_1 dimers in the ATP6-deficient cells. Mitochondrial complexes from KREPA6 and MRP2 knockdown cells, grown for six days in the absence (-) or presence (+) of 1 μg/ml tetracycline (tet), respectively, were separated by blue native gel electrophoresis and stained for ATPase activity as described in Fig. 1. 100 μg of protein was loaded per lane. 29-13, procyclic *T. brucei* strain 29-13. The position of the ferritin dimer molecular weight marker is indicated on the left; u – upper activity bands (~2700 kDa); l – lower activity bands (~900 kDa).
(**A**) In-gel ATPase activity. The % density of the lower activity bands relative to that of total density of the combined bands is indicated at the bottom.
(B) Western blot analysis with antibody against the F_1 β subunit under the conditions described in Fig. 1.
(C) Western blot analysis with antibody against the F_0 subunit ATP4 under the conditions described in Fig. 1.
(D) In-gel cytochrome *c* oxidase (complex IV) activity. The presence of complex IV on blue native gels, resolved as described in Fig. 1, was detected by incubation with the reaction buffer (see Experimental Procedures), which yields a dark precipitate upon catalysis by the complex. In both RNAi-silenced cells, the density of the activity band is reduced by ~70% compared to their non-induced counterparts.

Fig 5. Incorporation of F_0 ATP9 into complex V in the presence and absence of ATP6. Complex V from KREPA6 knockdown cell lines grown for six days in the absence (**A**) and presence (**B**) of 1 μg/ml tetracycline, were resolved by two-dimensional gel electrophoresis. Native mitochondrial complexes were separated on a 4-15% blue native gel (1^{st} D [first dimension]; top panel), as described in Fig. 1. Complex V was subsequently visualized by in-gel ATPase activity and the upper ~2700 kDa (u) and lower ~900 kDa (l) bands are indicated just below. The individual subunits of the complexes were separated by 10% Tricine SDS-PAGE (2^{nd} D [second dimension], three bottom panels), transferred to nitrocellulose and probed with antisera against the F_1 β-subunit, ATP4 and ATP9. These antibodies are indicated on the left. Molecular weight markers are specified on the right.

Fig. 6. ATP6-depleted cells exhibit reduced sensitivity to the F_0 inhibitor oligomycin. Inhibition of the *in vitro* ATPase activity of complex V by 10 μg/ml of oligomycin (grey bar), which targets the F_0 moiety, and 1 mM azide (striped bar), interfering with the F_1 moiety, was assayed. Hypotonically isolated mitochondria from the parental 29-13 cell line and from the KREPA6 and MRP2 knock-down cells, grown for six days in the absence (-) and presence (+) of 1 μg/ml tetracycline, were solubilized and incubated with 5 mM ATP. The release of free P_i was measured by absorbance at 610 nm. The vertical axis depicts average % inhibition of ATPase as compared to the untreated samples. For both inhibitors, three independent experiments using mitochondria from the 29-13 cells and six each from individual RNAi-induced and non-induced KREPA6 and MRP2 KDs were performed. Error bars indicate standard deviation among the ten individual experiments. The statistical significance of % inhibition of ATPase activity by oligomycin was $p= 0.0288$ and $p= 0.1810$ in between induced (+) and non-induced (-) KREPA6 and MRP2 KD cells, respectively, as determined by Student's t-test. P-values for changes in sensitivity to azide inhibition were $p= 0.4907$ and $p= 0.5199$ for KREPA6 and MRP2 tet+ and – samples.

Fig. 7. The prevelance of the F_1 moiety in naturally akinetoplastic *T. b. evansi* and dyskinetoplastic *T. b. equiperdum*.
(A) In-gel ATPase activity. Mitochondrial protein complexes were resolved and stained as described in Fig. 1. 100 μg of protein was loaded per lane. BS, bloodstream *T. brucei*, strain 920; PS, procyclic *T. brucei* strain 29-13; Ak, akinetoplastic bloodstream *T. brucei evansi*, strain 810; Dk, dyskinetoplastic bloodstream *T. brucei equiperdum*, strain 818; u – upper activity band (~2700 kDa); l – lower activity bands (~900 kDa).
(B) Western blot analysis with antibody against the F_1 β subunit under the conditions described in Fig. 2. The position of the ferritin dimer molecular weight marker is indicated on the left.
(C) Two-dimensional gel electrophoresis resolution of complex V in the bloodstream *T. brucei* (BS) was performed and labeled as described in detail in Fig. 5.
(**D**) Two-dimensional gel electrophoresis resolution of complex V in the akinetoplastic bloodstream *T. brucei evansi*, strain 810 (Ak) was performed and labeled as described in detail in Fig. 5.

