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Ph.D. Thesis

The role of RNA-binding proteins in post-transcriptional gene regulation of *Trypanosoma brucei*

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ANOTATION

This thesis characterizes RNA footprints of several RNA-binding proteins (RBPs) that are involved in U-insertion/deletion, A-to-I, and C-to-U RNA editing in *Trypanosoma brucei*. Relying on iCLIP data and biochemical methods it shows that two paralogs proteins from the MRB1 complex regulate distinct editing fates of the mitochondrial transcripts. Further, this thesis provides evidence where the combinatorial interplay of RBPs might fine-tune the levels of edited mRNA. Finally, the presented thesis adds to the growing evidence of the importance of RBPs in post-transcriptional gene regulation.

FINANCIAL SUPPORT

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DECLARATION

I declare that all the work presented in this thesis is carried out by myself or in collaboration with the presented co-authors by using cited literature.

České Budějovice, 2018 Sameer Dixit

PROHLÁŠENÍ

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STATEMENT REGARDING CONTRIBUTION

1. **Sameer Dixit,** Michaela Muller-McNicoll, Vojtěch David, Kathi Zarnack, Jernej Ule, Hassan Hashimi, Julius Lukeš. Differential binding of mitochondrial transcripts by MRB8170 and MRB4160 regulates distinct editing fates of mitochondrial mRNA in trypanosomes**. mBio**, 31 January 2017 mBio vol.8 no. *1 e02288-16.*

Sameer Dixit carried out all the wet-lab experiments, contributed in analyzing bioinformatics data and helped writing the manuscript.

2. **Sameer Dixit,** and Julius Lukeš. Combinatorial interplay of RNA-binding proteins fine-tune levels of edited mRNA in trypanosome mitochondrion. (Under submission).

Sameer Dixit carried out all experiments, analyzed data and participated in writing of the manuscript.

3. Zdeněk Verner, Somsuvro Basu, Corinna Benz, **Sameer Dixit,** Eva Dobáková, Drahomíra Faktorová, Hassan Hashimi, Eva Horáková, Zhenqui Huang, Zdeněk Paris, Priscilla Pena-Diaz, Lucie Ridlon, Jiří Týč, David Wildridge, Alena Ziková, Julius Lukeš. Malleable Mitochondrion of Trypanosoma brucei. Int Rev Cell Mol Biol. 2015;315:73-151.

Sameer Dixit co-wrote the "RNA editing" section.

Julius Lukeš, the corresponding author of all mentioned papers, approves the contribution of Sameer Dixit in these papers as described above.

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Prof. RNDr. Julius Lukeš CSc

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SUMMARY

Trypanosoma brucei belongs to the group of excavate protists of the kinetoplastid clade. It is the causative agent of human sleeping sickness and nagana in livestock. Trypanosomes are easy to maintain and amenable to a range of experimental techniques, which makes them by far the best-studied model organism from the early branching kinetoplastids. Studies of the trypanosome yielded many key biological insights, some of which were later extended to other eukaryotes, while some molecular and cell biology aspects remain unique to these parasitic flagellates. RNA editing is one such example, as first described in trypanosomes and only subsequently encountered widely across eukaryotes. Due to invariably polycistronic transcription, trypanosomes rely heavily on post-transcriptional regulation of gene expression. This unique feature makes trypanosomes an ideal model organism to study the regulatory rules governing the post-transcriptional gene regulation with the minimal transcriptional noise. The RNA binding proteins (RBPs) play a crucial role in post-transcriptional regulation, and this thesis focuses on their role in various pathways associated with RNA editing.

The thesis comprises of four chapters. The first three chapters focus on mitochondrial (mt) RNA editing, while the last one includes RNA binding footprints of trypanosome adenosine deaminase enzymes acting on tRNAs (TbADATs) that are involved in A-to-I and C-to-U RNA editing.

1) The first chapter deals with two novel RNA binding proteins named MRB8170 and MRB4160 that are implicated in mt RNA editing. We characterized RNA binding footprints of both the RBPs by using the *in vivo* UV-crosslinking and immunoprecipitation (iCLIP) methodology. Our study suggests MRB8170/MRB4160 marking of pre-edited mRNAs initiates their editing.
   (Published results, Dixit et al., 2017 mBio)

2) In the second chapter, we applied an iCLIP protocol on the RBP named MRP1. The rationale for the study was the interaction of MRB8170 with

both MRP1 and TbRGG2 that assists their capacity to bind poly (A) RNA. To explore if MRP1 and TbRGG2 compete for MRB8170, we made a quantitative comparison of bound RNA between MRP1, TbRGG2, and MRB8170. The study confirms their competitive binding and provides a mechanistic model to explain different levels of edited molecules (Currently under review)

3) The third chapter is a review describing various facets of trypanosome mitochondrial biology, where my contribution is limited to RNA editing section.
(Published review, Verner et al., 2015 Int Rev Cell Mol Biol)

4) In the fourth chapter, we characterized genome-wide RNA binding of trypanosome adenosine deaminase enzyme acting on tRNAs (TbADATs) that is involved in A-to-I and C-to-U RNA editing. The study demonstrates TbADAT2 binds 17 mRNAs and a single non-coding RNA, the functional relevance of which needs further validation. (Unpublished study)

1. OVERVIEW

T. brucei is a representative of the class Kinetoplastea. Members of the genus Trypanosoma pose a serious threat to the sub-Saharan countries as highly pathogenic parasites of humans and livestock. Their morbidity and mortality are aggravated by the lack of vaccines. The only treatment based on the chemotherapeutic drugs with low efficacy and toxic side effects argues for further efforts in drug discovery research (Steverding, 2010).

Trypanosome is well known to harbor a wide range of unique cellular and molecular features, exceeding in this respect to most other eukaryotes (Berriman et al., 2005; Fenn and Matthews, 2007; Horn, 2014; Matthews, 2005; Verner et al., 2015). The notable examples include; 1) antigenic variation using programmed DNA rearrangement; 2) compartmentalized glycolytic pathway in a membrane-bound organelle named glycosome; 3) polycistronic transcription of nuclear-encoded genes that is followed by their *trans*-splicing to generate mature mRNAs; 4) a single mitochondrion that requires import of all tRNAs due to their absence in mt DNA; 5) A prominent feature of their mitochondrion is an exceptionally complex RNA editing, which requires hundreds of non-coding guide RNAs and the dozens of proteins to edit just 12 transcripts; 6) the acidocalcisomes to store calcium. Additionally, the fully sequenced genome revealed that the ~60 % of *T. brucei* genes share no identity with other eukaryotes outside kinetoplastids, making trypanosomes an attractive model organism for basic research (Berriman et al., 2005).

T. brucei has a complex life cycle, which includes a mammalian host and a tsetse fly of the genus *Glossina*. It starts when an infected tsetse fly injects the metacyclic stage into mammalian skin tissues while taking the blood meal. Afterwards, metacyclics invade the lymphatic system and eventually reside in the bloodstream, a development that coincides with their transformation into the bloodstream trypomastigote stage. Meanwhile, they keep on replicating by binary fission, consuming glucose and evading immune

response of the host. Some cells transform into the stumpy form that is taken up by a tsetse fly during its blood feeding, marking the completion of the life cycle in the mammalian host.

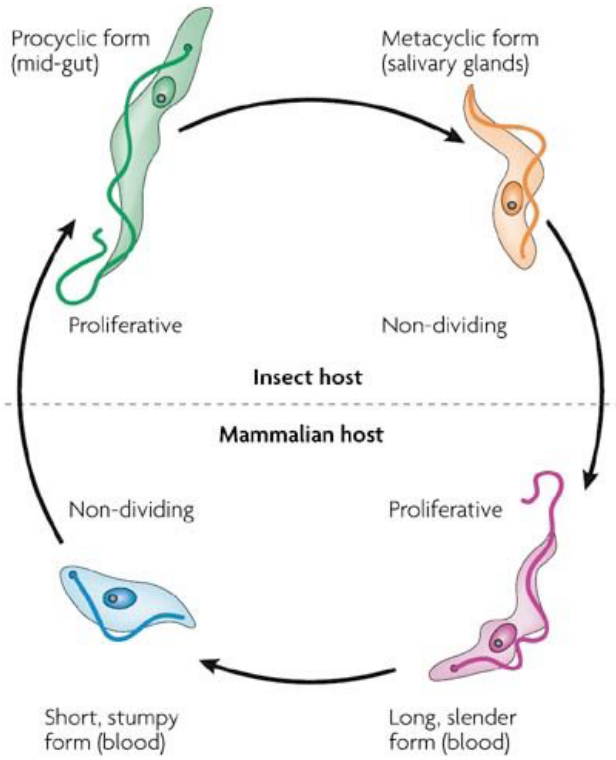

Figure1. Schematic depicting lifecycle of *T. brucei* taken from (Stephens et al., 2007)

The above-described life cycle means that trypanosomes encounter dramatically different environments. In response, they require a constant regulation of gene expression (Clayton, 2014; Shaw et al., 2016). The bulk of gene regulation is controlled at the post-transcriptional level, while transcription contributes little to the process (Clayton, 2013, 2014). In this context, RBPs emerge to be the key players in the trypanosome gene

regulation (Kolev et al., 2014). This is nicely exemplified by a recent study demonstrating that an over-expression of a single RBP, named TbRBP6, can trigger the developmental progression into the infective stage (Kolev et al., 2012).

The presented thesis includes characterization of RNA binding footprints of four proteins using *in vivo* UV cross-linking and immunoprecipitation (iCLIP) protocol. The first three proteins named MRB8170, MRB4160 and MRP1 are implicated in mt RNA editing, while TbADAT2 is an adenosine deaminase enzyme that is involved in A-to-I and C-to-U RNA editing in tRNAs (Rubio et al., 2017).

2. INTRODUCTION

2.1 Mitochondrial gene expression in *T. brucei*

The single mitochondrion of trypanosome carries a massive amount of DNA or known as kinetoplast (k) DNA, which is located close to its posterior end and in proximity to the flagellar basal body (Englund, 1978; Woodward and Gull, 1990). The kDNA replication is synchronized with the nuclear DNA and occurs once per cell cycle. The kDNA comprises of a dozen maxicircles and thousands of mutually concatenated minicircles (Read et al., 2016; Stuart, 1983). Each maxicircle is 23 kb long and is homologous to standard eukaryotic mt DNA. Maxicircle encodes two ribosomal (r) RNA and 18 protein-coding genes, the products of which mostly constitute subunits of the respiratory chain (Clement et al., 2004; Verner et al., 2015). To become translatable, 12 of 18 mt transcripts undergo an intricate process of uridine (U) insertions and deletions to generate correct open reading frames (ORFs). The entire process of U- insertion/deletion is called RNA editing (Read et al., 2016; Verner et al., 2015). Meanwhile, from each ~1 kb long minicircle, the short non-coding RNAs termed guide (g) RNAs are transcribed. Every gRNA contains the anchor domain that is antisense to the pre mRNAs region, while its information domain has a mismatch region, which guides the U insertions/deletions. Remarkably, the kDNA of trypanosomes lacks tRNA genes and therefore imports all the tRNAs from the cytosol into the mitochondrion (Alfonzo and Lukeš, 2011).

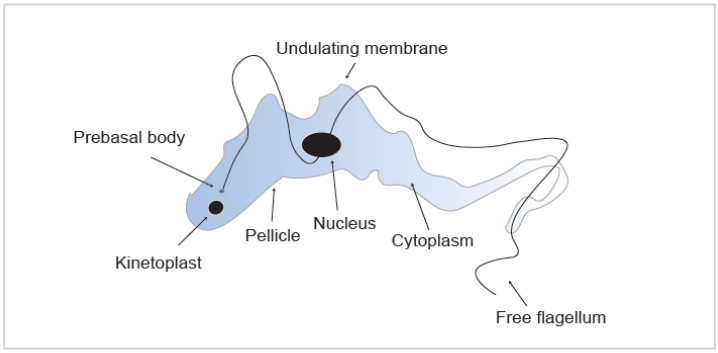

Figure 2. Basic morphology of *T. brucei* adapted from (Matthews, 2005)

2.2 kDNA replication and maintenance

The kDNA is a characteristic feature of the kinetoplastid flagellates with specific localization. The uniquely complex composition of the kDNA network requires several dozens of proteins for its replication and maintenance, most of which are confined exclusively to trypanosomes. Until now 30 proteins have been assigned to the kDNA replication and (or) maintenance, but the final list may contain more than 150 proteins (Jensen and Englund, 2012). The assigned proteins include well-conserved enzymes like ligases (Sinha et al., 2004, 2006) and topoisomerases (Bakshi and Shapiro, 2004; Li et al., 2008; Scocca and Shapiro, 2008; Wang et al., 2000), including six helicases (Klingbeil and Shapiro, 2009) and seven DNA polymerases, mostly unique to trypanosomes (Klingbeil et al., 2002; Rajão et al., 2009). Moreover, the list includes kinetoplastid histone-like proteins termed KAPs, which are critical for packaging and maintaining the structural integrity of kDNA (Avliyakulov et al., 2004), and a host of other proteins , the function of which remains unknown.

Replication of the kDNA minicircles has been dissected to a considerable detail, while the maxicircle replication remains a mystery primarily due to their low copy number. Primase Pri1 and polymerases Pol1C and Pol1D are considered to play a role in both maxicircle and minicircle replication (Hines and Ray, 2011; Liu et al., 2010). The binding of universal minicircle sequence binding protein to the conserved minicircle sequence element initiates the replication of minicircles, while a protein termed p38 helps in recognizing the origin of replication. The entire kDNA replicates once per cell cycle and the process is completed before the nuclear S phase (Jensen and Englund, 2012).

2.3 Mitochondrial transcription

In maxicircle, the conservative region of ~17 kb long contains rRNA and protein-coding genes, while remaining ~6 kb long divergent region carries repetitive sequences (Sloof et al., 1992). The transcription start site in maxicircle is ~1.2 kb upstream of the 12S rRNA on the major strand, while the same remains undetermined for the minor strand (Michelotti et al., 1992). In a small subset of mapped minicircles,

both strands are supposedly transcribed (Pollard et al., 1990). Both maxicircles and minicircles use a common mt RNA polymerase for their transcription, which presumably yields polycistronic transcripts and requires endonucleolytic cleavage for further processing (Clement and Koslowsky, 2001; Grams et al., 2002; Hashimi et al., 2009). After decades of substantial effort, the promoter region and proteins involved in transcription remain elusive. Another open question is if both kDNA molecules share cis-elements and transcriptional complexes or use a different array of promoters and transcription factors (Aphasizhev, 2007; Aphasizhev and Aphasizheva, 2011a).

Maxicircle-transcribed polycistronic transcripts require several RNA processing steps before translation, which is performed by hundreds of imported proteins (Lukeš et al., 2011). Presumably, an initial cleavage step needs endo- and exo-nuclease enzymes, but the identity of such proteins remains under investigation (Koslowsky and Yahampath, 1997). Most likely, the multi-protein complex named RNA editing core complex (RECC) contains three RNase III-type endonucleases and two 3`-5` exonucleases (Carnes et al., 2008; Ernst et al., 2009), and may therefore participate in these processing steps (Aphasizhev and Aphasizheva, 2011a, 2011b). Multiple endonucleolytic cleavages of the polycistronic maxicircle transcript give rise to 18 pre-mRNAs with short 5` and 3` UTR and no apparent ribosome-binding sites. Further journey of the pre-mRNAs in order to become translatable requires; 1) their stabilization; 2) U-insertion/deletion RNA editing in a subset of pre-mRNAs; 3) incorporation of structural features for ribosome binding.

Minicircles are highly heterogeneous, and can therefore be segregated into 200 to 400 sequence classes (Ochsenreiter et al., 2007). Each minicircle encodes 2 to 5 gRNAs, which adds up to an extremely diverse gRNA population available for editing. Additionally, minicircles also encode similarly sized gRNA-like molecules, the functional relevance of which is yet to be defined (Madej et al., 2008). In minicircles polycistronic transcripts are cleaved into individual gRNAs by mt endonucleolytic processing endonuclease 1 (Madina et al., 2011). Strikingly, unlike their maxicircle homologues, the minicircle-encoded RNAs retain 5` triphosphate, possibly reflecting the lack of 5` processing in them (Blum and Simpson, 1990; Blum et al., 1990; Zimmer et al., 2011).

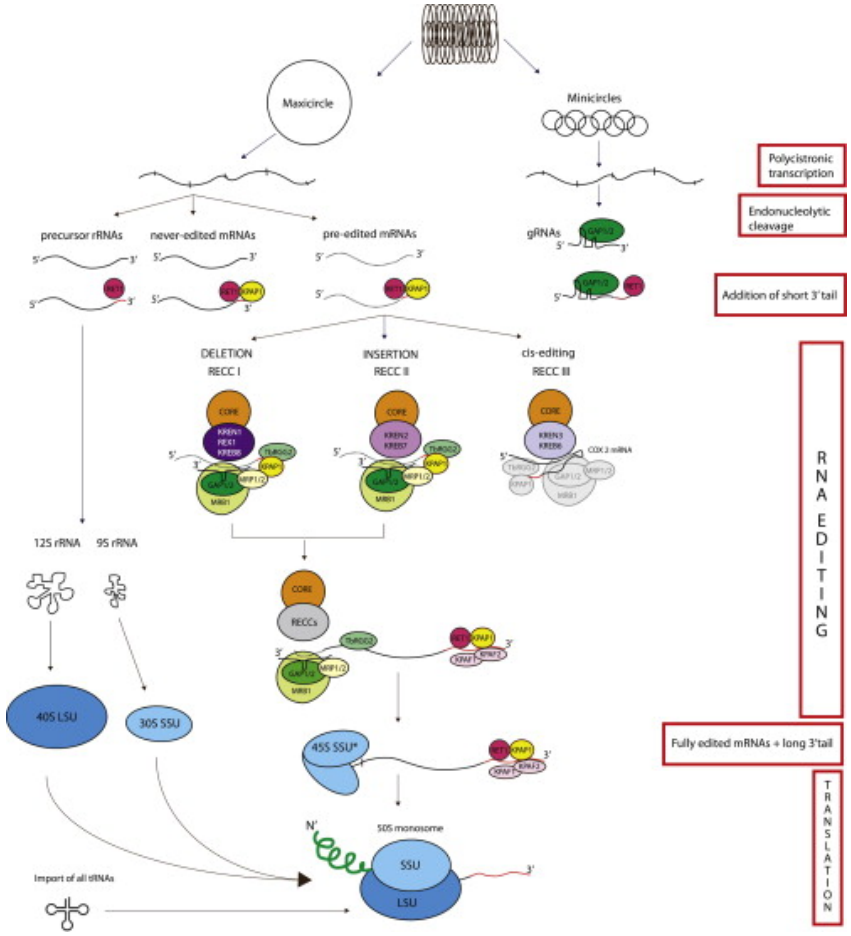

Figure 3. Mitochondrial gene expression.
The major steps in mitochondrial gene expression and translation are depicted in a schematic manner. Taken from (Verner et al., 2015).

2.4 Mitochondrial RNA processing

The next step after cleavage is an addition of short 3` tail to different categories of cleaved RNA molecules. The addition of short 3` U-tail to pre-rRNAs by RNA editing terminal uridylyl transferase RET1 allows its integration into the mt ribosome (Aphasizhev et al., 2003; Aphasizheva and Aphasizhev, 2010). Similarly, RET1 activity adds 3` oligo (U) tails to gRNAs (Blum and Simpson, 1990). However, it is not clear what role the oligo (U) tails play in gRNAs, since RET1 depletion does not impact the stability of gRNAs (Aphasizheva and Aphasizhev, 2010; Weng et al., 2008).

The 3` tail helps to determine mRNA stability and allows their access into the small ribosomal subunit (Read et al., 2011). In trypanosomes, the *in vitro* studies demonstrated that the short poly (A) tail in edited mRNAs protects them from 3`-5` degradation (Kao and Read, 2007; Ryan and Read, 2005). In contrast, a similar short tail leads to the degradation in pre-edited mRNAs (Kao and Read, 2005). To explain such perplexing results, the poly (A) tail switches from a destabilizing mode to a stabilizing when pre-edited mRNAs transforms into edited version, although this theory needs further validation. The addition of 3` tail requires several protein complexes (Paolo et al., 2009; Vaňáčová et al., 2005). In trypanosomes, the polyadenylation complex with its subunit named kinetoplastid poly (A) polymerases 1 and 2 (Etheridge et al., 2008; Kao and Read, 2007; Zimmer et al., 2012) synthesizes both short and long 3` tails. The next step after the addition of poly (A) tail is its elongation by attaching A/U tracks, by the action of kinetoplastid polyadenylation/uridylation factors 1 and 2, both of which belong to the pentatricopeptide repeat (PPR) protein family (Aphasizheva et al., 2011).

In pre-mRNAs, the addition of short tail is not as straightforward, since 12 mRNAs undergo RNA editing, while the other six do not. The mt mRNA either carries a short (20-50) or long (200-300) poly (A) tail, determined in a transcript-specific manner in both life stages (Bhat et al., 1992; Gazestani et al., 2016; Koslowsky et al., 1991). Astonishingly, there seems to be no simple correlation between the editing requirement, the length of poly (A) tail, and their mRNA abundance (Read et al., 1994). Consequently, fully- or never-edited mRNAs either possess long or short poly (A) tail, while mostly pre-edited mRNAs displays short (A) tails. After the addition of poly (A) tails, never-edited mRNAs further require two more rounds of 3` tail addition for their translation (Aphasizhev and Aphasizheva, 2011; Verner et al., 2015). The addition of short 20-25 nt long U/A/AU tails is followed by their elongation to 12-250 nt long A/U tails (Etheridge et al., 2008). Meanwhile, in pre-edited mRNA, the latter step of tail elongation is coupled with RNA editing.

Another pathway regulating mt RNA levels operates via RNA degradation. It provides a quality control mechanism by eliminating aberrant RNAs. A recently discovered degradosome-like complex composed of subunits TbSUV and TbDSS-1

was shown to have 3` to 5` exoribonuclease and RNA helicase activities (Mattiacio and Read, 2008, 2009; Penschow et al., 2004). This makes them suitable candidates for the poorly understood mt RNA degradation pathway.

2.5 U- insertion/deletion type of mt RNA editing

RNA editing was first described in the mitochondrion of *T. brucei* as a post-transcriptional addition of four uridine residues in the cytochrome c oxidase subunit 2 mRNA (Benne et al., 1986). Nowadays, RNA editing is recognized as any change in mRNA at the post-transcriptional level, however, excluding splicing and terminal RNA processing events. RNA editing comes in several flavors, the U-insertion/deletion RNA editing in the mitochondria of kinetoplastids is just one out of many types (Verner et al., 2015). As another example, the deaminase activity performs cytosine-to-uridine (C-to-U) and adenosine-to-inosine (A-to-I), which is mostly found in plants and metazoans, respectively (Nishikura, 2015) .

In the trypanosome mitochondrion, 12 out of 18 protein-coding transcripts require U-insertion/deletion to become translatable. The extent of editing varies, and consequently different number of gRNAs is employed (Read et al., 2016). Based on the varying editing level, mRNAs are divided into the following categories: i) pan-edited mRNAs, which requires massive U-insertion/deletion throughout the transcripts and employ multiple gRNAs; ii) minimally-edited mRNAs is a group of three transcripts that need only a few U-insertion/deletion over a small region and utilize just one or two gRNAs; iii) the remaining six mRNAs do not require editing and are known as never-edited mRNAs.

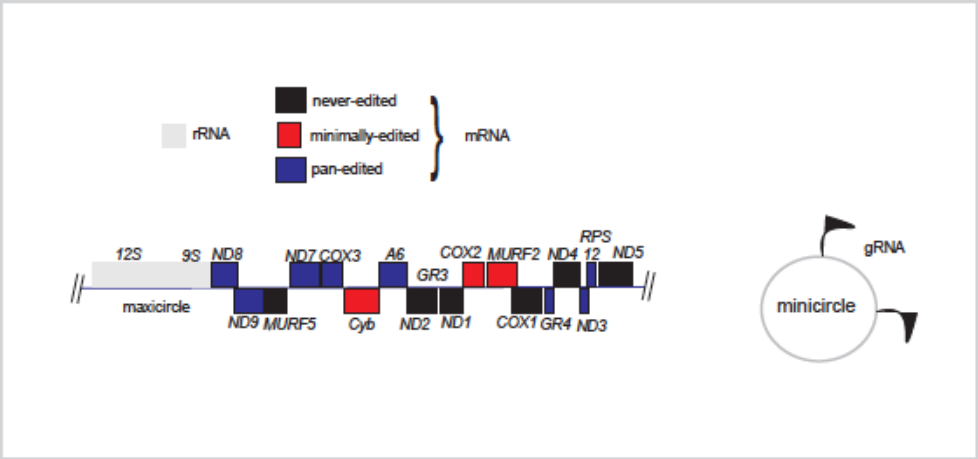

Figure 4. The schematic representation of genes belonging to maxicircle and minicircle DNA. *A6*, ATP synthase subunit 6; *COX1, 2 and 3*, cytochrome c oxidase subunit 1, 2 and 3; *Cyb*, cytochrome b; *GR*, G-rich region; *MURF1, 2, and 5*, maxicircle unidentified reading frame 1, 2 and 5; *ND1, 3, 4, 5, 7, 8, and 9,* NADH dehydrogenase subunit 1, 3, 4, 5, 7, 8, and 9; *RPS12*, 40S ribosomal protein S12. In minicircle DNA the flag represents the start of a gRNA gene.

Editing starts from the 3` end of a pre-edited mRNA that shares complementarity with the specific gRNA (Hashimi et al., 2013; Read et al., 2016). This first gRNA anneals to the most 3` edited region of the pre-edited mRNA by its anchor domain, which is 8-12 nucleotide long at the 5` end of the gRNA. The process of gRNA: mRNA annealing involves both canonical and non-canonical G: U base pairing; such discrete regions are termed the editing sites. Next, the first mismatch region between the above RNA duplex guides the U-insertion/deletion. This mismatch region in gRNA constitutes the so-called information domain. Interestingly, the transcribed gRNA population contains much more combinations then needed to execute editing, yet the reason for such redundancy remain unknown.

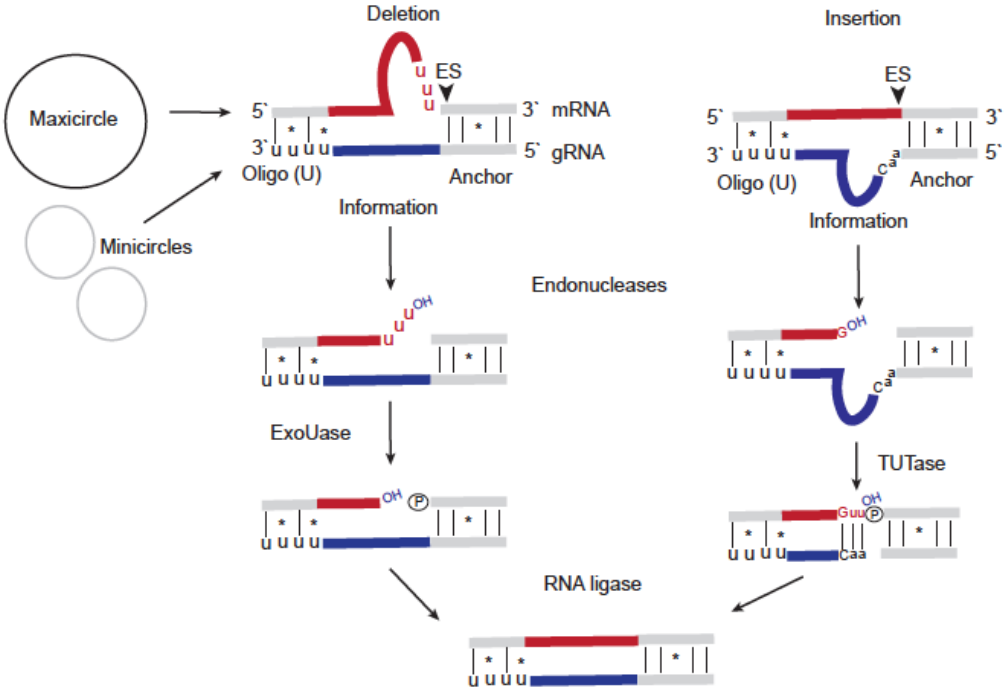

Figure 5. Mitochondrial RNA editing mechanism.
Two separate pathways of (U) insertion or deletion in a pre-edited mRNA are described for a single round of editing. The gRNA 5` anchor region (grey) is complimentary to the 3` region of pre-edited mRNA. The information region of gRNA (blue) contains mismatch regions that dictate U insertion or deletion. The RNA editing core complex (RECC) provides all the enzymatic activities that are required to execute single round of editing. The ES represents editing site. Adapted from (Read et al., 2016)

Another critical component of RNA editing is proteins, which execute a wide range of required activities. The ever-growing list includes more than ~100 proteins associated with the process (Lukeš et al., 2011). One such multiprotein complex is the RNA editing core complex (RECC), also known the 20S editosome (Aphasizhev and Aphasizheva, 2014; Aphasizheva et al., 2014). It contains proteins with the enzymatic activities for a single round of editing. RECC exists in three isoforms with a common core and several unique proteins that perform slightly different roles in editing. The first RECC isoform contains RNA editing endonuclease REN2 and selectively carries out U-insertions (Carnes et al., 2005), while the second isoform includes REN1 dedicated to U-deletions (Carnes et al., 2011). The third isoform with REN3

exclusively edits cox2 mRNA (Carnes et al., 2008). Another RECC subunit, the RNA editing exonuclease REX1, exclusively associated with the REN1 containing RECC isoform, carries out the removal of Us. Finally, an RNA editing ligase REL1 from RECC ligates two RNA fragments, thereby marking the end of single round editing (Gao and Simpson, 2003; Li et al., 2011a; Schnaufer et al., 2003).

The other extensively studied protein complex is the highly abundant mitochondrial RNA-binding protein 1 and 2 (MRP1/2) complex (Allen et al., 1998; Müller and Göringer, 2002). It is a heterotetramer with two molecules each of MRP1 and MRP2. Previously, MRP1/2 was shown to promote non-specific RNA binding through negatively charged phosphate backbone, while its depletion affected all three categories of mRNAs (Schumacher et al., 2006). The MRP1/2 proposed functions include RNA stability and mRNA: gRNA matchmaking (Fisk et al., 2009; Vondrušková et al., 2005; Zíková et al., 2006, 2008). However, the exact function of MRP1/2 in RNA editing remains poorly understood.

Another mitochondrial multiprotein complex labelled the RNA binding complex 1 (MRB1), also referred to as the RNA editing substrate binding complex (RESC), was shown to be essential for editing (Acestor et al., 2009a; Aphasizheva et al., 2014; Hashimi et al., 2013). It interacts with both the above-mentioned RECC and MRP1/2 complexes. MRB1 is hypothesized to play a critical role in recruiting RNA to RECC, the progression of 3` to 5` editing and coupling editing with other RNA processing steps (Hashimi et al., 2013; Read et al., 2016). The MRB1 complex consists of a stable core of six proteins also known as guide RNA binding complex (GRBC), as it includes the only known gRNA-stabilizing proteins GAP1 and GAP2 (Aphasizhev and Aphasizheva, 2011b; Weng et al., 2008). Two other proteins, MRB3010 and MRB11870, are implicated in the early stage of editing (Ammerman et al., 2011; Huang et al., 2015), while the role of remaining MRB8620 and MRB5390 proteins remains unclear (Huang et al., 2015; Read et al., 2016).

In total, 31 proteins have been identified to be associated with MRB1 complex, including t6 that constitutes its core. The numerous transient protein-protein or RNA-induced interaction with the MRB1 core have yet to be incorporated into the MRB1 architecture (Acestor et al., 2009b; Ammerman et al., 2012; Read et

al., 2016). One such stable MRB1 subcomplex, also known as the RNA editing mediator complex (REMC) includes TbRGG2 (Fisk et al., 2008). TbRGG2 has an RNA-binding RRM domain and has RNA annealing and unwinding activity. The depletion of TbRGG2 severely impacts editing of pan-edited mRNAs, possibly by stalling its progression (Ammerman et al., 2010; Foda et al., 2012). REMC include other novel RNA-binding proteins, MRB8170 and MRB4160, which were found to be functional paralogs (Kafkova et al., 2012). Interestingly, the simultaneous depletion of MRB8170/MRB4160 severely impacts editing of both pan- and minimally-edited transcripts, thereby making them strong candidates for future studies to enhance our understanding of REMC's role in editing.

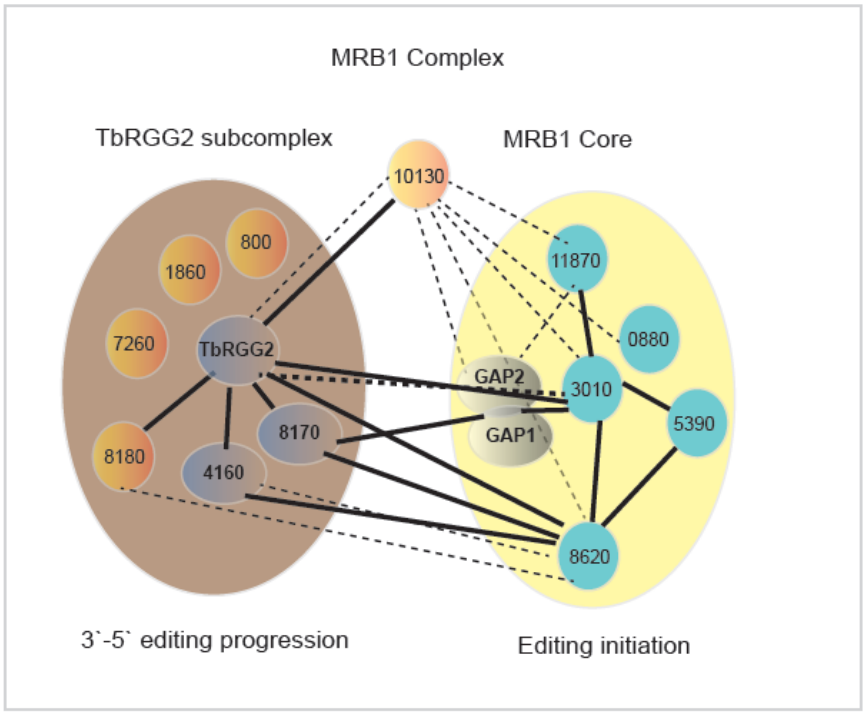

Figure 6. Mitochondrial RNA binding complex 1 (MRB1). The MRB1 complex consists of two subcomplexes. The six proteins including GAP1/2 heterotetramer constitute the MRB1 core subcomplex implicated in gRNA stability. The other so-called TbRGG2 subcomplex contains proteins with less known functions and is involved in 3`-5` editing progression, while MRB10130 is implicated in holding both subcomplexes together, possibly by protein-protein interactions. Solid lines mark strong interactions, while thin lines depict weak interactions. Adapted from (Read et al., 2016)

2.6 Mitochondrial translation

Trypanosome mt translation relies on the prokaryotic-like translation system adapted to the eukaryotic-type tRNAs all of which have to be imported into the organelle. The nuclear-encoded $tRNA^{Met}$ upon formylation by methionyl-$tRNA^{Met}$ formyltransferase initiates mt translation, while for the elongation step its non-formylated version is used (Cristodero et al., 2010). Other components are the translation initiation factor 2, and the four translation factors, namely the release factor 1, and elongation factors Tu, Ts, and G (Charrière et al., 2005; Cristodero et al., 2010, 2013).

