The University of South Bohemia

Faculty of Science

Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei*

Master thesis

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Annotation

The function of two subunits of the putative mitochondrial RNA binding complex (MRB1) associated with RNA editing in parasitic protist *Trypanosoma brucei* was studied using various *in vivo* and *in vitro* methods of molecular biology.

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Student's statement regarding her contributions to enclosed research article

A part of this thesis is a manuscript of a research article entitled "Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei*" in the same form that is currently submitted into RNA journal. My contributions to this work are as follows: generating data used for figures 1B, 2, 6, 7, 8B, and 9; writing text of several figure legends and experimental procedures; final design of most of figures; intellectual input to experimental design and data interpretation during the completion of the project.

Acknowledgements

My fathomless gratitude belongs to my supervisor in the past five years, Hassan Hashimi. Without his patience I would have probably given up, without his impatience I might have never finished. Thank you, Hassan, for both your guidance and an occasional lack of it, since the first one showed me new ways of thinking and the latter taught me to solve problems on my own.

Expressing thanks and not mentioning our enthusiastic leader, Julius Lukeš would be like talking about sea without mentioning water. Thank you, Jula, for giving me the chance to explore the world of science and always making time to talk about it.

Science can be a frustrating hobby sometimes. In those times when nothing seemed to work as it was supposed to I could always find a sympathetic ear to listen to my rants, an open mind to offer fresh ideas or a cheerful face to make me think that things will work out. Everyone in the lab has played a role in my life. Thank you, guys, you were great!

Mom and dad, thank you for everything. I couldn't have done this without you.

Contents

1. List of abbreviations

cDNA	complementary DNA
Co	cytochrome oxidase
Cyb	cytochrome b
dKD	double knockdown
dsRBD	dsRNA-binding domain
dsRNA	double-stranded RNA
ES	editing site
exoUase	U-specific exonuclease
GAP	guide RNA associated proteins
GRBC	gRNA binding complex
gRNA	guide RNA
KD	knockdown
kMAP	Kinetoplastid mRNA associated protein
KPAP1	kinetoplast poly(A)polymerase 1
MERS	mitochondrial edited mRNA stability factor
MP	mitochondrial protein
MRB1	Mitochondrial RNA binding complex 1
mRNA	messenger RNA
mRPN1	mitochondrial RNA precursor-processing endonuclease 1
mt	mitochondrial
mtRNAP	mitochondrial RNA polymerase
MURF	mitochondrial unidentified reading frame
ND	NADH hedydrogenase
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
qPCR	real time quantitative PCR
PPR	pentatricopeptide repeat
pre-mRNA	pre-edited mRNA
ProtC-TEV-ProtA	PTP
RBP	RNA binding protein

RECC	RNA editing core complex
REN	RNA editing endonuclease
RGG	arginine-glycine-glycine
RNAi	RNA interference
RNP	ribonucleoprotein particle
RPS12	ribosomal protein subunit 12
TAP	tandem afinity purification
TUTase	terminal uridynyl transferase
U	uridine
UTR	untranslated region
VSGs	variable surface glycoproteins
Y2H	yeast two-hybrid

1.1 Confusing terms

Moderately-edited mRNA: transcripts that need editing only in a small part of thein sequence, also called minimally-edited

Pan-edited mRNA: transcripts that need extensive editing to gain meaningful ORF

Never-edited mRNA: RNA that does not need editing, can also be used for a region on transcript that is not edited, even though editing occurs in other parts of said transcript

Pre-edited mRNA: any mRNA that needs editing to some degree, but editing has not yet been initiated

Partially-edited mRNA: 3‘ to 5‘ progression of editing on this mRNA has started, but has not yet finished

Fully-edited mRNA: editing of this transcript has finished

2. Introduction

2.1 Several words about *Trypanosoma brucei*

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

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

In both procyclic and bloodstream stages, there can be found a single mitochondrion, which takes up significant portion of the cell cytoplasm. Although the physiology of this organelle is dramatically different between these two forms, one of the biological features of my interest, mitochondrial DNA, is contained in the same structure in both stages: the kinetoplast.

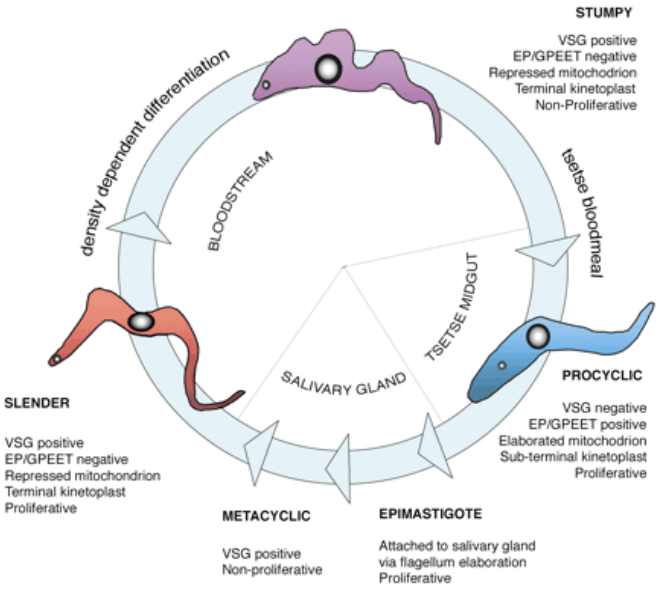

Fig. 1 *Trypanosoma brucei* life cycle

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

From Matthews, 2005

2.2 Kinetoplast DNA of *T. brucei*

The kinetoplast, a disc shaped structure located near the base of flagellum, is what gives the order Kinetoplastida its name. It is a tightly packed mitochondrial genome composed of circular DNA molecules concatenated into one large network (Englund, 1979), as shown in fig. 2B, that represents about 10-20% of total cell DNA. Two types of circles have been identified within this assembly: maxicircles and minicircles (Shapiro and Englund, 1995). Maxicircles, present in tens of copies, are about 20 kb in size and mostly encode genes that can be typically found in mitochondrial genomes. However, some of these genes are encrypted and need to be repaired at mRNA level in order to produce a functional protein. Minicircles, that are in *T. brucei* about 1 kb, contain the key to this repair. There are 5-10 thousands of them in a single kinetoplast and vary in sequence. Each encodes 2-5 so-called guide RNA (gRNA) genes (Hong and Simpson, 2003) that provide the genetic information for a process called uridine (U) insertion/deletion RNA editing (Sturm and Simpson, 1990), which will be further discussed in section 2.4.