Fig. 8. The mitochondrion in the ATP6-deficient cells has unaltered morphology and does not lose the cristae. Transmission electron micrographs taken from the KREPA6 knock-down cells grown for six days in the presence (+) of 1 μg/ml tetracycline (**A**), parental 29-13 cell line (procyclic *T. brucei*) (**B**), and the bloodstream stages of naturally akinetoplastic *T. b. evansi* (**C**). Typical discoidal cristae are labeled with arrows; n = nucleus; k = kinetoplast; f = flagellum. Bar – 500 nm (**A**), 200 nm (**B**) and 1 μm (**C**).

Table 1. Summary of mitochondrial transcripts affected by the RNAi silencing of the KREPA6 and MRP2 proteins.

	Effect on mitochondrial RNAs						
RNAi KD	I	III	IV	rRNA	RPS12	**ATP6**	Reference
KREPA6	E	E	E	ND	E	**E**	35
MRP2	NE & E	E	NE	0	E	**0**	33

Mitochondrial RNAs: I = subunit of complex I (NADH dehydrogenase); III = subunit of complex III (cytochrome *c* reductase); IV = subunit of complex IV (cytochrome *c* oxidase) ; rRNA = mitoribosomal RNA; RPS12 = ribosomal protein subunit 12.
Effect: E = decrease in edited mRNA; NE = decrease in never-edited RNA; 0 = no effect; ND = not determined.

Table 2. Number of cristae observed in mitochondrial sections by transmission electron microscopy.

Cell line	Mito section count	Number of cristae
29-13	239	231
KREPA6 KD tet+	227	213
BS	225	22

