THE FUNCTION OF A CENTRAL RNA HELICASE RNP

IN EDITOSOMES OF TRYPANOSOMES

A Dissertation

by

VIKAS KUMAR

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Chair of Committee,	Jorge Cruz-Reyes
Committee Members,	Gregory Reinhart
	Xiuren Zhang
	Julian Leibowitz
Head of Department,	Gregory Reinhart

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ABSTRACT

Trypanosoma brucei requires extensive remodeling of its mitochondrial gene expression during different stages of its life cycle. RNA editing is one of the vital processes for these mitochondrial changes. This process involves insertions and deletions of uridylates in mitochondrial mRNAs that is governed by a multi-protein RNA editing core complex (RECC). Editing proceeds in small blocks from 3' to 5' direction leaded by small *trans*-acting guide RNAs (gRNAs). RECC appears to lack both, the mRNA substrates and gRNAs, indicating a role of accessory proteins in RNA editing. Many other non-RECC proteins have been discovered that directly impact the editing process, including mitochondrial RNA-binding complex 1(MRB1) that was shown to contain gRNAs. Despite of all the progress made, central long-standing question still remains unanswered including the mechanism for substrate delivery and regulation of RNA editing process.

This dissertation presents the discovery of two variants of native MRB1 (mitochondrial RNA-binding complex 1) that we termed REH2-MRB and 3010-MRB. These MRB1 variants contain both mRNAs and gRNAs and show specialized functions, 3010-MRB and REH2-MRB seem to serve as scaffolds for RNA editing. REH2-MRB is defined by the critical RNA helicase termed REH2 (RNA editing helicase 2) that acts in *trans*, affecting multiple steps of the editing function in 3010-MRB. In addition, we discovered two cofactors of REH2. This novel RNA editing helicase 2-associated subcomplex (REH2C) binds mRNA substrates and products and therefore represents a stable mRNA-bound protein subcomplex (mRNP). Our working model is that MRB1

variant complexes are formed by the coupling of this mRNP with gRNA-bound subcomplexes (gRNPs). The mRNP/gRNP complexes form a platform for the assembly of functional mRNA-gRNA hybrids and catalytic RECC enzyme. Thus, in our proposed model editosomes are assembled in a stepwise process that involves the docking of mRNP and gRNP modules through specific base-pairing of respective mRNA and gRNAs. These subunits of the REH2C may control specific checkpoints in the editing pathway.

DEDICATION

I dedicate this dissertation to my beloved family for their love, affection and encouragement that guided me towards this achievement.

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Author contributions

Pages 51-84 are the original text and figures reported in reference (1). Native mitochondrial RNA-binding complexes in kinetoplastid RNA editing differ in guide RNA composition. Madina BR, Kumar V, Metz R, Mooers BHM, Bundschuh R, Cruz-Reyes J. RNA. 2014, 20:1142-1152. Author contributions are as follows:

Page 61 Figure 9: (panels A to D) Kumar V, (panels E & F) Madina BR; Page 65 Figure 10: (panels A to G) Cruz-Reyes J, (panel H) Madina BR. Metz R, Bundschuh R analyzed the Illumina libraries in collaborations with Madina BR and Kumar V; Page 66 Table 5: Cruz-Reyes J. Immunoprecipitation assays and RNA purification was performed by Madina BR and Kumar V; Page 73 Figure 11: Cruz-Reyes J; Page 75 Figure 12: Madina BR; Page 80 Figure 13: Cruz-Reyes J; Page 81 Figure 14: Kumar V; Page 82 Figure 15: Kumar V; Page 83 Figure 16: Madina BR; Page 84 Figure 17: Kumar V.