In several ways trypanosome mt ribosome differs from its bacterial counterpart, especially by its smaller size when fully assembled (Ban et al., 2000; Clemons Jr. et al., 1999; Maslov et al., 2006). Trypanosome mt ribosome consists of 30S small subunit (SSU) and large 40S large subunit (LSU) particles, with the smallest known 9S and 12S rRNA molecules encoded by the kDNA. Both 9S and 12S rRNA share some overlap with their bacterial homologs 16S and 23S rRNA, respectively (De la Cruz et al., 1985a, 1985b). A comparison between the 12S rRNA and 23S rRNA counterpart revealed the loss of some well-conserved stem-loop regions (Maslov et al., 2007; Sharma et al., 2003, 2009), as a consequence making a known prokaryotic translation inhibitor chloramphenicol ineffective in trypanosomes (Hashimi et al., 2016).

Chapter 1

Differential binding of mitochondrial transcripts by MRB8170 and MRB4160 regulates distinct editing fates of mitochondrial mRNA in trypanosomes

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RESEARCH ARTICLE

Differential Binding of Mitochondrial Transcripts by MRB8170 and MRB4160 Regulates Distinct Editing Fates of Mitochondrial mRNA in Trypanosomes

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ABSTRACT A dozen mRNAs are edited by multiple insertions and/or deletions of uridine residues in the mitochondrion of *Trypanosoma brucei*. Several protein complexes have been implicated in performing this type of RNA editing, including the mitochondrial RNA-binding complex 1 (MRB1). Two paralogous novel RNA-binding proteins, MRB8170 and MRB4160, are loosely associated with the core MRB1 complex. Their roles in RNA editing and effects on target mRNAs are so far not well understood. In this study, individual-nucleotide-resolution UV-cross-linking and affinity purification (iCLAP) revealed a preferential binding of both proteins to mitochondrial mRNAs, which was positively correlated with their extent of editing. Integrating additional *in vivo* and *in vitro* data, we propose that binding of MRB8170 and/or MRB4160 onto pre-mRNA marks it for the initiation of editing and that initial binding of both proteins may facilitate the recruitment of other components of the RNA editing/processing machinery to ensure efficient editing. Surprisingly, MRB8170 also binds never-edited mRNAs, suggesting that at least this paralog has an additional role outside RNA editing to shape the mitochondrial transcriptome.

IMPORTANCE *Trypanosoma brucei* mitochondrial mRNAs undergo maturation by RNA editing, a unique process involving decrypting open reading frames by the precise deletion and/or insertion of uridine (U) residues at specific positions on an mRNA. This process is catalyzed by multiprotein complexes, such as the RNA editing core complex, which provides the enzymatic activities needed for U insertion/deletion at a single editing site. Less well understood is how RNA editing occurs throughout an mRNA bearing multiple sites. To address this question, we mapped at single-nucleotide resolution the RNA interactions of two unique RNA-binding proteins (RBPs). These RBPs are part of the mitochondrial RNA-binding complex 1, hypothesized to mediate multiple rounds of RNA editing. Both RBPs were shown to mark mRNAs for the process in correlation with the number of editing sites on the transcript. Surprisingly, one also binds mRNAs that bypass RNA editing, indicating that it may have an additional role outside RNA editing.

Trypanosoma brucei, the causative agent of African sleeping sickness, is distinguished by a single reticulated mitochondrion containing an unusually large amount of mitochondrial DNA (mtDNA), termed kinetoplast DNA (kDNA). The kDNA comprises ~25 maxicircles and ~5,000 minicircles, mutually concatenated into a single network (1, 2). Maxicircles are homologs of classical mtDNA, containing two rRNAs and 18 protein-encoding genes, most of which constitute subunits of the mt respiratory complexes.

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Twelve out of 18 maxicircle mRNAs require numerous posttranscriptional insertions and/or deletions of uridine residues (U) to remove frameshifts and generate a correct open reading frame (3, 4). The kDNA minicircles are highly heterogeneous in sequence and carry small noncoding guide RNAs (gRNAs) (5). The binding of a gRNA to its cognate mRNA via Watson-Crick and G-U wobble base-pairing guides precise U insertions/deletions, eventually producing a fully edited mRNA (6).

The polycistronic maxicircle transcript is split into three differently processed transcript categories (7): (i) pan-edited mRNAs that undergo extensive editing mediated by several gRNAs in a 3′-to-5′ direction along the transcript (8), (ii) minimally edited mRNAs usually containing a single edited region, and (iii) never-edited mRNAs, which bypass editing and proceed directly to standard processing (9–11). However, little is known about how these individual transcripts, arising from a multicistronic precursor RNA, achieve distinct expression levels and how the abundance of these transcript categories is controlled in different life cycle stages of *T. brucei* (5, 10, 12).

Proteins are key components of the editing machinery, as they participate in all effector and regulatory steps in a highly coordinated manner (6, 10, 13). The RNA editing core complex (RECC), also called the editosome, is a large complex that contains the core enzymatic activities required for editing (14–16). Surprisingly, purified RECC is devoid of RNA and lacks processivity *in vitro* (17). Thus, additional proteins must cooperate with RECC to carry out multiple rounds of RNA editing *in vivo*. One such complex is the mtRNA-binding complex 1 (MRB1) (6). The MRB1 core complex is composed of six proteins: gRNA-associated proteins 1 and 2 (GAP1 and GAP2, respectively), plus MRB3010, MRB5390, MRB8620, and MRB11870 (18). This core is also referred to as the gRNA-binding complex (19) (for a guide to the different MRB1 protein nomenclatures in the field, see Table S1 in the supplemental material). The heterodimer of GAP1 and GAP2 was found to stabilize gRNAs (20, 21). Other vital MRB1 subunits are loosely associated with the core complex, including the accessory subunits MRB8170, MRB4160, and *T. brucei* RGG2 (TbRGG2) (6, 22). RNA interference (RNAi)-mediated depletion of most subunits leads to a profound decrease in pan-edited transcripts, while the effect on minimally edited mRNAs varies depending on the targeted subunits (18, 19, 23).

MRB8170 and MRB4160 are unique RNA-binding proteins (RBPs), which were recently shown to bind RNA via a novel and hitherto-undefined RNA-binding domain (24). These proteins are highly similar paralogs that are conserved within the kinetoplastid flagellates but without orthologs outside this clade (24). Simultaneous depletion of MRB8170 and MRB4160 results in a decrease of edited forms of pan-edited and minimally edited transcripts and a slight increase in never-edited transcripts (24).

In this study, we used biochemical and genomics approaches to dissect the functions of MRB8170 and MRB4160 in processing different categories of maxicircle transcripts. We applied individual-nucleotide-resolution UV-cross-linking and affinity purification (iCLAP) (25, 26) to investigate interactions of both proteins with mtRNAs in the procyclic stage of *T. brucei*. This quantitative binding assay revealed a high preference of both proteins for maxicircle transcripts. Moreover, binding of both proteins influenced the steady-state abundance of mt mRNAs, as demonstrated by the double knockdown (dKD) of MRB8170 and MRB4160. Rapid tandem affinity purification (TAP) confirmed interaction of both proteins with the core and accessory MRB1 subunits GAP1 and TbRGG2, respectively (22, 27), and detected interactions with mtRNA-binding protein 1 (MRP1), Nudix hydrolase (or MERS1), and TbRGG1, which belong to different RNA processing complexes (10). Furthermore, the dKD of MRB8170 and MRB4160 was also shown to affect the mRNA-binding efficiency of these proteins. By integrating iCLAP data with *in vivo* and *in vitro* data, we propose the working dynamics of the MRB1 complex in facilitating RNA editing and also reveal a potential, unexpected role in the expression of never-edited transcripts.

RESULTS

MRB8170 and MRB4160 preferentially bind mitochondrial mRNAs. We used the iCLAP protocol with the aim of identifying the direct RNA targets of the two accessory MRB1 subunits MRB8170 and MRB4160 in the mitochondrion of *T. brucei* (Fig. 1A). MRB4160 and MRB8170 were tagged with modified TAP tag (mTAP), bearing the His_6 epitope, and stably expressed in *T. brucei* procyclic cells. In order to cross-link *in vivo* the tagged proteins to RNA, three UV irradiation doses (1.6, 0.8, and 0.4 J/cm^2) were tested. Phosphorimaging of the cross-linked RNA revealed that UV cross-linking with a radiant energy ranging from 0.8 to 1.6 J/cm^2 was more efficient than 0.4 J/cm^2 (Fig. 1B; see also Fig. S1A in the supplemental material). Thus, a UV dose of 0.8 J/cm^2 was applied for preparation of the MRB4160 and MRB8170 iCLAP libraries (Fig. 1C and S1B and C). No RNA-protein complexes were detected in the two controls, the non-UV-cross-linked trypanosomes with MRB4160-mTAP and the UV-cross-linked parental cells (Fig. 1B and C).

Cross-linked and affinity-purified RNA from two independent iCLAP replicates with MRB8170-mTAP, MRB4160-mTAP, and the control (UV-cross-linked parental cells) was RNase I digested into 60- to 120-nucleotide (nt)-long fragments, reverse transcribed, and subjected to next-generation sequencing (Fig. S1D). The sequencing reads, henceforth referred to as iCLAP tags, were aligned against the preedited and fully edited versions of the kDNA maxicircle transcripts using Bowtie2 alignment software (28). The two replicates combined from MRB8170 and MRB4160 data sets yielded a total of 191,683 and 100,313 uniquely aligned iCLAP tags, respectively. The control library obtained from the UV-cross-linked parental cells contained only a negligible 483 unique iCLAP tags. This very low number of control iCLAP tags confirmed the high stringency of the applied iCLAP protocol.

Promiscuous binding of MRB8170 to all classes of mitochondrial mRNAs contrasts with restricted binding of MRB4160. To analyze the binding of MRB8170 and MRB4160 on maxicircle-derived transcripts, we divided the iCLAP tags into two categories according to their generation from preedited and fully edited transcripts (Fig. 2A). Since preedited iCLAP tags had been mapped directly to the maxicircle genome, they include all 18 maxicircle-derived pre-mRNAs (pan-edited, minimally edited, and never-edited mRNAs) before undergoing editing. In contrast, fully edited iCLAP tags had been mapped to 12 fully edited maxicircle mRNAs (pan-edited and minimally edited) in which all U insertions/deletions had been completed.

To dissect RNA interactions of RBPs that are part of large stable protein complexes, such as MRB1, it is necessary to use extended RNase I digests to generate small RNA fragments. Our protocol produced iCLAP tags ~30 to 50 nt long after the removal of the adaptor sequences. However, a drawback of the short read length is that iCLAP tags mapping to fully edited sequences can also be derived from partially edited mRNAs still undergoing the process. Vice versa, iCLAP tags mapping to preedited sequences can originate from RNAs not yet edited, or from already partially edited transcripts. Thus, in both cases, it is impossible to quantitate the amount of reads originating from partially edited mRNAs, which creates a bias in the numbers of preedited and fully edited iCLAP tags (Fig. S2A). Approximately 95.3% of MRB4160 iCLAP tags aligned to preedited mRNAs, while 4.6% aligned to fully edited mRNAs (Fig. S2A). Similarly, 90.7% and 9.2% of MRB8170 iCLAP tags mapped to preedited and fully edited mRNAs, respectively (Fig. S2A).

Next, we used our quantitative iCLAP data to establish the proportion of binding relative to the extent of RNA editing. For this, maxicircle mRNAs were divided into pan-edited (*COX3*, *ND7*, *ND8*, *A6*, *CR3*, *RPS12*, *ND9*, *ND3*, and *CR4*), minimally edited (*COX2*, *MURF2*, and *CYB*) and never-edited (*ND1*, *COX1*, *ND4*, *ND5*, *MURF5*, and *MURF1*) transcript categories. For those transcripts undergoing editing, preedited and fully edited iCLAP tags were combined. The distribution of MRB8170 and MRB4160 iCLAP tags on mtRNAs was compared to their expression level determined by publicly

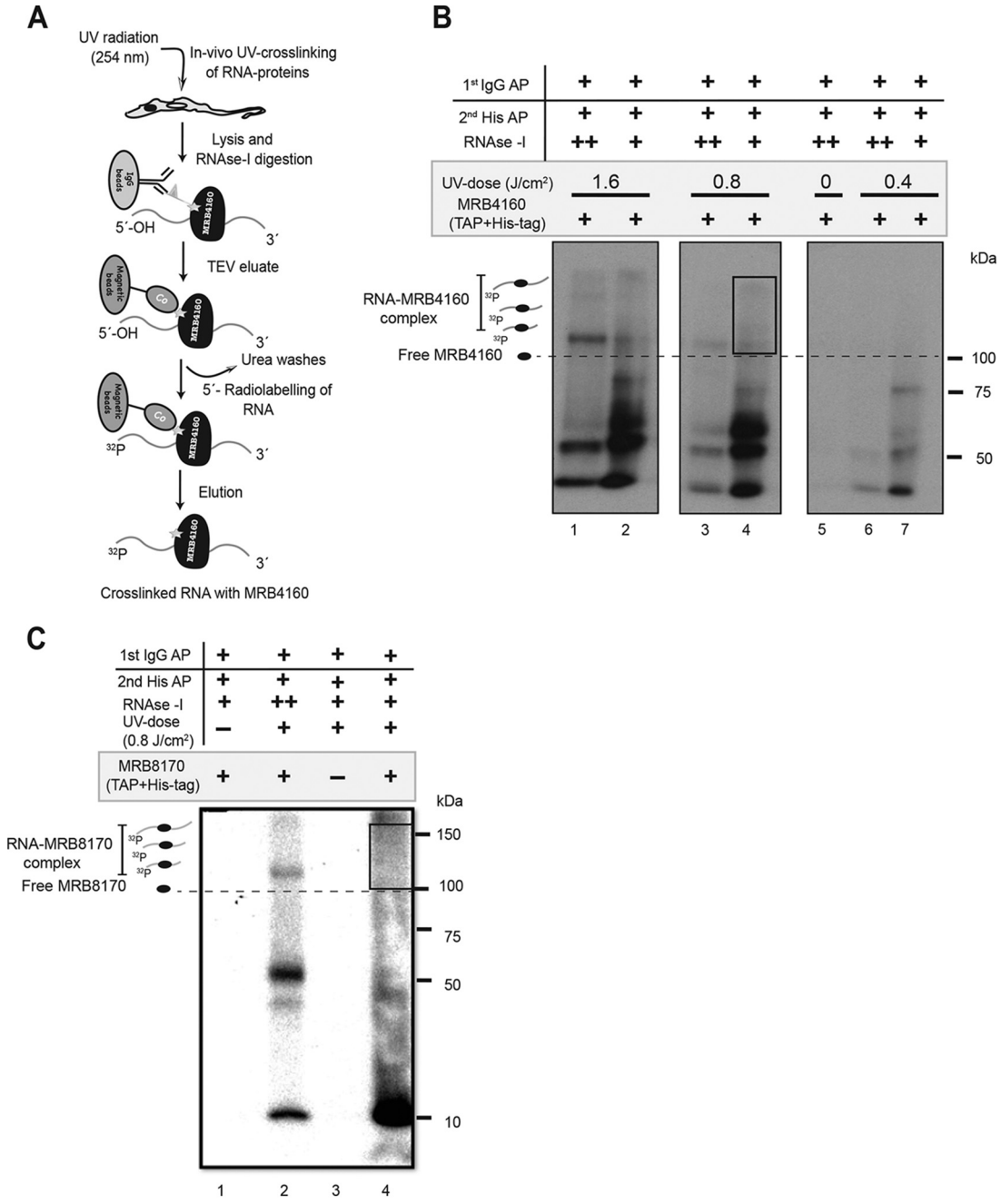

FIG 1 MRB4160 and MRB8170 iCLAP. (A) Schematic depiction of the iCLAP workflow to purify UV-cross-linked RNA-MRB4160-mTAP complex using two-step affinity purification. (B) Copurification of UV-cross-linked RNA-MRB4160-mTAP complex. Autoradiography of the ^{32}P-labeled complexes after two-step affinity purification (AP). Three UV irradiant fluences were used: 1.6 J/cm^2 (lanes 1 and 2), 0.8 J/cm^2 (lanes 3 and 4), and 0.4 J/cm^2 (lanes 6 and 7) to *in vivo* cross-link RNA with proteins, while non-UV-cross-linked cells (lane 5) were used as a control. The high (++)- and low (+)-RNase I treatments were applied to confirm the shift in the cross-linked RNA-MRB4160-mTAP complex under these conditions. The box marks the part that was cut out and used for RNA isolation. Two independent replicates were performed for preparation of the iCLAP library. (C) Copurification of UV-cross-linked RNA-MRB8170-mTAP complex. After two-step affinity purification, the ^{32}P-labeled complexes were monitored by autoradiography. The optimal 0.8-J/cm^2 UV radiant fluence was used to *in vivo* cross-link RNA to proteins. Non-UV-cross-linked mTAP-tagged MRB8170 (lane 1) and UV-cross-linked parental cell line (lane 3) yielded no signal. The high-RNase I treatment of UV-cross-linked MRB8170 (lane 2) showed a band at ~100-kDa size. The low-RNase treatment (boxed region in lane 4) was used to prepare the MRB8170 iCLAP libraries. Two independent replicates were performed for preparation of the iCLAP library.

available *T. brucei* RNA sequencing (RNA-seq) data, which also include transcripts originating from the organelle (29).

Interestingly, the proportion of MRB8170 iCLAP tags that map onto never-edited RNAs (2.6%) correlates with their occurrence in the RNA-seq data (2.2%) (Fig. 2B and C).

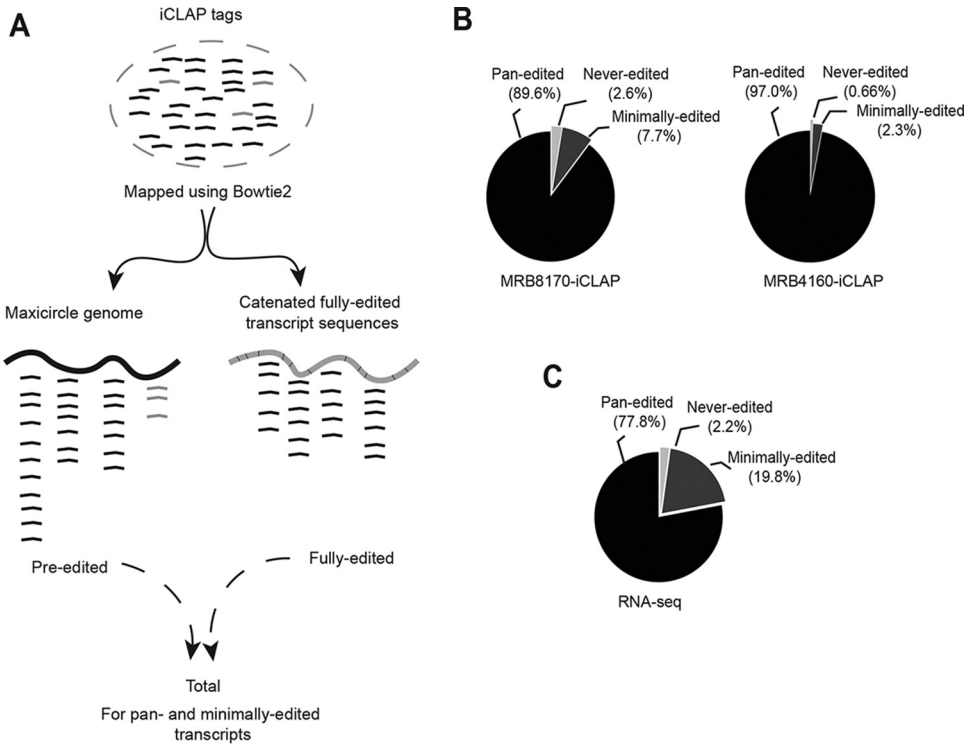

FIG 2 Distribution of MRB8170 and MRB4160 iCLAP tags on maxicircle transcripts. (A) Schema of the strategy to map iCLAP tags onto mitochondrial mRNAs. iCLAP tags were separately mapped to maxicircle genome and catenated sequences of fully edited transcripts (9 pan-edited mRNAs plus 3 minimally edited mRNAs). The tags uniquely mapped to maxicircle genome were named preedited, while iCLAP tags mapped to catenated sequences were categorized as fully edited (see text for explanation). For transcripts undergoing editing, preedited and fully edited mapped iCLAP tags (black) were combined for analysis. The iCLAP tags (gray) mapped to never-edited transcripts were limited to the preedited region. (B) Pie chart of uniquely mapped iCLAP tags on the maxicircle mRNAs. Percentages of MRB8170 and MRB4160 iCLAP tags uniquely mapped to three different classes of maxicircle mRNAs. Black, pan-edited mRNAs (preedited and fully edited iCLAP tags mapped to pan-edited region); dark gray, minimally edited mRNAs (preedited and fully edited iCLAP tags mapped to minimally edited region); light gray, never-edited mRNAs. Two independent iCLAP replicates each for MRB8170 and MRB4160 were combined for the analysis. (C) Percentage of RNA-seq tags uniquely mapped to the same classes of maxicircle mRNAs. The pie chart is shaded as in panel B.

In contrast, a surprisingly high fraction (~97%) of MRB4160 iCLAP tags mapped to pan-edited transcripts, while binding to never-edited transcripts was negligible (Fig. 2B). This result was confirmed by RNA immunoprecipitation (RIP)-quantitative real-time PCR (qPCR) (Fig. 3E). In summary, our data suggest that MRB8170 binds all classes of maxicircle mRNAs, while MRB4160 binding is restricted to pan-edited and minimally edited transcripts.

MRB8170 and MRB4160 binding on pan-edited and minimally edited transcripts correlates with their editing status. In order to understand the function(s) of MRB8170 and MRB4160 in editing, we quantified the binding of both proteins to nine individual pan-edited transcripts using iCLAP tags mapping to preedited and fully edited mRNAs (Fig. 3A). In agreement with being paralogs, the distributions of MRB8170 and MRB4160 iCLAP tags mapping onto pan-edited transcripts were very similar (Fig. 3A). For instance, both proteins massively bind to preedited *COX3* but have minimal binding to *ND9*, *ND3*, and *CR4*. Interestingly, the extent of binding correlates with the number of U insertions/deletions needed to be fully edited (Fig. 3B). Visual inspection of iCLAP tags in the genome browser showed that both proteins bind continuously along the entire preedited sequence of six out of nine pan-edited transcripts, including *A6*, *CR3*, *COX3*, *ND7*, *ND8*, and *RPS12* (Fig. 3C and S3 and S4). Since RNA editing proceeds in a stepwise manner in a 3′-to-5′ direction, the pronounced binding of MRB8170 and MRB4160 over the entire length of these preedited transcripts hinted at their role in flagging pan-edited RNAs for editing.

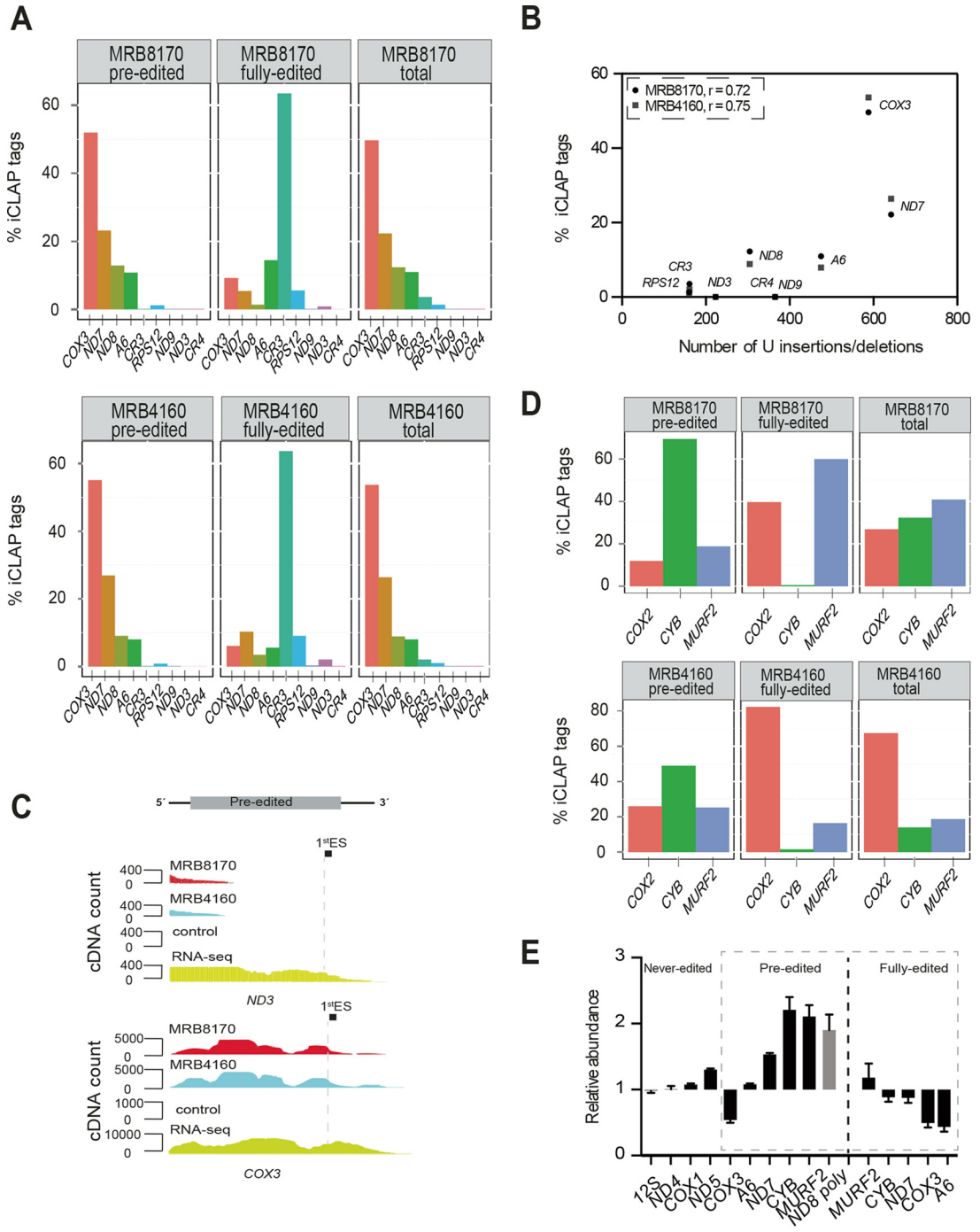

FIG 3 MRB8170 and MRB4160 binding to transcripts undergoing editing. (A) Preferential binding of MRB8170 and MRB4160 to pan-edited transcripts. Bar plots show the percent share of MRB8170 and MRB4160 iCLAP tags uniquely mapped to the preedited and fully edited regions of pan-edited transcripts and the total (preedited and fully edited), respectively, of pan-edited mRNAs indicated on the *x* axis. (B) Scatter plot depicting the correlation between total share of mapped iCLAP tags (*y* axis) and the number of U insertions or deletions, reflecting the extent of editing of individual transcripts (*x* axis). Each point represents a pan-edited transcript (*RPS12*, *CR3*, *ND3*, *CR4*, *ND8*, *ND9*, *A6*, *COX3*, and *ND7*) as indicated. Pearson's correlation coefficients (*r*) are shown for both MRB8170 and MRB4160. Black circles, MRB8170; gray squares, MRB4160. (C) Genomic browser view displays preferential binding of MRB8170 and MRB4160 to *ND3* and *COX3* transcripts. The unique cDNA count is depicted on the *y* axis, and the mapped tag position along a given transcript is on the *x* axis. MRB8170 iCLAP tags are in red, MRB4160 iCLAP tags are in blue, control iCLAP tags are in black, and RNA-seq reads are in yellow. ES, editing site. (D) Binding of MRB8170 and MRB4160 to minimally edited transcripts. Labeling as in panel A. (E) Relative abundance of maxicircle mRNAs compared between MRB8170/MRB4160 and ATM1 knockdown cells by qPCR analysis. 18S rRNA was used as an internal reference. The following maxicircle mRNAs were analyzed in triplicate: rRNA (12S), never-edited mRNA (*ND4*, *COX1*, and *ND5*), pan-edited mRNA (*COX3*, *A6*, and *ND7*), minimally edited mRNA (*CYB* and *MURF2*), and *ND8* poly (polycistronic *ND8* transcript). The dashed line separates preedited and fully edited versions of the transcripts. All mRNAs are in black except *ND8* poly, which is in gray.

In contrast, both MRB8170 and MRB4160 showed strong accumulation toward the 5′ end of the preedited *ND3* and *CR4* mRNAs and minimal binding to *ND9* mRNA (Fig. 3C and S3), although they are well expressed, as judged from RNA-seq data (Fig. S2B). This observation suggests that these transcripts are not flagged for editing by MRB8170 and MRB4160. The observation that the preedited forms of some pan-edited transcripts are completely covered by MRB8170 and MRB4160, while others show binding only toward the 5′ end, could explain previous reports on different editing states in the procyclic stage of *T. brucei*. Indeed, the two paralogs bind the entire length of preedited *COX3*, *RPS12*, and *A6* mRNAs, which are all fully edited in the procyclic stage. Furthermore, their limited binding onto preedited *ND3* and *CR4* mRNAs correlates with their not being edited in this stage (30–32).

Next, we dissected the binding of MRB8170 and MRB4160 to the minimally edited *COX2*, *CYB*, and *MURF2* transcripts, which have 4, 39, and 26 U insertions, respectively, plus four U deletions in the case of *MURF2*. Binding of MRB8170 and MRB4160 to fully edited *CYB* was extremely low (Fig. 3D). As this transcript also exhibits a low steady-state level in the mt transcriptome (Fig. S2B), low binding likely reflects the paucity of fully edited *CYB* in the procyclic stage. In contrast, both proteins bind over the entire length of preedited *COX2*, *CYB*, and *MURF2* transcripts (Fig. 3D and S5), suggesting that they mark all three minimally edited transcripts for editing, similarly to pan-edited mRNAs.

To further validate the impact of both proteins on editing of pan-edited and minimally edited transcripts, we assayed the relative abundance of maxicircle transcripts by quantitative real-time PCR (qPCR) in MRB8170/MRB4160 dKD cells. Control cells were depleted of ATM1 mRNA, encoding an inner membrane transporter that does not affect mt gene expression (33). Indeed, qPCR analysis showed that preedited forms of pan-edited and minimally edited mRNAs accumulated upon MRB8170/MRB4160 depletion, but not in control cells, while the relative abundance of fully edited transcripts was considerably reduced (Fig. 3E).

Taken together, iCLAP and knockdown data support a role for MRB8170 and MRB4160 in flagging mRNAs for editing, as their absence reduces the abundance of edited transcripts in the procyclic stage.

MRB8170 binds to a subset of less-abundant never-edited transcripts. We next investigated binding of MRB8170 to six never-edited transcripts. *ND4*, *ND5*, and *MURF5* mRNAs were represented in more than 90% of the iCLAP tags mapping to never-edited transcripts, while the remainder were derived from *ND1*, *COX1*, and *MURF1* (Fig. 4A). Normalization of the iCLAP tag number to gene length resulted in similar proportions of iCLAP tags (Fig. S2C).

Such biased binding of MRB8170 to a subset of never-edited transcripts was unexpected and prompted us to look into their steady-state relative abundances. Interestingly, *ND1* and *COX1* are the most abundant never-edited transcripts in procyclic trypanosomes (Fig. 4B and S6) (30–32). Hence, there is a notable discrepancy between the very low number of iCLAP tags and the high expression of these two genes. For *MURF1* on the other hand, the insignificant number of mapped iCLAP tags corresponds to its low abundance, rendering its detection difficult by both iCLAP and RNA-seq methods. In contrast, the enrichment of *ND4*, *ND5*, and *MURF5* bound to MRB8170 did not correspond to their relatively low steady-state levels as determined by RNA-seq (Fig. 4B, S2B, and S6). This result suggests that MRB8170 serves an additional role outside RNA editing by negatively regulating the expression of this subset of never-edited transcripts. This notion is supported by the accumulation of never-edited transcripts in MRB8170/MRB4160 dKD cells (Fig. 3E).

To validate the iCLAP data, we performed RNA immunoprecipitation (RIP) in cell lines expressing tagged MRB8170-mTAP or MRB4160-mTAP and a parental cell line lacking the mTAP-tag (mock IP). Immunoprecipitated RNA was reverse transcribed, and qPCR was performed using primers recognizing preedited and fully edited versions of pan-edited *RPS12* and *COX3*, minimally edited *CYB*, and never-edited *ND4* and *COX1*

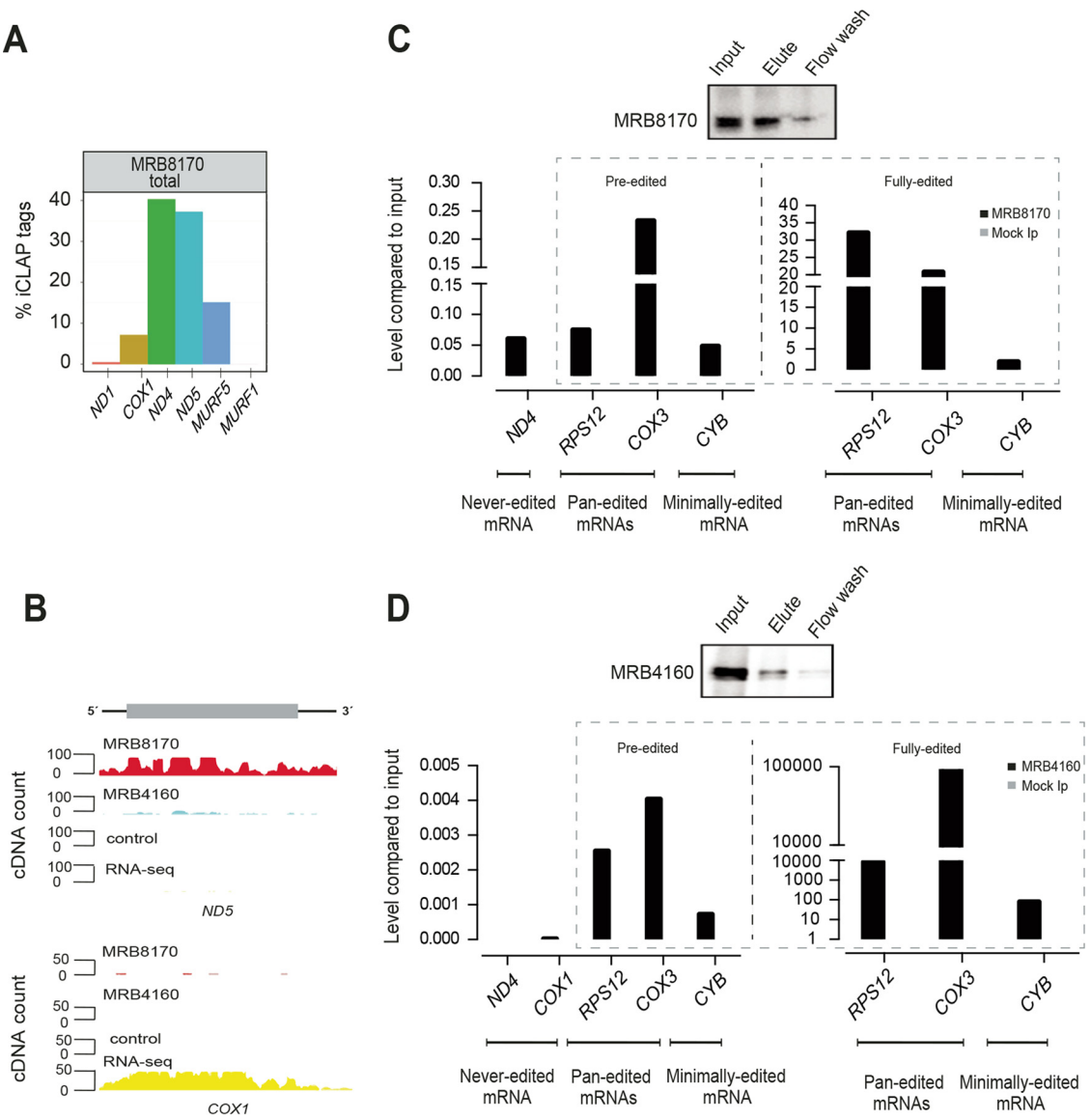

FIG 4 MRB8170 and MRB4160 binding to never-edited transcripts and iCLAP validation by RNA immunoprecipitation-quantitative PCR. (A) Preferential binding of MRB8170 to never-edited transcripts. The bar plot shows the percent share of MRB8170 iCLAP tags uniquely mapped to never-edited transcripts, as indicated on the *x* axis. (B) Genomic browser view displays preferential binding of MRB8170 and MRB4160 to *ND5* and *COX1* transcripts. The unique cDNA count is depicted on the *y* axis, and the mapped tag position along a given transcript is on the *x* axis. MRB8170 iCLAP tags are in red, MRB4160 iCLAP tags are in blue, control iCLAP tags are in black, and RNA-seq reads are in yellow. (C) MRB8170-associated maxicircle transcripts determined by RIP-qPCR. The top panel confirms MRB8170 purification using IgG beads by Western blotting. Bar plots below show the relative amount of never-edited (*ND4*), pan-edited (*RPS12* and *COX3*), and minimally edited (*CYB*) mRNAs pulled down with mTAP-tagged MRB8170 (black) and mock immunoprecipitation (Mock Ip, parental cell line, in gray). Data are presented relative to the input sample (RNA recovered and reverse transcribed using 10% of lysate). One representative set of measurements is shown. (D) MRB4160-associated maxicircle transcripts determined by RIP-qPCR. Labeling as in panel C.

transcripts. These data confirmed that MRB8170 binds to all three classes of maxicircle mRNAs similarly enriched for never-edited (*ND4*) and pan-edited (preedited *RPS12*) mRNAs. As seen before in the iCLAP data, MRB4160 failed to bind to never-edited transcripts (Fig. 4C and D).