Figure 1

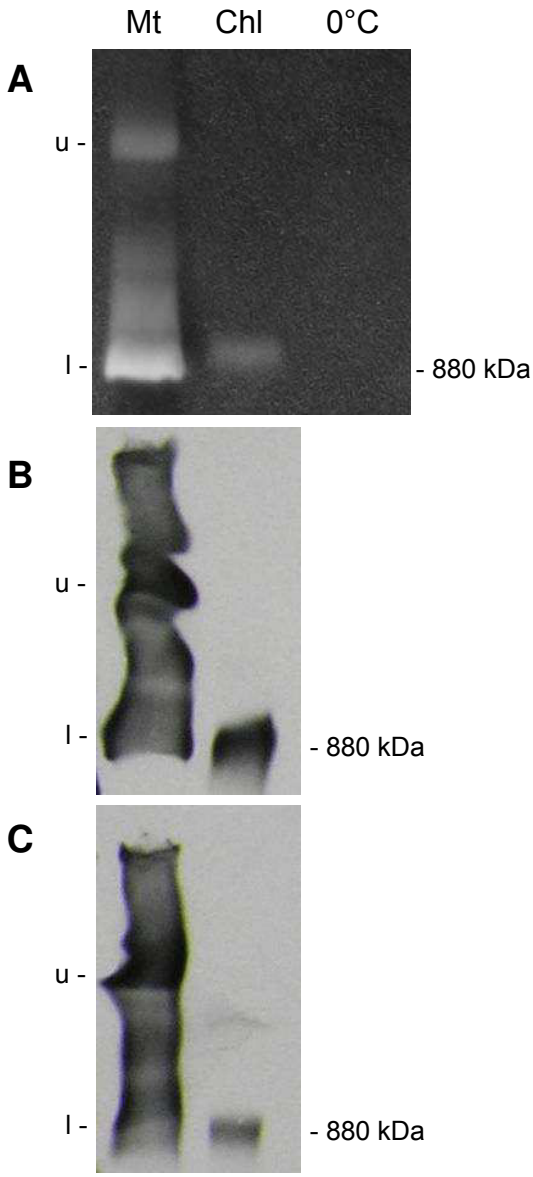
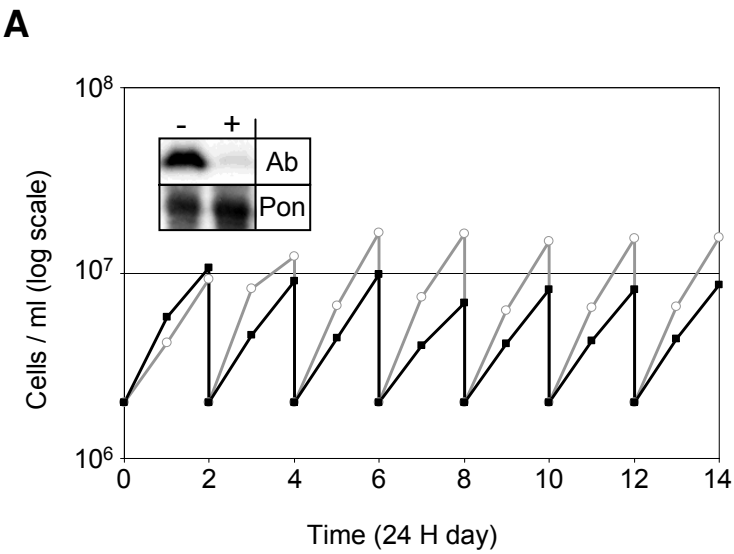
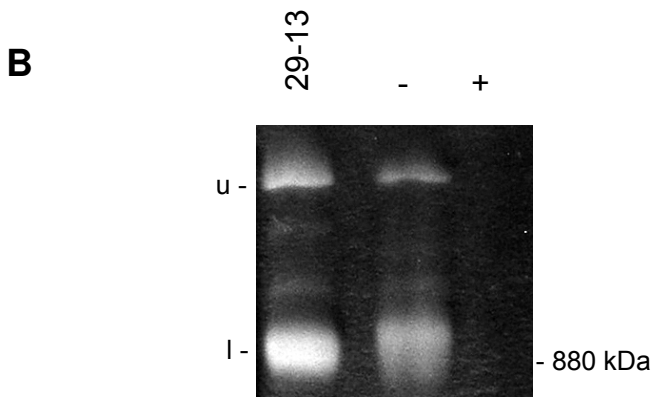


Figure 2


A
10^8
10^7
10^6
Cells / ml (log scale)
- +
Ab
Pon
0 2 4 6 8 10 12 14
Time (24 H day)