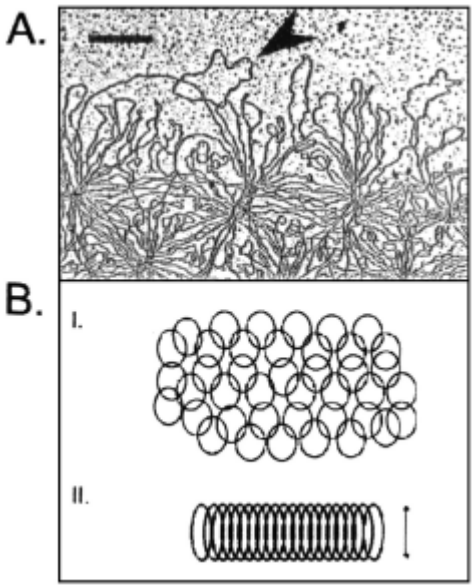

Fig. 2 kDNA network structure.

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

(B) Diagrams showing the organization of minicircles.

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

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

From Lukeš *et al.*, 2002

2.3 Mitochondrial transcripts of *T. brucei*

T. brucei maxicircles encode eighteen protein-coding genes, two ribosomal RNAs, and two gRNAs. The protein coding genes can be divided into three classes based on the level of encryption. Pan-edited genes require extensive editing utilizing multiple gRNAs and the insertion of hundreds and deletion of tens of Us to render a proper open reading frame (ORF). Moderately or minimally-edited genes undergo U-insertion editing only in a small part of their sequence, thus requiring just a few gRNAs. Finally, never-edited genes do not require any editing at all since they already encode the proper ORFs (for list of encoded genes and the degree to which they are edited see fig. 6). Out of the two maxicircle encoded gRNAs one is also found on minicircles, but the other one, belonging to the cytochrome oxidase subunit II (Co2) is unique not only by its position but also by the mechanism of function. It is positioned in the 3' untranslated region (UTR) of the said gene and it is the only cis-acting gRNA (Golden and Hajduk, 2005).

The gRNAs encoded on minicircles are heterogeneous in size, averaging 60 nt in length, and have a very distinct primary structure (fig. 3). The 5'-domain is called the anchor region and forms a duplex with pre-edited mRNA directly 3' to the sequence block that is to be edited. The middle region contains the information domain, dictating specifically which nucleotides to edit. The 3'-end contains an oligo (U) tail that is added post-transcriptionally (Sturm and Simpson, 1990) and is believed to stabilize gRNA:mRNA hybridization (Koslowsky *et al.*, 2004).

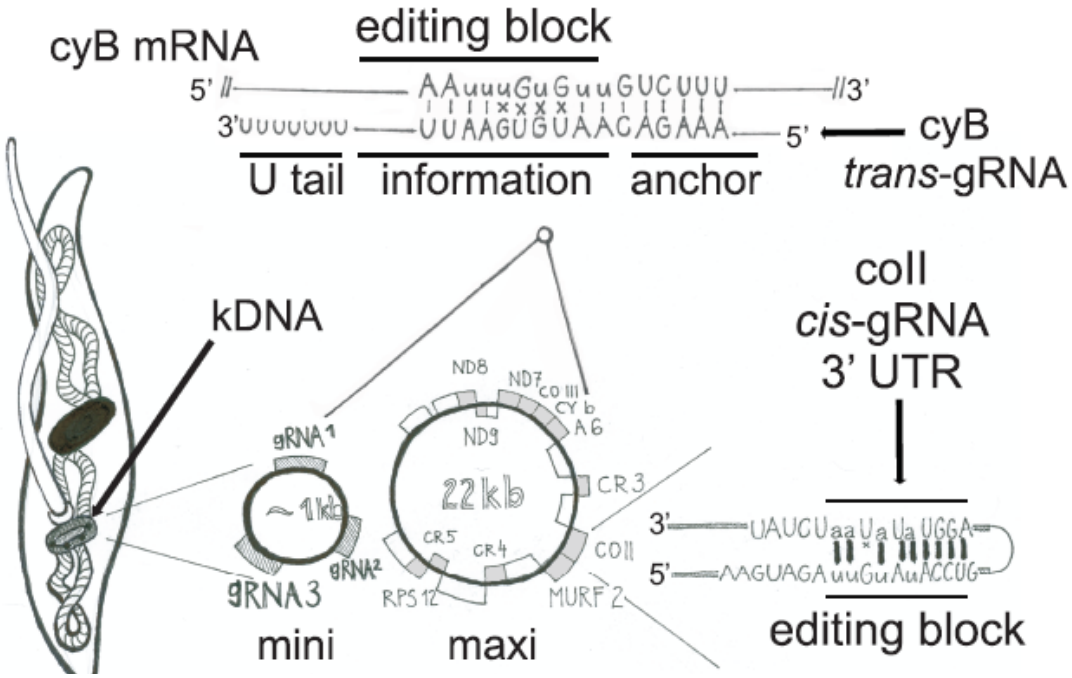

Fig. 3 Guide RNAs mediate RNA editing. A minicircle-encoded *trans*-acting gRNA forms a duplex with its cognate mRNA through hybridization of its anchor domain (**top**). The information domain guides U insertions into and deletions from the RNA until it complements the editing block. Non-canonical U:G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

From Hashimi, 2009

2.4 RNA editing *Trypanosoma brucei*

All forms of RNA editing have been defined as a post-transcriptional modification that changes an mRNA's coding capacity and is distinct from splicing, capping or processing (Keegan *et al.*, 2001). This process can happen either by insertion/deletion of nucleotides, as has been mentioned in trypanosome mitochondrial (mt) mRNAs, or their modification. The latter is a more widespread phenomenon and is found in higher eukaryotes such as plants and vertebrates (Keegan *et al.*, 2001).