MRB8170 and MRB4160 interact with non-MRB1 proteins. After identification of MRB8170 and MRB4160 RNA-binding sites and finding that the former binds to never-edited transcripts, we wondered about their interactions with non-MRB1 proteins involved in RNA editing or other RNA processing steps. For this purpose, we performed rapid tandem affinity purification using IgG-coated magnetic Dynabeads (34). RNase

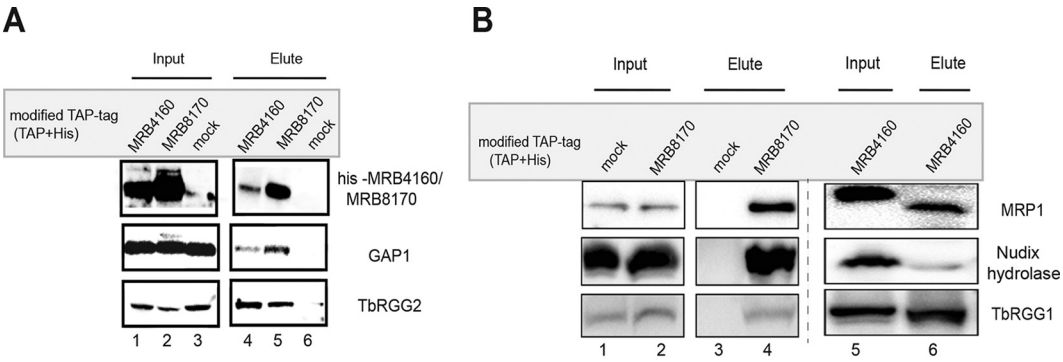

FIG 5 MRB8170- and MRB4160-associated proteins. (A) Rapid affinity purification of MRB8170- and MRB4160-associated proteins belonging to the MRB1 complex. Western blot analysis of proteins indicated on the right in total extracts (input; lanes 1 to 3) and eluates (lanes 4 and 5). The control includes a mock purification of the parental cell line (lanes 3 and 6). (B) Confirmation of rapid affinity purification of MRB8170- and MRB4160-associated proteins belonging to different RNA processing complexes. Western blot analysis of indicated proteins in total extracts (input, lanes 1, 2, and 5) and eluates (lanes 3, 4, and 6). The control includes a mock purification of the parental cell line (lanes 1 and 3).

l-digested supernatants from *T. brucei* containing mTAP-tagged MRB8170 or MRB4160, as well as the parental control cell line, were mixed with the beads. In order to validate this new protocol for its pulldown efficiency, the eluates were first probed with antibodies against GAP1 and TbRGG2 from MRB1, which are known to stably interact with MRB8170 and MRB4160 (6). Indeed, both GAP1 and TbRGG2 were detected in MRB8170 and MRB4160 pulldowns, while their absence in the control demonstrated the high stringency of this approach (Fig. 5A). Eluates were then probed with a panel of specific antibodies revealing additional interactions of both proteins with MRP1 from the MRP1/MRP2 complex and with Nudix hydrolase and TbRGG1 (Fig. 5B). All proteins are part of complexes with known roles in stabilizing RNA (13, 19, 35).

Depletion of MRB8170 and MRB4160 affects RNA-binding activity of interacting proteins. We modified the protocol for UV cross-linking and subsequent pulldown of RBPs using oligo(dT) magnetic beads (36) in *T. brucei*, using the same UV dose as applied in iCLAP (Fig. 6A). The modified protocol to capture the RBPs was applied to the procyclic stage, in which we depleted either MRB8170/MRB4160 by dKD or ATM1 as a negative control (33). Oligo(dT)-captured RBPs were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Subsequently, the mt mRNA interactome was probed with antibodies specific for Nudix hydrolase, TbRGG2, MRP1, TbRGG1, the RECC subunit RNA editing ligase 1 (REL1), and GAP1 (Fig. 6B and S7A). REL1 was the only examined protein without a significant reduction in the pulldown ratio between MRB8170/MRB4160 and ATM1 depletion (Fig. 6B). Nudix hydrolase and TbRGG2 exhibited the highest decrease in poly(A)$^+$ RNA binding upon MRB8170/MRB4160 depletion. Captured MRP1 and TbRGG1 proteins were reduced to a lesser degree but still by more than 50% (Fig. 6B). The absence of GAP1 in our cross-linked mt mRNA interactome pulldown suggests that its RNA binding *in vivo* is strictly limited to gRNAs. As a control, we assessed the poly(A)$^+$ RNA binding of the cytoplasmic mRNA-binding protein DRBD18 (29), which as expected was not affected by the depletion of MRB8170 and MRB4160.

The decrease in the mt mRNA-binding efficiency of TbRGG2 caused by the depletion of MRB8170 and MRB4160 was further validated using an *in vitro* cross-linking immunoprecipitation (CLIP) assay, using extracts from MRB8170/MRB4160-depleted cells lysed under mild conditions. The lysate was divided into four equal aliquots and subsequently supplemented with recombinant glutathione *S*-transferase (GST)-tagged MRB8170 (10 and 20 μM), bovine serum albumin (BSA) (20 μM), or buffer alone. The supplemented supernatant was incubated and subsequently *in vitro* UV cross-linked. TbRGG2 antibody-coated magnetic beads were used to pull down the protein-RNA adducts, followed by 5′ radioactive labeling of the bound nucleic acid. Upon resolution

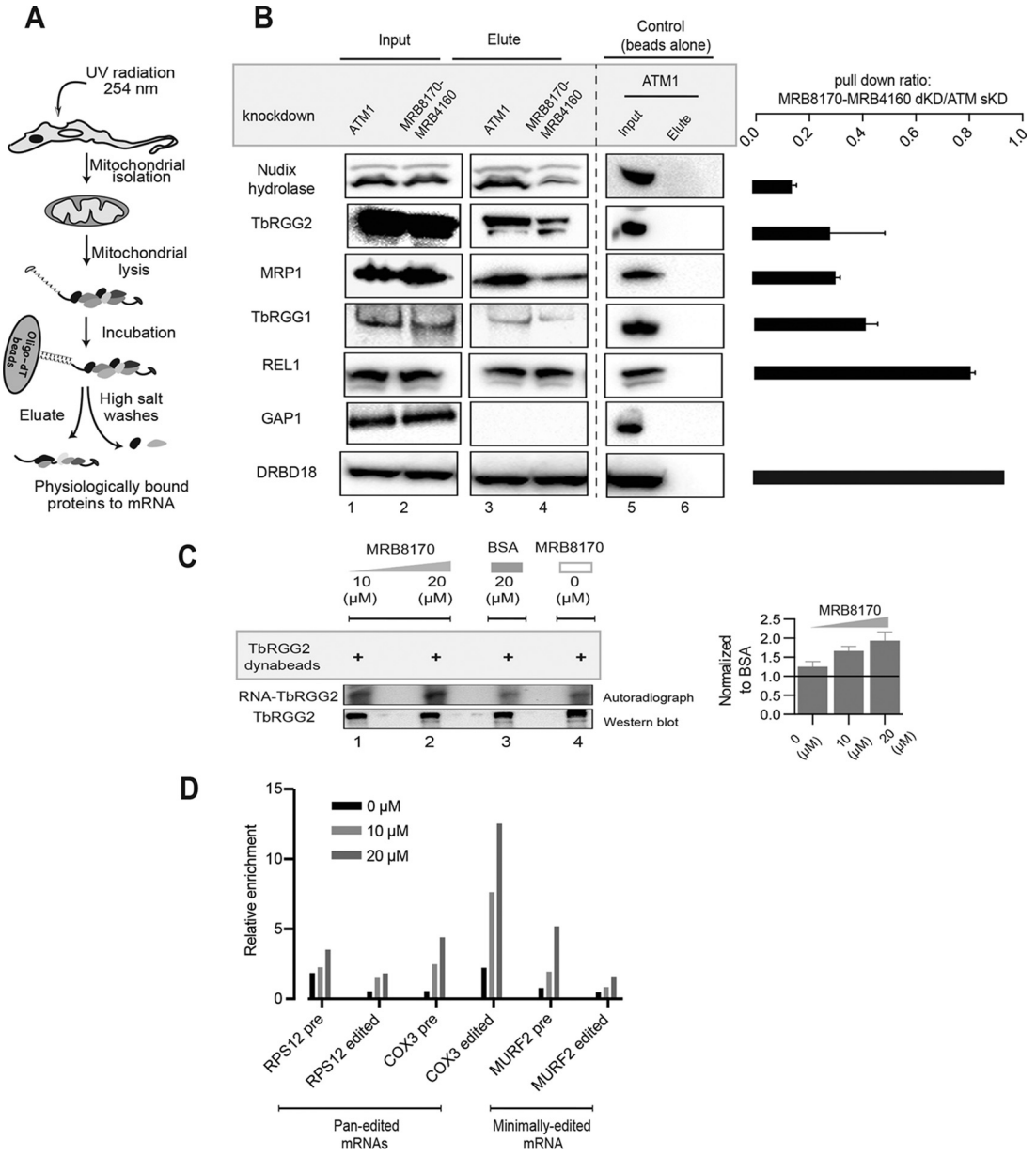

FIG 6 mRNA-binding efficiency of associated proteins following MRB8170/MRB4160 double knockdown. (A) Workflow of *in vivo* UV cross-linking and oligo(dT) magnetic bead pulldown of mitochondrial proteins associated with maxicircle mRNAs. (B) Western blot analysis of total extracts (lanes 1 and 2) and oligo(dT) eluates (lanes 3 and 4) from ATM1 and MRB8170/MRB4160 RNAi knockdown cells displaying levels of proteins indicated on the left. The control is beads not conjugated to oligo(dT) (lanes 5 and 6). Bar plots on the right show the ratio of lane 4 to lane 3 (MRB8170/MRB4160 versus ATM1 knockdown cell pulldown efficiency) signals for each protein. Error bars represent standard deviations ($n = 2$ to 3 for all proteins and $n = 1$ for DRBD18). sKD, single knockdown. (C) *In vitro* CLIP assay in MRB8170/MRB4160 double-knockdown cells. The ^{32}P-labeled RNA from the immunoprecipitated TbRGG2-RNA complex was visualized by autoradiography (autoradiograph; lanes 1 to 4). Below is a Western blot showing the eluted TbRGG2 (Western blot; lanes 1 to 4). A consistent amount of supernatant from MRB8170/MRB4160-depleted cells was supplemented with recombinant GST-MRB8170, indicated above in micromolar concentrations (lanes 1, 2, and 4) and BSA (20 μM; lane 3). The bar plot was calculated relative to the BSA ($n = 2$); error bars, standard deviations. (D) *In vitro* CLIP assay and subsequent qPCR analyses in MRB8170/MRB4160 double-knockdown cells labeled as in panel C. Bar plots show the relative amounts of *RPS12*, *COX3*, and *MURF2* in both preedited and fully edited forms as obtained from TbRGG2 pulldown in MRB8170/MRB4160-depleted cells supplemented with recombinant GST-MRB8170, indicated above in micromolar concentrations (lanes 1, 2, and 4) and BSA (20 μM; lane 3). The bar plot was calculated relative to BSA. A representative set of measurements is shown.

in an SDS-PAGE gel, the immunoprecipitated RNA-TbRGG2 complex was transferred onto a nitrocellulose membrane. The resulting autoradiogram depicted a direct relationship between the supplemented recombinant GST-MRB8170 in the supernatant and the amount of RNA bound to TbRGG2 (Fig. 6C and S7B). The addition of BSA into

the supernatant as a control also caused a slight decrease in the intensity of the autoradiogram signal, which may be a consequence of nonspecific binding of BSA onto the beads. The notion that recombinant GST-MRB8170 enhances TbRGG2 RNA binding *in vitro* was further substantiated by using RIP-qPCR to show TbRGG2 binding to several minimally and pan-edited mRNAs (Fig. 6D).

Taken together, the *in vivo* and *in vitro* data confirmed the role of MRB8170 and MRB4160 in mediating efficient binding of Nudix hydrolase, TbRGG1, TbRGG2, and MRP1 onto mt transcripts, qualifying MRB8170 and MRB4160 as crucial players in coordinating the cross talk between MRB1 and other mtRNA processing complexes in *T. brucei*.

DISCUSSION

In order to define the roles of MRB8170 and MRB4160 in RNA editing and/or processing *in vivo*, we captured their RNA-binding footprints using iCLAP. MRB8170 was shown to bind all three classes of maxicircle mRNAs, while MRB4160 was restricted to pan-edited and minimally edited transcripts. Thus, MRB8170 emerged as the more active paralog, which is consistent with the stronger phenotype caused by its depletion (24). Furthermore, while both proteins preferentially bind pan-edited mRNAs, there is a striking positive correlation between the amount of binding to a given transcript and the extent of editing. Moreover, the genomic snapshots of MRB8170/MRB4160 iCLAP tags demonstrated that both proteins bind over the entire length of preedited mRNAs, seemingly as a hallmark of their participation in this process. In support of this hypothesis, MRB8170 and MRB4160 iCLAP tags are absent on preedited versions of *ND3* and *CR4* mRNAs, which are transcribed but not edited in the procyclic stage examined here (30, 32). The iCLAP data are therefore compatible with a binding of both proteins to preedited transcripts as a prerequisite for editing. The sharp decrease in the abundance of fully edited versions of pan-edited and minimally edited transcripts upon simultaneous depletion of MRB8170 and MRB4160 further supports this argument (24). Combined with previous findings, our data show that MRB8170 and/or MRB4160 is indispensable for the editing of both pan-edited and minimally edited transcripts (24, 37).

In contrast to its binding to preedited forms of pan-edited and minimally edited mRNAs, the binding of MRB8170 to never-edited transcripts showed an inverse relationship with their abundance. This observation might indirectly explain the accumulation of never-edited transcripts in flagellates depleted of MRB8170 and MRB4160 (24). The negative impact of MRB8170 binding on the abundance of never-edited transcripts is intriguing and may also involve its interaction partner TbRGG2, which was reported to destabilize never-edited transcripts (27, 37, 38). Among all tested maxicircle transcripts, three showed an unexpected behavior. Although preedited *ND7* and *ND8* were extensively bound by both MRB8170 and MRB4160, suggesting their efficient editing, the low abundance of fully edited versions in the procyclic stage suggests that additional proteins are involved in their regulation (30, 32). Several subunits of the MRB1 core complex represent suitable candidates for such a function, as they were reported to affect a subset of pan-edited transcripts (18). Also, the RNA editing helicase 2 (REH2)-associated subcomplex was recently shown to act in parallel to MRB8170 and MRB4160 (17). Moreover, the stage-specific regulation of *MURF1* mRNA guided by its poly(A/U) tail implicates the polyadenylation mediator complex (PAMC) as yet another player in maintaining the steady-state level of some maxicircle transcripts (9, 11, 40).

We provide evidence that MRB8170 and MRB4160 are a nexus between RNA editing and other processing steps. Both proteins satisfy the following requirements to be considered for such a role: (i) they interact with MRB1 core proteins, and their simultaneous depletion compromises the overall integrity of MRB1; (ii) they share a number of RNase-resistant interacting partners outside MRB1 that belong to other processing complexes; (iii) they bind both preedited and edited mRNAs; and (iv)

their simultaneous depletion affects the steady-state abundance of all three categories of maxicircle mRNAs. Below, we elaborate on the basis for these conclusions, ultimately proposing a model of how MRB1 functions in shaping the mt transcriptome.

In agreement with a previous study, our data show that the MRB1 core component GAP1, as well as the accessory protein TbRGG2, is a stable interacting partner of MRB8170 and MRB4160 (24). Moreover, our analyses further support the idea that MRP1, TbRGG1, and Nudix hydrolase are associated with both proteins. To seek further support for this hypothesis, *in vivo* mt mRNA interactome pulldown experiments were carried out in the presence and absence of both MRB8170 and MRB4160. In the latter samples, TbRGG1, TbRGG2, MRP1, and Nudix hydrolase showed a substantial reduction in poly(A) RNA binding. These results allow us to postulate that TbRGG1, TbRGG2, MRP1, and Nudix hydrolase require the assistance of MRB8170 and MRB4160 to bind mRNA. The *in vivo* data were further supported by the observation that addition of recombinant MRB8170 was sufficient to enhance poly(A) RNA binding of TbRGG2 *in vitro*. Taken together, we provide strong evidence that MRB8170 and MRB4160 enhance the activity of other mt RBPs, presumably by attracting or stabilizing them to transcripts already decorated by one or both of these paralogs.

Based on the above results and previous studies (27), we propose a scenario for the regulatory interplay between MRB8170 and TbRGG2 in which the N-terminal RNA recognition motif (RRM) domain of TbRGG2 mediates its interaction with MRB8170 and/or MRB4160 (Fig. 7). This interaction frees the TbRGG2 C-terminal G-rich domain, which was previously sequestered by interaction with the RRM domain, to bind RNA (27, 37). This hypothesized interplay between MRB8170 and TbRGG2 brings a new perspective on how MRB1 is involved in RNA editing. In a model that attempts to integrate the iCLAP data with our *in vivo* and *in vitro* results, the preferential binding of MRB8170 and/or MRB4160 onto preedited mRNAs marks the initiation of RNA editing, followed by binding of TbRGG2 via its RRM domain (Fig. 7). Subsequently, the gRNA-loaded MRB1 core proteins dock into the MRB8170-TbRGG2 (or MRB4160-TbRGG2) subcomplex (also known as the RNA editing mediator complex [REMC]), bringing the MRB1 complex together (19). In the absence of MRB8170 and MRB4160, the bipartite module fails to form, leading to a general reduction in the abundance of fully edited transcripts and an eventual impact on parasite fitness.

MATERIALS AND METHODS

iCLAP protocol. For a single purification, 500 ml of cells expressing mTAP-tagged MRB8170 or MRB4160 was harvested after 2 days of induction. For *in vivo* UV-cross-linking experiments, cells were washed once and then resuspended in 25 ml of ice-cold phosphate-buffered saline (PBS) and placed in a petri dish 5 cm from the light source for UV irradiation (0.8 J/cm^2 at 254 nm for iCLAP library preparation) in a Stratalinker 1800 machine (Stratagene). After a quick spin, the cells were snap-frozen in liquid nitrogen and stored at −80°C until further use. Cell pellets (~1.0 to 1.5 g [dry weight]) were resuspended in 5 ml of lysis buffer (50 mM Tris, pH 7.6, 1.5 mM $MgCl_2$, 10% glycerol, 250 mM NaCl, 2.5 mM β-mercaptoethanol, 0.5% NP-40, 0.1% SDS) containing Complete EDTA-free protease inhibitor cocktail for 10 min on ice. The cell suspension was lysed and spun down by centrifugation (20 min at 20,000 × *g* at 4°C). The supernatant was treated with Turbo DNase (Life Technologies) and RNase I at 37°C for 3 min and then incubated on ice for 3 min as recommended in the published protocol (26). The recovered RNA was used to prepare iCLAP libraries using a previously published protocol (26). The specificity and efficiency of the affinity purification were confirmed by SDS-PAGE and Western blot analysis using anti-His antibody to detect the mTAP-tagged MRB8170 and MRB4160, which also bear this epitope.

Next-generation sequencing and computational analysis. MRB8170, MRB4160, and control (UV-cross-linked parental cells) iCLAP cDNA libraries were sequenced using Illumina Hi-Seq 2000 (single-end sequencing, 75-nt length). Raw reads were trimmed of 3′ adaptor sequences (Tag cleaner version 0.16), and PCR duplicates were collapsed (Fastx collapser version 0.13). The remaining reads were ~30 to 50 nt long. The reads were divided into individual replicates using 4-nt experimental barcodes and mapped first onto preedited (GenBank sequence accession no. M94286) and then to fully edited (39) sequences using Bowtie (Bowtie2 version 0.2) with "very sensitive" preset and a mismatch penalty tightened to 1. More details are in Text S1 in the supplemental material.

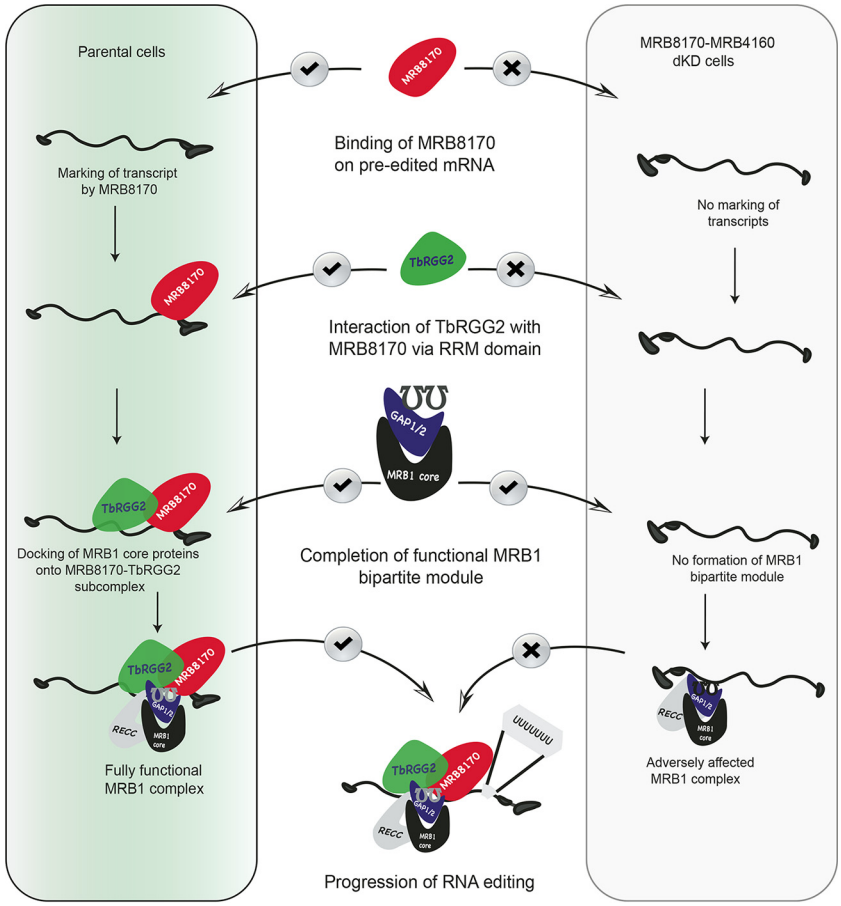

FIG 7 Schematic depiction of the formation of MRB1 bipartite modules in wild-type and MRB8170/MRB4160-depleted cells. In wild-type *T. brucei* (green), the preferential binding of MRB8170 and/or MRB4160 to preedited mRNAs marks the initiation of RNA editing, followed by their binding of TbRGG2 via its RRM domain. Subsequently, the gRNA-loaded MRB1 core proteins dock into the MRB8170-TbRGG2 (or MRB4160-TbRGG2 [data not shown]) subcomplex, eventually completing the MRB1 complex. In contrast, in MRB8170/MRB4160-depleted cells (light gray), MRB1 complex fails to come together, consequently undermining the RNA editing process.

Accession number(s). All the iCLAP sequences are available at ArrayExpress with accession number E-MTAB-4934.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02288-16.

TEXT S1, DOCX file, 0.05 MB.
FIG S1, PDF file, 1.3 MB.
FIG S2, PDF file, 1 MB.
FIG S3, PDF file, 1.5 MB.
FIG S4, PDF file, 1.6 MB.
FIG S5, PDF file, 1.1 MB.
FIG S6, PDF file, 1 MB.
FIG S7, PDF file, 1.6 MB.
TABLE S1, PDF file, 0.05 MB.

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REFERENCES

1. Jensen RE, Englund PT. 2012. Network news: the replication of kinetoplast DNA. Annu Rev Microbiol 66:473–491. https://doi.org/10.1146/annurev-micro-092611-150057.
2. Povelones ML. 2014. Beyond replication: division and segregation of mitochondrial DNA in kinetoplastids. Mol Biochem Parasitol 196:53–60. https://doi.org/10.1016/j.molbiopara.2014.03.008.
3. Benne R, Van den Burg J, Brakenhoff JP, Sloof P, Van Boom JH, Tromp MC. 1986. Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. Cell 46:819–826. https://doi.org/10.1016/0092-8674(86)90063-2.
4. Alfonzo JD, Thiemann O, Simpson L. 1997. The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. Nucleic Acids Res 25:3751–3759. https://doi.org/10.1093/nar/25.19.3751.
5. Aphasizhev R, Aphasizheva I. 2014. Mitochondrial RNA editing in trypanosomes: small RNAs in control. Biochimie 100:125–131. https://doi.org/10.1016/j.biochi.2014.01.003.
6. Read LK, Lukeš J, Hashimi H. 2016. Trypanosome RNA editing: the complexity of getting U in and taking U out. Wiley Interdiscip Rev RNA 7:33–51. https://doi.org/10.1002/wrna.1313.
7. Koslowsky DJ, Yahampath G. 1997. Mitochondrial mRNA 3′ cleavage/polyadenylation and RNA editing in *Trypanosoma brucei* are independent events. Mol Biochem Parasitol 90:81–94. https://doi.org/10.1016/S0166-6851(97)00133-3.
8. Horváth A, Berry EA, Maslov DA. 2000. Translation of the edited mRNA for cytochrome b in trypanosome mitochondria. Science 287:1639–1640. https://doi.org/10.1126/science.287.5458.1639.
9. Aphasizheva I, Maslov D, Wang X, Huang L, Aphasizhev R. 2011. Pentatricopeptide repeat proteins stimulate mRNA adenylation/uridylation to activate mitochondrial translation in trypanosomes. Mol Cell 42:106–117. https://doi.org/10.1016/j.molcel.2011.02.021.
10. Aphasizhev R, Aphasizheva I. 2011. Mitochondrial RNA processing in trypanosomes. Res Microbiol 162:655–663. https://doi.org/10.1016/j.resmic.2011.04.015.
11. Zimmer SL, McEvoy SM, Menon S, Read LK. 2012. Additive and transcript-specific effects of KPAP1 and TbRND activities on 3′ non-encoded tail characteristics and mRNA stability in *Trypanosoma brucei*. PLoS One 7:e37639. https://doi.org/10.1371/journal.pone.0037639.
12. Klingbeil MM, Shapiro TA. 2009. Unraveling the secrets of regulating mitochondrial DNA replication. Mol Cell 35:398–400. https://doi.org/10.1016/j.molcel.2009.08.007.
13. Verner Z, Basu S, Benz C, Dixit S, Dobáková E, Faktorová D, Hashimi H, Horáková E, Huang Z, Paris Z, Peña-Diaz P, Ridlon L, Týč J, Wildridge D, Zíková A, Lukeš J. 2015. Malleable mitochondrion of *Trypanosoma brucei*. Int Rev Cell Mol Biol 315:73–151. https://doi.org/10.1016/bs.ircmb.2014.11.001.
14. Madison-Antenucci S, Grams J, Hajduk SL. 2002. Editing machines: the complexities of trypanosome RNA editing. Cell 108:435–438. https://doi.org/10.1016/S0092-8674(02)00653-0.
15. Stuart KD, Schnaufer A, Ernst NL, Panigrahi AK. 2005. Complex management: RNA editing in trypanosomes. Trends Biochem Sci 30:97–105. https://doi.org/10.1016/j.tibs.2004.12.006.
16. Schnaufer A, Ernst NL, Palazzo SS, O'Rear J, Salavati R, Stuart K. 2003. Separate insertion and deletion subcomplexes of the *Trypanosoma brucei* RNA editing complex. Mol Cell 12:307–319. https://doi.org/10.1016/S1097-2765(03)00286-7.
17. Kumar V, Madina BR, Gulati S, Vashisht AA, Kanyumbu C, Pieters B, Shakir A, Wohlschlegel JA, Read LK, Mooers BHM, Cruz-Reyes J. 2016. REH2C helicase and GRBC subcomplexes may base pair through mRNA and small guide RNA in kinetoplastid editosomes. J Biol Chem 291:5753–5764. https://doi.org/10.1074/jbc.M115.708164.
18. Huang Z, Faktorová D, Křížová A, Kafková L, Read LK, Lukeš J, Hashimi H. 2015. Integrity of the core mitochondrial RNA-binding complex 1 is vital for trypanosome RNA editing. RNA 21:2088–2102. https://doi.org/10.1261/rna.052340.115.
19. Aphasizheva I, Zhang L, Wang X, Kaake RM, Huang L, Monti S, Aphasizhev R. 2014. RNA binding and core complexes constitute the U-insertion/deletion editosome. Mol Cell Biol 34:4329–4342. https://doi.org/10.1128/MCB.01075-14.
20. Weng J, Aphasizheva I, Etheridge RD, Huang L, Wang X, Falick AM, Aphasizhev R. 2008. Guide RNA-binding complex from mitochondria of trypanosomatids. Mol Cell 32:198–209. https://doi.org/10.1016/j.molcel.2008.08.023.
21. Hashimi H, Číčová Z, Novotná L, Wen YZ, Lukeš J. 2009. Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex 1 and mitochondrial RNA polymerase. RNA 15:588–599. https://doi.org/10.1261/rna.1411809.
22. Hashimi H, Zimmer SL, Ammerman ML, Read LK, Lukeš J. 2013. Dual core processing: MRB1 is an emerging kinetoplast RNA editing complex. Trends Parasitol 29:91–99. https://doi.org/10.1016/j.pt.2012.11.005.
23. Ammerman ML, Downey KM, Hashimi H, Fisk JC, Tomasello DL, Faktorová D, Kafková L, King T, Lukeš J, Read LK. 2012. Architecture of the trypanosome RNA editing accessory complex, MRB1. Nucleic Acids Res 40:5637–5650. https://doi.org/10.1093/nar/gks211.
24. Kafková L, Ammerman ML, Faktorová D, Fisk JC, Zimmer SL, Sobotka R, Read LK, Lukeš J, Hashimi H. 2012. Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei*. RNA 18:1846–1861. https://doi.org/10.1261/rna.033852.112.
25. König J, Zarnack K, Rot G, Curk T, Kayikci M, Zupan B, Turner DJ, Luscombe NM, Ule J. 2010. iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution. Nat Struct Mol Biol 17:909–915. https://doi.org/10.1038/nsmb.1838.
26. Huppertz I, Attig J, D'Ambrogio A, Easton LE, Sibley CR, Sugimoto Y, Tajnik M, König J, Ule J. 2014. iCLIP: protein-RNA interactions at nucleotide resolution. Methods 65:274–287. https://doi.org/10.1016/j.ymeth.2013.10.011.
27. Foda BM, Downey KM, Fisk JC, Read LK. 2012. Multifunctional G-rich and RRM-containing domains of TbRGG2 perform separate yet essential functions in trypanosome RNA editing. Eukaryot Cell 11:1119–1131. https://doi.org/10.1128/EC.00175-12.
28. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie2. Nat Methods 9:357–359. https://doi.org/10.1038/nmeth.1923.
29. Lott K, Mukhopadhyay S, Li J, Wang J, Yao J, Sun Y, Qu J, Read LK. 2015. Arginine methylation of DRBD18 differentially impacts its opposing effects on the trypanosome transcriptome. Nucleic Acids Res 43:5501–5523. https://doi.org/10.1093/nar/gkv428.
30. Read LK, Göringer HU, Stuart K. 1994. Assembly of mitochondrial ribonucleoprotein complexes involves specific guide RNA (gRNA)-binding proteins and gRNA domains but does not require preedited mRNA. Mol Cell Biol 14:2629–2639. https://doi.org/10.1128/MCB.14.4.2629.
31. Read LK, Wilson KD, Myler PJ, Stuart K. 1994. Editing of *Trypanosoma brucei* maxicircle CR5 mRNA generates variable carboxy terminal predicted protein sequences. Nucleic Acids Res 22:1489–1495. https://doi.org/10.1093/nar/22.8.1489.
32. Corell RA, Myler P, Stuart K. 1994. *Trypanosoma brucei* mitochondrial CR4 gene encodes an extensively edited mRNA with completely edited sequence only in bloodstream forms. Mol Biochem Parasitol 64:65–74. https://doi.org/10.1016/0166-6851(94)90135-X.
33. Horáková E, Changmai P, Paris Z, Salmon D, Lukeš J. 2015. Simultaneous depletion of ATM and Mdl rebalances cytosolic Fe-S cluster assembly but not heme import into the mitochondrion of *Trypanosoma brucei*. FEBS J 282:4157–4175. https://doi.org/10.1111/febs.13411.
34. Oeffinger M, Wei KE, Rogers R, DeGrasse JA, Chait BT, Aitchison JD, Rout MP. 2007. Comprehensive analysis of diverse ribonucleoprotein complexes. Nat Methods 4:951–956. https://doi.org/10.1038/nmeth1101.
35. Hashimi H, Zíková A, Panigrahi AK, Stuart KD, Lukeš J. 2008. TbRGG1, an essential protein involved in kinetoplastid RNA metabolism that is associated with a novel multiprotein complex. RNA 14:970–980. https://doi.org/10.1261/rna.888808.