B
29-13
- +
u -
l -
- 880 kDa

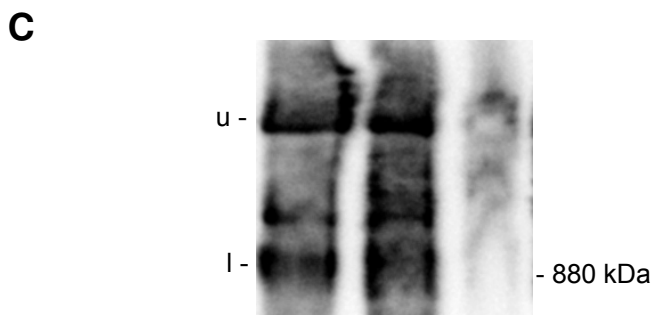

C
u -
l -
- 880 kDa

Figure 3

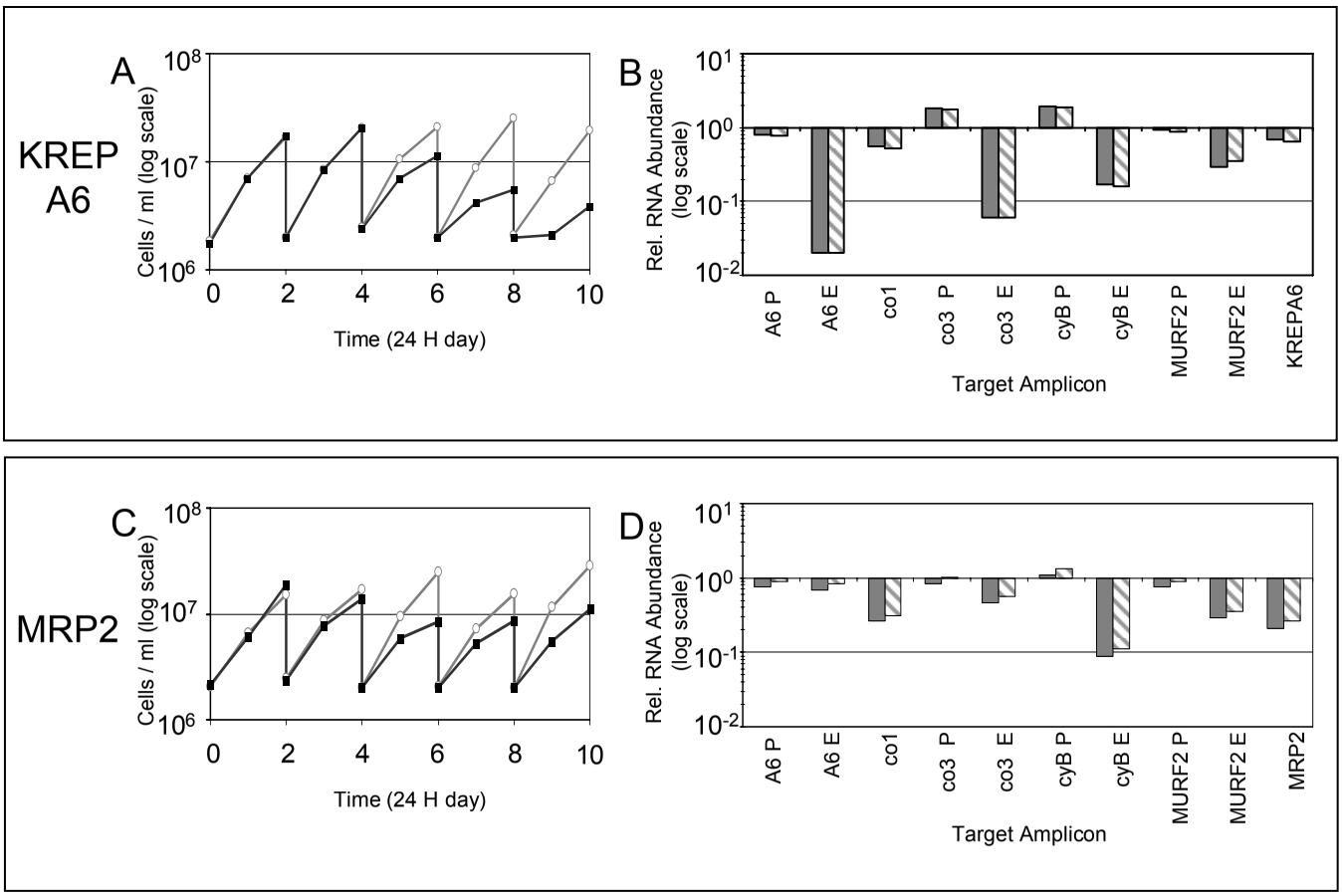

A
KREP A6
Cells / ml (log scale)
10^8
10^7
10^6
0
2
4
6
8
10
Time (24 H day)
B
Rel. RNA Abundance (log scale)
10^1
10^0
10^{-1}
10^{-2}
A6 P
A6 E
co1
co3 P
co3 E
cyB P
cyB E
MURF2 P
MURF2 E
KREPA6
Target Amplicon
C
MRP2
Cells / ml (log scale)
10^8
10^7
10^6
0
2
4
6
8
10
Time (24 H day)
D
Rel. RNA Abundance (log scale)
10^1
10^0
10^{-1}
10^{-2}
A6 P
A6 E
co1
co3 P
co3 E
cyB P
cyB E
MURF2 P
MURF2 E
MRP2
Target Amplicon