The first mention of insertion/deletion editing came in 1986 when Benne and colleagues described the insertion of four uridines into the Co2 transcript in both *T. brucei* and the closely related trypanosomatid *Crithidia fasciculata (Benne et al., 1986)* which is a commensal of the mosquito midgut. The scepticism of the scientific community that went along with this discovery changed into curiosity about the subject two years later when Feagin and co-workers published extensive pan-editing events that occur with the Co3 transcript (Feagin *et al.*, 1988). The mature RNA of this gene contains hundreds of inserted uridines in addition to the deletion of tens as compared to the gene-encoded sequence on

the kDNA maxicricle. To this day, the basic mechanism of RNA editing are known, although the details are still a subject of intense studies.

2.4.1 Uridine insertion/deletion RNA editing mechanism

Guide RNA (gRNA) hybridyzes with its cognate pre-edited mRNA (pre-mRNA) via its anchor region as shown in fig. 3 and 4, creating what is called an anchor duplex. Several catalytic steps follow: the endonuclease cleaves the pre-mRNA at the editing site (ES) where the uridine is to be added by a terminal uridynyl transferase (TUTase) or deleted by U-specific exonuclease (exoUase). The mRNA is then ligated back together (Kable *et al.*, 1996; Seiwert *et al.*, 1996; Igo *et al.*, 2000). A region edited with a single gRNA is called an editing block. Most RNAs require multiple gRNAs in order to create a meaningful ORF. Editing starts at 3' end and proceeds to 5' of the mRNA (Maslov and Simpson, 1992), often creating a new anchor sites at it proceeds (fig. 5).

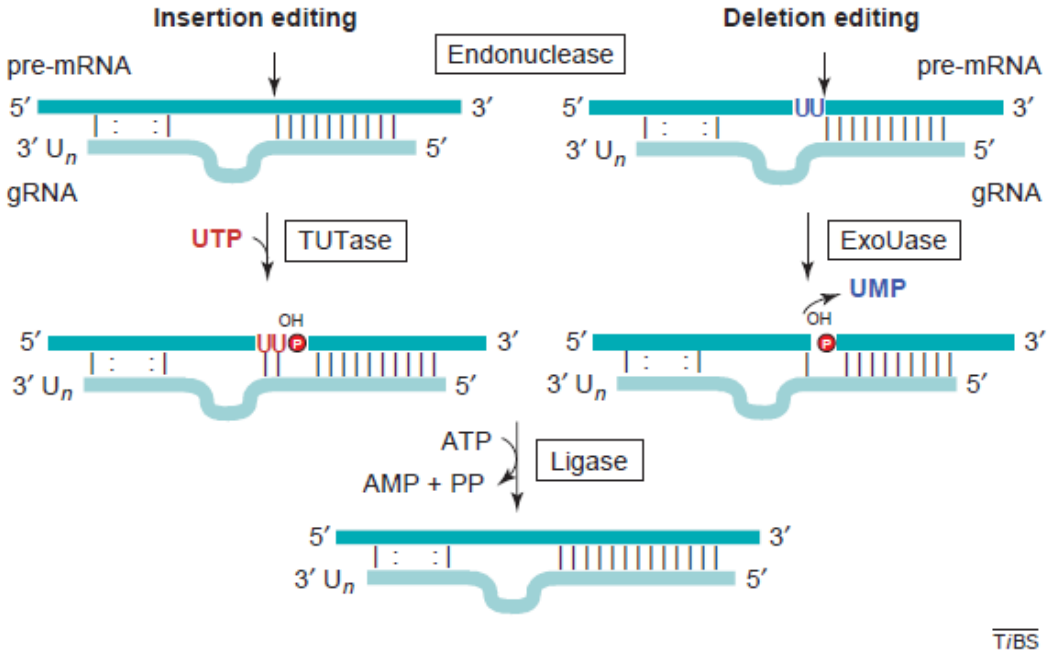

Fig. 4 *General mechanism of RNA editing*

Pre-mRNA (dark blue strand) hybridizes with gRNA (light blue strand) specifying the editing of several sites. Sequence that is edited using single gRNA is called editing block. Interaction between the RNAs by Watson–Crick base-pairs (unbroken lines) and G-U base-pairs (colons) determines the sites of cleavage and number of U nucleotides that are added or removed. Editing occurs by a series of coordinated catalytic steps. Endonucleolytic cleavage of the pre-mRNA occurs upstream of the anchor duplex (arrow). Us are either added to the 5' cleavage fragment by a TUTase in insertion editing or removed by an ExoUase in deletion editing, as specified by the sequence of the gRNA. The resultant mRNA fragments are then ligated by an RNA ligase.

From Stuart et al. 2005

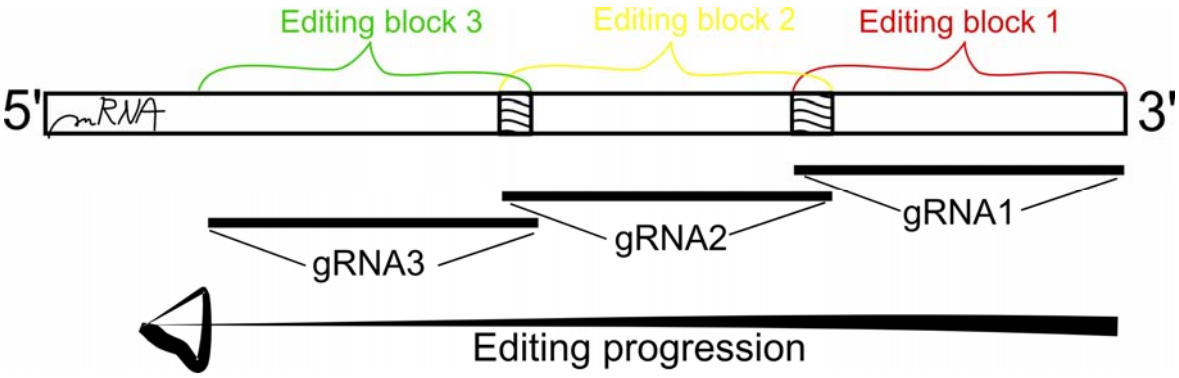

Fig. 5 *Scheme showing the 3'->5' progression of editing through multiple editing blocks*

The editing block is a part of mRNA that is edited using a single gRNA. Overlaps between editing blocks are represented by a wavy square. As gRNA1 finishes editing, an anchor region for gRNA2 is created in the area shown as wavy square.