Pages 85-119 are the original text and figures reported in reference (2). Native Variants of the MRB1 Complex Exhibit Specialized Functions in Kinetoplastid RNA Editing. Madina BR*, Kumar V*, Mooers BHM, Cruz-Reyes J. PLoS ONE. 2015. 10(4): e0123441. Madina and Kumar share first-authorship (*). Author contributions are as follows:

Page 96 Figure 18: Madina BR; Page 97 Figure 19: (panels A to D) Madina BR and Kumar V, (panel E) Kumar V; Page 99 Figure 20: (panels A to C) Kumar V, (panel D) Madina BR; Page 101 Figure 21: Madina BR; Page 102 Figure 22: Madina BR; Page

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Pages 120-153 are the original text and figures reported in reference (3). REH2C Helicase and GRBC Subcomplexes may Base Pair through mRNA and Small Guide RNA in Kinetoplastid Editosomes. Kumar V*, Madina BR*, Gulati S, Vashisht AA, Kanyumbu C, Pieters B, Shakir A, Wohlschlegel JA, Read LK, Mooers BHM, Cruz-Reyes J. *J Biol Chem*. 2016. 291(11):5753-64. Kumar and Madina share first-authorship (*). Author contributions are as follows:

Page 131 Figure 31: Cruz-Reyes J; Page 134 Figure 32: Kumar V; Page 137 Figure 33: Cruz-Reyes J, 3-D structures by Mooers BHM; Page 140 Figure 34: Kumar V; Page 143 Figure 35: (panel A) Kumar V in collaboration with Madina BR, (panel B-F) Kumar V; Page 145 Figure 36: Kumar V; Page 146 Figure 37: Kumar V; Page 148 Figure 38: (panel A) Gulati S, (panel B-F) Kumar V; Page 153 Figure 39: Cruz-Reyes.

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CHAPTER I

INTRODUCTION

Kinetoplastids are considered evolutionarily among the most ancestral eukaryotic parasites (4). They belong to a diverse group of unicellular flagellated eukaryotes that consist of a dense granule of DNA known as "kinetoplast". Despite the similarities in genomic organization, cellular structure, these kinetoplastid pathogens can cause different diseases in humans through different insect vectors (Table 1). These diseases includes African sleeping sickness also known as human African Trypanosomiasis (HAT), chagas disease and leishmaniasis that are caused by *Trypanosoma brucei* (*T. brucei*), *Trypanosoma cruzi* (*T. cruzi*) and species of *Leishmania* respectively (5).

HAT consists of two forms: a chronic form caused by *T. brucei gambiense,* which occurs in central and West Africa; and an acute form caused by *T. brucei rhodesiense,* which occurs in eastern and Southern Africa (6). This disease has infected over 80,000 people per annum and the results are fatal if not treated (5). Current treatments for Trypanosomiasis are inadequate because the drugs are highly toxic and are difficult to administer. At present, there are no evident prospects for developing a vaccine due to a remarkable mechanism to evade host immune response in *T. brucei*. This mechanism involves remodeling of the surface proteins, antigenic variability of the surface proteins, clearing host antibodies off its surface and finally consuming antibodies through endocytosis (7).

Chagas disease, a chronic infection caused by *T. cruzi* can result into cardiomyopathy, digestive megasyndromes, or both (8). This disease has affected central

and south America, causing 14,000 deaths annually (5). At present, available diagnostics include multiple serological tests for this disease. However, the test results can be inconclusive which make these diagnostics incompetent but imperative simultaneously (9–11). Leishmaniasis, caused by over 20 species of *Leishmania*; kills 51,000 people worldwide per year (5). On the basis of clinical manifestations, this disease can cause cutaneous, mucocutaneous, and visceral infections (also known as kala-azar); the visceral form is the most severe and is potentially fatal if left untreated (12, 13). Unlike *T. cruzi* and *Leishmania*, *T. brucei* possesses a functional RNAi pathway which makes these parasites a suitable model in the research field (14).

Table 1. The kinetoplastid diseases

Disease	HAT	Chagas Disease	The leishmaniases
Causative organisms	*T. brucei gambiense*, *T. brucei rhodiense*	*T. cruzi*	~21 *Leishmania* spp.
Vectors of medical importance	Tsetse flies (~20 Glossina spp.)	Reduviid bugs (~12/~138 Triatominae spp.)	Phebotomine sandflies (~70 spp.)
Geographic distribution	Sub-Saharan Africa, (~20 countries)	South and Central America (19 countries)	South and Central America, Europe, Africa, Asia (88 endemic countries)
Infected	70,000–80,000	8–11 million	12 million
Deaths (per annum)	~30,000	14,000	51,000

Data reproduced from ref.(5) and http://www.who.int/neglected_diseases/diseases/en/.