Figure 4

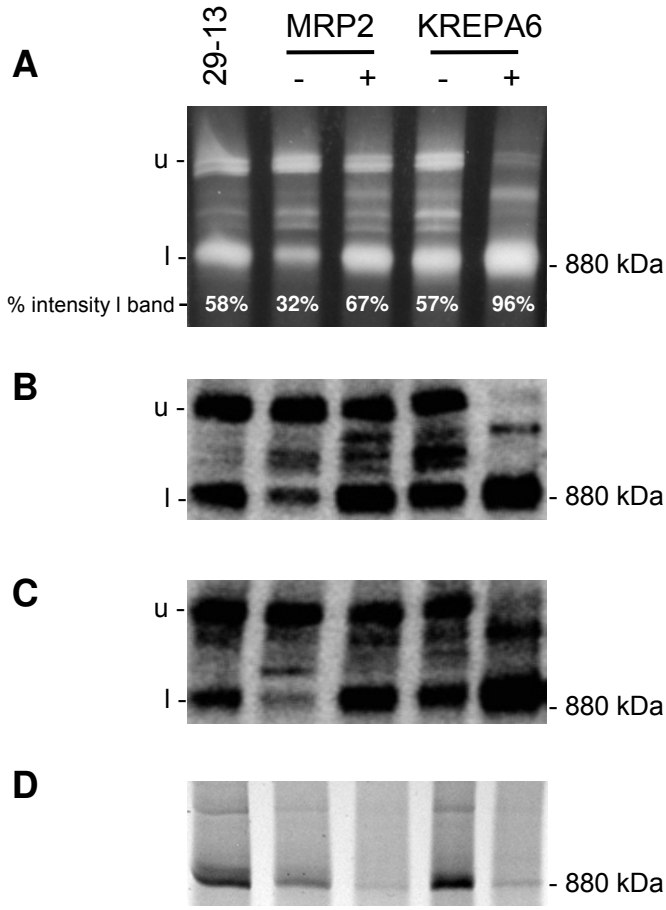

A
29-13
MRP2
KREPA6
-
+
-
+
u
l
- 880 kDa
% intensity l band
58%
32%
67%
57%
96%
B
u
l
- 880 kDa
C
u
l
- 880 kDa
D
- 880 kDa

Figure 5

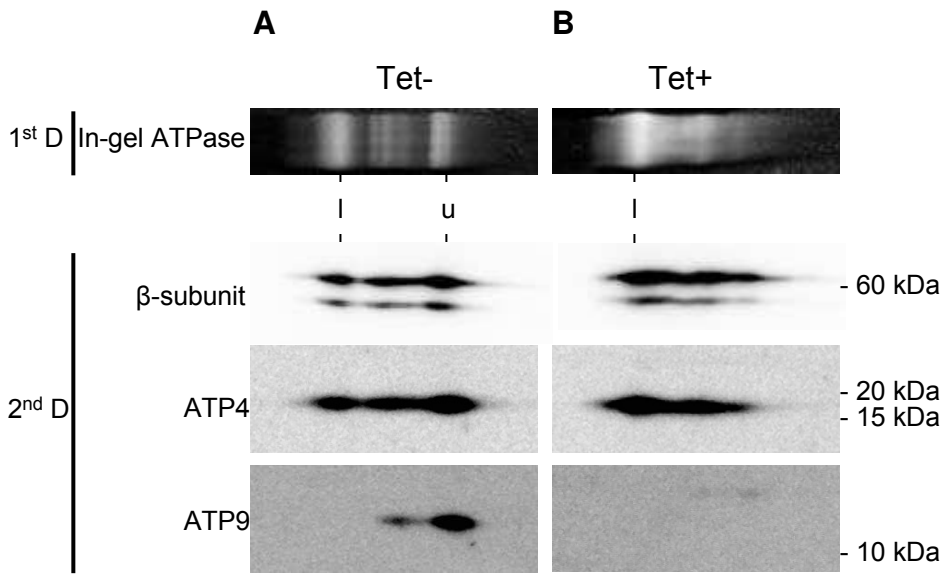

A
B
Tet-
Tet+
1st D
In-gel ATPase
l
u
l
2nd D
β-subunit
ATP4
ATP9
- 60 kDa
- 20 kDa
- 15 kDa
- 10 kDa