These catalytic activities are carried out by a complex sometimes known as the 20S editosome. Another name, RNA editing core complex (RECC) has been proposed (Simpson *et al.*, 2010) to reflect that this complex, even though capable of mediating RNA editing *in vitro* (Corell *et al.*, 1996; Kable *et al.*, 1996; Seiwert *et al.*, 1996), is only part of a larger machinery that facilities RNA editing. There are actually three functionally and compositionally distinct RECCs that are distinguished by which RNA editing endonuclease (REN) it contains. While REN1 cleaves RNA at U deletion sites, REN2 does so at U insertion sites. REN3 specifically cleaves the Co2 mRNA, which contains its own gRNA within its 3' UTR (see fig. 3).

2.5 Mitochondrial RNA metabolism in *Trypanosoma brucei*

The whole RNA metabolism of the *T.brucei* mitochondrion, which includes RNA editing, is not still completely understood. In the last two decades many proteins and complexes associated with RNA processing were identified. However, there is still a long way to go to having a textbook diagram of this overall process. While both maxi- and minicircles are polycistronically trascribed by a single mitochondrial RNA polymerase (mtRNAP) (Grams *et al.*, 2002; Hashimi *et al.*, 2009), the maturation pathways of these transcripts dramatically differ afterwards.

Polycistronic minicircle transcripts are thought to be cleaved into a single gRNA molecule by a complex that sediments in a glycerol gradient fractionation at 19 Svedberg units (19S) (Grams *et al.*, 2000). A recently discovered mitochondrial RNA precursor-processing endonuclease 1 (mRPN1) that has these sedimentation properties is reposible for this process, as its RNAi silencing leads to an accumulation of pre-processed gRNAs (Madina *et al.*, 2011). This protein is of particular interest to this thesis due to its

interactions with several proteins belonging to mitochondrial RNA binding complex 1 (MRB1), which will be discussed in section 3.1. The gRNA molecule is then polyuridylylated by kinetoplastid RNA editing 3' terminal uridylyltransferase (TUTase1, RET1) (Aphasizhev *et al.*, 2002; Aphasizhev *et al.*, 2003) and stabilized by guide RNA associated proteins (GAP) 1/2 (Hashimi *et al.*, 2009), also called gRNA binding complex (GRBC) (Weng *et al.*, 2008). RET1 also uridylylates the 3'-ends of the 9 and 12S rRNAs (Aphasizheva and Aphasizhev, 2010).

Maxicircle genes are tightly packed. For many of them, the 5' and 3' ends of flanking ORFs on the immature transcript overlap and the formation of these proper ends means the elimination of UTR or even a portion of the coding sequence of the adjacent gene (Koslowsky and Yahampath, 1997). However, once the polycistronic transcripts are properly cut into single cistron mRNAs, the need to distinguish those that should be translated from those requiring editing arises. Populations of the same transcript that had either short (20-25 nt) or long (120-250 nt) 3' extensions were previously observed (Bhat *et al.*, 1992). While the short tails comprise of adenosine monophosphates added by kinetoplast poly(A)polymerase 1 (KPAP1), the long tails consist of A/U heteropolymers that seem to be synthesized only after the transcript has undergone proper maturation and is ready for translation (Etheridge *et al.*, 2008) The synthesis of these tails requires the cooperation of RET1 and KPAP1. After these tails are formed, the pentatricopeptide repeat (PPR) proteins come into the picture. In *T. brucei* PPR1 (also known as KPAF1) RNAi-mediated depletion leads to selective loss of long tailed mitochondrial mRNA populations (Mingler *et al.*, 2006; Pusnik *et al.*, 2007). But recently, a study has shown that RET1 and KPAP1 cooperation is actually modulated by a heterotetramer formed by KPAF1 and 2 (KPAF1/2) (Aphasizheva *et al.*, 2011). The current hypothesis summarizing the whole process discussed in this section is depicted in fig. 6.