T. brucei: life cycle, morphology and immune evasion

T. brucei has a complex digenetic life cycle that includes two morphologically distinct forms, the procyclic form (PCF) and the bloodstream form (BSF). The PCF that live in the midgut of the tsetse fly undergoes stage-specific transitions while making its way up to the salivary glands of the fly. These transitions include switching from PCF to epimastigote form in the foregut, followed by metacyclic form that attaches itself to the salivary gland epithelium of the tsetse fly (Figure 1). This whole development process takes about 20-30 days before the parasite reaches its mature form and is ready to be transmitted (15). Once injected into the mammalian host, the parasite transitions into bloodstream form. Furthermore, in mammalian host, two main BSFs exist; a proliferative long slender form and a latent stumpy form (16). While taking a blood meal from the infected host, the fly ingests both these BSFs but only the stumpy form is able to survive this journey due to presence of a potent protease in the saliva and midgut of the tsetse fly (17). Once the stumpy form reaches the midgut, it differentiates into the PCF within 24 hours and the whole development process recommences (18).

Proliferating long slender form establishes and maintains a blood stream infection. This form migrates to the extravascular tissues that includes invasion of the central nervous system (CNS). Slender forms transition into stumpy forms based on the density of the parasite and not because of the immune response since this change is observed in the hosts with suppressed immunity (19–21). This change is thought to be stimulated by stumpy induced factor (SIF), which is accumulates when the parasitemia

increases. In addition, SIF is shown to be responsible for cell cycle arrest in G1/G0 phase and cell density sensing through a cAMP signal pathway (22, 23).