Figure 6

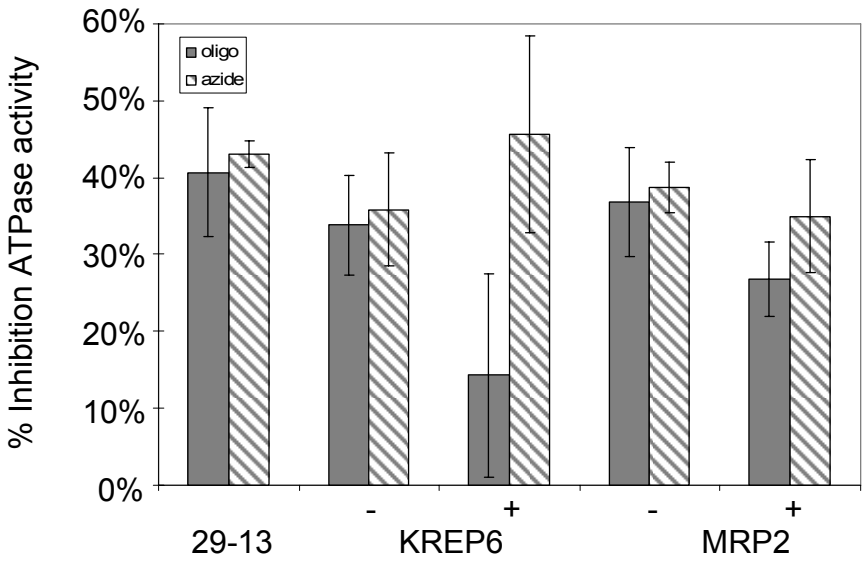

% Inhibition ATPase activity
60%
50%
40%
30%
20%
10%
0%
oligo
azide
-
+
-
+
29-13
KREP6
MRP2

Figure 7

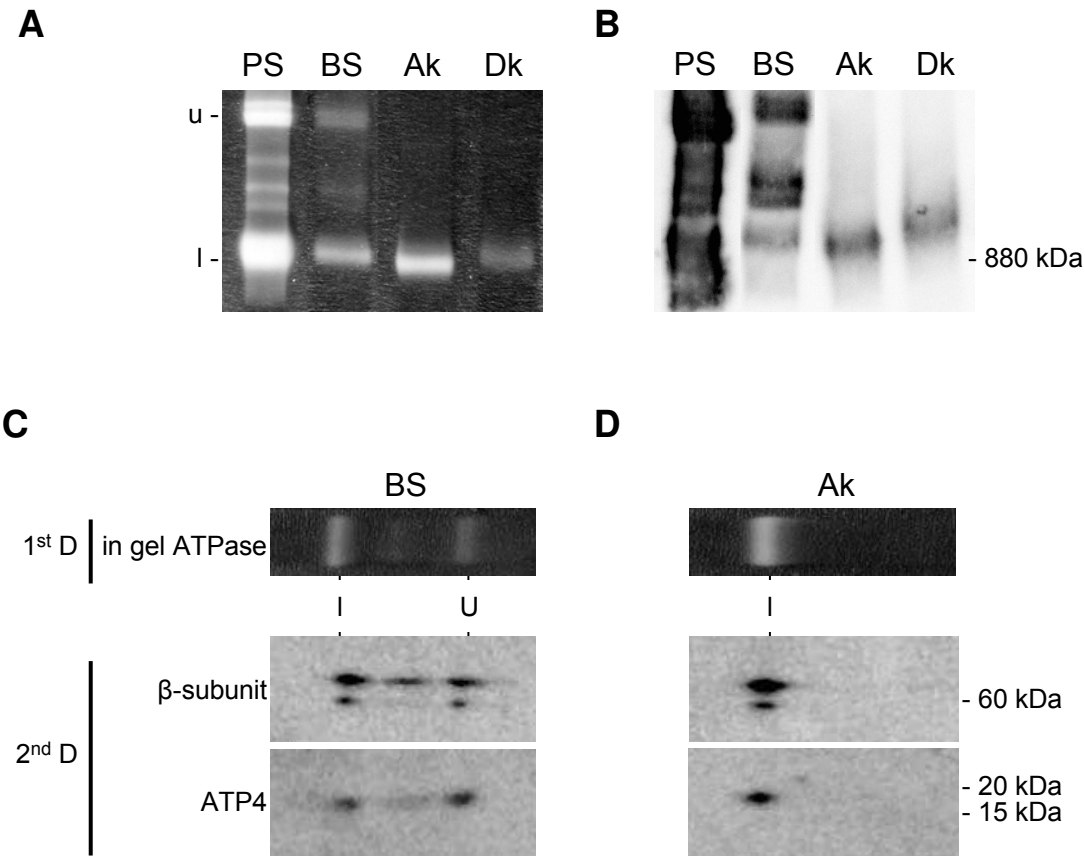
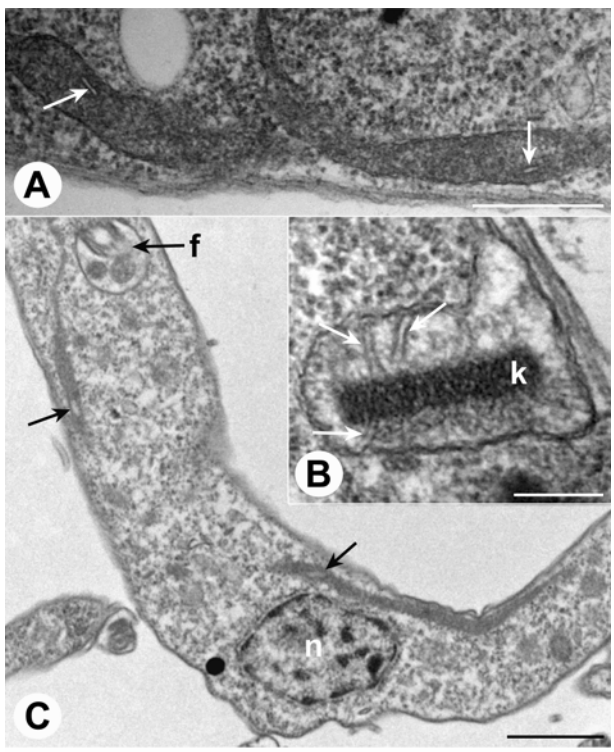