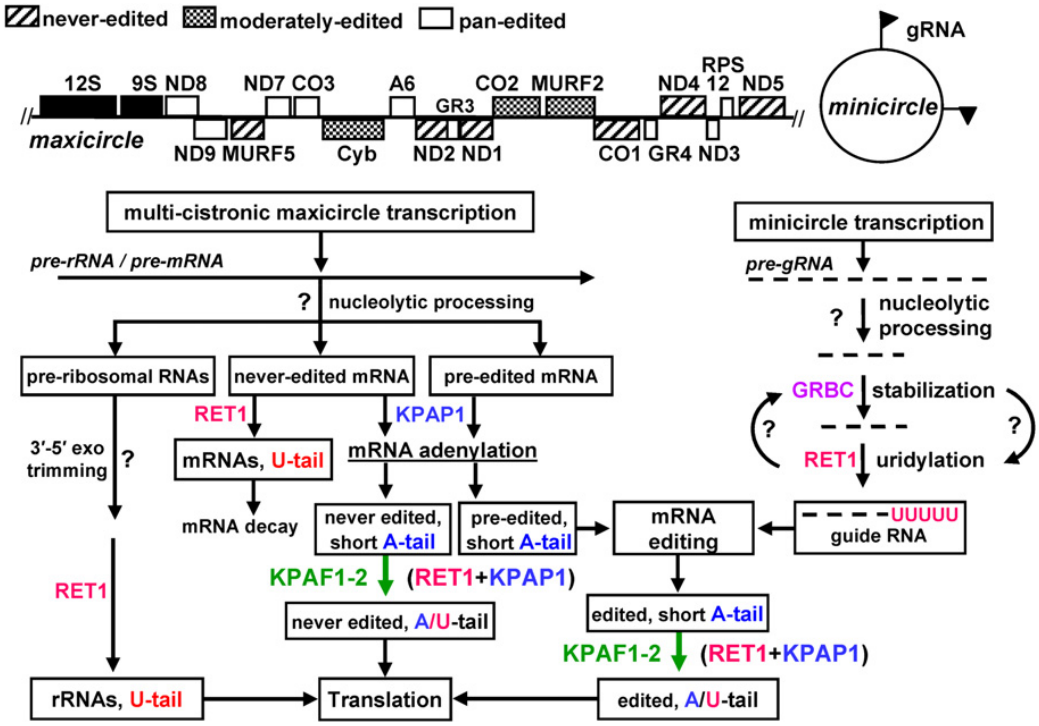

Fig. 6 *General outline of mitochondrial RNA processing in trypanosomes.*

Polycistronic transcripts are produced by mitochondrial RNA polymerase from maxicircle and minicircle components of the kinetoplast DNA. Gene abbrevations are as follows: 12S and 9S, rRNA subunits; ND, NADH dehydrogenase; CO, cytochrome oxidase; Cyb, cytochrome b; MURF, maxicircle unidentified reading frame; CR, C-rich reagion; RPS12, ribosomal protein subunit 12. Processed guide RNAs are stabilized by binding to the GAP1/2 (GRB) complex and 3' uridylated by RET1 TUTase, but the order of events is unclear. Pre-rRNAs are also uridylated by RET1. Editing events in the 3' region confer a requirement for the short A-tail as cis-element necessary for mRNA stability. The completion of editing triggers extension of the pre-existing A-tail into A/U-heteropolymer by KPAF1-2-coordinated, KPAP1/RET1-catalyzed reaction.

From Aphasizhev and Aphasizheva, 2011

To conclude this section I would like to briefly mention mitochondrial edited mRNA stability (MERS1) factor, also known as Nudix hydrolase due to its possessing such a signature motif. Proteins belonging to the Nudix hydrolase superfamily are found in all classes of organisms and hydrolyse a wide range of organic pyrophosphates, including nucleoside di- and triphosphates, dinucleoside and diphosphoinositol polyphosphates, nucleotide sugars and RNA caps, with varying degrees of substrate specificity (McLennan, 2006). The function of this particular hydrolase in the mitochondrion of *T. brucei* remains unclear. Its downregulation led either to drastic decrease of abundance of edited mitochondrial transcripts (Weng *et al.*, 2008) or in another case almost all mitochondrial

transcripts, including never-edited and pre-edited ones (Hashimi *et al.*, 2009). Thus at best we can make a vague conclusion that it affects mRNA stability.

2.6 RNA editing accessory factors

RECC only provides the catalytic activity needed for editing As it it can perform one round of editing *in vitro* (Corell *et al.*, 1996; Kable *et al.*, 1996; Seiwert *et al.*, 1996). A smooth run of pan-editing from the 3' to 5' end of mRNA throughout multiple editing blocks most likely requires many more proteins to mediate the whole process. These proteins are sometimes called RNA editing accessory factors.

Among the peptdides confirmed to have a role in RNA editing are the so-called mitochondrial RNA-binding proteins MRP1/2, which exist as a heterotetramer and facilitate hybridization between maxicircle transcript and gRNA (Schumacher *et al.*, 2006; Zikova *et al.*, 2008). RNAi knockdowns of MRP1/2 have virtually the same phenotype as those of another RNA binding protein, RBP16 (Vondruskova *et al.*, 2005; Pelletier *et al.*, 2000). Their silencing has an adverse effect on cytochrome b (Cyb) editing and decreased stability of never-edited cytochrome oxidase subunit 1 (Co1) and NADH dehydrogenase (ND4) mRNAs. Simultaneous depletion of both factors seems to have a lethal effect in the procyclic stage of *T. brucei*, as opposed to an inhibition of growth, and downregulates several maxicircle mRNAs in a synergistic fashion (Fisk *et al.*, 2009). Interestingly, RPB16 appears to enhance *in vitro* editing activities, perhaps suggesting that it facilitates gRNA/mRNA interactions(Ammerman *et al.*, 2008).

RNA editing helicase 1 (REH1) also known as Hel61 is a DEAD-box protein. *In vitro* experiments have shown that it posseses ATP-dependant double strand (ds)RNA unwinding activity. It is essential for 3'-5' progression of editing through multiple overlapping editing blocks (Li *et al.*, 2011)

TbRGG1 was named for arginine-glycine-glycine (RGG) tripeptide, which is repeated within close proximity of each other and interspersed with aromatic residues, comprising a motif that is present in a number of RNA-binding proteins (Burd and Dreyfuss, 1994). This protein has high binding affinity for oligo-(U) (Vanhamme *et al.*, 1998). RNA interference of TbRGG1 leads to a moderate decrease of abundance of almost all edited mRNAs.

2.6.1 Mitochondrial RNA binding complex 1

MRB1, also known as GRBC, was isolated in 2008 intependantly by three different groups (Hashimi *et al.*, 2008; Panigrahi *et al.*, 2008; Weng *et al.*, 2008). Even though tandem afinity purification (TAP) approach was employed in all three cases, the composition of the complex varied, albeit with enough overlap to support the existence of MRB1 (Fig. 7).