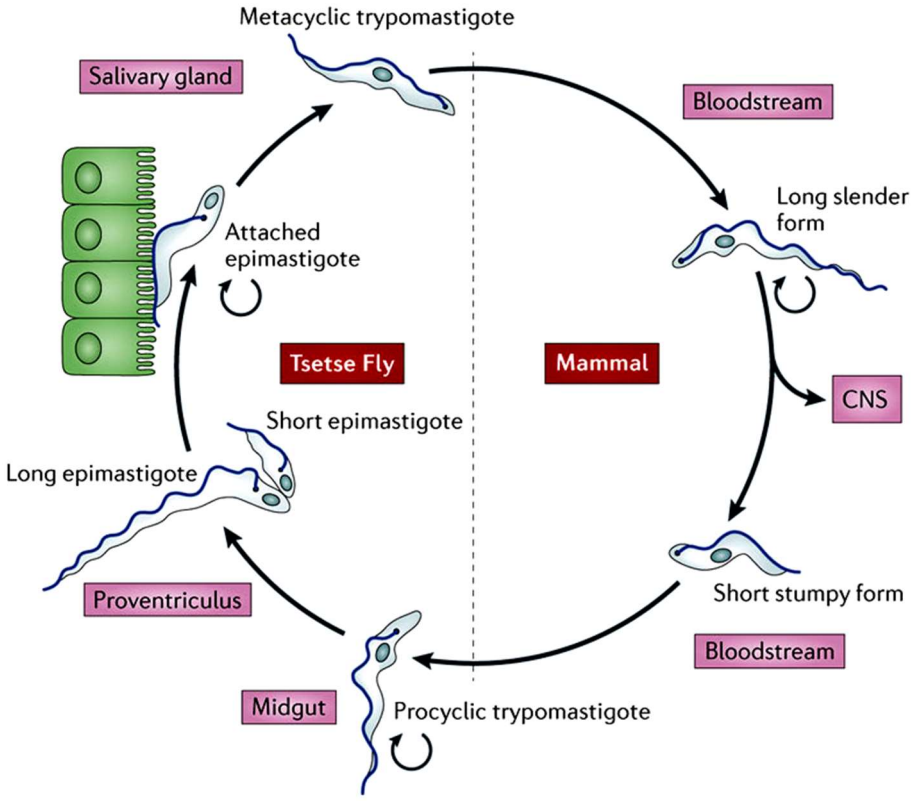

Figure 1. *T. brucei* life cycle inside insect vector and mammalian hosts. This figure summarizes the life cycle of *T. brucei*. The infection of a mammalian host initiates upon the bite of tsetse fly, delivering the metacyclic trypomastigotes in the bloodstream of mammalian host. This parasite differentiates into proliferating long slender forms that is capable of maintaining bloodstream infection and eventually penetrates the blood vessel endothelium and invades extravascular tissues, including the central nervous system (CNS). The long slender forms differentiate to stumpy forms (non-proliferative) that are picked up by tsetse fly into the midgut during the bloodmeal, where the parasite is differentiated into PCF. The PCF migrates from midgut along the foregut to proventriculus, transitioning into epimastigote forms. The parasite reaches its final destination inside the fly when it reaches the salivary glands where it attaches itself to epithelial cells of salivary gland. The parasite ultimately completes its life cycle by differentiating into mammalian infective metacyclic trypomastigotes. The dividing forms that replicate via binary fission are indicated with circular arrows. (Used with permission from (24)).

In order to survive the different environments in distinct host systems, the parasite must evade the acquired and innate immune system of the host. During its life cycle, the transition of *T. brucei* between insect and mammalian hosts requires extensive remodeling of cellular processes and morphology that includes transitioning between major proliferative forms. An essential change in *T. brucei* while adapting between the insect and mammalian hosts, is remodeling of its surface proteins. Inside the midgut of the insect, PSF contains protease-resistant procyclin proteins that are attached to the parasite's surface by a glycosylphosphatidylinositol (GPI) anchor (7, 25, 26). The surface coat changes to *brucei* alanine-rich protein (BARP) during the morphological switch into epimastigote form. Metacyclic forms obtain a dense coat of single variant surface glycoprotein (VSG) over its entire surface before its transmission into the mammalian host, where BSF will possess the same VSG coat (7). Each VSG contains a hypervariable alpha-helical N-terminal domain exposed to the immune system, and a conserved C-terminal domain connected to the plasma membrane of the parasite through a GPI-anchor.

T. brucei can survive several waves of host immune response through acquiring a thick VSG coat but eventually, VSG becomes a target for host antibodies. Therefore, to ensure survival *T. brucei* has developed a unique mechanism of sporadically switching the surface VSG with another antigenic variant VSG (27–29). There are approximately 2000 VSG and VSG-related gene that this parasite can chose from to express over its surface (7, 30). This VSG switching is spontaneous and occurs at a rapid rate (about 10^{-2}-10^{-3} per population doubling) (31). This process happens so often that by the time the

human immune system assembles appropriate antibodies; there will be a distinct antigenic form of VSG coating the plasma membrane of parasite. The amino acid identity shared between VSG variants is low (15%) which facilitates evasion by existing host immunity and prolonging the infection (32).

This parasite continuously recycles its surface coat, which facilitates rapid removal of VSG-antibody complexes (33). The flagellum on the parasite's surface and its mobility is also known to contribute in clearing VSG-antibody complexes from the surface. Hydrodynamic forces generated by flagellum mobility results in surface drag which is further increased by VSG-antibody complexes, resulting in the shift of the complex towards the posterior of the cells (34). This mechanism explains why the downregulation of flagellar proteins is lethal to the bloodstream form (35). Antibody bound VSGs are engulfed into the endosome by a clatherin-coated endocytosis process, where the antibodies are targeted to the lysosomes and are destroyed, whereas VSG is recycled back to the cell surface (36, 37).

Evasion of host immunity is one of the greatest challenges that this parasite encounters but there are even more threats to the parasites perhaps even greater than immune evasion. Transmission between mammalian host and tsetse fly, parasite faces different surroundings with regard to temperature, pH, nutrients, and osmotic stress. To overcome these environmental obstacles, *T. brucei* undergoes metabolic adaptations, especially in the mitochondria (7).

Mitochondrion of *T. brucei*: change in morphology and energy metabolism

Mitochondrion of *T. brucei* undergoes significant morphological changes while switching between insect and mammalian hosts. In PCF, the mitochondrion is highly branched and cristae-rich and contains all the enzymes required for the tricarboxylic acid cycle (TCA). Conversely, in BSF, mitochondrion is less developed with considerably less branches; and substantially lacks cristae, cytochrome-containing complexes and crucial enzymes for TCA (38, 39).