36. Castello A, Horos R, Strein C, Fischer B, Eichelbaum K, Steinmetz LM, Krijgsveld J, Hentze MW. 2013. System-wide identification of RNA-binding proteins by interactome capture. Nat Protoc 8:491–500. https://doi.org/10.1038/nprot.2013.020.
37. Fisk JC, Ammerman ML, Presnyak V, Read LK. 2008. TbRGG2, an essential RNA editing accessory factor in two *Trypanosoma brucei* life cycle stages. J Biol Chem 283:23016–23025. https://doi.org/10.1074/jbc.M801021200.
38. Ammerman ML, Presnyak V, Fisk JC, Foda BM, Read LK. 2010. TbRGG2 facilitates kinetoplastid RNA editing initiation and progression past intrinsic pause sites. RNA 16:2239–2251. https://doi.org/10.1261/rna.2285510.
39. Ochsenreiter T, Cipriano M, Hajduk SL. 2007. KISS: the kinetoplastid RNA editing sequence search tool. RNA 13:1–4. https://doi.org/10.1261/rna.232907.
40. Etheridge RD, Aphasizheva I, Gershon PD, Aphasizhev R. 2008. 3′ adenylation determines mRNA abundance and monitors completion of RNA editing in *T. brucei* mitochondria. EMBO J 27:1596–1608. https://doi.org/10.1038/emboj.2008.87.

Chapter 2

Combinatorial interplay of RNA-binding proteins fine-tune levels of edited mRNA in trypanosome mitochondrion

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(Under submission)

Combinatorial interplay of RNA-binding proteins fine-tune levels of edited mRNA in trypanosome mitochondrion

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ABSTRACT

MRP1/2 is an abundant and well-studied heteromeric protein complex that functions in kinetoplastids RNA editing. MRP1/2 was shown to interact with the MRB8170 protein, which initiates RNA editing by marking pre-edited mRNAs, while it requires TbRGG2 for 3′ to 5′ progression of editing. To test whether MRP1/2 interaction with MRB8170 influences the levels of edited mRNAs by interfering with the progression of editing, we applied iCLIP on MRP1 and compared its RNA binding with MRB8170 and TbRGG2. We show that MRP1 competes against TbRGG2 to bind MRB8170 on pre-edited mRNAs. Moreover, we provide a mechanistic framework based on the combinatorial interplay of RNA-binding proteins (RBPs), which can fine-tune levels of fully-edited mRNAs in trypanosome mitochondrion. This provides another compelling case of the involvement of RBPs in highly adaptable post-transcriptional regulatory networks.

INTRODUCTION

The trypanosomatid flagellates constitute a major clade within the Kinetoplastea

group of excavates. Their best-studied representative is *Trypanosoma brucei*, the causative agent of African sleeping sickness in humans and livestock. Probably due to their long independent evolutionary history, numerous molecular processes of the kinetoplastid cell show a complexity exceeding their norm in a typical eukaryotic cell (1). RNA editing is a prominent example, as in these parasitic protists it was not only discovered for the first time (2), but also reached so far unparalleled complexity (3). Being of the uridine (U) insertion and/or deletion type, it is confined to mitochondrial (mt) mRNAs. These are transcribed from mt DNA, also termed the kinetoplast (k) DNA, which is composed of dozens of large maxicircles and thousands of small minicircles, mutually catenated into a single densely packed network (4).

Each ~27 kb-long maxicircle carries 18 protein-coding genes, most of which constitute subunits of the respiratory complexes (1, 4). Intriguingly, to create a standard open reading frame, a dozen of mt mRNAs require post-transcriptional addition and/or removal of a few up to hundreds of U's per mRNA molecule. The function of ~ 1kb-long minicircles is to encode 50 to 70 bp-long non-coding RNAs termed guide (g) RNAs, which provide trans-acting templates to specify the precise insertion/deletion of U's (1, 3). Nine out of twelve mRNAs edited throughout their entire length and employing multiple gRNAs are classified as " pan-edited" transcripts. The remaining three mRNAs require only a few insertions and/or deletions, hence need just one or two gRNAs and are termed "minimally-edited". Finally, the remaining six mRNAs do not undergo editing and constitute the "never-edited" category.

The enzymatic activities required during editing are provided by the RNA editing core complex (RECC or editosome) (5, 6) which, however, lacks RNAs and processivity *in vitro* and therefore requires the assistance of several additional protein

complexes (7–9). One of them is the mitochondrial RNA binding complex (MRP1/2), a heterotetramer consisting of two molecules each of the MRP1 and MRP2 proteins (10–13), which were implicated in mRNA stability and editing. Additionally, the crystal structure of apoMRP1/2 and with gRNAs revealed the nature of extensive non-specific electrostatic-based RNA interactions (14). Moreover, the natively purified MRP1/2 complex, as well as the reconstituted proteins were shown to efficiently promote annealing of mRNAs with gRNAs (13, 15). Still, despite its abundance and conservation in the kinetoplastid flagellates, the function of the MRP1/2 complex in RNA editing and processing remains elusive to the extent that this complex is often ignored in the increasingly intricate editing schemes (3, 16, 17).

Mitochondrial RNA binding complex 1 (MRB1), another protein complex interacting with both RECC and MRP1/2 (18), has two subcomplexes. The first one, termed gRNA-binding complex (GRBC), is composed of six proteins involved in gRNA stability and availability, while the other subcomplex, known as RNA editing mediator complex (REMC) (17), is loosely defined probably due to many transient interactions (19, 20). Lately, functionally redundant MRB8170 and MRB4160 subunits of REMC were shown to act as editing initiators by selectively marking the pre-edited mRNAs (21). To allow the progression of editing in the 3′ to 5′ direction, MRB8170 requires the assistance of another REMC subunit, TbRGG2, which exhibits both RNA melting and annealing properties (22, 23). Interestingly, MRB8170 not only binds TbRGG2 (24) but also interacts with MRP1/2, and its depletion affects poly (A) RNA binding efficiency of both proteins (21). This made us wonder whether the function of MRP1/2 in editing is to compete with TbRGG2 over the binding of MRB8170 on a given mRNA. Such an interaction would influence the levels of fully-edited mRNAs by interfering with the progression of editing.

To test this hypothesis, we used individual nucleotide resolution UV cross-linking and immuno- or affinity- precipitation (iCLIP/iCLAP) to characterize the maxicircle transcriptome-wide binding of MRP1 and generated a quantitative comparison of its bound RNA with MRB8170 and TbRGG2. Our study suggests that MRP1 fine-tunes the levels of fully-edited mRNAs by competing against TbRGG2. Moreover, we provide a mechanistic framework to explain the complex spatial and temporal changes in the fully-edited mRNA population in the trypanosome mitochondrion.

MATERIALS AND METHODS

T.brucei culture conditions and generation of cell lines

Previously constructed cell lines procyclic form (PF) *T. brucei* (strain 29-13) harboring MRB8170/MRB4160 (24), MRP1 with mTAP tags (15), plus those for simultaneously inducible RNAi silencing of MRB8170 and MRB4160 (24), ATM1 (25) or TbRGG2 (22) were used as previously described. RNAi was induced by the addition of 1 μg/ml tetracycline to culture media. Cell densities were measured by using a Z2 cell counter (Beckman Coulter Inc) and were maintained in the exponential mid-log growth phase at 27°C with constant shaking.

In-vivo UV-cross linking and immunity purification (iCLIP) protocol

29-13 cells (Parental cell line) were irradiated once with 800 J/cm^2 UV light (254 nm), and subsequently MRP1 iCLIP was performed as previously described for MRB8170 and MRB4160 proteins (21). Protein G Dynabeads coupled with anti-MRP1 antibody were used for immunoprecipitation (IP). The specificity and efficiency of IP was confirmed by western blot. Cross-linked, immunopurified RNA

was digested of 60-150 nt, reverse transcribed to generate iCLIP libraries. The adapter oligo-nucleotides, reverse-transcription primers for amplification were as described (26). The iCLIP libraries were sequenced using Illumina Hi-Seq 2000 (single-end sequencing, 75-nt length).

Computational analysis of Next Generation Sequencing data

Two replicates each for MRP1 (UV-crosslinked 29-13 cells) and negative control (non-UV crosslinked 29-13 cells) were sequenced using Illumina Hi-Seq 2000 (single-end sequencing, 75-nt length). The processing of iCLIP tags was done as previously described (21). For all subsequent analyses, replicates were merged into one iCLAP dataset. In total, we obtained 613,7846 uniquely aligned iCLIP tags for MRP1 (123,8207and 589,9639 for replicates 1 and 2, respectively) The control library from Non UV-crosslinked parental cells contained 12856 uniquely mapping iCLIP tags (7588 and 5268 for replicates 1 and 2, respectively). Already published MRB8170, MRB4160 and mRNA-seq libraries were used in this study (21, 27). Mapping of iCLIP data was done using Bowtie 2 (28), while data normalization was carried out using a Bioconductor package DESeq2 (29). The data visualization of iCLIP and RNA-seq was done using Integrative Genomics Viewer (https://www.broadinstitute.org/igv/) (30).

RNA immunoprecipitation and quantitative real-time PCR

TbRGG2 and ATM1 knockdown cells were harvested after 24 hr of RNAi induction were resuspended in RIP lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 1 mM DTT, 10% glycerol, 0.5% NP-40) containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and RNaseOUT (100 units; Life Technologies). The supernatant

was Turbo DNase treated (Life Technologies) and subjected to immunoprecipitation using anti-MRP1 antibodies coated with Protein G Dynabeads for 2 hr at 4°C. Ten percent of the supernatant was taken as input to generate cDNA for normalizing the respective immunoprecipitated samples. After immunoprecipitation, the beads were washed with RIP wash buffer (50 mM Tris pH7.5, 1 M NaCl, 0.5% NP40, and 0.1% SDS) three times before phenol extraction of RNA. RNA obtained from the supernatant (input) and eluate (output) was transcribed into cDNA and further analyzed by qPCR (Light Cycler, Roche). The relative ratios were calculated for each immunoprecipitated sample normalized against their respective input. Construction of cDNA was done using the QuantiTect Reverse Transcription Kit (Qiagen). Previously designed primers that anneal to the specified maxicircle mRNA sequences were used in qPCR (31). The PCR conditions were 95°C for 10 min, followed by 40 cycles [95°C for 15 sec, 60°C for 1 min]. Separate immunoprecipitation strategy is used concerning MRP1 mTAP tag RIP-qPCR by employing IgG-Sepharose beads (GE Healthcare); the rest of the protocol remains same. Similarly, TbRGG2 RIP-qPCR immunoprecipitation is done using antibody against TbRGG2 coated Protein G Dynabeads.

Statistical Analysis

All plots, graphs and statistical analyses were done using GraphPad Prism 5.00 (GraphPad, San Diego, CA, USA).

RESULTS

MRP1 efficiently binds to minimally- and never-edited mRNAs

Recently described iCLAP protocol on MRB8170 and MRB4160 (21) was herein adapted for the MRP1 immunoprecipitation (IP), with the aim to quantify RNA molecules bound by MRP1 *in vivo* (Figure 1A*)*. The UV-crosslinked cells showed a strong signal of radiolabelled RNAs at the expected size of ~21-50 kDa (Figure S1A; lane 3). Two conditions used as negative controls, namely a mock IP and a non-UV-crosslink, yielded no radiolabelled signal (Figure S1A; lanes 1 and 2). Next, from the UV- and non-UV-crosslinked lanes, the labelled smears (~21-50 kDa) were excised and processed for iCLIP libraries (Figure S1A; lanes 2 and 3). The size-selected iCLIP libraries were verified using gel electrophoresis (Figure S1B).

Two biological replicates of the UV- and non-UV-crosslinked iCLIP libraries were processed, and afterwards mapped against pre- and fully-edited maxicircle transcripts (Figure 1B). The uniquely mapped reads from two replicates, from now on called iCLIP tags, were used for comparative purposes. A strong correlation (r= 0.99) between MRP1 replicates (Figure S2A) allowed us to combine them into a single dataset. The MRP1 replicates yielded a total of 6,137,846 iCLIP tags, while the control produced only 12,856 of them. The ~500 fold difference in the mapped iCLIP tags demonstrated high stringency of the applied protocol. For subsequent analyses, the published MRB8170 and MRB4160 iCLIP libraries were pooled together (Figure S2B). To further verify iCLIP stringency, we generated correlation plots between MRP1, MRB8170 and MRB4160, which confirmed that MRB8170 and MRB4160 are functional paralogs (r=0.94) (24), while MRP1 demonstrated no such correlation (r=0.19) (Figure S2C).

To examine MRP1 binding across mRNAs, we categorized the mapped iCLIP tags into three categories composed of 9 pan-edited, 3 minimally-edited, and 6 never-edited mRNAs (Figure 1B). In order to account for their editing status, we clustered

iCLIP tags mapped on both pre- and fully-edited regions of the pan- and minimally-edited mRNAs (Figure 1B). The total of 31.6% and 51.1% iCLIP tags belonged to pan- and minimally-edited mRNAs, respectively, while the remaining 17.1% went into the never-edited category (Figure 1C). Next, we normalized the dataset to account for varied gene lengths, which resulted in the enrichment of 43.5%, 50.5%, and 5.85% over pan-, minimally-, and never-edited mRNAs, respectively (Figure 1D).

Both normalized and non-normalized results depict the higher binding of MRP1 over minimally- and never-edited mRNAs, as compared to its interacting partner MRB8170, which dedicates ~90% of its iCLIP tags to pan-edited mRNAs (21). To quantify and compare MRP1 and MRB8170 binding across the three mRNA classes, we plotted the mapped normalized iCLIP tags of these proteins relative to the published mRNA-seq mapped read counts (20, 21). In the case of MRP1, this resulted in ~5 and ~2 fold enrichment over never- and minimally-edited mRNAs, respectively, while pan-edited mRNAs were close to their transcription level (Figure 1E). On the contrary, the enrichment of MRB8170 and MRB4160 iCLIP tags for pan-edited mRNAs was ~2 and ~1.5 fold higher, respectively, as compared to the other two transcript categories. Furthermore, visual inspection of protein binding on mRNAs revealed higher enrichment of bound MRP1 with respect to MRB8170 on minimally- and never-edited mRNAs (Figures 1F and S4-S6). Combined, these results reveal that when compared to its interacting partner MRB8170 or functionally redundant MRB4160, MRP1 efficiently binds minimally- and never-edited mRNAs, while the binding to pan-edited mRNAs is less efficient.

MRP1 binding of pan-edited mRNAs correlates neither with MRB8170 nor with their editing status

The iCLIP protocol generates ~30-50 nt-long iCLIP tags, which in the case of *T. brucei* mt mRNAs creates an inherent bias in identifying the genesis of mapped iCLIP tags, due to the varying extent of their editing (Figure 2A). To circumvent this bias, we merged iCLIP tags from pre- and fully-edited regions into a single "total" category. Next, to dissect repressed binding of MRP1 to pan-edited mRNAs and to explore its binding relationship with respect to its interacting partner MRB8170. We calculated their individual proportions mapped on pan-edited mRNAs and used them for further analyses (Figure S3A). Total bar-plots of MRB8170 and MRP1 differed in that 90% of MRB8170 iCLIP tags were derived from *COX3*, *ND7*, *ND8*, and *A6* mRNAs (21), while the same mRNAs represented only ~50% of the latter dataset (Figure S3A).

To analyze the binding relationship between MRP1 and MRB8170 over pan-edited mRNAs, we calculated the Pearson's correlation coefficient by plotting total iCLIP tag counts mapped on all 9 pan-edited mRNAs (Figures 2 B and C). The strong correlation between MRB8170 and MRB4160 (r= 0.99) served as a positive control. The essential role of both paralogs in their editing is reflected by correlation-plot with mRNA-seq (r= 0.98). However, the comparison of MRP1 with that of MRB8170 and mRNA-seq revealed a poor correlation with both (r= 0.30 and r = 0.261, respectively) (Figure 2C). Next, we explored whether MRP1 binding correlates with the extent of U insertions/deletions in a given transcripts, as is the case of MRB8170 and MRB4160 (r= 0.72, r= 0.75, respectively) (Figure 2D). However, the plot failed to identify such a correlation (r = -0.001). Taken together, these analyses indicate that the extent of MRP1 binding to pan-edited mRNAs is neither governed by the requirement for U's, nor does it correlate with the binding of pan-edited mRNAs by MRB8170.

MRP1 binding of minimally- and never-edited mRNAs correlates with MRB8170

Since the above-described analysis showed no correlation between the binding of MRP1 and MRB8170 to pan-edited mRNAs, we explored their relationship with minimally- and never-edited mRNAs. Firstly, we used the above approaches to assess MRP1 binding on minimally- and never-edited mRNAs by generating bar-plots for them (Figures S3B and C). Next, we tested the binding correlation between MRP1 and MRB8170 over minimally-edited mRNAs by plotting their cDNA counts (r= 0.99) (Figure 2E). MRP1 cDNA counts were also plotted against mRNA-seq for minimally-edited mRNAs, revealing a good correlation (r= 0.85) (Figure S3D). Since from both paralogs only MRB8170 binds never-edited mRNAs (21), we generated a binding correlation-plot between MRP1 and MRB8170 on this category of transcripts. It also revealed a high correlation between them (r=0.99) (Figure 2F), although MRP1 showed a negative correlation when assayed against mRNA-seq on them (r= - 0.41) (Figure 3SE). Finally, we validated the MRP1 iCLIP results by RNA immunoprecipitation (RIP) in combination with quantitative (q) PCR on the MRP1-tagged cells. Indeed, MRP1 RIP-qPCR revealed strong binding to minimally- and never- edited mRNAs, as compared to pan-edited ones (Figure 2G). Combined, a quantitative comparison of MRP1 and MRB8170 binding on mRNAs shows a strong correlation over minimally- and never-edited mRNAs, which is not the case with pan-edited mRNAs.

TbRGG2 and MRP1 compete against each other

Since MRB8170 interacts not only with MRP1 but also with TbRGG2 (21, 24), we wondered whether MRP1/2 competes with TbRGG2 to bind MRB8170. Firstly, we tested if TbRGG2, which is indispensable for pan-editing (22), preferentially binds pan-edited mRNAs over the other two mRNA categories. This was likely since the depletion of TbRGG2 does not impact minimally- and never-edited mRNAs (22, 32).

For that end, we applied TbRGG2 RIP-qPCR (Figure 3A). Remarkably, this method revealed that TbRGG2 binds almost exclusively pan-edited mRNAs, while the binding of other two mRNA categories is highly repressed (Figure 3A). Next, we tested whether TbRGG2 and MRP1 interfere with each other over their binding of MRB8170. For this we used an indirect method by relying on MRP1 and MRB8170 iCLIP data. The transcripts were divided into two classes, based on their levels in the TbRGG2-depleted cells. The first class contains all 9 pan-edited mRNAs, their fully-edited counterparts being negatively influenced in the TbRGG2-depleted cells (22, 33). The second class brings together 9 unaffected mRNAs comprising minimally- and never-edited mRNAs.

Our aim was to analyze whether between these two classes, there is a different enrichment ratio of MRB8170 versus MRP1. In iCLIP data the enrichment ratio of MRB8170 versus MRP1 significantly differs for both classes (Figure 3B). Transcripts affected by the depletion of TbRGG2 show higher variability in the enrichment ratio, while there is almost no variability in the other class. Similar analyses using MRB4160 instead of MRP1 revealed negligible variability and served as a negative control (Figure 3C). In the case of MRB4160, the second class of mRNAs was not analyzed, because this protein does not bind never-edited mRNAs (21). From this we conclude that by residual binding on pan-edited mRNAs, MRP1 likely interferes with TbRGG2 in binding of MRB8170.

Next, we measured the relative abundance of immunoprecipitated RNA by MRP1 in cells depleted of TbRGG2 (Figure 3D). Cells with down-regulated Atm1, an inner mt membrane protein involved in iron-sulfur cluster assembly that neither affects mt gene expression (25), nor competes with MRP1, was used as a control. In the Atm1-depleted cells, MRP1 showed a similar level of bound mRNAs as in the

parental cells. However, we found a significant increase in the mRNAs pulled down by MRP1 in the absence of TbRGG2 (Figure 3D), reflecting their mutual competition. Strikingly, mRNAs unaffected by the lack of TbRGG2 also participate in this increase. This can be attributed to TbRGG2 and MRB8170 RNA-independent interaction, which might release more MRB8170 to bind mRNAs in TbRGG2-depleted cells (Figure 3E).

The pan-edited mRNAs represent the only category where MRP1 competes with TbRGG2, which is essential for their editing. Therefore, we investigated mutual relationships among these two RNA-binding proteins (RBPs) and MRB8170. For this purpose a model is considered, which is based on the following assumptions. Firstly, MRP1 and TbRGG2 compete with each other to bind MRB8170. If so, a higher ratio of bound MRP1 versus MRB8170 will inversely affect the TbRGG2 versus MRB8170 ratio, consequently blocking the progression of editing (20, 23, 33). Secondly, based on previous studies, MRP1 may also play a role in mRNA stability of pre-edited mRNAs. Consequently, both higher and lower enrichment of bound MRP1 versus MRB8170 on pre-edited mRNAs will result in lower mRNA-seq counts. To test this "competition" model, we plotted the enrichment ratio of bound MRP1 versus MRB8170 for each pan-edited mRNA against their corresponding mRNA-seq read counts (Figure 4A). Strikingly, we observed a bell-shaped graph, wherein the tail corresponds to the high and low enrichment ratio of MRP1 on mRNAs. The high mRNA-seq read counts of *ND7* and *COX3* mRNAs with an intermediate enrichment ratio appear at the peak of the graph. Taken together, our results favor a situation where MRP1 and TbRGG2 compete with one another to bind MRB8170.

DISCUSSION

Recently, MRB8170 was shown to act in the initiation of editing by selectively marking pre-edited mRNAs (21), yet it requires the assistance of TbRGG2 for the progression of editing in the 3′ to 5′ direction (20, 23, 33). Moreover, MRB8170 not only interacts with TbRGG2 but also with the MRP1/2 complex, and its depletion affect mRNA binding capability of both these proteins (21). This prompted us to test whether the function of MRP1/2 in editing is to compete with TbRGG2 over mRNAs bound by MRB8170, and if this interference might influence the progression of editing.

In this study, we use iCLIP on MRP1 in order to dissect RNA interactions of the MRP1/2 complex. A tight mutual dependence between MRP1 and MRP2 makes their phenotypic effects highly similar (13–15), although this shall eventually be addressed by a dedicated MRP2 iCLIP. We report that MRP1 efficiently binds minimally- and never-edited mRNAs as compared to pan-edited ones. Consequently, the repressed binding of MRP1 to pan-edited mRNAs suggests a more efficient binding of TbRGG2 to MRB8170 on this category of transcripts. In support of this conclusion, our study showed an almost exclusive binding of TbRGG2 to pan-edited molecules, as compared to the other two mRNA categories. Furthermore, a quantitative comparison of the binding by MRP1 and MRB8170 revealed a strong correlation between these two proteins over the binding of minimally- and never-edited mRNAs, while no correlation was apparent over the binding of pan-edited mRNAs. Our analyses also failed to detect any relationship between the binding of MRP1 and the extent of editing of a given mRNA. Combined, these results suggest that MRB8170 teams up with MRP1 over the binding of minimally- and never-edited

mRNAs, while its preferred partner for the binding of pan-edited mRNAs is TbRGG2.

Perhaps the most striking observation of our study is that the enrichment ratio of MRP1 with respect to MRB8170 showed a huge variability exclusively for pan-edited mRNAs, where TbRGG2 is supposed to compete. This sharply contrasts with almost no variability in the enrichment ratio of MRP1 versus MRB8170 on minimally- and never-edited mRNAs, likely because of the absence of competing TbRGG2. To further test the hypothesized competition between these proteins over the binding of pan-edited mRNAs, we built a model based on the role of MRB8170 in the initiation of editing, while TbRGG2 is required for its progression (23). This means that a higher ratio of bound MRP1 with respect to MRB8170 on a given mRNA will inversely affect the TbRGG2 versus MRB8170 ratio, halting editing at the pause sites, while lower binding of MRP1 on an mRNA molecule will impact its stability (34). This way, MRP1 may tweak editing in either positive or negative way, depending upon its enrichment ratio on the pre-edited mRNAs (Figure 4B). Simulations conducted to test this “competition” model faithfully recapitulated such dynamics. We found further support for this model by measuring the amount of RNA bound by MRP1 in cells depleted of either TbRGG2 or Atm1. Indeed, we observed a significant increase in mRNAs immunoprecipitated by MRP1 solely in the background of depleted TbRGG2, strongly advocating for the above-described model.

Our study puts several unexplained observations from previous studies on a firmer ground. The observed unimpeded editing of minimally-edited mRNAs in the TbRGG2-depleted cells correlates with its lack of binding to this category of transcripts (22, 33). At the same time, the highly correlated binding of MRP1 and MRB8170 of minimally-edited mRNAs sheds light on their role in editing of

minimally-edited *Murf2* and *Cyb* transcripts (24, 34). Moreover, previously implied role of MRP1/2 in mRNA stability of never-edited mRNAs (34, 35) is supported by MRP1 highly enriched binding on them. Finally, pan-edited transcripts emerged as the only category where MRP1 competes with TbRGG2, which is essential for their editing.

The RNA binding protein, RBP16, adds another layer of complexity by having a similar function in RNA stability and annealing as that of MRP1 (34). Consequently, such functional redundancy between MRP1/2 and RBP16 was used to explain the somewhat limited effects of their individual depletion, while when depleted simultaneously; the effect is dramatic (34). A strong support for our "competition" model of fine-tuning the levels of mRNAs proposed here explains the complex positive and negative effects of RBP16 on mRNA levels in the MRP1/2-depleted cells (34). Furthermore, it also explains the increased levels of pre-edited form of pan-edited mRNAs and the parallel decrease of their fully-edited counterparts in TbRGG2-depleted cells (32, 36). Consequently, the lack of TbRGG2 will cause an enrichment of MRP1 on pre-edited mRNAs, while the absence of TbRGG2 will halt editing at pause sites leading to diminished levels of fully-edited mRNA. Intriguingly, both MRP1/2 and TbRGG2 exhibit gRNA/mRNA annealing activity and share similar RNA modulated interactions with the GRBC sub-complex of MRB1 (14, 15, 36). Therefore, we speculate that MRP1/2 can replace TbRGG2 during editing by binding to MRB8170, while narrowing the indispensability of TbRGG2 exclusively to the editing pause sites. If correct, it further explains why TbRGG2 mostly affects editing of pan-edited mRNAs and much less that of minimally-edited ones (22), as the former category harbors more editing pause sites by having larger editing regions. The same reasoning might explain the recently observed lack of overlap in positions

where editing gets stalled on mRNAs in the MRB8170 and TbRGG2-depleted cells (20). Reflecting its role in the initiation of editing, the process indeed gets stalled at the initial editing sites in the MRB8170-depleted cells, while TbRGG2-depletion halts editing at the editing pause sites.

In conclusion, we provide a mechanistic framework based on the combinatorial interplay among several RBPs (Figure 4B), which likely allows a fine-tuned control of the levels of fully-edited mRNAs in response to the life cycle stage and/or environmental signals. Only recently, we started to appreciate the complex regulatory roles exercised by RBPs from several studies related to alternative splicing (37, 38). Here we show that the same category of proteins participates in the complex regulation of mitochondrial RNA editing in trypanosomes.

Data availability

Accession number: All the iCLIP sequences are available at ArrayExpress with accession number E-MTAB-4934.
(http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-4934/)

SUPPLEMENTARY DATA

Supplementary Data includes six figures.

AUTHORS CONTRIBUTION

SD performed the experiments. Both authors designed experiments, analyzed data and wrote the manuscript.

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Conflict of interest statement. None declared

REFERENCES

1. Verner,Z., Basu,S., Benz,C., Dixit,S., Dobáková,E., Faktorová,D., Hashimi,H., Horáková,E., Huang,Z., Paris,Z., *et al.* (2015) Malleable mitochondrion of *Trypanosoma brucei*. *Int. Rev. Cell. Mol. Biol.*, **315**, 73-151

2. Benne,R., Van den Burg,J., Brakenhoff,J.P., Sloof,P., Van Boom,J.H. and Tromp,M.C. (1986) Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell*, **46**, 819–826.

3. Read,L.K., Lukeš,J. and Hashimi,H. (2016) Trypanosome RNA editing: The complexity of getting U in and taking U out. *Wiley Interdiscip. Rev. RNA*, **7**, 33–51.

4. Jensen,R.E. and Englund,P.T. (2012) Network news: the replication of kinetoplast DNA. *Annu. Rev. Microbiol.*, **66**, 473–491.

5. Schnaufer,A., Panigrahi,A.K., Panicucci,B., Igo,R.P., Salavati,R. and Stuart,K. (2001) An RNA ligase essential for RNA editing and survival of the bloodstream form of *Trypanosoma brucei*. *Science*, **291**, 2159–2162.

6. Schnaufer,A., Ernst,N.L., Palazzo,S.S., O'Rear,J., Salavati,R. and Stuart,K. (2003)

Separate insertion and deletion subcomplexes of the *Trypanosoma brucei* RNA editing complex. *Mol. Cell*, **12**, 307–319.

7. Rusché,L.N., Cruz-Reyes,J., Piller,K.J. and Sollner-Webb,B. (1997) Purification of a functional enzymatic editing complex from *Trypanosoma brucei* mitochondria. *EMBO J.*, **16**, 4069–81.
8. Li,F., Ge,P., Hui,W.H., Atanasov,I., Rogers,K., Guo,Q., Osato,D., Falick,A.M., Zhou,Z.H. and Simpson,L. (2009) Structure of the core editing complex (L-complex) involved in uridine insertion/deletion RNA editing in trypanosomatid mitochondria. *Proc. Natl. Acad. Sci.*, **106**, 12306–12310.
9. Suematsu,T., Zhang,L., Aphasizheva,I., Monti,S., Huang,L., Wang,Q., Costello,C.E. and Aphasizhev,R. (2016) Antisense transcripts delimit exonucleolytic activity of the mitochondrial 3' processome to generate guide RNAs. *Mol. Cell*, **61**, 364–378.
10. Allen,T.E., Heidmann,S., Reed,R., Myler,P.J., Göringer,H.U. and Stuart,K.D. (1998) Association of guide RNA binding protein gBP21 with active RNA editing complexes in *Trypanosoma brucei*. *Mol. Cell. Biol.*, **18**, 6014–22.
11. Müller,U.F. and Göringer,H.U. (2002) Mechanism of the gBP21-mediated RNA/RNA annealing reaction: matchmaking and charge reduction. *Nucleic Acids Res.*, **30**, 447–55.
12. Aphasizhev,R., Aphasizheva,I., Nelson,R.E. and Simpson,L. (2003) A 100-kD complex of two RNA-binding proteins from mitochondria of *Leishmania tarentolae* catalyzes RNA annealing and interacts with several RNA editing components. *RNA*, **9**, 62–76.
13. Vondrušková,E., Van Den Burg,J., Zíková,A., Ernst,N.L., Stuart,K., Benne,R. and Lukeš,J. (2005) RNA interference analyses suggest a transcript-specific

regulatory role for mitochondrial RNA-binding proteins MRP1 and MRP2 in RNA editing and other RNA processing in *Trypanosoma brucei*. *J. Biol. Chem.*, **280**, 2429–2438.

14. Schumacher,M.A., Karamooz,E., Zíková,A., Trantírek,L. and Lukeš,J. (2006) Crystal structures of *T. brucei* MRP1/MRP2 guide-RNA binding complex reveal RNA matchmaking mechanism. *Cell*, **126**, 701–711.
15. Zíková,A., Kopečná,J., Schumacher,M.A., Stuart,K., Trantírek,L. and Lukeš,J. (2008) Structure and function of the native and recombinant mitochondrial MRP1/MRP2 complex from *Trypanosoma brucei*. *Int. J. Parasitol.*, **38**, 901–912.
16. Aphasizhev,R. and Aphasizheva,I. (2011) Uridine insertion/deletion editing in trypanosomes: A playground for RNA-guided information transfer. *Wiley Interdiscip. Rev. RNA*, **2**, 669–685.
17. Aphasizheva,I. and Aphasizhev,R. (2016) U-Insertion/Deletion mRNA-Editing Holoenzyme: Definition in Sight. *Trends Parasitol.*, **32**, 144–56.
18. Weng,J., Aphasizheva,I., Etheridge,R.D., Huang,L., Wang,X., Falick,A.M. and Aphasizhev,R. (2008) Guide RNA-binding complex from mitochondria of trypanosomatids. *Mol. Cell*, **32**, 198–209.
19. Hashimi,H., Zimmer,S.L., Ammerman,M.L., Read,L.K. and Lukeš,J. (2013) Dual core processing: MRB1 is an emerging kinetoplast RNA editing complex. *Trends Parasitol.*, **29**, 91–99.
20. Simpson,R.M., Bruno,A.E., Chen,R., Lott,K., Tylec,B.L., Bard,J.E., Sun,Y., Buck,M.J. and Read,L.K. (2017) Trypanosome RNA editing mediator complex proteins have distinct functions in gRNA utilization. *Nucleic Acids Res.*, **45**, 7965–7983.

21. Dixit,S., Müller-McNicoll,M., David,V., Zarnack,K., Ule,J., Hashimi,H. and Lukeš,J. (2017) Differential binding of mitochondrial transcripts by MRB8170 and MRB4160 regulates distinct editing fates of mitochondrial mRNA in trypanosomes. *mBio*, **8**, e02288-16.

22. Fisk,J.C., Ammerman,M.L., Presnyak,V. and Read,L.K. (2008) TbRGG2, an essential RNA editing accessory factor in two *trypanosoma brucei* life cycle stages. *J. Biol. Chem.*, **283**, 23016–23025.

23. Ammerman,M.L., Presnyak,V., Fisk,J.C., Foda,B.M. and Read,L.K. (2010) TbRGG2 facilitates kinetoplastid RNA editing initiation and progression past intrinsic pause sites. *RNA*, **16**, 2239–2251.

24. Kafková,L., Ammerman,M.L., Faktorová,D., Fisk,J.C., Zimmer,S.L., Sobotka,R., Read,L.K., Lukes,J. and Hashimi,H. (2012) Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei. RNA*, **18**, 1846-1861.

25. Horáková,E., Changmai,P., Paris,Z., Salmon,D. and Lukeš,J. (2015) Simultaneous depletion of Atm and Mdl rebalances cytosolic Fe-S cluster assembly but not heme import into the mitochondrion of *Trypanosoma brucei*. *FEBS J.*, **282**, 4157-4175.

26. Huppertz,I., Attig,J., D'Ambrogio,A., Easton,L.E., Sibley,C.R., Sugimoto,Y., Tajnik,M., König,J. and Ule,J. (2014) iCLIP: protein-RNA interactions at nucleotide resolution. *Methods*, **65**, 274–87.

27. Lott,K., Mukhopadhyay,S., Li,J., Wang,J., Yao,J., Sun,Y., Qu,J. and Read,L.K. (2015) Arginine methylation of DRBD18 differentially impacts its opposing effects on the trypanosome transcriptome. *Nucleic Acids Res.*, **43**, 5501–5523.

28. Langmead,B., Trapnell,C., Pop,M., Salzberg,S.L., Dettling,M., Dudoit,S., Ellis,B.,

Gautier,L., Ge,Y., Gentry,J., *et al.* (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.*, **10**, R25.