A
PS
BS
Ak
Dk
u -
l -
B
PS
BS
Ak
Dk
- 880 kDa
C
BS
1st D
in gel ATPase
I
U
2nd D
β-subunit
ATP4
D
Ak
I
- 60 kDa
- 20 kDa
- 15 kDa

Figure 8



Conclusions and perspectives

This thesis concerns two aspects of RNA editing in *Trypanosoma brucei*: 1) functional analysis of accessory proteins playing some role in this process; 2) understanding the phenomena of dyskinetoplasty. This section will discuss the conclusions and perspectives of each of these parts separately.

1. Functional analysis of mitochondrial RNA binding complex 1.

RNAi-silencing of the protein TbRGG1 results in a decrease in the steady state levels of the assayed edited RNAs without affecting those of gRNAs. The tandem affinity purification (TAP) of TbRGG1 led to the discovery of ~14 proteins that we provisionally named mitochondrial (mt) RNA binding complex 1 (MRB1), although its status as a *bona fide* complex remains in doubt. This notion is supported by the overlapping yet diverse MRB1 compositions reported by other groups identifying it with the same technique. Furthermore, TbRGG1 associates with this complex in a RNA-mediated manner, an indication that MRB1 may represent a group of smaller complexes and/or monomers.

Four subunits of MRB1, guide RNA associated proteins (GAPs) 1 and 2 as well as the predicted Nudix hydrolase and RNA helicase, were chosen for further analysis by reverse genetics. RNAi silencing of the GAPs and RNA helicase resulted in a downregulation of *trans*-gRNA mediated RNA editing due to a depletion of the minicircle encoded gRNAs, while *cis*-editing of cox2 persisted. Furthermore, the GAPs appear to be interacting partners, are essential in the bloodstream stage and have an interesting, punctuate localization in the mitochondrion of the procyclic stage. In our study, Nudix hydrolase knockdowns exhibited a general destability of maxicircle encoded RNAs without affecting gRNAs. Thus, a subset of MRB1 appears to have a role in gRNA biogenesis. A single mt RNA polymerase seems to transcribe both maxicircle and minicircle transcripts, seemingly precluding such a role of the GAPs and RNA helicase in gRNA expression.