In the bloodstream of mammalian host glucose is present in abundance. Therefore, BSF takes advantage of it and derives most of its energy needs through substrate-level phosphorylation events in glycolysis. Glycolysis in *T. brucei*, takes place in a specialized peroxisome-like organelle, the glycosomes. However, the last few steps of glycolysis are catalyzed in the cytosol and produce pyruvate as the end product, which is eventually excreted (38, 39). Inside the midgut of the tsetse fly where nutrients are limited, the parasite has developed its energy metabolism that produces ATP via mitochondrial catabolic pathways. This includes the consumption pyruvate, the end product of glycolysis, which is further broken down into acetate and ATP inside the mitochondria via substrate level phosphorylation instead of being excreted (39, 40).

Due to scarcity of glucose inside midgut of tsetse fly, PCFs utilize amino acids such as threonine and proline as an important carbon sources for ATP production. Threonine is metabolized into glycine and acetate in this glucose-depleted environment, whereas proline is broken down into alanine, succinate, glutamate and CO_2 (41, 42). Although, all the enzymes that drives citric acid cycle are present in the PCFs; however,

this pathway does not follow its usual route in PCF. For example, acetyl-CoA, the main substrate for the citric acid cycle, is consumed in fatty acid synthesis pathway rather being fed into citric acid cycle (43–45). In addition, Krebs cycle is not the main pathway in terms of energy production in *T. brucei,* but mitochondrial electron transport chain (ETC) is essential for its survival.

Oxidative phosphorylation in *T. brucei* includes three additional enzymes that transport electron through the respiratory chain. The first two enzymes include a mitochondrial glycerol-3-phosphate dehydrogenase (G3PDH) and an alternative rotenone-insensitive NADH dehydrogenase (NDH2) (Figure 2) (46). These enzymes are responsible for passing electrons from glycerol-3-phosphate and NADH to ubiquinone respectively. Another enzyme that is part of the electron transport chain is trypanosome-specific alternative oxidase (TAO) that takes electrons from ubiquinol and passing it to oxygen (47–49). Oxidative phosphorylation in mitochondria of *T. brucei*, changes drastically between two life stages of the parasite in distinct hosts. In PSFs, oxidative phosphorylation is similar to other eukaryotes, following the classical respiratory chain. On the other hand, In BSFs, the less developed mitochondria does not express cytochrome-containing complexes and its ETC is missing III and IV respiratory complex (Figure 2a) (50, 51). Unexpectedly, complex I and II are present in this stage, but their function in the BSF still remains a question (52, 53). Furthermore, F_0/F_1-ATP synthetase has a distinct function in BSF, where it maintains the membrane potential of the mitochondria through ATP hydrolysis instead of ATP production. Subunits of the

respiratory complex are encoded in the DNA of the mitochondrion, known as kinetoplast DNA or kDNA.