29. Love,M.I., Huber,W. and Anders,S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.*, ***15***, 550.
30. Thorvaldsdottir,H., Robinson,J.T. and Mesirov,J.P. (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief. Bioinform.*, **14**, 178–192.
31. Carnes,J., Trotter,J.R., Ernst,N.L., Steinberg,A. and Stuart,K. (2005) An essential RNase III insertion editing endonuclease in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. U. S. A.*, **102**, 16614–9.
32. Acestor,N., Panigrahi,A.K., Carnes,J., Zíková,A. and Stuart,K.D. (2009) The MRB1 complex functions in kinetoplastid RNA processing. *RNA,* **15**, 277–286.
33. Foda,B.M., Downey,K.M., Fisk,J.C. and Read,L.K. (2012) Multifunctional G-rich and RRM-containing domains of TbRGG2 perform separate yet essential functions in trypanosome RNA editing. *Eukaryot. Cell*, **11**, 1119–1131.
34. Fisk,J.C., Presnyak,V., Ammerman,M.L. and Read,L.K. (2009) Distinct and overlapping functions of MRP1/2 and RBP16 in mitochondrial RNA metabolism. *Mol. Cell. Biol.*, **29**, 5214–5225.
35. Hashimi,H., Cicová,Z., Novotná,L., Wen,Y.-Z. and Lukeš,J. (2009) Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex 1 and mitochondrial RNA polymerase. *RNA*, **15**, 588–99.
36. Aphasizheva,I., Zhang,L., Wang,X., Kaake,R.M., Huang,L., Monti,S. and Aphasizhev,R. (2014) RNA binding and core complexes constitute the U-insertion/deletion editosome. *Mol. Cell. Biol.*, **34**, 4329–42.

37. Zarnack,K., König,J., Tajnik,M., Martincorena,I., Eustermann,S., Stévant,I., Reyes,A., Anders,S., Luscombe,N.M. and Ule,J. (2013) Direct competition between hnRNP C and U2AF65 protects the transcriptome from the exonization of Alu elements. *Cell*, **152**, 453–466.

38. Fu,X.-D., Ares,M. and Jr (2014) Context-dependent control of alternative splicing by RNA-binding proteins. *Nat. Rev. Genet.*, **15**, 689–701.

FIGURE LEGENDS

Figure 1. MRP1 iCLIP and its efficient binding to minimally- and never-edited mRNAs

(A) Schematic depiction of the MRP1 iCLIP workflow to purify UV-crosslinked RNA-MRP1 complexes.

(B) Illustration of a strategy used to map MRP1 iCLIP data. Pre-processed iCLIP reads were mapped against maxicircle mRNAs to its pre- and fully-edited forms. The uniquely mapped iCLIP tags from both forms were combined and named as total iCLIP tags. Never-edited mRNAs contributed only to pre-edited iCLIP tags since they lack editing. Pan-, minimally and never-edited mRNAs are marked in blue, grey and black, respectively.

(C) Percentage of total MRP1 iCLIP tags contribution over three different categories of mRNAs.

(D) The fold enrichment of iCLIP tags density on dividing the number of iCLIP tags by their length of each RNA feature.

(E) Comparison of fold enrichment of iCLIP tags over three different classes of mRNAs from MRB8170, MRB4160 and MRP1 iCLIP libraries. The data is expressed relative to mapped mRNA-seq read counts.

(F) Genomic browser snapshots of protein binding by MRP1 and MRB8170 on pre-edited form of *ND8* (pan-edited), *CYB* (minimally-edited) and *ND4* (never-edited) mRNA. White lines show the approximate distribution of editing sites on *ND8* and *CYB*.

See also Figures S1, S2, and S4-6.

Figure 2. Quantitative comparison of MRP1 and MRB8170 binding on different class of mRNAs

(A) Schematic representation of intrinsic bias in iCLIP mapping due to the progression of 3` to 5` editing. We used prolong RNAse treatment to get rid of bound non-specific RNA and generate small RNA fragments for sequencing. This results in ~ 30 to 50 nt-long iCLIP tags after the removal of adaptors. However, mapping short iCLIP tags create a drawback as it becomes difficult to decipher the origin of uniquely mapped iCLIP tags on pre- and fully-edited forms which might be contributed by their partially-edited forms. This creates a bias in interpreting the quantitative data. In response, we combined uniquely mapped iCLIP tags on pre-edited and fully-edited regions and called them total and use them for later analysis.

(B) Plots comparing the total number of mapped iCLIP tags on nine pan-edited mRNAs from MRB8170, MRB4160 and mRNA-seq data. Pearson correlation coefficient are (r) indicated

(C) As in B, from MRP1, MRB8170 and mRNA-seq data.

(D) Plot displaying the correlation between total mapped iCLIP tags (*y*-axis) on pan-edited transcripts and the respective number of U insertions/deletions required by them (x-axis). Pearson correlation coefficients (r) are indicated.

(E) Plots comparing the total number of mapped iCLIP tags on three minimally-edited mRNAs from MRB8170 and MRP1. Pearson correlation coefficients (r) are indicated.

(F) Plots comparing the total number of mapped iCLIP tags on six never-edited mRNAs from MRB8170 and MRP1. Pearson correlation coefficients (r) are indicated.

(G) MRP1 RIP-qPCR. Bar-plots show the relative amount of pre- and never-edited mRNAs bound by MRP1. Data is presented relative to the RNA recovered from 10% of input lysate. One representative set of measurement is shown.

See also Figure S3

Figure 3. MRP1 and TbRGG2 competes with one another to bind MRB8170

(A) TbRGG2 RIP-qPCR. Bar-plots show the relative amount of pre-and never-edited mRNAs bound by TbRGG2. Data is presented relative to the RNA recovered from 10% of input lysate. One representative set of measurement is shown.

(B) Comparing variability in the enrichment ratio of bound MRB8170 with respect to MRP1 (y-axis) over individual mRNAs. Transcripts were separated into two categories based on their levels in TbRGG2 depleted cells. First category contains nine pan-edited mRNAs, which are affected by TbRGG2 depletion (TbRGG2 affected mRNAs), while the other nine mRNAs consist of minimally- and never-edited mRNAs that are marked as TbRGG2 unaffected mRNAs (Mann-Whitney test, **P <0.005).

(C) As in C, for comparing MRB8170 with respect to MRB4160. TbRGG2 unaffected mRNAs were not shown since MRB4160 lacks binding over

never-edited mRNAs.

(D) Bar-plot displays the relative amount of immunoprecipitated mRNAs by MRP1 in three different conditions using RIP-qPCR (Tukey's multiple comparison test, *P <0.05, ***P <0.001, ****P< 0.0001, n=2-3). Data was presented relative to the RNA recovered from 10% of input.

(E) In parental cells, MRB8170 is bound to mRNAs, thereby initiated editing. Meanwhile, TbRGG2 and MRP1 are competing with one another to bind MRB8170 on pan-edited mRNAs, while MRP1 alone is its preferred binding partner on other mRNA classes. The situation differs in TbRGG2-depleted cells, as lack of TbRGG2 halts editing of pan-edited mRNAs, while there is no effect on other mRNA classes due to MRP1 availability. Moreover, TbRGG2-depletion releases more MRB8170 that was previously bound to it by RNAse-independent interaction.

Figure 4. "Competition" model involving three RNA-binding proteins

(A) Plot displays enrichment ratio of bound proteins MRB8170 vs. MRP1 on each pan-edited mRNAs (x-axis), against their respective total mapped read-counts (y-axis). Data was fitted using nonlinear model for Gaussian function.

(B) Schematized competition between TbRGG2 and MRP1 for MRB8170, over pan-edited mRNAs. TbRGG2 is essential for the progression of editing at the pause sites, whereas MRP1 provides RNA stability. Therefore, on a given pre-edited mRNA higher amount of bound MRP1 will cease editing. On the contrary, mRNA will be destabilized if TbRGG2 outcompetes MRP1. Consequently, the combinatorial interplay among these RBPs by deploying their different ratios on a pre-edited mRNA will allow the fine-tuning of fully-

edited mRNA levels.

SUPPLEMENTARY FIGURES

Figure S1. MRP1 iCLIP library verification

(A) Co-purification of UV crosslinked RNA-MRP1 complexes. The ^{32}P-labelled complexes were monitored by autoradiography. Mock IP (lane1) and Non-UV crosslinked parental cell line (lane 2) yielded no signal. The low RNAse treated (boxed region in lane 3) was used to prepare the iCLIP libraries. Two independent replicates were sequenced. Western blot confirmed the presence of MRP1 (21 kDa).

(B) TBE-6% urea gel showing amplified MRP1 iCLIP libraries. The cDNAs were size fractionated and amplified separately to reduce any size biases during PCR. Three sizes were cut out and PCR amplified, which are indicated as high (H) (150-300 nt), medium (M) (80-150 nt) and low (L) (60-80 nt). The H and M nucleotide long libraries were submitted for sequencing. RT –ve, represent reverse transcriptase controls.

Figure S2. Correlation-plots

(A) Plots comparing the total number of mapped iCLIP tag on mRNAs from different replicates for MRB4160 and MRP1. Pearson correlation coefficients (r) are indicated.

(B) As in A, for MRB8170 and MRB4160

(C) Plots comparing the total number of mapped iCLIP tag on mRNAs between MRB8170, MRB4160 and MRP1. Pearson correlation coefficients (r) are indicated.

Figure S3. MRP1 iCLIP tags distribution on different categories of mRNAs

(A) Bar-plot shows the MRP1 iCLIP tags distribution over individual pan-edited mRNAs. The percentage is calculated separately for pre- fully- and total-mapped iCLIP tags in their respective regions.

(B) As in A, for minimally edited mRNAs.

(C) As in A, for never-edited mRNAs

(D) Plots comparing the total number of mapped iCLIP tag on minimally-edited mRNAs between MRP1 and mRNA-seq. Pearson correlation coefficients (r) are indicated.

(E) As in D, for never-edited mRNAs

Figure S4. Genomic browser snapshots for 9 pan-edited transcripts

The unique cDNA count is depicted on the y-axis and the mapped tag position along a given transcript is on the x-axis.

Figure S5. Genomic browser snapshots for 3 minimally-edited transcripts

The unique cDNA count is depicted on the y-axis and the mapped tag position along a given transcript is on the x-axis.

Figure S6. Genomic browser snapshots for 6 never-edited transcripts

The unique cDNA count is depicted on the y-axis and the mapped tag position along a given transcript is on the x-axis.

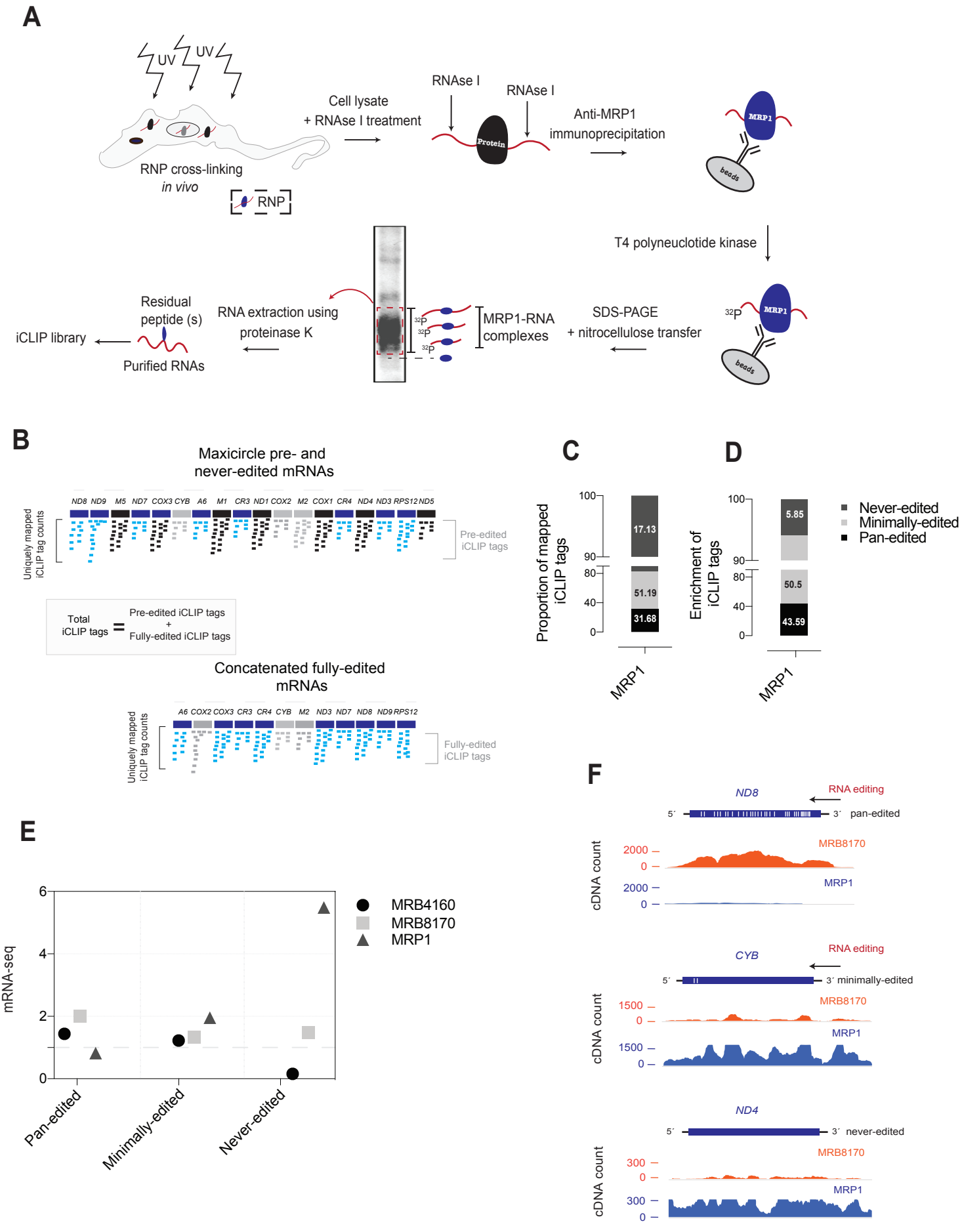

A
UV
UV
RNP cross-linking
in vivo
RNP
Cell lysate
+ RNAse I treatment
RNAse I
RNAse I
Protein
Anti-MRP1
immunoprecipitation
MRP1
beads
T4 polyneuclotide kinase
32P
MRP1
beads
SDS-PAGE
+ nitrocellulose transfer
MRP1-RNA
complexes
RNA extraction using
proteinase K
Residual
peptide (s)
Purified RNAs
iCLIP library
B
Maxicircle pre- and
never-edited mRNAs
ND8 ND9 M5 ND7 COX3 CYB A6 M1 CR3 ND1 COX2 M2 COX1 CR4 ND4 ND3 RPS12 ND5
Uniquely mapped
iCLIP tag counts
Pre-edited
iCLIP tags
Total
iCLIP tags
=
Pre-edited iCLIP tags
+
Fully-edited iCLIP tags
Concatenated fully-edited
mRNAs
A6 COX2 COX3 CR3 CR4 CYB M2 ND3 ND7 ND8 ND9 RPS12
Uniquely mapped
iCLIP tag counts
Fully-edited
iCLIP tags
C
Proportion of mapped
iCLIP tags
100
90
80
40
0
17.13
51.19
31.68
MRP1
D
Enrichment of
iCLIP tags
100
90
80
40
0
5.85
50.5
43.59
MRP1
Never-edited
Minimally-edited
Pan-edited
E
Enrichment relative to
mRNA-seq
6
4
2
0
Pan-edited
Minimally-edited
Never-edited
MRB4160
MRB8170
MRP1
F
ND8
RNA editing
5′
3′ pan-edited
cDNA count
2000
0
MRB8170
2000
0
MRP1
CYB
RNA editing
5′
3′ minimally-edited
1500
0
MRB8170
1500
0
MRP1
ND4
5′
3′ never-edited
300
0
MRB8170
300
0
MRP1

Figure 1.

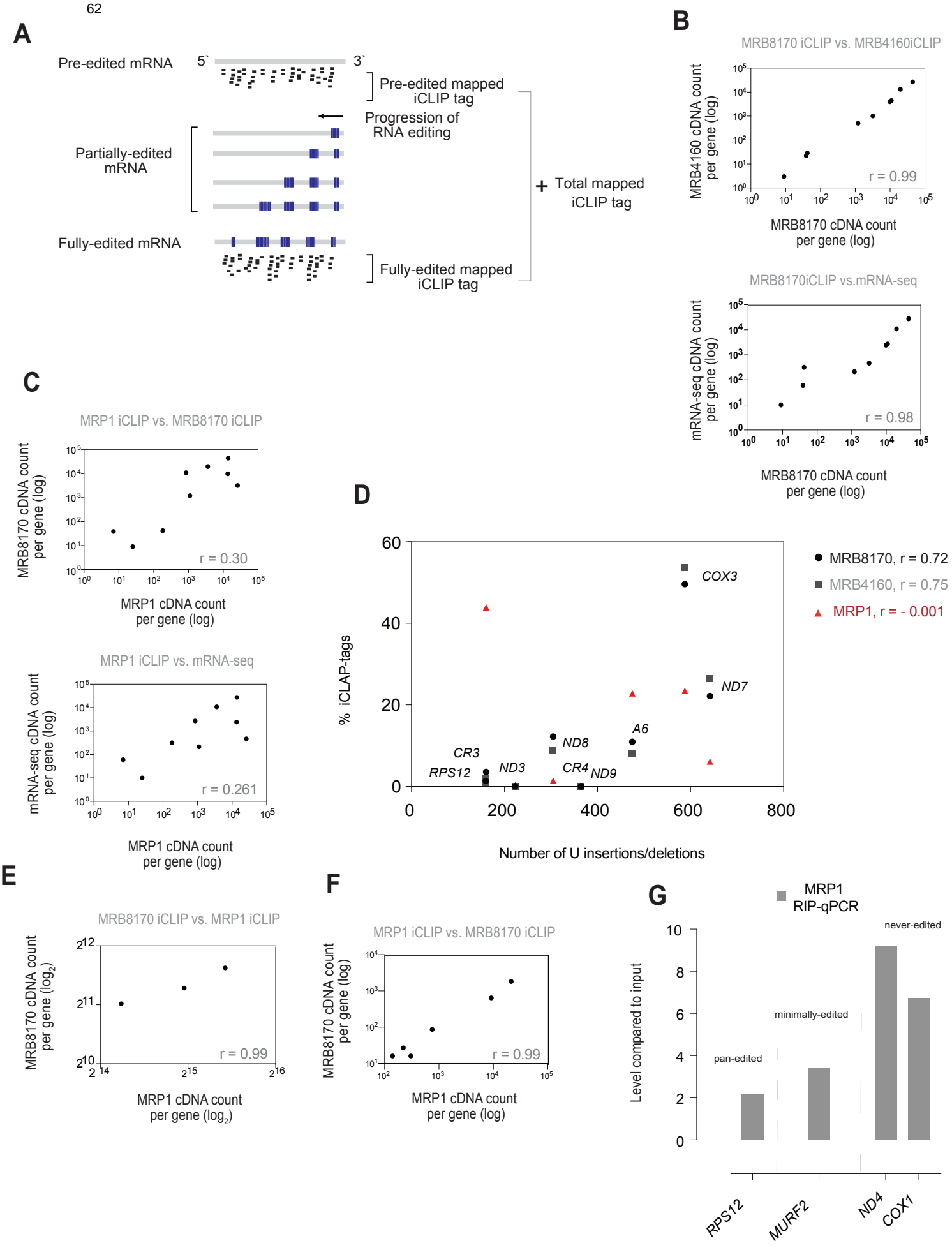

A
Pre-edited mRNA
5`
3`
Pre-edited mapped iCLIP tag
Progression of RNA editing
Partially-edited mRNA
+ Total mapped iCLIP tag
Fully-edited mRNA
Fully-edited mapped iCLIP tag
B
MRB8170 iCLIP vs. MRB4160iCLIP
MRB4160 cDNA count per gene (log)
MRB8170 cDNA count per gene (log)
r = 0.99
MRB8170iCLIP vs.mRNA-seq
mRNA-seq cDNA count per gene (log)
MRB8170 cDNA count per gene (log)
r = 0.98
C
MRP1 iCLIP vs. MRB8170 iCLIP
MRB8170 cDNA count per gene (log)
MRP1 cDNA count per gene (log)
r = 0.30
MRP1 iCLIP vs. mRNA-seq
mRNA-seq cDNA count per gene (log)
MRP1 cDNA count per gene (log)
r = 0.261
D
% iCLAP-tags
Number of U insertions/deletions
COX3
ND7
A6
ND8
CR3
RPS12
ND3
CR4
ND9
MRB8170, r = 0.72
MRB4160, r = 0.75
MRP1, r = - 0.001
E
MRB8170 iCLIP vs. MRP1 iCLIP
MRB8170 cDNA count per gene (log2)
MRP1 cDNA count per gene (log2)
r = 0.99
F
MRP1 iCLIP vs. MRB8170 iCLIP
MRB8170 cDNA count per gene (log)
MRP1 cDNA count per gene (log)
r = 0.99
G
MRP1 RIP-qPCR
Level compared to input
never-edited
minimally-edited
pan-edited
RPS12
MURF2
ND4
COX1

Figure 2.

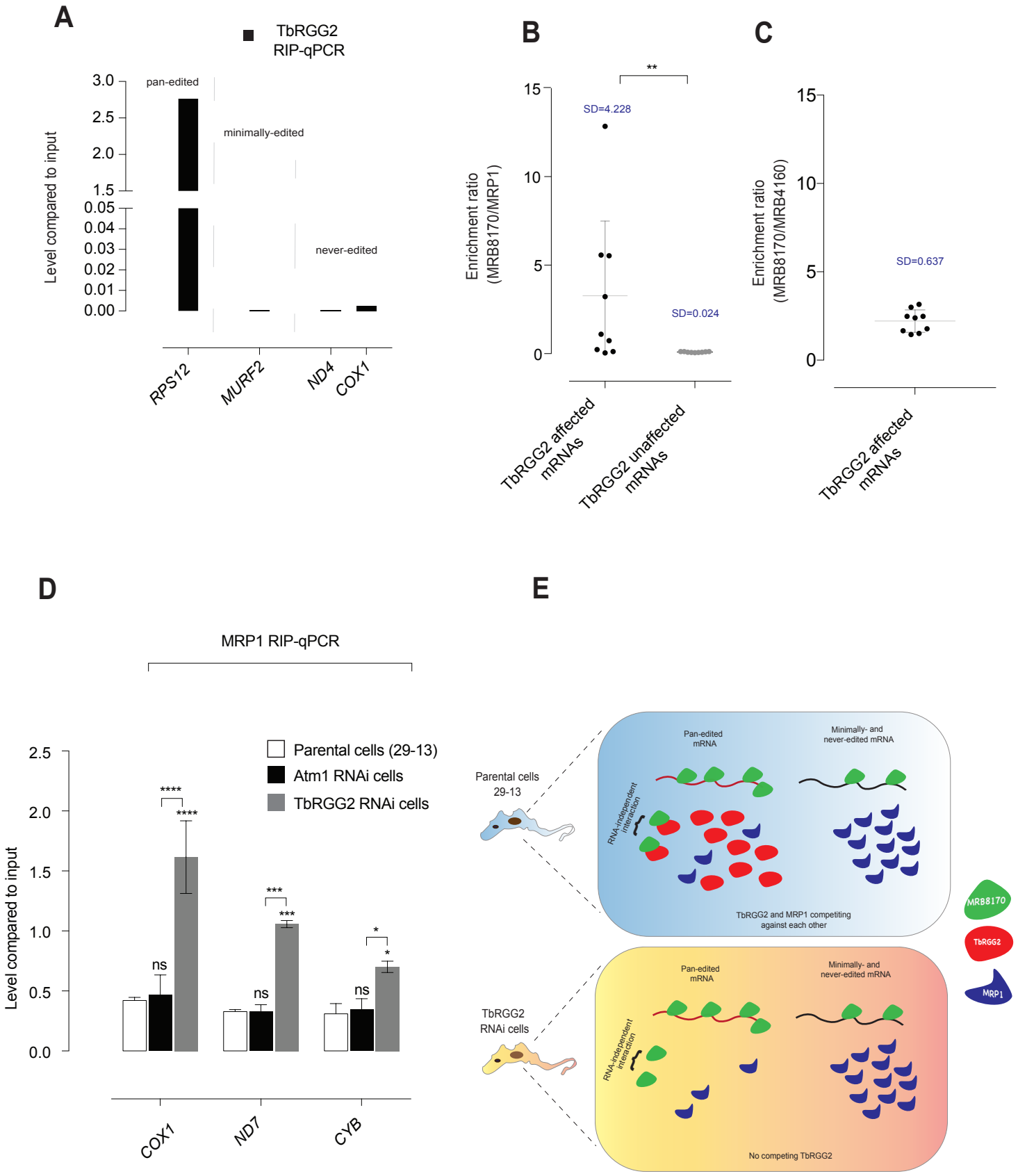
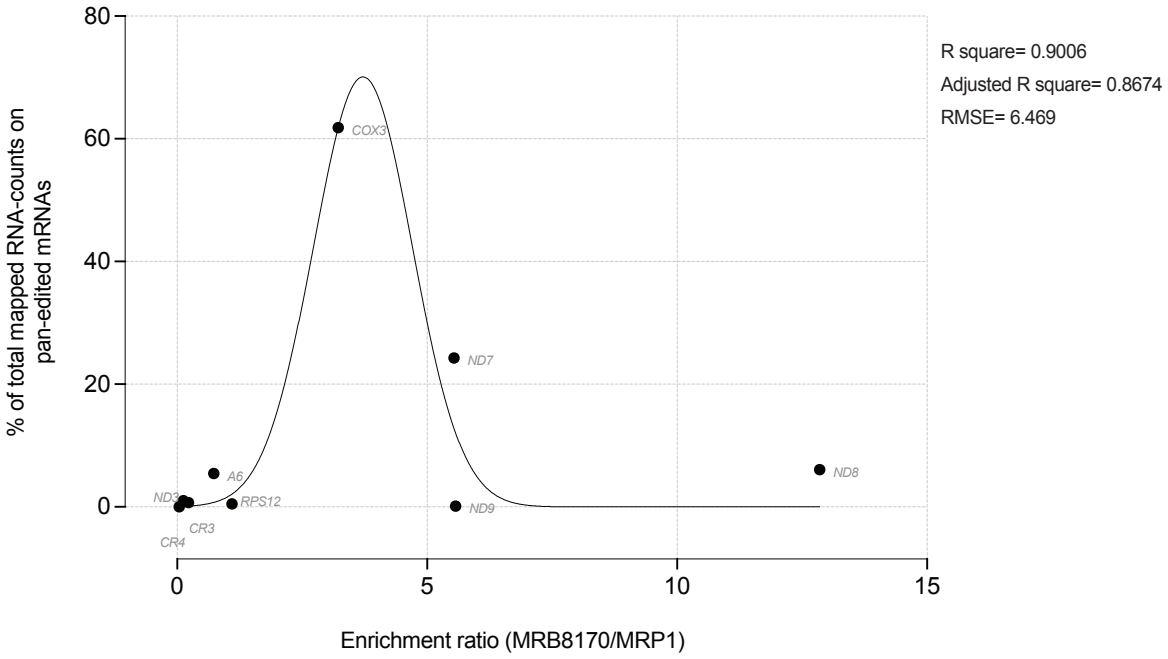

A
TbRGG2
RIP-qPCR
Level compared to input
pan-edited
minimally-edited
never-edited
RPS12
MURF2
ND4
COX1
B
Enrichment ratio
(MRB8170/MRP1)
SD=4.228
SD=0.024
TbRGG2 affected mRNAs
TbRGG2 unaffected mRNAs
C
Enrichment ratio
(MRB8170/MRB4160)
SD=0.637
TbRGG2 affected mRNAs
D
MRP1 RIP-qPCR
Parental cells (29-13)
Atm1 RNAi cells
TbRGG2 RNAi cells
Level compared to input
COX1
ND7
CYB
E
Parental cells 29-13
Pan-edited mRNA
Minimally- and never-edited mRNA
RNA-independent interaction
TbRGG2 and MRP1 competing against each other
TbRGG2 RNAi cells
No competing TbRGG2
MRB8170
TbRGG2
MRP1

Figure 3.

A


% of total mapped RNA-counts on pan-edited mRNAs
Enrichment ratio (MRB8170/MRP1)
R square= 0.9006
Adjusted R square= 0.8674
RMSE= 6.469
COX3
ND7
ND8
A6
ND3
RPS12
ND9
CR3
CR4

B

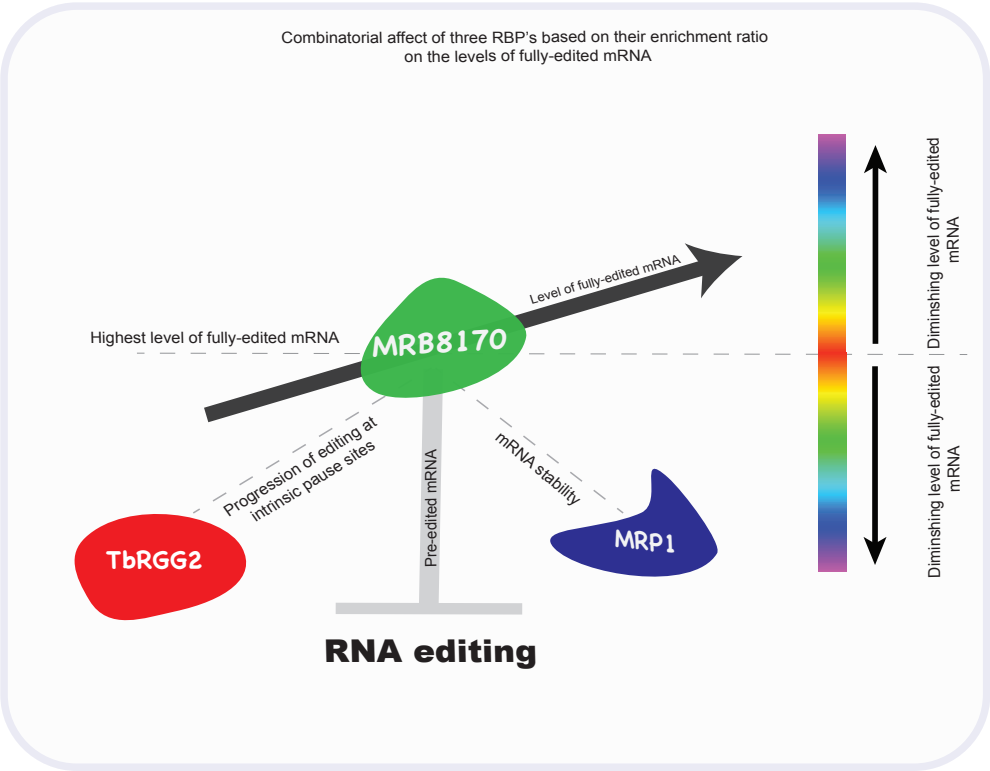

Combinatorial affect of three RBP's based on their enrichment ratio on the levels of fully-edited mRNA
Highest level of fully-edited mRNA
Level of fully-edited mRNA
MRB8170
Progression of editing at intrinsic pause sites
Pre-edited mRNA
mRNA stability
TbRGG2
MRP1
RNA editing
Diminshing level of fully-edited mRNA
Diminshing level of fully-edited mRNA

Figure 4.

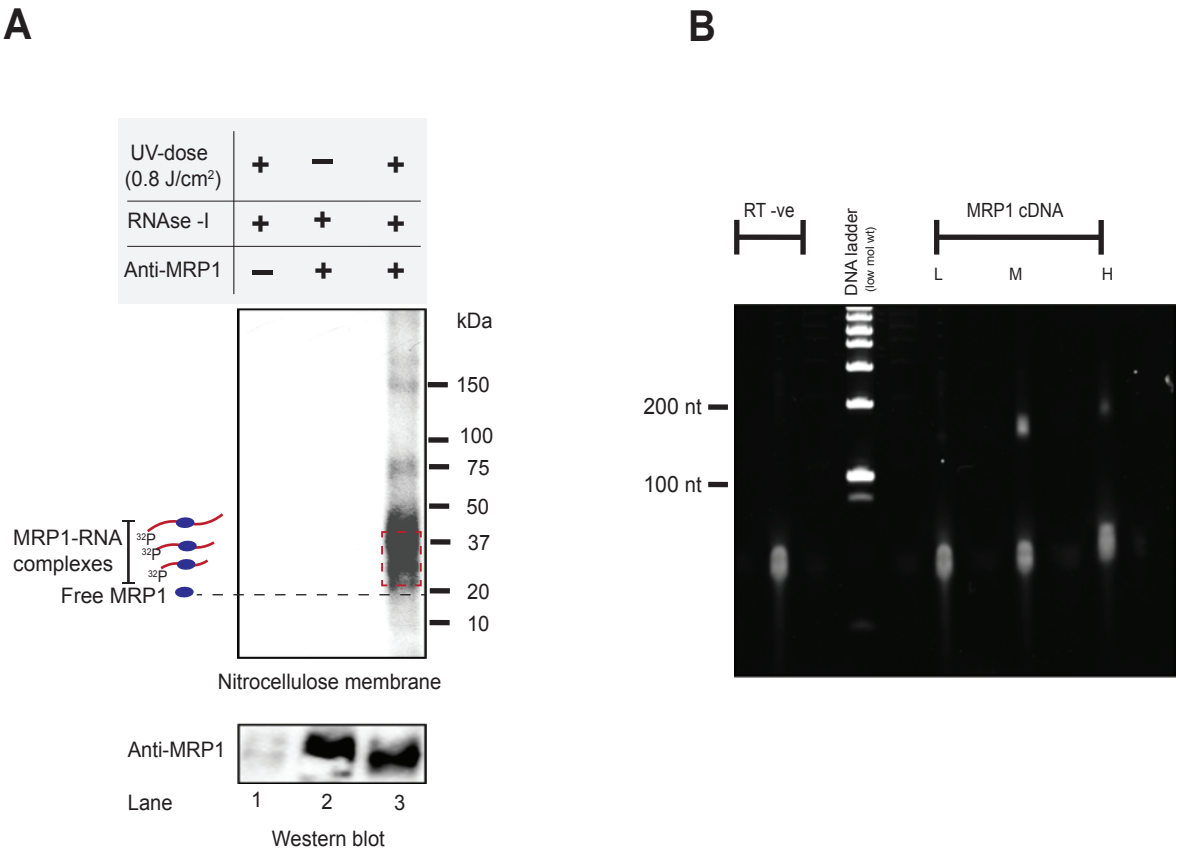
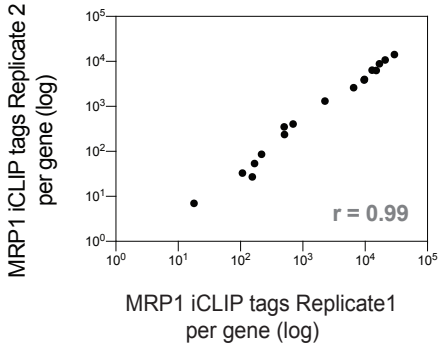
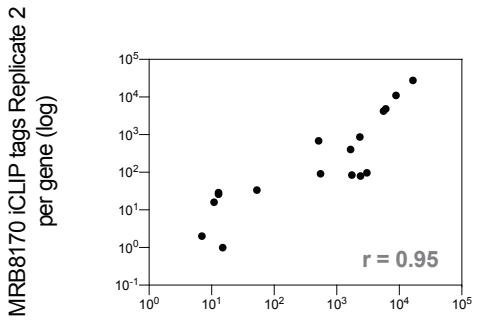

A
UV-dose (0.8 J/cm²)
RNAse -I
Anti-MRP1
kDa
150
100
75
50
37
20
10
MRP1-RNA complexes
Free MRP1
Nitrocellulose membrane
Anti-MRP1
Lane
1
2
3
Western blot
B
RT -ve
DNA ladder (low mol wt)
MRP1 cDNA
L
M
H
200 nt
100 nt

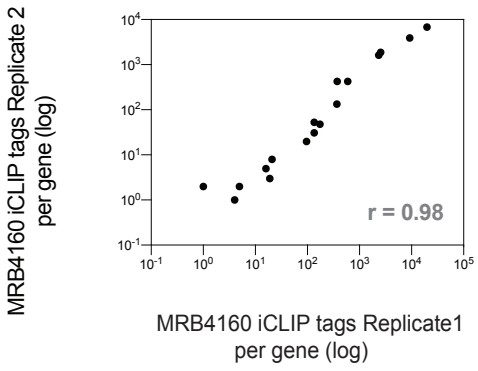
Figure S1.