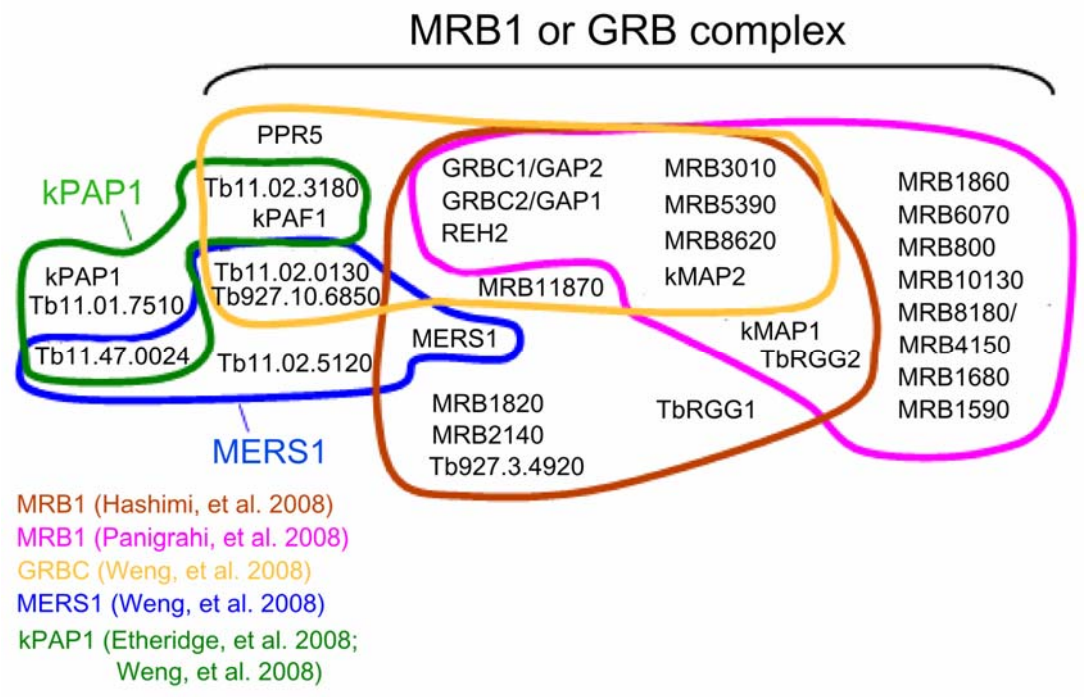

Fig. 7 *Overlapping composition of putative MRB1 and GRB complex*

Putative complexes isolated by different groups using the TAP approach. Overlap occurs not only between MRB1 and GRBC but as well covers some complexes involved in mRNA metabolism. Names of genes were uprated to fit the nomenclature used in this thesis.

From Hashimi, 2009

This discovery was followed by multiple studies that were trying to assign this complex a function and a place in mitochondrial RNA metabolism, possibly among RNA editing accessory factors. This task turned out to be quite complicated, mostly because RNAi on different subunits of MRB1 resulted in variable phenotypes rather than one common to all of them, which would be expected if MRB1 was a *bona-fide* complex. The functional disunity of these proteins taken together with the difficulty to define a precise composition of the complex lead to the current theory that MRB1 are in fact several subcomplexes

linked together by transient interactions. The neccessity to carry out a study that would explore these interactions in detail emerged.

2.6.1.1 Functional studies of MRB1 proteins

Guide RNA associated protein (GAP) 1 and 2 (also known as GRBC) are two homologous proteins forming a heterotetramer (Hashimi *et al.*, 2009; Weng *et al.*, 2008). Their stability is dependant on their mutual association, thus the silencing of any of the two genes resulted in the same phenotype: the overall decrease in gRNAs abundance. The levels of edited mRNAs were subsequently also decreased, with only Co2 edited mRNA remaining unaffected due to its cis-acting gRNA. Thus it seems GAP 1/2 play a role in gRNA stabilization (Hashimi *et al.*, 2009; Weng *et al.*, 2008).

Unlike GAP 1/2, MRB3010 downregulation does not affect gRNAs. It has overall negative effect on edited transcripts that is more severe in the case of pan-edited ones. It seems to facilitate an early step of RNA editing (Ammerman *et al.*, 2011).

TbRGG2 carries the same RNA binding motif as TbRGG1 that was already discussed in previous section. As TbRGG1, it also preferentially binds oligo(U) *in vitro*. Its downregulation leads to a massive decrease in the abundance of pan-edited mRNAs while moderately-edited mRNAs seem to be unaffected. (Fisk *et al.*, 2008; Acestor *et al.*, 2009) Pan-editing in wild-type cells seems to slow down at certain points, mostly at the 3' end of gRNA. These "pause sites" were much more prominent upon TbRGG2 downregulation. *In vitro* assays showed a capacity of TbRGG2 for RNA-RNA interaction modulation. This data taken together with unaffected levels of gRNAs point to a role in progression of pan-editing through gRNA utilization (Ammerman *et al.*, 2010).

RNA editing associated helicase (REH2) has a conserved dsRNA-binding domain (dsRBD) as well as several sequence motifs shared by DExH box protein family. The repression of the translation of this protein leads to overall decrease of gRNA abundance (Hashimi *et al.*, 2009; Hernandez *et al.*, 2010). The effect of REH2 on gRNAs is supported by quantitative real-time (q)PCR showing decrease of all edited transcripts but Co2, that has maxicircle encoded cis-acting gRNA (Hashimi *et al.*, 2009). REH2 has been shown to interact with MRB1, which also appears to associate with mitochondrial ribosomes and some other RNA editing accessory factors; however most of these interactions are purely RNA dependant (Hernandez *et al.*, 2010).

Kinetoplastid mRNA associated proteins (kMAPs) 1 and 2 functional analysis is a topic of the enclosed article. Genes coding these two proteins share 77.1% sequence

identity and a product of recent gene duplication event. However, the data suggest that they are not fully functionally redundant, even though absence of one can be somewhat compensated by the other. They are both novel RNA binding proteins that preferentially bind mRNA despite a lack of any previously described RNA binding motifs. Their simultaneous downregulation leads to decrease of abundance of edited transcripts and Co3 pre-edited one. This effect is more drastic for pan-edited mRNAs and is not caused by gRNA destabilization, since levels of gRNAs remain unaffected upon knockdown. They are most likely to play a role in mRNA stability. The exact mechanism of their function remains as a topic of future study.