Because studies of the proteins comprising MRB1 is just starting, there are several directions for future research. The issue with most priority is the heterogeneity of data pertaining to MRB1. The diverse compositions of isolated MRB1 is striking, although they contain a significant overlap of identified subunits, such as the GAPs,

TbRGGs, Nudix hydrolase and RNA helicase. Work by Weng and colleagues (2008) state that these proteins are divided into four smaller complexes that are linked by RNA. The complexes are: 1) guide RNA binding complex (GRBC), which contains the RNA helicase and GAPs; 2) MERS, which contains the Nudix hydrolase; 3) the heterotetrameric MRB complex (Schumacher et al., 2006; Zíková et al., 2008); 4) KPAP complex, containing TbRGG1 (Etheridge et al., 2008). Data presented in this thesis, such as the variability of RNAi-silencing phenotypes, supports the notion that MRB1 represents smaller complexes. However, the division into these four complexes does not take into account all of the isolated MRB1 proteins or glaring absences of key proteins in other preparations, such as KPAP, MRB1 and 2 or the pentatricopeptide repeat proteins.

Clearly, a systematic approach is needed to define true protein-protein interactions between MRB1 proteins, such as a yeast-2-hybrid based approach, as utilized for defining interactions among 20S editosome subunits (Schnaufer et al., 2003). This effort in conjunction with the continued screening of other MRB1-subunit knockdowns for phenotypes such as gRNA depletion may allow for definition of smaller complexes and/or monomers in this collection of proteins.

The Nudix hydrolase phenotype described in this work counters that reported by Weng and co-workers (2008). However, our results are consistent with a role that these enzymes typically have in the repair of oxidative damage to nucleotides (McLennan, 2006). We verified that maxicircle DNA was not mutagenized in the Nudix-hydrolase knockdowns by sequencing several clones of PCR-amplified cox1 (data not shown). To address whether this Nudix hydrolase has the capacity to repair oxidized ribonucleosides, it can be expressed in bacteria containing Nudix null-mutations to determine whether it rescues this repair pathway by measuring growth.

2. Understanding dyskinetoplasty

The dyskinetoplastic (dk) *Trypanosoma equipped* and akinetoplastic (ak) *Trypanosoma evansi* are important veterinarian pathogens that have a worldwide distribution despite exhibiting aberrant or complete loss of kDNA, respectively. Analysis of these trypanosomes employing molecular and classical-parasitology approaches indicated that they are strains of *T. brucei* and should be given subspecies status. The dk state seems to

arise spontaneously with the unfaithful replication minicircles. Although the kDNA network appears intact, it is only comprised of a few minicircle classes and a drastically diminished repertoire of gRNA genes. Since the selection pressure for maintaining maxicircles is no more, these molecules accumulate mutations such as deletions and are eventually lost. Eventually, the ak state is attained, in which all kDNA is lost. Because dk/ak cells prosper by fermentation of glucose, they can be considered to be petite mutants of *T. brucei*, which may arise repeatedly in an autonomous fashion in nature.

The existence of these trypanosomes ostensibly contradicts the finding that RNA editing is essential in the bloodstream stage (Schnaufer et al., 2001), presumably for the extensive editing of a transcript provisionally assigned to be F_OF_1 ATP synthase (complex V) subunit 6 (A6). This respiratory complex is essential in the bloodform to maintain membrane potential of the mitochondrion. Elegant studies have shown that compensatory mutations to another subunit in a laboratory-induced dk strain compensates for the absence of this mt encoded subunit in these cells (Schnaufer et al., 2005). We addressed the issue of whether this subunit is incorporated into complex V indirectly. By comparing the differential RNAi phenotypes of MRB1 knockdowns, affecting only mt encoded subunits of the electron transport chain, with KREPA6, which affects all respiratory chain complexes including complex V. We show the accumulation of the matrix F_1 moiety in the latter cells, while the assembly of the complex is not affected in the former. The oligomycin sensitivity of complex V ATPase activity is reduced in KREPA6-silenced cells as well. The accumulation F_O is also observed in dk and ak cell lines as compared to *T. brucei* bloodforms. We conclude from these results that A6 is indeed incorporated into complex V. Furthermore, we propose that the F_1 moiety exists as a dimer.

The ongoing sequencing of the *T. evansi* ak strain used in these studies may identify candidate mutations that may compensate for the absence of A6 in these cell lines. Such mutations can be verified by expressing these in a RNA-editing incompetent cell line, such as the RNA ligase KREL1 knockouts (Schnaufer et al., 2005) or GAP knockdowns, to see whether growth is rescued.

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