A

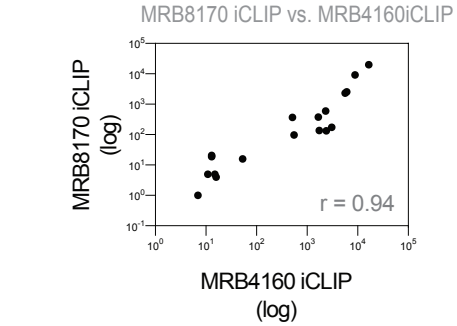


B





C



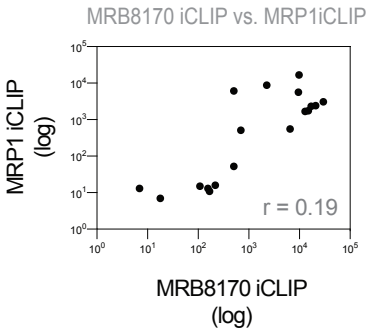
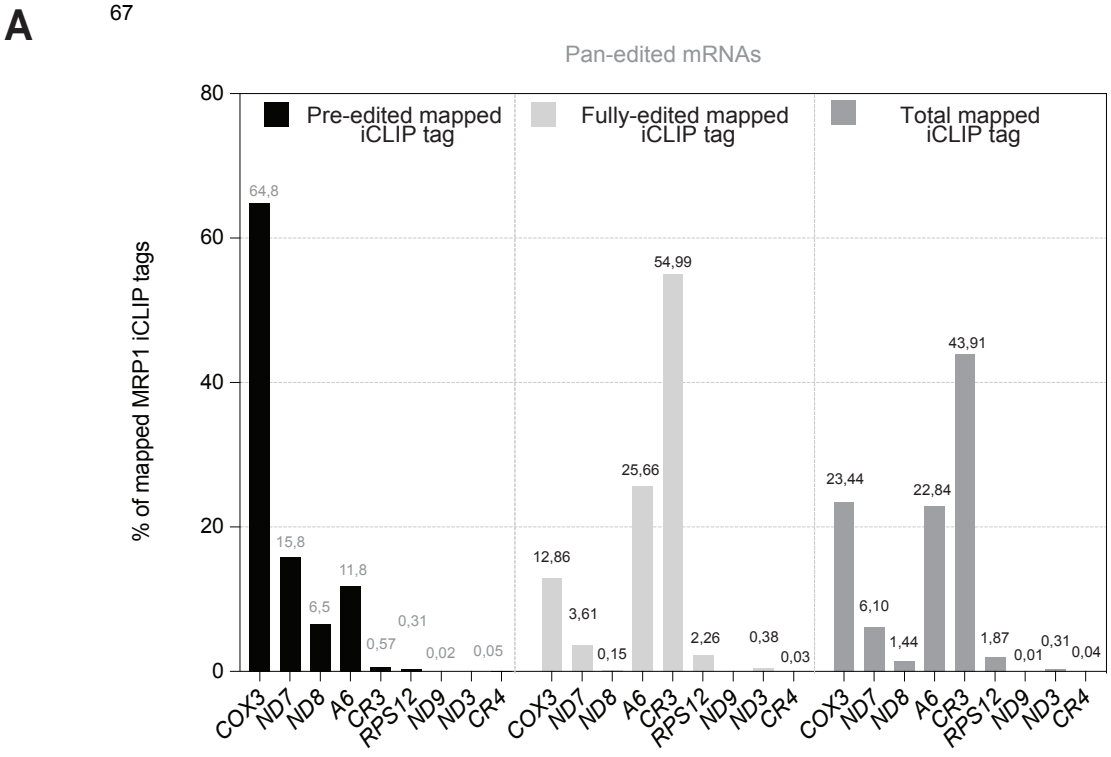
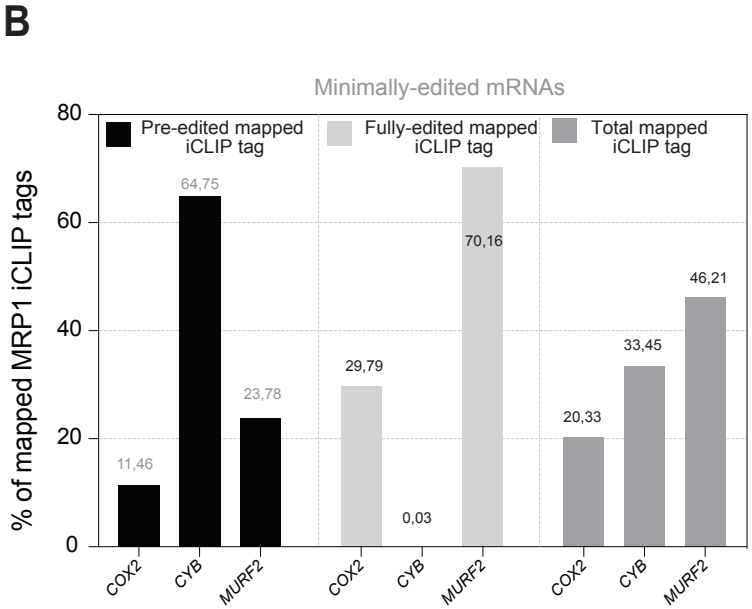
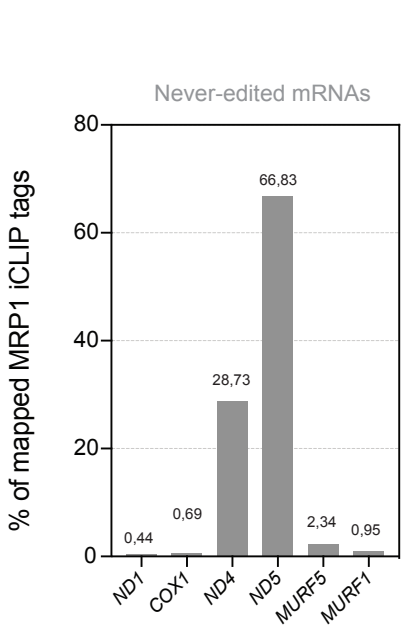
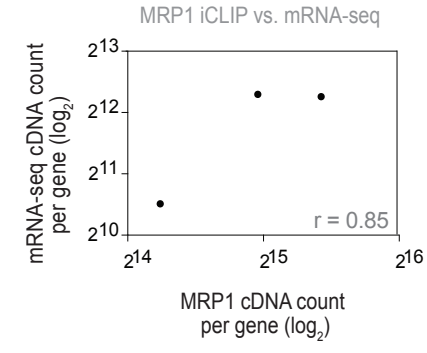


Figure S2.


A
Pan-edited mRNAs
Pre-edited mapped iCLIP tag
Fully-edited mapped iCLIP tag
Total mapped iCLIP tag
% of mapped MRP1 iCLIP tags
64,8
15,8
6,5
11,8
0,57
0,31
0,02
0,05
12,86
3,61
0,15
25,66
54,99
2,26
0,38
0,03
23,44
6,10
1,44
22,84
43,91
1,87
0,01
0,31
0,04
COX3 ND7 ND8 A6 CR3 RPS12 ND9 ND3 CR4


B
Minimally-edited mRNAs
Pre-edited mapped iCLIP tag
Fully-edited mapped iCLIP tag
Total mapped iCLIP tag
% of mapped MRP1 iCLIP tags
11,46
64,75
23,78
29,79
0,03
70,16
20,33
33,45
46,21
COX2 CYB MURF2


C
Never-edited mRNAs
% of mapped MRP1 iCLIP tags
0,44
0,69
28,73
66,83
2,34
0,95
ND1 COX1 ND4 ND5 MURF5 MURF1


D
MRP1 iCLIP vs. mRNA-seq
mRNA-seq cDNA count per gene (log2)
r = 0.85
MRP1 cDNA count per gene (log2)

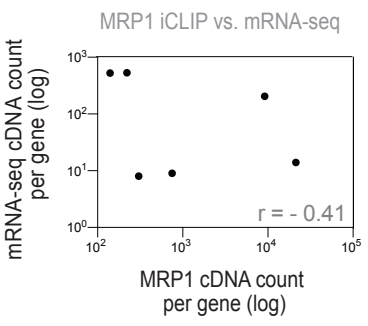

E
MRP1 iCLIP vs. mRNA-seq
mRNA-seq cDNA count per gene (log)
r = - 0.41
MRP1 cDNA count per gene (log)

Figure S3.

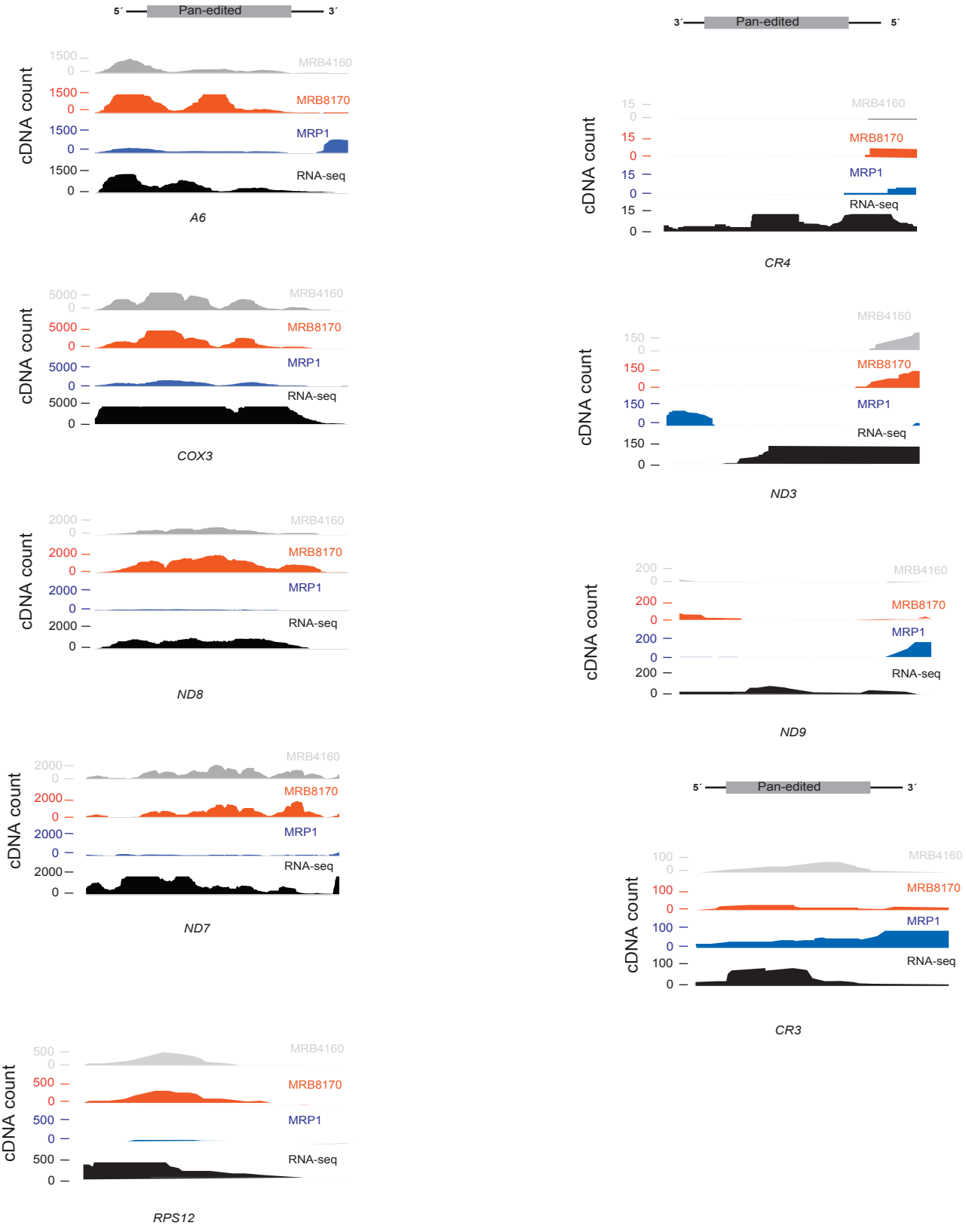

5´ Pan-edited 3´
cDNA count
MRB4160
MRB8170
MRP1
RNA-seq
A6
COX3
ND8
ND7
RPS12
3´ Pan-edited 5´
CR4
ND3
ND9
5´ Pan-edited 3´
CR3

Figure S4.

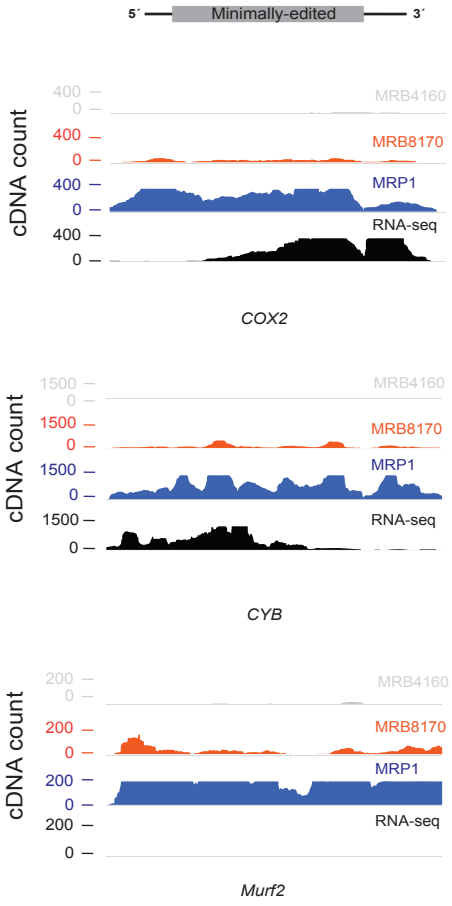

5´
Minimally-edited
3´
cDNA count
400
0
MRB4160
MRB8170
MRP1
RNA-seq
COX2
1500
CYB
200
Murf2

Figure S5.

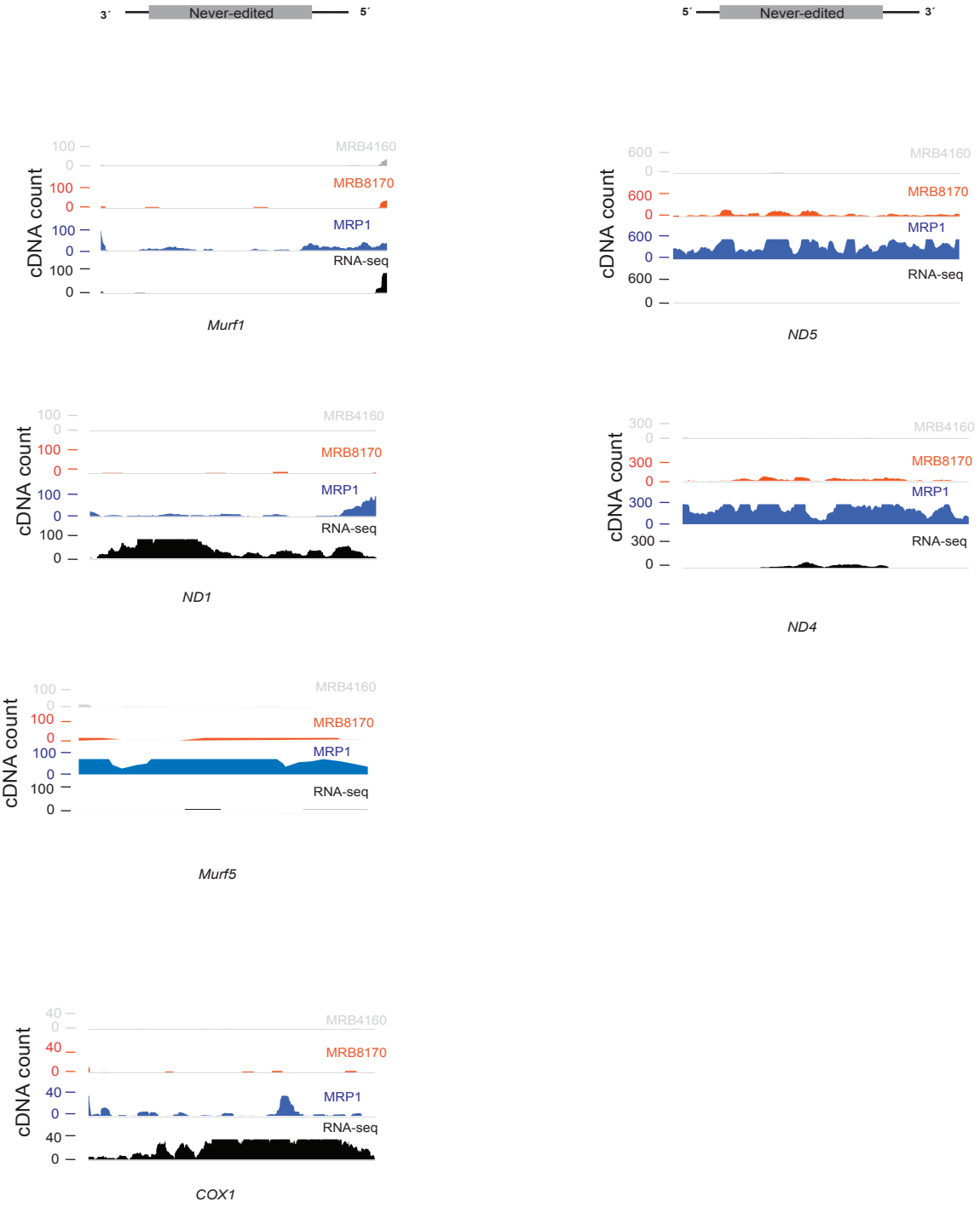

3´
Never-edited
5´
5´
Never-edited
3´
cDNA count
MRB4160
MRB8170
MRP1
RNA-seq
Murf1
ND5
ND1
ND4
Murf5
COX1

Figure S6.

Chapter 3

Malleable Mitochondrion of Trypanosoma brucei
(2.2.3 section- RNA editing)

Zdeněk Verner, Somsuvro Basu, Corinna Benz, **Sameer Dixit,**

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(This section includes only part of the review that was co-written by me)

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CHAPTER THREE

Malleable Mitochondrion of *Trypanosoma brucei*

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Contents

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Abstract

The importance of mitochondria for a typical aerobic eukaryotic cell is undeniable, as the list of necessary mitochondrial processes is steadily growing. Here, we summarize the current knowledge of mitochondrial biology of an early-branching parasitic protist, *Trypanosoma brucei*, a causative agent of serious human and cattle diseases. We present a comprehensive survey of its mitochondrial pathways including kinetoplast DNA replication and maintenance, gene expression, protein and metabolite import, major metabolic pathways, Fe-S cluster synthesis, ion homeostasis, organellar dynamics, and other processes. As we describe in this chapter, the single mitochondrion of *T. brucei* is everything but simple and as such rivals mitochondria of multicellular organisms.

1. INTRODUCTION

The importance of mitochondria for a typical eukaryotic cell cannot be exaggerated, as the list of processes in which they are involved is steadily growing (Scheffler, 2007). In this chapter, we demonstrate that this is also true for the mitochondrion of a well-studied parasitic protist, and that in terms of complexity, its organelle matches that of multicellular organisms. It is clear that all extant mitochondria are of singular origin. Although numerous protist lineages harbor organelles such as mitosomes and hydrogenosomes that were thought to have emerged independently of the mitochondrion, the available evidence points to all of them being derived from it (Tachezy, 2008). Some mitosomes were reduced to a mere shadow of the organelle from which they evolved, and currently the only function shared by all known mitochondria and mitochondrion-derived organelles (sometimes also labeled mitochondrion-related organelles (MROs)) is the synthesis of iron-sulfur (Fe-S) clusters (Lill, 2009; Tachezy, 2008).

Out of the six to seven currently recognized eukaryotic supergroups, the exclusively unicellular Excavata brings together protists with a groove-shaped central cytostome (Adl et al., 2012). They carry the most diverse forms of aerobic and anaerobic mitochondria, as well as MROs. However, with more excavates being examined, it appears that there are no strict boundaries defining these categories, but rather a continuum of organelles, ranging from a conventional mitochondrion to a massively reduced MRO (Jedelský et al., 2011). Indeed, excavates arguably evolved higher diversity of their mitochondrial (mt) genomes than all the other eukaryotes combined (Flegontov et al., 2011).

et al., 2008). The processing of pre-edited RNAs that have to undergo RNA editing to decrypt a translatable sequence also undergoes short and long 3'-tail additions as in the case of never-edited mRNAs. However, the latter processing step is intertwined with RNA editing (Section 2.2.3).

The polyadenylation complex plays a critical role in the 3′ tailing process in mRNA. The kinetoplastid poly(A) polymerase 1 (KPAP1) is involved in the synthesis of both short and long 3′ tails (Etheridge et al., 2008). RET1 has been experimentally proved to be involved in the generation of long 3′ tails as well (Aphasizheva and Aphasizhev, 2010; Ryan and Read, 2005). Pentatricopeptide repeat (PPR) proteins called kinetoplast polyadenylation/uridylation factors 1 and 2 (KPAF1 and KPAF2) coordinate long 3′ A/U tail synthesis (Aphasizheva et al., 2011). The 3′ poly A/U tails play an important role in the regulation of RNA stability and diverse effects on these mRNAs at various stages of processing. The pre-edited, partially edited, and fully edited transcripts are stabilized by the addition of a short 3′ tail (Etheridge et al., 2008; Kao and Read, 2007, 2005), although only never-edited and fully edited mRNAs bearing long 3′ A/U tails are translated (Aphasizheva et al., 2011). These long-tailed mRNAs interact with the small ribosomal subunit (SSU) (Section 2.3). After assembly of the SSU and large ribosomal subunit (LSU), tRNAs are recruited to the mRNA and protein synthesis can begin.

2.2.3 RNA editing

RNA editing refers to any posttranscriptional processing step introducing changes in a transcript sequence relative to the corresponding gene, thus changing the information content of the RNA, except for splicing and terminal processing (Gott and Emeson, 2000). This process occurs throughout eukaryotes in different forms, such as the substitution adenosine-to-inosine editing that is prevalent in mammals. Yet RNA editing was originally discovered as four U residues inserted posttranscriptionally into cytochrome *c* oxidase subunit 2 (cox2) mRNA of *T. brucei* (Benne et al., 1986). Transcripts of several maxicircle genes were revealed to undergo more extensive pan-editing, in which hundreds of Us are inserted and tens of Us are deleted (Feagin et al., 1988). In general, RNA editing in trypanosomes generates open reading frames in edited mRNAs that serve as templates for translation. The role of this process remains poorly defined, although some transcripts are differentially edited between the BSF and PCF, suggesting its additional regulatory role in controlling the expression of maxicircle genes (Feagin and Stuart, 1988; Souza et al., 1992). The limited phylogenetic distribution of

U-insertion/deletion editing indicates that it evolved after the kinetoplastid clade of the Excavata branched off the eukaryotic tree.

U-insertion/deletion editing has been recapitulated at a single editing site with synthetic mRNA, gRNA, and crude mt extract, providing all necessary cofactors (Seiwert and Stuart, 1994). An editing site on a pre-edited mRNA is defined by the so-called anchor domain of a gRNA, 8–12 nt of sequence on the 5′-end that anneals to its cognate transcript. This hybridization between gRNA and mRNA also employs noncanonical G:U base pairs. The mRNA editing site starts at the first base pair mismatch within the RNA duplex, which also defines the beginning of the information domain of the gRNA. It is this part of the gRNA that actually specifies U-insertions and deletions in a small region of mRNA until this molecule is complementary with the information domain. The 3'-oligo(U) tail that is adjacent to the gRNA information domain has been proposed to interact with the downstream purine-rich sequence of the mRNA that remains to be edited (McManus et al., 2000).

In the case of pan-edited mRNAs, several gRNAs are needed for their editing, which proceeds in the 3′ to 5′ direction (Maslov and Simpson, 1992). This polarity eliminates the co-occurrence of editing and translation. There is a higher number of gRNAs encoded in the minicircles than is required for the decoding of all mRNAs, as gRNAs with slightly varied sequences seem to be able to decode part of an edited mRNA (Koslowsky et al., 2014). Thus, a large and redundant population of gRNAs is encoded by minicircle kDNA. The cox2 transcript was the first example of RNA editing, although it does not utilize the aforementioned gRNAs for its sole four U-insertions. Its editing is mediated by a *cis*-acting gRNA-like element located in its 3′ untranslated region, making it a unique substrate for the editing machinery that performs the enzymatic steps of this process (Golden and Hajduk, 2005).

Numerous protein complexes coordinating the highly complex editing process have been described. The well-studied heterotetrameric complex consisting of mt RNA-binding proteins 1 and 2 (MRP1 and MRP2) (Schumacher et al., 2006) has been proposed to act in gRNA–mRNA annealing, a necessary association for the initiation of editing (Müller et al., 2001; Zíková et al., 2008a). This complex has an electropositive face that facilitates the nonspecific binding of RNAs via their negatively charged sugar-phosphate backbone, exposing their base moieties to potential hybridizing transcripts (Schumacher et al., 2006).

The multiprotein complex, called RECC or the 20S editosome, provides the core enzymatic activities needed to achieve a single round of RNA editing (Panigrahi et al., 2001; Seiwert and Stuart, 1994), and also contains proteins with just a structural role. There are at least three RECC isoforms bearing different endonucleases, each partnered with a unique protein that selectively cleaves U-insertion (by RNA-editing endonuclease 2 (REN2)), deletion (REN1), and cox2 mRNA (REN3) editing sites (Carnes et al., 2011, 2008). After cleavage of an mRNA at an insertion site into 5′- and 3′-cleavage products, Us are added to the 3′-end of the former by terminal uridylyl transferase 2 (RET2), a less processive enzyme than RET1 (Ernst et al., 2003). When a deletion site is cleaved by REN1, excess Us on the 5′-cleavage fragment are removed by RNA-editing exonuclease 1 (REX1) (Carnes et al., 2012; Ernst et al., 2009). Interestingly, REX1 exclusively associates with REN1 in this RECC isoform (Carnes et al., 2011). Once the appropriate number of Us has been added or deleted from the 5′-cleavage fragment as dictated by the gRNA, the two mRNA fragments are resealed by RNA-editing ligase 1 (REL1) to complete a single round of editing. All RECC isoforms also contain less-dominant REL2 and RNA-editing exonuclease 2 (REX2), whose roles in the process remain unclear (Carnes et al., 2012; Ernst et al., 2009; Gao and Simpson, 2003).

The mt RNA-binding complex 1 (MRB1) has emerged as another key player in RNA editing. Thirty one proteins have been found in various preparations of MRB1, many of which bear motifs or domains that have been associated with RNA binding and processing, giving the complex its name. In addition, several of these proteins have known protein–protein interaction motives, further evidence that MRB1 represents a macromolecular assembly (Etheridge et al., 2008; Hashimi et al., 2008; Hernandez et al., 2010; Panigrahi et al., 2008; Weng et al., 2008). The architecture of this complex appears to be made up of a core of six proteins that is present in all MRB1 purifications (Ammerman et al., 2012). It contains the aforementioned GAP1/2 heterotetramer that binds and stabilizes gRNAs (Hashimi et al., 2009; Weng et al., 2008), MRB3010, and MRB11870, which are involved in early steps of RNA editing (Ammerman et al., 2013, 2011), and MRB8620 and MRB5390, whose role in the process remains undefined.

This core interacts with the TbRGG2 subcomplex, which is named after a residing RNA-binding protein (Ammerman et al., 2012). TbRGG2 has an N-terminal region with an annealing activity, and a C-terminal part, which

confers double-stranded RNA unwinding activity (Foda et al., 2012). This protein interacts with MRB8180 as well as two novel RNA-binding proteins, MRB8170 and MRB4160, in a mutually exclusive manner (Ammerman et al., 2012; Kafková et al., 2012). The downregulation of TbRGG2, MRB8170, and MRB4160 leads to a preferential decrease in pan-editing (Fisk et al., 2008; Kafková et al., 2012), which along with the biochemical properties of TbRGG2 seems to indicate that this subcomplex mediates multiround editing (Hashimi et al., 2013b).

The MRB complex also associates with RNA-editing helicase 2 (REH2), which has been found to interact with RECC in an RNA-dependent fashion (Hernandez et al., 2010). This protein has double-stranded RNA unwinding activity and appears to play a role in dislodging gRNA from an edited mRNA. Another RNA helicase dubbed REH1, which does not interact with MRB1 but has been found to associate with RECC, has been proposed to play a role in this process, too (Li et al., 2011; Missel et al., 1997). MRB1 subunits interact with the PPR protein KPAF1, which is involved in the addition of long 3'-tails on mRNAs (Ammerman et al., 2010). Due to this association with proteins in other mt RNA processing steps, MRB1 may be involved in integrating the RNA-editing process with the general mt RNA metabolism from transcription to translation (Hashimi et al., 2013b).

2.2.4 RNA turnover

RNA maturation pathways are not the only elements in the regulation of expression of mt-encoded genes. Directed RNA degradation also contributes to this process by controlling the abundance of a given RNA, and also serves in quality control, as aberrant transcripts are eliminated. As previously discussed, various RNAs are appended with 3′-extensions that affect their stability (Section 2.2.2). Several proteins involved in RNA turnover have been characterized. A degradosome-like complex (containing TbSUV and TbDSS-1) has been described in the *T. brucei* mitochondrion, the first detection of this complex outside of the yeast *Saccharomyces cerevisiae*, where it has 3′ to 5′ exoribonuclease and RNA helicase activities (Mattiacio and Read, 2009). Indeed, the *T. brucei* ortholog TbDSS-1 appears to affect the stability of a subset of mt mRNAs (Penschow et al., 2004) and process the 3′-end of 12 S rRNA (Mattiacio and Read, 2008). However, it still remains to be seen whether this endonuclease is truly involved in the bulk turnover of mt RNAs, indirectly or in conjunction with other factors.

Another mt 3′ to 5′ exonuclease bearing an RNase D domain has been shown to preferentially act on poly(U) (Zimmer et al., 2011). Consistent with this finding, the enzyme trims 3′-oligo(U) tails of small gRNAs. The biological relevance of this activity is still a mystery, although it seems that the enzyme does not act on bulk gRNA turnover, a process that would presumably also be beneficial in maintaining this pool of RNA species. Furthermore, the 3′-oligo(U) tails are not essential for the stability of gRNAs (Aphasizheva and Aphasizhev, 2010).

2.2.5 Mitochondrial tRNA import and modifications

In contrast to protein-coding genes, mitochondria generally encode all structural RNAs (rRNAs and tRNAs) that are needed for organellar translation (Adams and Palmer, 2003). However, the loss of mt-encoded tRNA genes apparently occurred multiple times during eukaryotic evolution. In such cases, the corresponding nuclear-encoded tRNAs have to be imported into the mitochondrion to sustain organellar translation (Rubio and Hopper, 2011). Most, perhaps even all, organisms are able to import tRNAs from the cytosol into mitochondria regardless of whether a complete set of tRNAs is encoded by the organellar genome or not (Rubio et al., 2008). The most extreme situation evolved in two groups of parasitic protozoa, namely the apicomplexan and kinetoplastid parasites, as both groups lost their full complement of mt tRNA genes, and as a consequence must import them from the cytosol (Esseiva et al., 2004; Hancock and Hajduk, 1990; Tan et al., 2002).

A number of studies have investigated necessary factors or mechanisms that perform and control tRNA import. In general, there are two tRNA import pathways. One utilizes the protein import pathway, requiring the mt membrane potential for tRNA translocation. The other process, which is present in *T. brucei*, is not dependent on the protein import pathway and does not require mt membrane potential (Paris et al., 2009). The only common feature for both import pathways is a need for ATP. Despite laudable efforts, both mechanisms remain poorly understood.

Transfer RNA molecules are of course crucial for protein synthesis. A typical tRNA does not represent a nude structure as it carries a high number of modified nucleotides. Over 100 naturally occurring chemical modifications have been described in tRNAs, with each tRNA molecule containing an average of 12 of them (Grosjean, 2009), and it is hypothesized that these modifications carry more information than tRNA genes themselves (Björk et al., 2001). The comprehensive distribution and roles of tRNA

(Niemann et al., 2013; Wideman et al., 2013). However, neither Mdm12 nor Mdm34 localizes to the *T. brucei* mitochondrion or mitochondrion-ER contact sites, indicating that a functional ERMES-like complex is most probably not formed in the parasite (Schnarwiler et al., 2014). In the same study, a novel β-barrel protein functionally homologous to Mdm10 was identified; this protein localizes to the TAC and is not essential for mt division but instead required for kDNA segregation (Schnarwiler et al., 2014). Thus far, trypanosome Gem1, the yeast Miro GTPase regulating mt morphology, has not been functionally analyzed but was found to associate with the OM (Niemann et al., 2013).

Overall, despite conservation of parts of the mt fission machinery, the trypanosome must have evolved unique regulatory mechanisms to ensure proper segregation of this single-copy organelle during cytokinesis and to effect morphological changes during its life cycle. Several unique and trypanosome-specific proteins with functions in mt morphology regulation are indeed beginning to emerge, for example, the POMP proteins (present in outer membrane proteome) POMP9, POMP14, and POMP40 (Niemann et al., 2013), as well as TbLOK1 (Loss of kDNA), initially identified in a screen for loss of kDNA as the name implies (Povelones et al., 2013). Silencing of the POMPs caused specific morphological changes to the mitochondrion. While RNAi-mediated ablation of POMP40 creates a BSF-like mitochondrion in PCF cells, ablation of POMP9 and POMP14 causes collapse of the mt network (Niemann et al., 2013). Similarly, depletion of OM protein TbLOK1 in PCF cells results in mt morphology that resembles that of the BSF flagellates (Povelones et al., 2013).

6. CONCLUDING REMARKS

In this chapter we provide a comparison between *T. brucei* and the more established eukaryotic model systems and reveal numerous similarities and differences in mt processes at the DNA, RNA, and protein levels. Trypanosomes contain surprisingly complex machineries for many of the classical biological mt features including energy metabolism via oxidative phosphorylation, RNA editing, Fe-S cluster biogenesis, and mt fission machinery. Apart from highly conserved mechanisms, these processes also contain differences, which is not surprising given that *T. brucei* is one of the earliest known diverging eukaryotes. Consequently, these conserved features between diverse eukaryotic clades, and the presence of a single

mitochondrion make *T. brucei* an attractive model to further enhance our general understanding of both evolution and cellular biology of the mitochondrion. It is also important to explore the differences in the mt proteome and processes between this parasite and mammalian host. An essential aspect of drug development is the ability to target diverse or novel proteins, which include those found in kDNA replication and FA biosynthesis, in addition to a substantial repertoire of kinetoplastid genes that currently have no known function.

ACKNOWLEDGMENTS

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REFERENCES

Acestor, N., Zíková, A., Dalley, R.A., Anupama, A., Panigrahi, A.K., Stuart, K.D., 2011. *Trypanosoma brucei* mitochondrial respiratome: composition and organization in procyclic form. Mol. Cell. Proteomics 10. http://dx.doi.org/10.1074/mcp.M110.006908. M110.006908.