2.6.1.2 MRB1 structure

As it was mentioned earlier, the composition of different MRB1 isolations, while seemingly containing core of proteins always present, also varied (Fig. 6). A recent study by Ammerman and colleagues employed several different approaches in order to elucidate protein and RNA mediated interactions within MRB1 complex. A pairwise yeast two-hybrid (Y2H) screen of 31 MRB1 proteins was undertaken. In this method one protein fused with a DNA binding domain and another one with the activation domain of a transcription factor are expressed in yeast; if they interact, transcription of a reporter gene is triggered. To complement yeast two-hybrid data several subunits of MRB1 were ProtC-TEV-ProtA (PTP) tagged, a variation of the TAP methods (Schimanski *et al.*, 2005), and pull-downs were analyzed by mass spectrometry and Western analysis. These experiments revealed that MRB1 most likely has a core composed of MRB3010, GAP 1/2, MRB11870, MRB5390 and MRB8620. This core has RNA enhanced interaction with TbRGG2 that forms a subcomplex with kMAP1/2; for details see fig. 8. Furthermore, numerous weak interactions of MRB10130 with components of MRB1 as well as kPAP1 and MERS1 complexes were identified. MRB10130 is almost entirely composed of ARM/HEAT repeat units, which often act as a protein – protein interaction platforms (Ammerman *et al.*, 2012). Whether MRB10130 functions as a coordinator between all these complexes remains a question to be resolved.

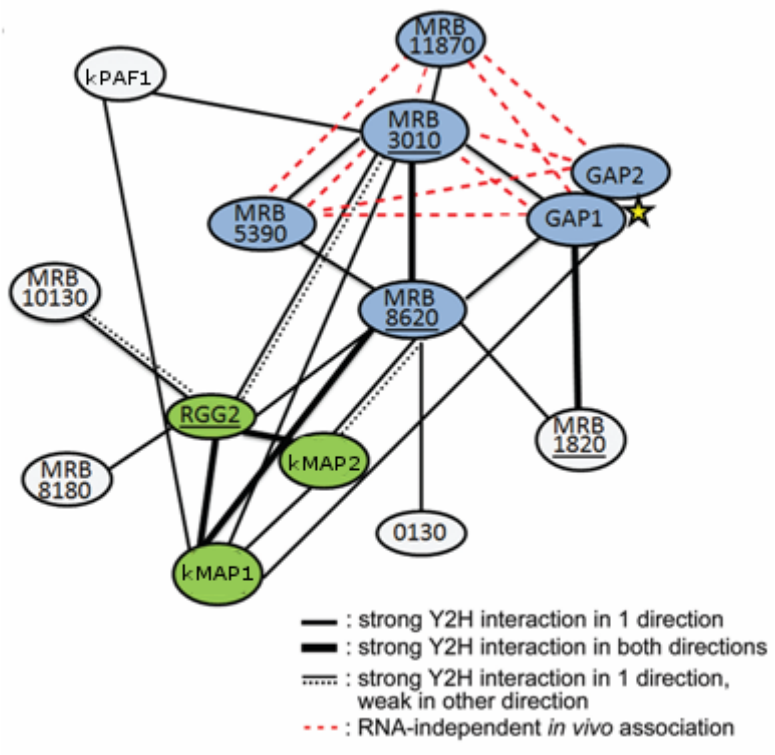

Fig. 8 *Schematic representation of all yeast two-hybrid (Y2H) strong interactions*

Thin black lines represent a strong interaction in one direction, thick black lines represent strong interactions in both directions, a thin black line with a dashed line represents a strong interaction in one direction and a weak interaction in the other direction, and an underline represents self-interaction. The yellow star is used to indicate that the GAP1 and GAP2 interaction is based on previous work, not yeast two-hybrid results. The MRB1 core complex is shown in blue and the TbRGG2 subcomplex is shown in green. Red dashed lines represent RNA-independent interactions between core proteins identified in *in vivo* purifications

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3. References for Introduction

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4. Aims of the project

- apply bioinformatics to gain information about possible conserved domains and other features of two highly similar hypothetical proteins associated with putative MRB1 complex (here already named kMAP1 and 2)
- create single and double knockdown cell line of kMAP1 and 2
- assay growth of knockdown cell lines
- perform quantitative RT-PCR to see whether these KDs affect levels of mitochondrial transcripts
- assay levels of gRNAs in the dKD background to explore the possibility that these proteins share function in gRNA stability with previously described components of MRB1: GAP1/2.
- assay the cosedimentation of kMAPs with other MRB1 components in glycerol gradient
- create a cell line with TAP-tagged kMAP 1 and kMAP2, isolate the RNP they are part of and assay its composition using mass-spectrometry
- explore the RNA binding capacity of kMAP1 and 2
- use the data to elucidate the role of kMAP 1 and 2 within MRB1 complex

Research article currently submitted to RNA journal

Lucie Kafková, Michelle L. Ammerman, Drahomíra Faktorová, John C. Fisk, Sara L. Zimmer, Roman Sobotka, Laurie K. Read, Julius Lukeš and Hassan Hashimi. Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei.* Submitted to RNA

ABSTRACT

A majority of *Trypanosoma brucei* proteins have unknown functions, a consequence of its independent evolutionary history within the order Kinetoplastida that allowed for the emergence of several unique biological properties. Among these is RNA editing, needed for expression of mitochondrial-encoded genes. The recently discovered mitochondrial RNA binding complex 1 (MRB1) is comprised of proteins with several functions in processing organellar RNA. We characterize two MRB1 subunits that are paralogs arisen from a large chromosome duplication occurring only in *T. brucei*. As many other MRB1 proteins, both have no recognizable domains, motifs or orthologs outside the order. They are both novel RNA binding proteins that we show to preferentially bind mRNA. They associate with a similar subset of MRB1 subunits but not directly with each other. We generated cell lines that ether individually or simultaneously target the mRNAs encoding both proteins using RNAi. Their dual silencing results a differential effect on moderately- and pan-edited RNAs, suggesting a functional separation of the two proteins. Cell growth persists upon RNAi-silencing of each protein individually in contrast to the dual knockdown. Yet, the notion that they are redundant is refuted as one of these knockdowns results in the general destabilization of pan-edited RNAs while the other has no discernable effect. Our results have prompted us to name them kinetoplastid mRNA associated proteins (kMAP) 1 and 2. While the kMAPs share a considerable degree of conservation, their recent sequence divergence has led to them influencing mitochondrial mRNAs to differing degrees.