Ackrell, B.A., 2000. Progress in understanding structure-function relationships in respiratory chain complex II. FEBS Lett. 466, 1–5.

Adamec, J., Kalousek, F., Isaya, G., 2002. Mitochondrial processing peptidase/mitochondrial intermediate peptidase. Enzymes 22, 77–100.

Adams, K.L., Palmer, J.D., 2003. Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Mol. Phylogenet. Evol. 29, 380–395.

Adinolfi, S., 2002. A structural approach to understanding the iron-binding properties of phylogenetically different frataxins. Hum. Mol. Genet. 11, 1865–1877. http://dx.doi.org/10.1093/hmg/11.16.1865.

Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., Bowser, S.S., Brown, M.W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., Le Gall, L., Lynn, D.H., McManus, H., Mitchell, E.A.D., Mozley-Stanridge, S.E., Parfrey, L.W., Pawlowski, J., Rueckert, S., Shadwick, R.S., Shadwick, L., Schoch, C.L., Smirnov, A., Spiegel, F.W., 2012. The revised classification of eukaryotes. J. Eukaryot. Microbiol. 59, 429–493. http://dx.doi.org/10.1111/j.1550-7408.2012.00644.x.

Alfonzo, J.D., Blanc, V., Estévez, A.M., Rubio, M.A., Simpson, L., 1999. C to U editing of the anticodon of imported mitochondrial tRNA(Trp) allows decoding of the UGA stop codon in *Leishmania tarentolae*. EMBO J. 18, 7056–7062. http://dx.doi.org/10.1093/emboj/18.24.7056.

Allen, J.W., Ferguson, S.J., Ginger, M.L., 2008. Distinctive biochemistry in the trypanosome mitochonrial intermembrane space suggests a model for stepwise evolution of the MIA pathway for import of cysteine-rich proteins. FEBS Lett. 582, 2817–2825.

Allmann, S., Morand, P., Ebikeme, C., Gales, L., Biran, M., Hubert, J., Brennand, A., Mazet, M., Franconi, J.-M., Michels, P.A.M., Portais, J.-C., Boshart, M., Bringaud, F., 2013. Cytosolic NADPH homeostasis in glucose-starved procyclic

A Proposed plan for a new peak-calling tool for RBPs involved in (U)-insertion/deletion RNA editing

New tool for the peak calling in iCLIP data of RBPs involved in U-insertion/deletion RNA editing

The advent of various crosslinking and immunoprecipitation (CLIP) protocols allowed characterizing the genome-wide RNA crosslinking positions of RBPs, as evident by the recent surge in published CLIP data. However, the complexity of mt transcriptome and limitations in the mapping of iCLIP reads, primarily in trypanosomes due to their extensive RNA editing, makes identification of protein crosslink positions challenging (Gerasimov et al., 2017).

An initial versions of high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) (Ule et al., 2003) and photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP) protocols rely on a higher probability of base transitions, deletions, and truncations during the reverse transcription step to detect the crosslink positions. However, the above protocols had a major drawback since they employ the 5` end adapter to immunoprecipitated RNAs and, therefore, capture only limited cDNAs that were entirely read by the reverse transcriptase. This failure to capture truncated cDNAs may amount to ~80% from the total immunoprecipitated RNA, due to halted reverse transcriptase at crosslink positions. An improved version of CLIP termed iCLIP uses the 3` end adaptor to the immunoprecipitated RNAs and an additional circularization step, thus allowing the majority of the cDNA molecules to be captured (Huppertz et al., 2014; König et al., 2010).

The technical improvements of the initial CLIP protocols accompanied the simultaneous advancement in bioinformatic tools allowing deciphering crosslink positions of RBPs. In contrast, to analyze HITS-CLIP and PAR-CLIP data, far fewer tools are available (De and Gorospe, 2017). The most commonly used bioinformatics tools for the iCLIP data include iCLIPpro, Piranha and CLIPper (Hauer et al., 2015; Van Nostrand et al., 2016; Uren et al., 2012). The published Piranha peak-calling tool performs strand-specific peak searches by segregating bin-wise read counts and then uses an arbitrary threshold value to call peaks, without normalizing for non-specific background noise. When analyzing the iCLIP data one needs to take into account several biases in order to identify the correct crosslink positions, namely the

differential abundance of transcripts, the sequence preference for crosslinking, background noise from sticky RNA fragments, non-specific crosslinking within crosslinking motifs, and the binding of background proteins. Most of the above-mentioned tools have various limits and suffers from identifying low-affinity binding regions.

Including the above bottlenecks, mapping and peak-calling in the generated iCLIP data of RBPs involved in U insertion/deletion RNA editing poses an extra challenge, hence demanding new bioinformatics tools. The 3` to 5` progression of editing creates three different states of mRNA at a time; 1) pre-edited mRNAs awaiting the initiation of editing; 2) a diverse set of partially-edited mRNAs that reflect different degree of editing progression; moreover, the majority of partially-edited sequences contains also some non-canonical modifications; 3) fully-edited mRNAs with the correct ORF, ready to be translated.

These three states of mRNA have different abundance. In general, the intermediate mRNAs are most abundant and more than 90% of these sequences contain junction regions with some non-canonical modifications, while the fully-edited mRNAs are rare. Consequently, any peak-calling tool requires taking into account the differential abundance of pre-, partially- and fully-edited mRNAs, which is currently not available among the existing tools. The other problem arises due to the necessary step of RNase treatment during the iCLIP protocol that results in shorter iCLIP reads. The resulting short iCLIP reads might map to the pre-, partially-, and fully-edited portions of the mRNA, or be unmappable if they contain non-canonical modifications. As a consequence, locating the origin of the mapped iCLIP reads becomes difficult. Taken together, RNA editing related RBPs iCLIP data requires a new tool that can meaningfully resolve the above issues.

Recently published Trypanosome RNA editing alignment tool (TREAT) aligns the full-length RNA sequences to identify the extent of editing across the population of pre-, partially- and fully-edited transcripts (Simpson et al., 2016). TREAT successfully captured the stalled editing positions in the MRB8170/MRB4160 depleted cells (Simpson et al., 2017). Therefore, to develop a new peak-calling tool, we want to start from an already published

MRB8170/MRB4160 TREAT and iCLIP data (Dixit et al., 2017). The plan includes creating an additional reference library encapsulating complex mt transcriptome using parental-cell line TREAT data to map the MRB8170/MRB4160 iCLIP data. This will be followed by the restricted peak-calling search to editing stalled regions derived from MRB8170/MRB4160 RNAi TREAT data. In conclusion, the proposed tool utilizes expanded mt transcriptome for mapping and refined the peak-calling search to the edited regions impacted by protein depletion. In the process, we hope to broaden the scope of the powerful iCLIP methodology to better understand the role of RBPs role in RNA editing.

Functional characterization of mt edited mRNA stability factor MERS1

Sameer Dixit, and Julius Lukeš

(Unpublished study)

INTRODUCTION

The Nudix family of proteins is widespread in all three domains of life, mostly engaged in hydrolyzing nucleoside diphosphate linked to another moiety X (NDP-X) into nucleoside monophosphate (NMP) and P-X (Bessman et al., 1996). The 23 amino acids-long conserved motif (Gx_5Ex_5[UA]xREx_2EExGU), where U is either an aliphatic or a hydrophobic residue and x can represents any amino acid, constitute the catalytic domain of the Nudix proteins (Mildvan et al., 2005). The metal ion coordination maintenance requires two glutamic acid residues that are present in the core of the conserved motif (REx_2EE). Generally, the Nudix hydrolases are small size (16-21 kDa) proteins; if large, they mostly carry other functional domains. The Nudix hydrolase family genes are proposed to play housecleaning roles by either eliminating toxic metabolites or maintaining intermediates to be available for metabolic pathways (Bessman et al., 1996). Additional roles include participation in RNA processing, activation of alcohol dehydrogenase, extracellular signal-regulated kinase signaling, and Ca^{2+} ion channel gates (McLennan, 2006). Overall, however, the capacities of Nudix proteins to bind a wide range of substrates make it challenging to define all their roles.

In mammals, 22 genes and at least five pseudogenes have been identified to contain the Nudix hydrolase domain (Song et al., 2013). Among them, only Dcp2 and Nudt16 are involved in mRNA decapping *in vivo* (Li et al., 2011b; Song et al., 2010). Several studies in yeast and mammals indicate that the mRNA decapping is a highly regulated process that maintains RNA abundance suitable for proper translation, especially under stress conditions. In case of *T. brucei*, the bioinformatic analyses suggested five proteins to carry Nudix hydrolase domain: TbNudix1 (GenBank accession: EU711412.1), TbNudix2 (Tb927.5.4350), TbNudix3 (Tb11.01.11570), TbNudix4 (Tb927.6.2670), TbNudix5 (Tb10.70.2530) (Ignatochkina et al., 2015). Among them, TbNudix1 is exclusively located in the mitochondrion and suggested to function as the mt edited mRNA stability factor MERS1 (Weng et al., 2008). The other two Nudix hydrolases, TbNudix2 and TbNudix3, were localized into the glycosomes but their catalytic activity remains undefined (Güther et al., 2014). A study carried out on four Nudix proteins (excluding MERS1), showed that TbNudix4

is the only member that possesses the RNA decapping activity, and was therefore named TbDcp2 (Ignatochkina et al., 2015).

In the trypanosome mitochondria, MERS1 interacts stably with guide RNA binding complex GRBC via RNA-mediated interaction (Aphasizheva and Aphasizhev, 2016). The MERS1 ablation causes downregulation of edited mRNAs, while gRNAs population remains unaffected (Hashimi et al., 2009; Weng et al., 2008). To learn more about the role of MERS1, we carried out *in vivo* RNA binding studies that were followed up by oligo (dT) pull-down studies in MERS1-depleted cells.

AIM

1) To find the *in vivo* RNA binding partner of MERS1 using iCLIP.
2) To characterize the effect of MERS1 depletion on the mRNA binding capability of proteins involved in RNA editing.

RESULTS

We wanted to test if MERS1 binds RNA as a substrate *in vivo*. For that purpose, we applied UV-crosslinking to 29-13 cells (UV254 nm and 800 J/cm^2). The MERS1-RNA complex was immunopurified from UV and non-UV crosslinked 29-13 cells. Next, the immunopurified RNA was 5` radiolabelled and verified using SDS-PAGE. Our analysis shows that the radiolabelled RNA smear is present exclusively in the UV-crosslinked cells, while it is absent from the non-UV crosslinked cells. Next, we wanted to characterize RNA binding partner of putative MERS1, however, in several experiments the amount of immunopurified RNA from MERS1 IP was very low, and hence we failed to make a successful iCLIP library.

Next, we applied the previously optimized oligo (dT) pull-down protocol to capture UV-crosslinked RBPs in MERS1 and Atm1-depleted cells (Dixit et al., 2017). The cells in which Atm1 was down regulated served as a negative control as this protein does not affect mitochondrial gene expression. The oligo (dT) captured RBPs

from both MERS1 and Atm1-depleted cells were resolved using SDS PAGE and then probed for the presence of MRP1, TbRGG1, KPAP1, MRB8170, MRB3010 and TbRGG2 using corresponding antibodies. When comparing the pull-down ratio between MERS1 and Atm1 captured RBPs, we observed a more than 50% decrease of TbRGG1 and KPAP, while the other probed proteins displayed a negligible decrease.

DISCUSSION

Our study demonstrates that MERS1 binds RNA *in vivo*. Our oligo (dT) pull-down studies show MERS1 depletion affects TbRGG1 and KPAP1 mRNA binding ability, while the MRP1, MRB8170 and TbRGG2 mRNA binding capability remains unaltered. The immediate goals require probing of MERS1 for an RNA decapping activity, and to successfully apply the iCLIP protocol to capture MERS1-bound RNAs. In conclusion, a considerable amount of future work is required to define the exact role of MERS1 in the regulation of mitochondrial RNA metabolism.

Figure 1. MERS1 iCLIP library

Co-purification of UV crosslinked RNA-MERS1 complexes. The ^{32}P-labelled complexes were monitored by autoradiography. Non-UV crosslinked parental cell line (lane 2) yielded no signal. The low RNAse treated (lane1) was used to prepare the iCLIP libraries. Western blot confirmed the presence of MERS1 (~54 kDa).

Figure 2. The mRNA binding efficiency of associated proteins following MERS1 depletion.

Western blot analysis of total extracts (lanes 1 and 2) and oligo (dT) eluates (lanes 3 and 4) from Atm1 and MERS1 knockdown cells displaying levels of proteins indicated in left. Bar-plot indicates the ratio of pull down proteins from MERS1 versus Atm1 knockdown cells.

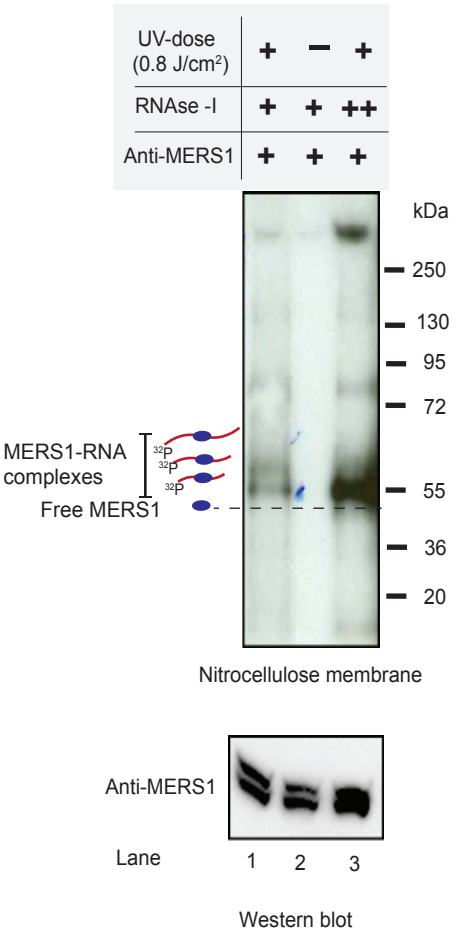


Figure 1.

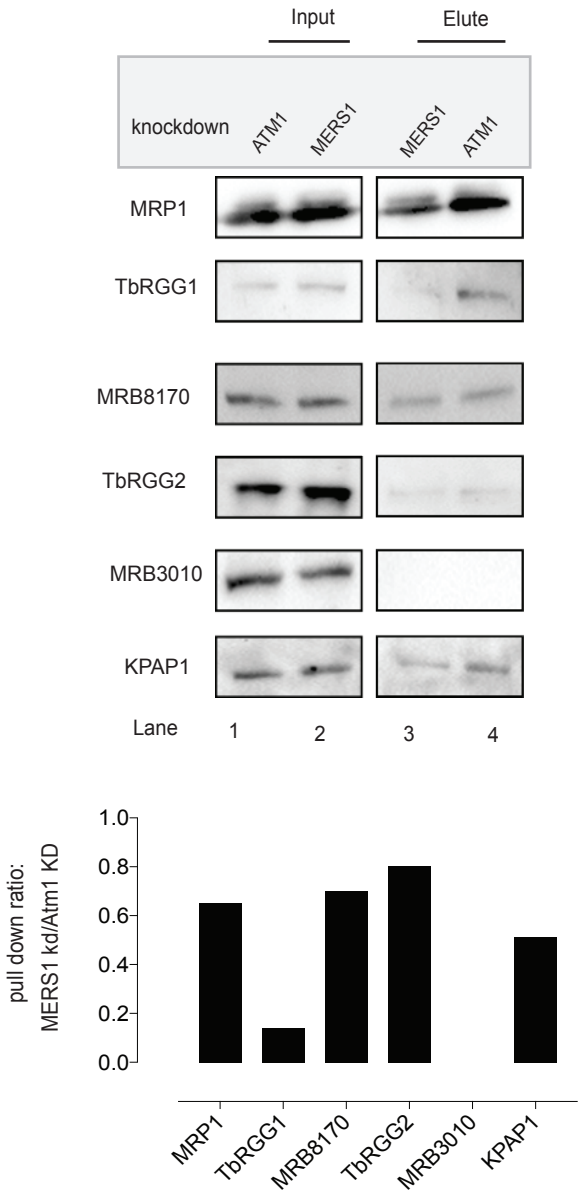


Figure 2.

Chapter 4

RNA binding footprints of TbADAT2 analyzed by CLIP

Mary Anne T. Rubio, **Sameer Dixit**, Julius Lukeš, and Juan D. Alfonzo

(Unpublished study)

INTRODUCTION

The central dogma of molecular biology states that DNA gets transcribed to mRNA, which is eventually translated into proteins. The scheme requires a set of tRNAs enabling decoding of mRNA codons (triplets of nucleotides) into corresponding amino acids. The Universal genetic code includes 64 codons of which 61 code for 20 canonical amino acid and the remaining three are used as termination codons (UAA, UAG, UGA) to signal the end of translation (Grosjean et al., 2010). The genetic code is "degenerated" as multiple synonymous codons codes for similar amino acid. In all three domains of life, there exists a far less diverse set of tRNAs with anticodons complementary to 61 sense codons as required for their translation. For example, *E. coli* contains only 39 tRNAs with distinct anticodons to decode the entire genetic code (Agris, 2004; Schimmel, 2017). To resolve this perplexing situation the cell allows a certain flexibility in the base pairing between tRNA anticodons and mRNA codons, as initially proposed by Crick and better known as the “wobble rules” (Crick, 1966). The "wobble rules" postulate that a standard Watson-Crick base pairing of A-U or G-C is permissible at first two positions of the codon, while at the third positions “wobbling” is allowed through stable non-Watson-Crick base pairing (e.g. G-U) without compromising translational fidelity.

The wobble rules manifest themselves in several ways, both in the case of standard nucleotides (G: U or U: G base pairing), as well as with modified nucleotides (Agris, 2004). Interestingly, maximum relaxation in the base pairing can only be achieved through the use of guanosine nucleotide analog called inosine, as it can base pair with A, C or U. That is why tRNAs carrying inosine at the first anticodon position (I^{34}) can decode three different codons (Agris, 1991; Fabret et al., 2011). Hence, both bacteria and eukaryotes rely heavily on inosine-forming enzymes to circumvent the need for greater diversity of their tRNAs (Curran, 1995). The tRNA with adenosine at the wobble base (I^{34}) decodes every U-ending codon but not the C-ending codons. Therefore, to expand the tRNAs capability in decrypting C-ending codons as well, the eukaryotes use A-to-I editing at the wobble base (Sprinzl, 1998), while the archaea employ genomically encoded G^{34}-containing tRNA. The essential A-to-I editing at the wobble base in eukaryotes and bacteria (but not in archaea) perform by an enzyme deaminating polynucleotides (Gerber, 1999; Sprinzl and Vassilenko, 2005).

Based on the deaminating polynucleotide enzyme's metal-binding pocket, they are divided into two major families. The first one includes adenosine deaminases acting on RNAs, so-called ADARs (Sprinzl, 1998). The second family brings together the polynucleotide cytidine deaminases, or CDAs, which are further sub-divided into the APOBEC enzymes that act on RNA, and the activation-induced deaminases (AID) that bind to DNA (Auxilien et al., 1996; Gerber, 1999; Grosjean et al., 1996; Kuratani et al., 2005; Wolf, 2002). The adenosine deaminase acting on tRNAs (ADATs) structurally resembles CDAs, but functionally modifies adenosines (Gaston et al., 2007; Rubio et al., 2006). In trypanosomes, the ablations of TbADAT2/3 impacts A-to-I editing at the wobble bases and C-to-U editing at the position 32 of $tRNA^{Thr}_{AGU}$. A recent study provides a mechanistic model to interpret a surprising involvement of TbADAT2/3 in both the A-to-I and C-to-U editing, which argues that cytosine 32 in $tRNA^{Thr}$ is methylated (m^3C) as a precondition for C-to-U editing. It is the initial step of methylation for which TbADAT2/3 along with methyltransferase TRM140 are critical for the deamination of 3-methylcytosine (m^3C) into 3-methyluridine (m^3U). Additionally, the complex between TRM140 and ADAT2/3 was suggested to safeguard the genome from wholesale deamination (Rubio et al., 2017).

AIM

We wanted to characterize the genome-wide TbADAT2/3-bound RNAs. The main aim was to search for the bound RNA substrates other than tRNAs with the hope to explain the functional relevance of such binding.

RESULTS

We applied similar UV-conditions as used in MRB8170 iCLAP (UV254 nm and 800 J/cm2) to UV-crosslink TbADAT2 with the bound RNA molecules in procyclic *T. brucei*. Next, the immunopurified TbADAT2-RNA complex from the UV- and non-UV crosslinked (control) trypanosomes were verified using the SDS-PAGE. In the UV-crosslinked cells the radiolabelled RNA migrated at the expected size, while it was absent from the control cells. Next, the size-selected RNAs were used to prepare

CLIP libraries, which were subsequently sequenced using the Illumina platform (single-end, 75 bp).

Next, the TbADAT2 CLIP libraries were pre-processed and then mapped against the *T. brucei* nuclear genome. The TbADAT2/3 CLIP data show the target protein binds 18 transcripts (Figure 1). The highest amount of the CLIP tag counts belonged to the THT1-hexose transporter transcript (Tb11.v5.0333). Unexpectedly, the data showed that TbADAT2/3 binds to two variant surface glycoprotein transcripts (Tb11.v5.0380, Tb11.v5.0595) and one noncoding RNA (Tb3.NT.36). Since about 60% of all *T. brucei* genes share no similarity with other eukaryotes the other bound transcripts represent hypothetical proteins.

Finally, we wanted to examine the crosslink position of TbADAT2/3 on the bound mRNAs. Unlike the MRB8170, MRB4160 or MRP1 binding over the whole transcripts, our analyses clearly demonstrate that TbADAT2/3 binds exclusively at the 3` end of the transcripts (Figures 1A, B, and C). Finally, we clustered all the bound transcripts in our mapped iCLIP data in order to perform gene ontology (GO) term analysis using TriTrypDB site. Our GO term analysis found mostly energy-related metabolic pathways (Table 1).

DISCUSSION

The presented analysis reveals that the TbADAT2 protein binds a small binds a small subset of 17 mRNAs and a single non-coding RNA in the procyclic stage of *T. brucei*. In the non-normalized iCLIP data, we found highest binding to the hexose transporter (THT1) mRNA. The very interesting preferential binding of THT1 by TbADAT2 needs further functional validation, more so in the bloodstream stage, as the glucose metabolism is essential for this life cycle stage (Azema et al., 2004).

The DNA deaminating activity of TbADAT2 as well as its structure puts the enzyme relatively close to the activation-induced cytidine deaminase (AID). The AID deaminates immunoglobin receptors and hence functions in the immune system. Drawing a parallel, we hypothesize that the TbADAT's plays a role in either the regulation or maintenance of variants surface glycoproteins (VSGs) in trypanosomes.

Indeed, even after performing CLIP in the procyclic stage where VSGs are repressed, two-bound VSG mRNAs were recovered during the analysis. The deaminase family proteins preferentially bind 3` UTR region of the RNA (Bahn et al., 2015; Rosenberg et al., 2011). Similarly, TbADAT2 CLIP showed its pronounced preference for the 3` UTR region. Next, GO term enrichment analysis revealed a significant over-representation of the metabolic pathways centered around the carbohydrate, energy and lipid metabolism pathways. Interestingly, one of the two bound VSGs is an atypical form and in our GO term analysis is implicated with purine metabolism.

The GO term analysis results assume additional significance, since VSGs anchoring in cell membrane requires glycosylphosphatidylinositol (GPI). Remarkably, the bloodstream trypanosomes represent the only organism known to date that differentiates between the *de novo* synthesized and extracellular myo-inositol (Martin and Smith, 2006). Hence, its *de novo* synthesis is essential for survival. The rate-limiting step in the *de novo* synthesis is the isomerization of glucose-6-phosphate to 1-myo-inositol-3-phosphate (Martin and Smith, 2006). Therefore, the observed 3` UTR binding of mRNAs implicated in carbohydrate metabolism by TbADAT2/3 possibly allows post-transcriptional regulation of the GPI production through a feedback mechanism. If so, TbADAT2/3 might regulate VSGs by limiting the GPI production through the *de novo* synthesis of inositol.

In conclusion, while this study demands further validation of the stated hypothesis, the obtained results are unexpected and quite exciting. They will be even more exciting if the ongoing experiments show that TbADAT2 has the anticipated function in the bloodstream *T. brucei*.

Figure 1. TbADAT2/3 iCLIP

(A) Plot comparing the total number of mapped iCLIP tags on various transcripts.

(B) Genomic browser snapshots of protein binding by TbADAT2/3 on THT1-hexose transporter (gene_id: Tb11.v5.0333); VSG, atypical, (gene_id: Tb11.v5.0380); variant surface glycoprotein (VSG, gene_id: Tb11.v5.0595)

Figure 2. TbADAT2/3 iCLIP

Gene ontology (GO) term analysis of TbADAT2/3 bound transcripts in iCLIP data.

Figure 1. (A)

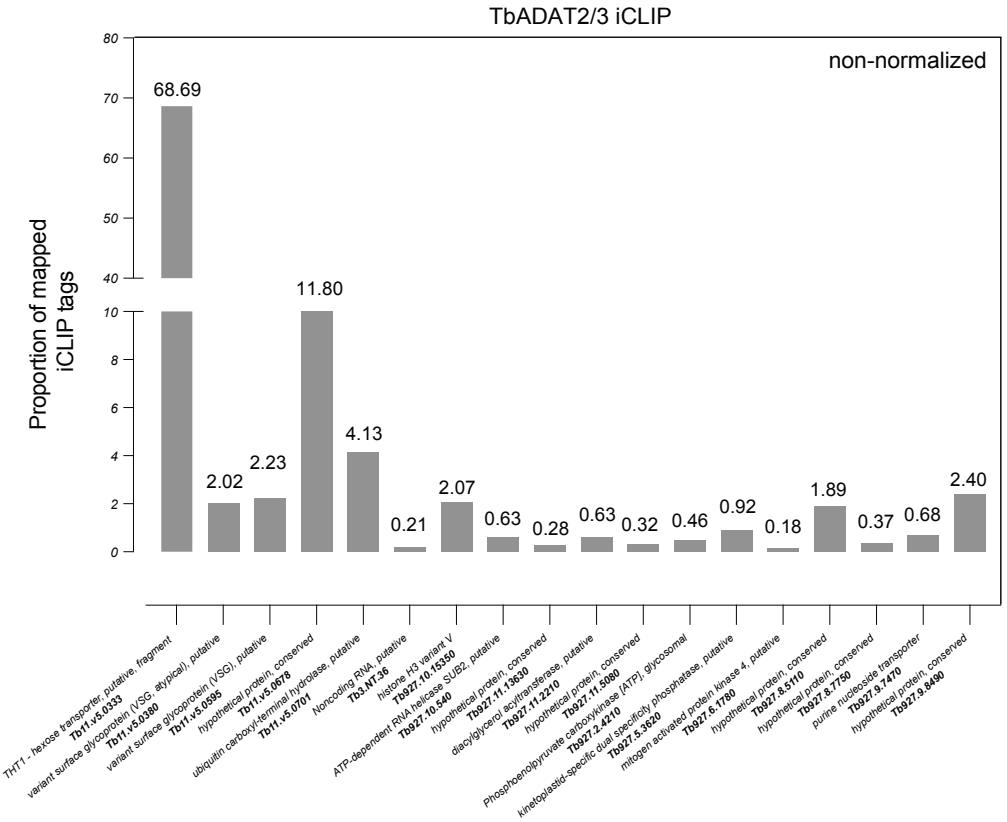
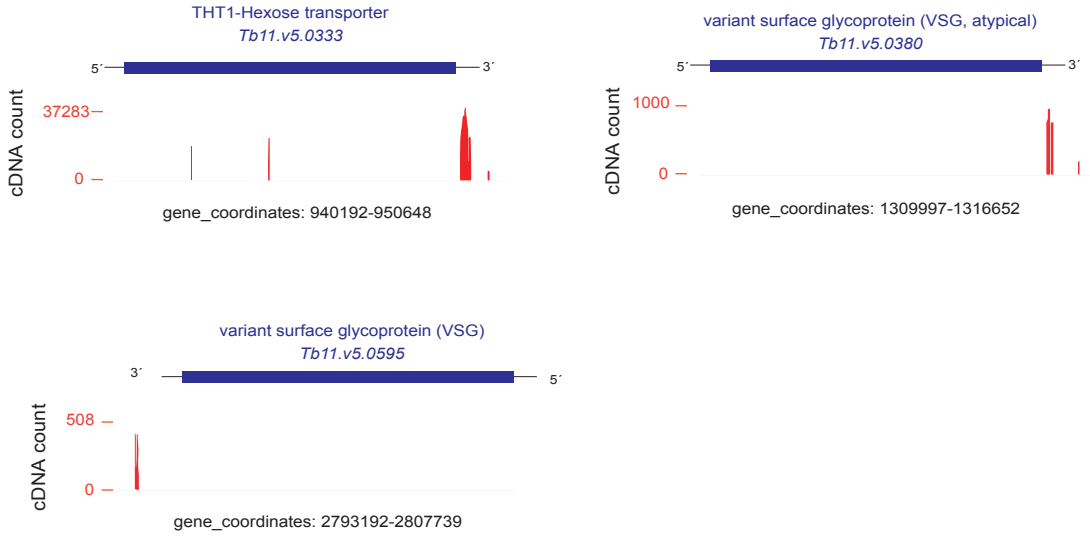


Figure 1. (B)



Pathway Id	Pathway	Genes ID
ec00010	Glycolysis / Gluconeogenesis	Tb927.2.4210
ec00020	Citrate cycle (TCA cycle)	Tb927.2.4210
ec00230	Purine metabolism	Tb11.v5.0380
ec00540	Lipopolysaccharide biosynthesis	Tb927.6.1780
ec00561	Glycerolipid metabolism	Tb927.11.2210
ec00620	Pyruvate metabolism	Tb927.2.4210
ec00680	Methane metabolism	Tb927.6.1780
ec00710	Carbon fixation in photosynthetic organisms	Tb927.2.4210
ec00900	Terpenoid backbone biosynthesis	Tb927.6.1780
GLUCONEO-PWY	gluconeogenesis I	Tb927.2.4210
PWY3IU-0	succinic fermentation pathway	Tb927.2.4210
PWY3IU-99	superpathway of central carbon metabolism	Tb927.2.4210
SPHINGOLIPID-SYN-PWY	sphingolipid metabolism	Tb927.6.1780
TRIGLSYN-PWY	triacylglycerol biosynthesis	Tb927.11.2210
GLUCONEO-PWY	gluconeogenesis I	Tb927.2.4210
LIPA-CORESYN-PWY	Lipid A-core biosynthesis	Tb927.6.1780
LPSSYN-PWY	superpathway of lipopolysaccharide biosynthesis	Tb927.6.1780
PWY-5129	sphingolipid biosynthesis (plants)	Tb927.6.1780
PWY-561	superpathway of glyoxylate cycle and fatty acid degradation	Tb927.2.4210
PWY-6322	phosphinothricin tripeptide biosynthesis	Tb927.6.1780
PWY-6369	inositol pyrophosphates biosynthesis	Tb927.6.1780
PWY-6371	superpathway of inositol phosphate compounds	Tb927.6.1780
PWY-6577	farnesylcysteine salvage pathway	Tb927.6.1780
PWY-6626	cytidine-5'-diphosphate-glycerol biosynthesis	Tb927.6.1780
PWY-6682	dehydrophos biosynthesis	Tb927.6.1780
PWY-7077	N-acetyl-D-galactosamine degradation	Tb927.6.1780
PWY-7117	C4 photosynthetic carbon assimilation cycle, PEPCK type	Tb927.2.4210
PWY-7321	ecdysteroid metabolism (arthropods)	Tb927.6.1780
PWY-7548	methylthiolincosamide biosynthesis	Tb927.6.1780
PWY-7769	phosalacine biosynthesis	Tb927.6.1780
PWY0-1517	sedoheptulose bisphosphate bypass	Tb927.6.1780
SPHINGOLIPID-SYN-PWY	sphingolipid biosynthesis (yeast)	Tb927.6.1780
TRIGLSYN-PWY	diacylglycerol and triacylglycerol biosynthesis	Tb927.11.2210
GLUCONEO-PWY	gluconeogenesis I	Tb927.2.4210
PWY1V8-4	Succinate shunt	Tb927.2.4210
PWY1V8-9	Succinate and Acetate/Acetyl CoA metabolism	Tb927.2.4210
TRIGLSYN-PWY	triacylglycerol biosynthesis	Tb927.11.2210

Figure 2.

REFERENCES

Acestor, N., Panigrahi, A.K., Carnes, J., Zíková, A., and Stuart, K.D. (2009). The MRB1 complex functions in kinetoplastid RNA processing. RNA *15*, 277–286.

Agris, P.F. (1991). Wobble position modified nucleosides evolved to select transfer RNA codon recognition: A modified-wobble hypothesis. Biochimie *73*, 1345–1349.

Agris, P.F. (2004). Decoding the genome: a modified view. Nucleic Acids Res. *32*, 223–238.

Alfonzo, J.D., and Lukeš, J. (2011). Assembling Fe/S-clusters and modifying tRNAs: ancient co-factors meet ancient adaptors. Trends Parasitol. *27*, 235–238.

Allen, T.E., Heidmann, S., Reed, R., Myler, P.J., Göringer, H.U., and Stuart, K.D. (1998). Association of guide RNA binding protein gBP21 with active RNA editing complexes in *Trypanosoma brucei*. Mol. Cell. Biol. *18*, 6014–6022.

Ammerman, M.L., Presnyak, V., Fisk, J.C., Foda, B.M., and Read, L.K. (2010). TbRGG2 facilitates kinetoplastid RNA editing initiation and progression past intrinsic pause sites. RNA *16*, 2239–2251.

Ammerman, M.L., Hashimi, H., Novotná, L., Číčová, Z., McEvoy, S.M., Lukeš, J., and Read, L.K. (2011). MRB3010 is a core component of the MRB1 complex that facilitates an early step of the kinetoplastid RNA editing process. RNA *17*, 865–877.

Ammerman, M.L., Downey, K.M., Hashimi, H., Fisk, J.C., Tomasello, D.L., Faktorová, D., Kafková, L., King, T., Lukeš, J., and Read, L.K. (2012). Architecture of the trypanosome RNA editing accessory complex, MRB1. Nucleic Acids Res. *40*, 5637–5650.

Aphasizhev, R. (2007). RNA editing. Mol. Biol. *41*, 227–239.

Aphasizhev, R., and Aphasizheva, I. (2011a). Mitochondrial RNA processing in trypanosomes. Res. Microbiol. *162*, 655–663.

Aphasizhev, R., and Aphasizheva, I. (2011b). Uridine insertion/deletion editing in trypanosomes: A playground for RNA-guided information transfer. Wiley Interdiscip. Rev. RNA *2*, 669–685.

Aphasizhev, R., and Aphasizheva, I. (2014). Mitochondrial RNA editing in trypanosomes: small RNAs in control. Biochimie *100*, 125–131.

Aphasizhev, R., Aphasizheva, I., and Simpson, L. (2003). A tale of two TUTases. Proc. Natl. Acad. Sci. *100*, 10617–10622.

Aphasizheva, I., and Aphasizhev, R. (2010). RET1-catalyzed uridylylation shapes the mitochondrial transcriptome in *Trypanosoma brucei*. Mol. Cell. Biol. *30*, 1555–1567.

Aphasizheva, I., and Aphasizhev, R. (2016). U-Insertion/Deletion mRNA-Editing Holoenzyme: Definition in Sight. Trends Parasitol. *32*, 144–156.

Aphasizheva, I., Maslov, D., Wang, X., Huang, L., and Aphasizhev, R. (2011). Pentatricopeptide Repeat Proteins Stimulate mRNA Adenylation/Uridylation to activate mitochondrial translation in trypanosomes. Mol. Cell *42*, 106–117.

Aphasizheva, I., Zhang, L., Wang, X., Kaake, R.M., Huang, L., Monti, S., and Aphasizhev, R. (2014). RNA binding and core complexes constitute the U-insertion/deletion editosome. Mol. Cell. Biol. *34*, 4329–4342.

Auxilien, S., Crain, P.F., Trewyn, R.W., and Grosjean, H. (1996). Mechanism, specificity and general properties of the yeast enzyme catalysing the formation of inosine 34 in the anticodon of transfer RNA. J. Mol. Biol. *262*, 437–458.

Avliyakulov, N.K., Lukeš, J., and Ray, D.S. (2004). Mitochondrial histone-like DNA-binding proteins are essential for normal cell growth and mitochondrial function in *Crithidia fasciculata*. Eukaryot. Cell *3*, 518–526.

Azema, L., Claustre, S., Alric, I., Blonski, C., Willson, M., Perié, J., Baltz, T., Tetaud, E., Bringaud, F., Cottem, D., et al. (2004). Interaction of substituted hexose analogues with the *Trypanosoma brucei* hexose transporter. Biochem. Pharmacol. *67*, 459–467.

Bahn, J.H., Ahn, J., Lin, X., Zhang, Q., Lee, J.-H., Civelek, M., and Xiao, X. (2015). Genomic analysis of ADAR1 binding and its involvement in multiple RNA processing pathways. Nat. Commun. *6*, 6355.

Bakshi, R.P., and Shapiro, T.A. (2004). RNA interference of *Trypanosoma brucei* topoisomerase IB: Both subunits are essential. Mol. Biochem. Parasitol. *136*, 249–255.

Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. Science *289*, 905–920.

Benne, R., Van Den Burg, J., Brakenhoff, J.P.J., Sloof, P., Van Boom, J.H., and Tromp, M.C. (1986). Major transcript of the frameshifted coxll gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. Cell *46*, 819–826.

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D.C., Lennard, N.J., Caler, E., Hamlin, N.E., Haas, B., et al. (2005). The genome of the African trypanosome *Trypanosoma brucei*. Science *309*, 416–422.

Bessman, M.J., Frick, D.N., and O'Handley, S.F. (1996). The MutT proteins or

"Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes. J. Biol. Chem. *271*, 25059–25062.

Bhat, G.J., Souza, A.E., Feagin, J.E., and Stuart, K. (1992). Transcript-specific developmental regulation of polyadenylation in *Trypanosoma brucei* mitochondria. Mol. Biochem. Parasitol. *52*, 231–240.

Blum, B., and Simpson, L. (1990). Guide RNAs in kinetoplastid mitochondria have a nonencoded 3' oligo(U) tail involved in recognition of the preedited region. Cell *62*, 391–397.

Blum, B., Bakalara, N., and Simpson, L. (1990). A model for RNA editing in kinetoplastid mitochondria: "guide" RNA molecules transcribed from maxicircle DNA provide the edited information. Cell *60*, 189–198.

Carnes, J., Trotter, J.R., Ernst, N.L., Steinberg, A., and Stuart, K. (2005). An essential RNase III insertion editing endonuclease in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. *102*, 16614–16619.

Carnes, J., Trotter, J.R., Peltan, A., Fleck, M., and Stuart, K. (2008). RNA editing in *Trypanosoma brucei* requires three different editosomes. Mol. Cell. Biol. *28*, 122–130.

Carnes, J., Soares, C.Z., Wickham, C., and Stuart, K. (2011). Endonuclease associations with three distinct editosomes in *Trypanosoma brucei*. J. Biol. Chem. *286*, 19320–19330.

Charrière, F., Tan, T.H.P., and Schneider, A. (2005). Mitochondrial initiation factor 2 of *Trypanosoma brucei* binds imported formylated elongator-type $tRNA^{Met}$. J. Biol. Chem. *280*, 15659–15665.

Clayton, C. (2013). The Regulation of Trypanosome gene expression by RNA-binding proteins. PLoS Pathog. *9*, e1003680.

Clayton, C.E. (2014). Networks of gene expression regulation in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *195*, 96–106.

Clement, S.L., and Koslowsky, D.J. (2001). Unusual organization of a developmentally regulated mitochondrial RNA polymerase (TBMTRNAP) gene in *Trypanosoma brucei*. Gene *272*, 209–218.

Clement, S.L., Mingler, M.K., and Koslowsky, D.J. (2004). An intragenic guide RNA location suggests a complex mechanism for mitochondrial gene expression in *Trypanosoma brucei*. Eukaryot. Cell *3*, 862–869.

Clemons Jr., W.M., May, J.L.C., Wimberly, B.T., McCutcheon, J.P., Capel, M.S., and

Ramakrishnan, V. (1999). Structure of a bacterial 30S ribosomal subunit at 5.5 Å resolution. Nature *400*, 833–840.

Crick, F.H.C. (1966). Codon—anticodon pairing: The wobble hypothesis. J. Mol. Biol. *19*, 548–555.

Cristodero, M., Seebeck, T., and Schneider, A. (2010). Mitochondrial translation is essential in bloodstream forms of *Trypanosoma brucei*. Mol. Microbiol. *78*, 757–769.

Cristodero, M., Mani, J., Oeljeklaus, S., Aeberhard, L., Hashimi, H., Ramrath, D.J.F., Lukeš, J., Warscheid, B., and Schneider, A. (2013). Mitochondrial translation factors of *Trypanosoma brucei*: Elongation factor-Tu has a unique subdomain that is essential for its function. Mol. Microbiol. *90*, 744–755.

Curran, J.F. (1995). Decoding with the A:I wobble pair is inefficient. Nucleic Acids Res. *23*, 683–688.

De, S., and Gorospe, M. (2017). Bioinformatic tools for analysis of CLIP ribonucleoprotein data. Wiley Interdiscip. Rev. RNA *8*.

Dixit, S., Müller-McNicoll, M., David, V., Zarnack, K., Ule, J., Hashimi, H., and Lukeš, J. (2017). Differential binding of mitochondrial transcripts by MRB8170 and MRB4160 regulates distinct editing fates of mitochondrial mRNA in Trypanosomes. mBio *8*, e02288-16.

Englund, P.T. (1978). The replication of kinetoplast DNA networks in *Crithidia fasciculata*. Cell *14*, 157–168.

Ernst, N.L., Panicucci, B., Carnes, J., and Stuart, K. (2009). Differential functions of two editosome exoUases in *trypanosoma brucei*. RNA *15*, 947–957.

Etheridge, R.D., Aphasizheva, I., Gershon, P.D., and Aphasizhev, R. (2008). 3' adenylation determines mRNA abundance and monitors completion of RNA editing in *T. brucei* mitochondria. EMBO J. *27*, 1596–1608.

Fabret, C., Dervyn, E., Dalmais, B., Guillot, A., Marck, C., Grosjean, H., and Noirot, P. (2011). Life without the essential bacterial tRNA Ile2-lysidine synthetase TilS: A case of tRNA gene recruitment in Bacillus subtilis. Mol. Microbiol. *80*, 1062–1074.

Fenn, K., and Matthews, K.R. (2007). The cell biology of *Trypanosoma brucei* differentiation. Curr. Opin. Microbiol. *10*, 539–546.

Fisk, J.C., Ammerman, M.L., Presnyak, V., and Read, L.K. (2008). TbRGG2, an essential RNA editing accessory factor in two *Trypanosoma brucei* life cycle stages. J. Biol. Chem. *283*, 23016–23025.

Fisk, J.C., Presnyak, V., Ammerman, M.L., and Read, L.K. (2009). Distinct and

overlapping functions of MRP1/2 and RBP16 in mitochondrial RNA metabolism. Mol. Cell. Biol. *29*, 5214–5225.

Foda, B.M., Downey, K.M., Fisk, J.C., and Read, L.K. (2012). Multifunctional G-rich and RRM-containing domains of TbRGG2 perform separate yet essential functions in trypanosome RNA editing. Eukaryot. Cell *11*, 1119–1131.

Gao, G., and Simpson, L. (2003). Is the *Trypanosoma brucei* REL1 RNA ligase specific for U-deletion Rna editing, and is the REL2 RNA ligase specific for U-insertion editing? J. Biol. Chem. *278*, 27570–27574.

Gaston, K.W., Rubio, M.A.T., Spears, J.L., Pastar, I., Papavasiliou, F.N., and Alfonzo, J.D. (2007). C to U editing at position 32 of the anticodon loop precedes tRNA 5' leader removal in trypanosomatids. Nucleic Acids Res. *35*, 6740–6749.

Gazestani, V.H., Hampton, M., Abrahante, J.E., Salavati, R., and Zimmer, S.L. (2016). circTAIL-seq, a targeted method for deep analysis of RNA 3' tails, reveals transcript-specific differences by multiple metrics. RNA *22*, 477–486.

Gerasimov, E.S., Gasparyan, A.A., Kaurov, I., Tichý, B., Logacheva, M.D., Kolesnikov, A.A., Lukeš, J., Yurchenko, V., Zimmer, S.L., and Flegontov, P. (2017). Trypanosomatid mitochondrial RNA editing: dramatically complex transcript repertoires revealed with a dedicated mapping tool. Nucleic Acids Res. *gkx1202*.

Gerber, A.P. (1999). An adenosine deaminase that generates inosine at the wobble position of tRNAs. Science *286*, 1146–1149.

Grams, J., Morris, J.C., Drew, M.E., Wang, Z., Englund, P.T., and Hajduk, S.L. (2002). A trypanosome mitochondrial RNA polymerase is required for transcription and replication. J. Biol. Chem. *277*, 16952–16959.

Grosjean, H., Auxilien, S., Constantinesco, F., Simon, C., Corda, Y., Becker, H.F., Foiret, D., Morin, A., Jin, Y.X., Fournier, M., et al. (1996). Enzymatic conversion of adenosine to inosine and to N1-methylinosine in transfer RNAs: A review. Biochimie *78*, 488–501.

Grosjean, H., de Crécy-Lagard, V., and Marck, C. (2010). Deciphering synonymous codons in the three domains of life: Co-evolution with specific tRNA modification enzymes. FEBS Lett. *584*, 252–264.

Güther, M.L.S., Urbaniak, M.D., Tavendale, A., Prescott, A., and Ferguson, M.A.J. (2014). High-confidence glycosome proteome for procyclic form *trypanosoma brucei* by epitope-tag organelle enrichment and SILAC proteomics. J. Proteome Res. *13*, 2796–2806.

Hashimi, H., Číčová, Z., Novotná, L., Wen, Y.-Z., and Lukeš, J. (2009). Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex 1 and mitochondrial RNA polymerase. RNA *15*, 588–599.

Hashimi, H., Zimmer, S.L., Ammerman, M.L., Read, L.K., and Lukeš, J. (2013). Dual core processing: MRB1 is an emerging kinetoplast RNA editing complex. Trends Parasitol. *29*, 91–99.

Hashimi, H., Kaltenbrunner, S., Zíková, A., and Lukeš, J. (2016). Trypanosome mitochondrial translation and tetracycline: no sweat about tet. PLoS Pathog. *12*, e1005492.

Hauer, C., Curk, T., Anders, S., Schwarzl, T., Alleaume, A.-M., Sieber, J., Hollerer, I., Bhuvanagiri, M., Huber, W., Hentze, M.W., et al. (2015). Improved binding site assignment by high-resolution mapping of RNA–protein interactions using iCLIP. Nat. Commun. *6*, 7921.

Hines, J.C., and Ray, D.S. (2011). A second mitochondrial DNA primase is essential for cell growth and kinetoplast minicircle DNA replication in *Trypanosoma brucei*. Eukaryot. Cell *10*, 445–454.

Horn, D. (2014). Antigenic variation in African trypanosomes. Mol. Biochem. Parasitol. *195*, 123–129.

Huang, Z., Faktorová, D., Křížová, A., Kafková, L., Read, L.K., Lukeš, J., and Hashimi, H. (2015). Integrity of the core mitochondrial RNA-binding complex 1 is vital for trypanosome RNA editing. RNA *12*, 2088-2102.

Huppertz, I., Attig, J., D'Ambrogio, A., Easton, L.E., Sibley, C.R., Sugimoto, Y., Tajnik, M., König, J., and Ule, J. (2014). iCLIP: protein-RNA interactions at nucleotide resolution. Methods *65*, 274–287.

Ignatochkina, A. V., Takagi, Y., Liu, Y., Nagata, K., and Ho, C.K. (2015). The messenger RNA decapping and recapping pathway in trypanosoma. Proc. Natl. Acad. Sci. *112*, 6967–6972.

Jensen, R.E., and Englund, P.T. (2012). Network news: the replication of kinetoplast DNA. Annu. Rev. Microbiol. *66*, 473–491.

Kafková, L., Ammerman, M.L., Faktorová, D., Fisk, J.C., Zimmer, S.L., Sobotka, R., Read, L.K., Lukeš, J., and Hashimi, H. (2012). Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei*. RNA *18*, 1846–1861.

Kao, C.-Y., and Read, L.K. (2005). Opposing effects of polyadenylation on the

stability of edited and unedited mitochondrial RNAs in *Trypanosoma brucei*. Mol. Cell. Biol. *25*, 1634–1644.

Kao, C.-Y., and Read, L.K. (2007). Targeted depletion of a mitochondrial nucleotidyltransferase suggests the presence of multiple enzymes that polymerize mRNA 3′ tails in *Trypanosoma brucei* mitochondria. Mol. Biochem. Parasitol. *154*, 158–169.

Klingbeil, M.M., and Shapiro, T. A. (2009). Unraveling the secrets of regulating mitochondrial DNA replication. Mol. Cell *35*, 398–400.

Klingbeil, M.M., Motyka, S.A., and Englund, P.T. (2002). Multiple mitochondrial DNA polymerases in *Trypanosoma brucei*. Mol. Cell *10*, 175–186.

Kolev, N.G., Ramey-Butler, K., Cross, G.A.M., Ullu, E., and Tschudi, C. (2012). Developmental progression to infectivity in *Trypanosoma brucei* triggered by an RNA-binding protein. Science *338*, 1352–1353.

Kolev, N.G., Ullu, E., and Tschudi, C. (2014). The emerging role of RNA-binding proteins in the life cycle of *T rypanosoma brucei*. Cell. Microbiol. *16*, 482–489.

König, J., Zarnack, K., Rot, G., Curk, T., Kayikci, M., Zupan, B., Turner, D.J., Luscombe, N.M., and Ule, J. (2010). iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution. Nat. Struct. Mol. Biol. *17*, 909–915.

Koslowsky, D.J., and Yahampath, G. (1997). Mitochondrial mRNA 3' cleavage/polyadenylation and RNA editing in *Trypanosoma brucei* are independent events. Mol. Biochem. Parasitol. *90*, 81–94.

Koslowsky, D.J., Jayarama Bhat, G., Read, L.K., and Stuart, K. (1991). Cycles of progressive realignment of gRNA with mRNA in RNA editing. Cell *67*, 537-46.

Kuratani, M., Ishii, R., Bessho, Y., Fukunaga, R., Sengoku, T., Shirouzu, M., Sekine, S., and Yokoyama, S. (2005). Crystal structure of tRNA adenosine deaminase (TadA) from *Aquifex aeolicus*. J. Biol. Chem. *280*, 16002–16008.

de la Cruz, V.F., Simpson, A.M., Lake, J.A., and Simpson, L. (1985). Primary sequence and partial secondary structure of the 12S kinetoplast (mitochondrial) ribosomal RNA from *Leishmania tarentolae*: Conservation of peptidyl-transferase structural elements. Nucleic Acids Res. *13*, 2337–2356.

De la Cruz, V.F., Lake, J.A., Simpson, A.M., and Simpson, L. (1985). A minimal ribosomal RNA: Sequence and secondary structure of the 9S kinetoplast ribosomal RNA from *Leishmania tarentolae*. Proc. Natl. Acad. Sci. *82*, 1401–1405.

Li, F., Herrera, J., Zhou, S., Maslov, D.A., and Simpson, L. (2011). Trypanosome

REH1 is an RNA helicase involved with the 3'-5' polarity of multiple gRNA-guided uridine insertion/deletion RNA editing. Proc. Natl. Acad. Sci. *108*, 3542–3547.

Li, Y., Song, M., and Kiledjian, M. (2011). Differential utilization of decapping enzymes in mammalian mRNA decay pathways. RNA *17*, 419–428.

Li, Z., Lindsay, M.E., Motyka, S.A., Englund, P.T., and Wang, C.C. (2008). Identification of a bacterial-like HslVU protease in the mitochondria of *Trypanosoma brucei* and its role in mitochondrial DNA replication. PLoS Pathog. *4*, e1000048.

Liu, B., Yildirir, G., Wang, J., Tolun, G., Griffith, J.D., and Englund, P.T. (2010). TbPIF1, a *Trypanosoma brucei* mitochondrial DNA helicase, is essential for kinetoplast minicircle replication. J. Biol. Chem. *285*, 7056–7066.

Lukeš, J., Archibald, J.M., Keeling, P.J., Doolittle, W.F., and Gray, M.W. (2011). How a neutral evolutionary ratchet can build cellular complexity. IUBMB Life *63*, 528–537.

Madej, M.J., Niemann, M., Hüttenhofer, A., and Göringer, H.U. Identification of novel guide RNAs from the mitochondria of *Trypanosoma brucei*. RNA Biol. *5*, 84–91.

Madina, B.R., Kuppan, G., Vashisht, A.A., Liang, Y.-H., Downey, K.M., Wohlschlegel, J.A., Ji, X., Sze, S.-H., Sacchettini, J.C., Read, L.K., et al. (2011). Guide RNA biogenesis involves a novel RNase III family endoribonuclease in *Trypanosoma brucei*. RNA *17*, 1821–1830.

Martin, K.L., and Smith, T.K. (2006). The glycosylphosphatidylinositol (GPI) biosynthetic pathway of bloodstream-form *Trypanosoma brucei* is dependent on the *de novo* synthesis of inositol. Mol. Microbiol. *61*, 89–105.

Maslov, D.A., Sharma, M.R., Butler, E., Falick, A.M., Gingery, M., Agrawal, R.K., Spremulli, L.L., and Simpson, L. (2006). Isolation and characterization of mitochondrial ribosomes and ribosomal subunits from *Leishmania tarentola*e. Mol. Biochem. Parasitol. *148*, 69–78.

Maslov, D.A., Spremulli, L.L., Sharma, M.R., Bhargava, K., Grasso, D., Falick, A.M., Agrawal, R.K., Parker, C.E., and Simpson, L. (2007). Proteomics and electron microscopic characterization of the unusual mitochondrial ribosome-related 45S complex in *Leishmania tarentolae*. Mol. Biochem. Parasitol. *152*, 203–212.

Matthews, K.R. (2005). The developmental cell biology of *Trypanosoma brucei.* J. Cell Sci. *118*, 283–290.

Mattiacio, J.L., and Read, L.K. (2008). Roles for TbDSS-1 in RNA surveillance and

decay of maturation by-products from the 12S rRNA locus. Nucleic Acids Res. *36*, 319–329.

Mattiacio, J.L., and Read, L.K. (2009). Evidence for a degradosome-like complex in the mitochondria of *Trypanosoma brucei.* FEBS Lett. *583*, 2333–2338.

McLennan, A.G. (2006). The Nudix hydrolase superfamily. Cell. Mol. Life Sci. *63*, 123–143.

Michelotti, E.F., Harris, M.E., Adler, B., Torri, A.F., and Hajduk, S.L. (1992). *Trypanosoma brucei* mitochondrial ribosomal RNA synthesis, processing and developmentally regulated expression. Mol. Biochem. Parasitol. *54*, 31–41.

Mildvan, A.S., Xia, Z., Azurmendi, H.F., Saraswat, V., Legler, P.M., Massiah, M.A., Gabelli, S.B., Bianchet, M.A., Kang, L.-W., and Amzel, L.M. (2005). Structures and mechanisms of Nudix hydrolases. Arch. Biochem. Biophys. *433*, 129–143.

Müller, U.F., and Göringer, H.U. (2002). Mechanism of the gBP21-mediated RNA/RNA annealing reaction: matchmaking and charge reduction. Nucleic Acids Res. *30*, 447–455.

Nishikura, K. (2015). A-to-I editing of coding and non-coding RNAs by ADARs. Nat. Rev. Mol. Cell Biol. *17*, 83–96.

Van Nostrand, E.L., Pratt, G.A., Shishkin, A.A., Gelboin-Burkhart, C., Fang, M.Y., Sundararaman, B., Blue, S.M., Nguyen, T.B., Surka, C., Elkins, K., et al. (2016). Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). Nat. Methods *13*, 508–514.

Ochsenreiter, T., Cipriano, M., and Hajduk, S.L. (2007). KISS: the kinetoplastid RNA editing sequence search tool. RNA *13*, 1–4.

Paolo, S.S., Vanacova, S., Schenk, L., Scherrer, T., Blank, D., Keller, W., and Gerber, A.P. (2009). Distinct roles of non-canonical poly(A) polymerases in RNA metabolism. PLoS Genet. *5*, e1000555.

Penschow, J.L., Sleve, D.A., Ryan, C.M., and Read, L.K. (2004). TbDSS-1, an essential *Trypanosoma brucei* exoribonuclease homolog that has pleiotropic effects on mitochondrial RNA metabolism. Eukaryot. Cell *3*, 1206–1216.

Pollard, V.W., Rohrer, S.P., Michelotti, E.F., Hancock, K., and Hajduk, S.L. (1990). Organization of minicircle genes for guide RNAs in *Trypanosoma brucei.* Cell *63*, 783–790.

Rajão, M.A., Passos-Silva, D.G., DaRocha, W.D., Franco, G.R., Macedo, A.M., Pena, S.D.J., Teixeira, S.M., and Machado, C.R. (2009). DNA polymerase kappa from

Trypanosoma cruzi localizes to the mitochondria, bypasses 8-oxoguanine lesions and performs DNA synthesis in a recombination intermediate. Mol. Microbiol. *71*, 185–197.

Read, L.K., Wilson, K.D., Myler, P.J., and Stuart, K. (1994). Editing of *Trypanosoma brucei* maxicircle CR5 mRNA generates variable carboxy terminal predicted protein sequences. Nucleic Acids Res *22*, 1489–1495.

Read, L.K., Zimmer, S.L., and Ammerman, M.L. (2011). Marked for Translation: long A/U tails as an interface between completion of RNA editing and ribosome recruitment. Mol. Cell *42*, 6–8.

Read, L.K., Lukeš, J., and Hashimi, H. (2016). Trypanosome RNA editing: The complexity of getting U in and taking U out. Wiley Interdiscip. Rev. RNA *7*, 33–51.

Rosenberg, B.R., Hamilton, C.E., Mwangi, M.M., Dewell, S., and Papavasiliou, F.N. (2011). Transcriptome-wide sequencing reveals numerous APOBEC1 mRNA-editing targets in transcript 3′ UTRs. Nat. Struct. Mol. Biol. *18*, 230–236.

Rubio, M.A.T., Ragone, F.L., Gaston, K.W., Ibba, M., and Alfonzo, J.D. (2006). C to U Editing Stimulates A to I Editing in the Anticodon Loop of a Cytoplasmic Threonyl tRNA in *Trypanosoma brucei*. J. Biol. Chem. *281*, 115–120.

Rubio, M.A.T., Gaston, K.W., McKenney, K.M., Fleming, I.M.C., Paris, Z., Limbach, P.A., and Alfonzo, J.D. (2017). Editing and methylation at a single site by functionally interdependent activities. Nature *542*, 494–497.

Ryan, C.M., and Read, L.K. (2005). UTP-dependent turnover of *Trypanosoma brucei* mitochondrial mRNA requires UTP polymerization and involves the RET1 TUTase. RNA *11*, 763–773.

Schimmel, P. (2017). The emerging complexity of the tRNA world: mammalian tRNAs beyond protein synthesis. Nat. Rev. Mol. Cell Biol. *19*, 45–58.

Schnaufer, A., Ernst, N.L., Palazzo, S.S., O'Rear, J., Salavati, R., and Stuart, K. (2003). Separate insertion and deletion subcomplexes of the *Trypanosoma brucei* RNA editing complex. Mol. Cell *12*, 307–319.

Schumacher, M.A., Karamooz, E., Zíková, A., Trantírek, L., and Lukeš, J. (2006). Crystal structures of *T. brucei* MRP1/MRP2 guide-RNA binding complex reveal RNA matchmaking mechanism. Cell *126*, 701–711.

Scocca, J.R., and Shapiro, T.A. (2008). A mitochondrial topoisomerase IA essential for late theta structure resolution in African trypanosomes. Mol. Microbiol. *67*, 820–829.

Sharma, M.R., Koc, E.C., Datta, P.P., Booth, T.M., Spremulli, L.L., and Agrawal, R.K. (2003). Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. Cell *115*, 97–108.

Sharma, M.R., Booth, T.M., Simpson, L., Maslov, D.A., and Agrawal, R.K. (2009). Structure of a mitochondrial ribosome with minimal RNA. Proc. Natl. Acad. Sci. *106*, 9637–9642.

Shaw, A.K., Kalem, M.C., and Zimmer, S.L. (2016). Mitochondrial gene expression is responsive to starvation stress and developmental transition in *Trypanosoma cruzi*. mSphere *1*, e00051-16.

Simpson, R.M., Bruno, A.E., Bard, J.E., Buck, M.J., and Read, L.K. (2016). High-throughput sequencing of partially edited trypanosome mRNAs reveals barriers to editing progression and evidence for alternative editing. RNA *5*, 677-95.

Simpson, R.M., Bruno, A.E., Chen, R., Lott, K., Tylec, B.L., Bard, J.E., Sun, Y., Buck, M.J., and Read, L.K. (2017). Trypanosome RNA editing mediator complex proteins have distinct functions in gRNA utilization. Nucleic Acids Res. *45*, 7965–7983.

Sinha, K.M., Hines, J.C., Downey, N., and Ray, D.S. (2004). Mitochondrial DNA ligase in *Crithidia fasciculata*. Proc. Natl. Acad. Sci. *101*, 4361–4366.

Sinha, K.M., Hines, J.C., and Ray, D.S. (2006). Cell cycle-dependent localization and properties of a second mitochondrial DNA ligase in *Crithidia fasciculata.* Eukaryot. Cell *5*, 54–61.

Sloof, P., de Haan, A., Eier, W., van Iersel, M., Boel, E., van Steeg, H., and Benne, R. (1992). The nucleotide sequence of the variable region in *Trypanosoma brucei* completes the sequence analysis of the maxicircle component of mitochondrial kinetoplast DNA. Mol. Biochem. Parasitol. *56*, 289–299.

Song, M.-G., Li, Y., and Kiledjian, M. (2010). Multiple mRNA decapping enzymes in mammalian cells. Mol. Cell *40*, 423–432.

Song, M.-G., Bail, S., and Kiledjian, M. (2013). Multiple Nudix family proteins possess mRNA decapping activity. RNA *19*, 390–399.

Sprinzl, M. (1998). Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. *26*, 148–153.

Sprinzl, M., and Vassilenko, K.S. (2005). Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. *33*, D139–D140.

Stephens, J.L., Soo, H.L., Paul, K.S., and Englund, P.T. (2007). Mitochondrial fatty

acid synthesis in *Trypanosoma brucei.* J. Biol. Chem. *282*, 4427–4436.

Steverding, D. (2010). The development of drugs for treatment of sleeping sickness: a historical review. Parasit. Vectors *3*, 15.

Stuart, K. (1983). Mitochondrial DNA of an African trypanosome. J. Cell. Biochem. *23*, 13–26.

Ule, J., Jensen, K.B., Ruggiu, M., Mele, A., Ule, A., and Darnell, R.B. (2003). CLIP identifies Nova-regulated RNA networks in the brain. Science *302*, 1212–1215.

Uren, P.J., Bahrami-Samani, E., Burns, S.C., Qiao, M., Karginov, F. V., Hodges, E., Hannon, G.J., Sanford, J.R., Penalva, L.O.F., and Smith, A.D. (2012). Site identification in high-throughput RNA–protein interaction data. Bioinformatics *28*, 3013–3020.

Vaňáčová, Š., Wolf, J., Martin, G., Blank, D., Dettwiler, S., Friedlein, A., Langen, H., Keith, G., and Keller, W. (2005). A new yeast poly(A) polymerase complex involved in RNA quality control. PLoS Biol. *3*, e189.

Verner, Z., Basu, S., Benz, C., Dixit, S., Dobáková, E., Faktorová, D., Hashimi, H., Horáková, E., Huang, Z., Paris, Z., et al. (2015). Malleable mitochondrion of *Trypanosoma brucei* *315*, 73-151.

Vondrušková, E., Van Den Burg, J., Zíková, A., Ernst, N.L., Stuart, K., Benne, R., and Lukeš, J. (2005). RNA interference analyses suggest a transcript-specific regulatory role for mitochondrial RNA-binding proteins MRP1 and MRP2 in RNA editing and other RNA processing in *Trypanosoma brucei.* J. Biol. Chem. *280*, 2429–2438.

Wang, Z., Morris, J.C., Drew, M.E., and Englund, P.T. (2000). Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. *275*, 40174–40179.

Weng, J., Aphasizheva, I., Etheridge, R.D., Huang, L., Wang, X., Falick, A.M., and Aphasizhev, R. (2008). Guide RNA-Binding complex from mitochondria of Trypanosomatids. Mol. Cell *32*, 198–209.

Wolf, J. (2002). tadA, an essential tRNA-specific adenosine deaminase from *Escherichia coli*. EMBO J. *21*, 3841–3851.

Woodward, R., and Gull, K. (1990). Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei.* J. Cell Sci. *95*, 49–57.

Zíková, A., Horáková, E., Jirků, M., Dunajčíková, P., and Lukeš, J. (2006). The effect

of down-regulation of mitochondrial RNA-binding proteins MRP1 and MRP2 on respiratory complexes in procyclic *Trypanosoma brucei.* Mol. Biochem. Parasitol. *149*, 65–73.

Zíková, A., Kopečná, J., Schumacher, M.A., Stuart, K., Trantírek, L., and Lukeš, J. (2008). Structure and function of the native and recombinant mitochondrial MRP1/MRP2 complex from *Trypanosoma brucei*. Int. J. Parasitol. *38*, 901–912.

Zimmer, S.L., McEvoy, S.M., Li, J., Qu, J., and Read, L.K. (2011). A novel member of the RNase D exoribonuclease family functions in mitochondrial guide RNA metabolism in *Trypanosoma brucei*. J. Biol. Chem. *286*, 10329–10340.

Zimmer, S.L., McEvoy, S.M., Menon, S., and Read, L.K. (2012). Additive and transcript-specific effects of KPAP1 and TbRND activities on 3' non-encoded tail characteristics and mRNA stability in *Trypanosoma brucei*. PLoS One *7*, e37639.

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RESEARCH EXPERIENCE

Institute of Parasitology 2012-Present
PhD-fellowship; Advisor: Julius Lukeš

Characterizing the role of RNA-binding proteins involved in mitochondrial RNA editing of *Trypanosoma brucei*

I optimized *in vivo* crosslinking and immunoprecipitation (iCLIP) method that is optimal to crosslink mitochondrial (mt) RNA-protein complexes in trypanosomes. Latter on iCLIP was applied to study three RNA-binding proteins (RBPs), which are implicated in mt RNA editing.

Research includes first Bioinformatics pipeline targeted specifically towards analyzing mt iCLIP data that requires tweaking in order to address changes introduced to mRNAs by Uridine insertion and or deletion.

ICGEB 2010-2012
Master-student; Advisor: Amit Sharma

Studying the components of malaria parasite protein secretion machinery known as PTEX translocon assembly.

Helped with protein purification of Thioredoxin-2 and setting-up of the X-crystallization trays. ELISA and Isothermalcalorimetry based assays to study protein-ligand interactions.

EXPERTISE ACQUIRED

During Bachelor and Master period

Different aspects of X-ray crystallography lab:

Protein Expression, rotein Crystalization, molecular biology and biochemistry techniques, and setting-up of X-ray crystalization plates

During Doctoral period

Different aspects of Functional Genomics lab dealing in with mitcohondrial biology:

Generating RNAi and knock-out cell-lines in trypanosomes, studying RNA-protein interactions: by iCLIP and other NGS related techniques, bioinformatic analyses of iCLIP data, and biochemistry related assays

Mitochondrial biology: Organelllar purifications and assays to measure mitochondrion membrane potential

COURSES ATTAINED

RNPnet Summerschool for Strutural and Chemical Biology	EMBL Heidelberg 2013

To study RNA-protein interactions using structural and chemical biology techniques

Research Carrer in the Private Sector	Novartis Basel

2013

Scientific Writing and Presentation — ETH Zurich, 2013

RNPnet Summerschool for Cell and System Biology approches in RNA biology — CNRS Montpelier, 2014

To study RNA-protein interctions using Cell and System biology techniques

Ethics in Scinece — CNRS Montpelier, 2014

CONFERENCE PRESENTATIONS

Dixit S, and Lukes J. Quantitative binding of MRP1 on mitochondrial mRNA determines its RNA editing level in Trypanosomes. Short talk; Czech Society of parasitologist, Czech Republic 2017 (**awarded as best talk**)

Dixit S, and Lukes J. Differential binding of mitochondrial transcripts by MRB8170 and MRB4160 regulates distinct editing fates of mitochondrial mRNA in trypanosomes. Poster presentation delivered in a conference; 12th International Congress of Cell Biology, Prague, Czech Republic, 2016.

Dixit S, and Lukes J. Unravelling complexities of RNA Editing in Trypanosomes. Poster presentation delivered in a conference; RNP structure, function and mechanism of action, Marrakesh, Morocco, 2015.

Dixti S, and Lukes J. MRB8170 and MRB4160 are a Nexus between RNA Editing and other RNA procesisng complexes for mitochondrial transcripts in Trypanosomes. Short talk delivered in a conference; RNA Surveillance, editing and splicing: RNP: Strucutre meets function, Ceske Budejovice, Czech Republic, 2014.

PEER-REVIEWED PUBLICATIONS

Dixit S, and Lukeš J. Combinatorial interplay of RNA-binding proteins fine-tune levels of edited mRNA in trypanosomes (Under Submission)

Dixit S, Muller-McNicoll, David V, Zarnack K, Ule J, Hashimi H, and Lukeš J. 2017. Differential binding of mitochondrial transcripts by MRB8170 and MRB4160 regulates distinct editing fates of mitochondrial mRNA in trypanosomes. mBio **8**: 02288-16.
http://mbio.asm.org/content/8/1/e02288-16.full.pdf

Sharma A, Sharma A, **Dixit S**, and Sharma A. 2011. Structural insights into Thioredoxin-2: component of malaria parasite protein secretion machinery. Scientific Reports **1**:179
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3240959/pdf/srep00179.pdf

REVIEW ARTICLES

Verner Z, Basu S, Benz C, **Dixit S**, Dobáková E, Faktorová D, Hashimi H, Horáková E, Huang Z, Paris Z, Peña-Diaz P, Ridlon L, Týč J, Wildridge D, Zíková A, and Lukeš J. 2015. Malleable mitochondrion of *Trypanosoma brucei*. Int Rev Cell Mol Biol **315**:73–151.
http://www.sciencedirect.com/science/article/pii/S1937644814000094?via%3Dihub