6. Discussion

The striking sequence similarity between kMAP1 and kMAP2 would point to redundancy of these two proteins. This hypothesis would be supported by the absence of a growth phenotype in the single KD cell lines, similar composition of the RNP they are associated with and their capacity to bind RNA *in vitro*. However, while kMAP1 sKD affects the levels of pan-edited mRNAs to some degree, kMAP2 single KD does not alter levels of mRNA transcripts. Also, the glycerol gradients performed on kMAP1 sKD show a slight shift in MRB3010 and GAP1 sedimentation unlike sKD of kMAP2. This leads us to believe that the 5' end sequence divergence between these two proteins actually affects their function.

According to the work done by Ammerman and colleagues (Ammerman *et al.*, 2012) on MRB1 architecture, both kMAPs bind to TbRGG2 and MRB8620 in the yeast two hybrid system, but kMAP1 has additional interactions with MRB3010 and kPAF1, a PPR protein that is thought to mediate creation of long A/U tails. We have to be careful about interpretation of negative results from Y2H, because of technical reasons such as the tag inhibiting protein interaction or low expression of the tagged protein. However, it is possible, that while the conserved part of their sequence fulfills the same function, the diverged region is responsible for these additional interactions. These interactions might also be responsible for the different effect of single knockdowns (sKDs) on mitochondrial transcript abundancy. Regardless of these subtle differences, the phenotype fully manifests upon simultaneous depletion of both kMAPs. The kMAP1/2 dKD cell line grows slower. Pan-edited mRNAs are severly downregulated in their fully edited form, but we do not observe any accumulation of pre-edited form of these transcripts. In contrast with this result is an effect on moderately-edited transcripts that accumulate in their pre-edited form and are only slighty downregulated in their fully edited form. The question is, whether the latter effect is caused by disruption of protein-protein interactions which decreases the effectivity of editing process or it is a direct effect of kMAPs downregulation.

There are several possiblities why the edited mRNA abundancy is altered upon RNAi of any RNA editing accessory factor. First, we explored the possibility that the effect would be caused by destabilization of gRNAs. However, we did not see any change in abundancy of gRNAs by Northern analysis or by the guanylyltransferase labeling method. Also, the Co2 edited transcript that carries in its 3'-UTR its cis-

acting gRNA is affected in kMAPs dKD cell line. The kMAPs thefore do not share the gRNA stability function with two core MRB1 subunits GAP1/2.

The next possibility is that the kMAPs affect the progression of pan-editing as TbRGG2 does. The build up of partially-edited transcripts would not be visible by qPCR, since the primers we use apmlify either transcripts completely unedited or fully edited. To test this possibility we used gene-specific PCR. This method uses a primer at the very 3' end of the gene in a never-edited region and amplifies the whole population of targeted transcript. The reaction was run on a polyacrylamide gel. However, we did not see any change between induced and non-induced cells, which could either mean that there is no accumulation of intermediates or that the resolution of the gel was not sufficient. The result was thefore evaluated as inconclusive, was not included in the manuscript and this function remains a possibility until a clear result can be obtained.

It seems that mRNAs of pan-edited transcripts are destabilized upon kMAP1/2 dKD induction, but we lack option to prove this hypothesis *in vivo*. The levels of unedited transcripts can be measured in the course of time after the transcription has been inhibited by actinomycin D. However, we do not have a tool to stop editing in these cells. Therefore the persisting editing would shift the pre-edited/edited mRNA ratio, which would make any measurements of edited mRNA levels highly unreliable.

We could speculate that since pre-edited versions of pan-edited transcripts are not accumulated, as in the case of MRB3010 that is believed to facilitate an early step in editing (Ammerman *et al.*, 2011) and preliminary results suggest a role in progression of editing to be rather unlikely, that kMAP1/2 could play a role in the final stage of editing. As it has already been suggested in the introduction, MRB1 probably does not contain many strong direct protein-protein interactions, leaving the possiblity that the complex is flexible and changes its composition. There is no evidence, that moderately-edited mRNAs and pan-edited mRNAs would be handled in a different way, although it is possible that certain conditions have to be met for editing to start and end. These conditions could be achieved differently for transcripts that require gRNAs to overlap (pan-editing) and moderately edited transcripts. The kMAPs could only be essential for the initiation of editing under a condition that the editing block in question does not create an anchor site for another gRNA. This would mean that in the case of moderately-edited mRNAs, the editing would not even start in the kMAPs dKD background, resulting in an accumulation of pre-edited transcripts

and downregulation of edited ones. For pan-edited mRNAs, the kMAP role would be played by a different protein, TbRGG2 would then facilitate progression of editing, but at the very last gRNA the same situation as with moderately-edited mRNAs would arise and the editing would not even initiated for the last editing block. The primers we used to see edited mRNAs either by qPCR or northern are designed to anneal to this very last part of editing, therefore these RNAs would not be visible by these methods and the result would look the same way as if they were degraded. To explore whether this hypothesis is valid we could employ an RNA-seq approach that would allow us to properly quantify all RNA editing intermediates. Another approach would be to resolve the aforementioned gene-specific PCR using a high resolution gel in order to clearly see a potential difference due to a few added nucleotides.

The last thing I would like to consider is the role of kMAP1 interaction with kPAF1. kPAF1/2 have been proposed to modulate creation of long A/U tails on the 3' end of fully-edited transcripts (Aphasizheva *et al.,* 2011). It is unclear, how are these fully-edited transcripts recogized. kMAP1 could be the factor that stabilizes mRNA in the course of editing and signals kPAF1 once it has finished.

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