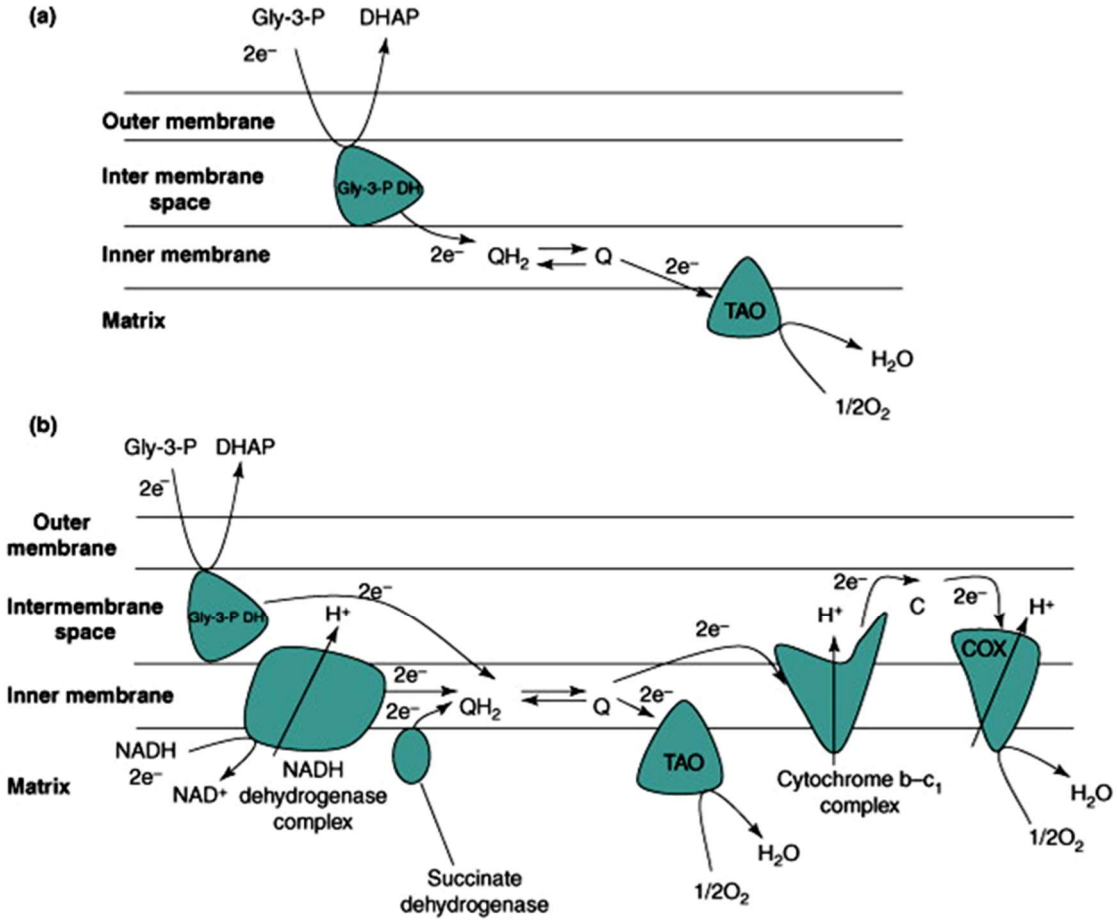

Figure 2. The mitochondrial respiratory chain complexes in BSF and PCF of *T. brucei*. (a) BSF lacks the cytochrome-containing complexes in comparison to PCF. (b) In PCF, oxidative phosphorylation is similar to mammals, with all the necessary components required for a working ETC. Abbreviations: DHAP, dihydroxyacetone phosphate; Gly-3-PDH, glycerol-3-phosphate dehydrogenase; QH_2, ubiquinol; Q, ubiquinone; TAO, trypanosome-specific alternative oxidase; C, cytochrome c; COX, cytochrome oxidase. (Used with permission from (46)).

Kinetoplast: kDNA, its transcription and RNA editing

Kinetoplast

Another very interesting morphological feature of *T. brucei,* is its mitochondrial genome that is organized into a structure known as kinetoplast (Figure 3) (54–56). A single mitochondrion of every cell consists of a massive network of kDNA, which is comprised of dozens of ~23 kb-long maxicircles and thousands of ~1 kb-long minicircles (54, 57–59). Within a maxicircle sequence, there is a highly conserved ~17kb long coding region, while the rest of the sequence contains an extremely variable region with long repeats. Maxicircles contain traditional mitochondrial genes including 2 ribosomal RNAs (rRNAs), and subunits of respiratory complexes (60–64). The transcripts encoded by maxicircle are summarized in Figure 4 (65). This array of these mitochondrial genes is observed in all the species of trypanosomes; however, for some genes the level of editing may vary between distinct species (66).

Minicircles exclusively encode *trans*-acting guide-RNAs (gRNAs), which are partially complementary to the mRNA transcripts that are encoded by maxicircles. The large population of minicircles accounts for over 95% of the kDNA network. Each minicircle can transcribe 3-5 gRNA transcripts that help in altering the sequence of mRNA through a unique mechanism known as RNA editing (66–69), which is the focus of my studies and will be discussed later in this section.

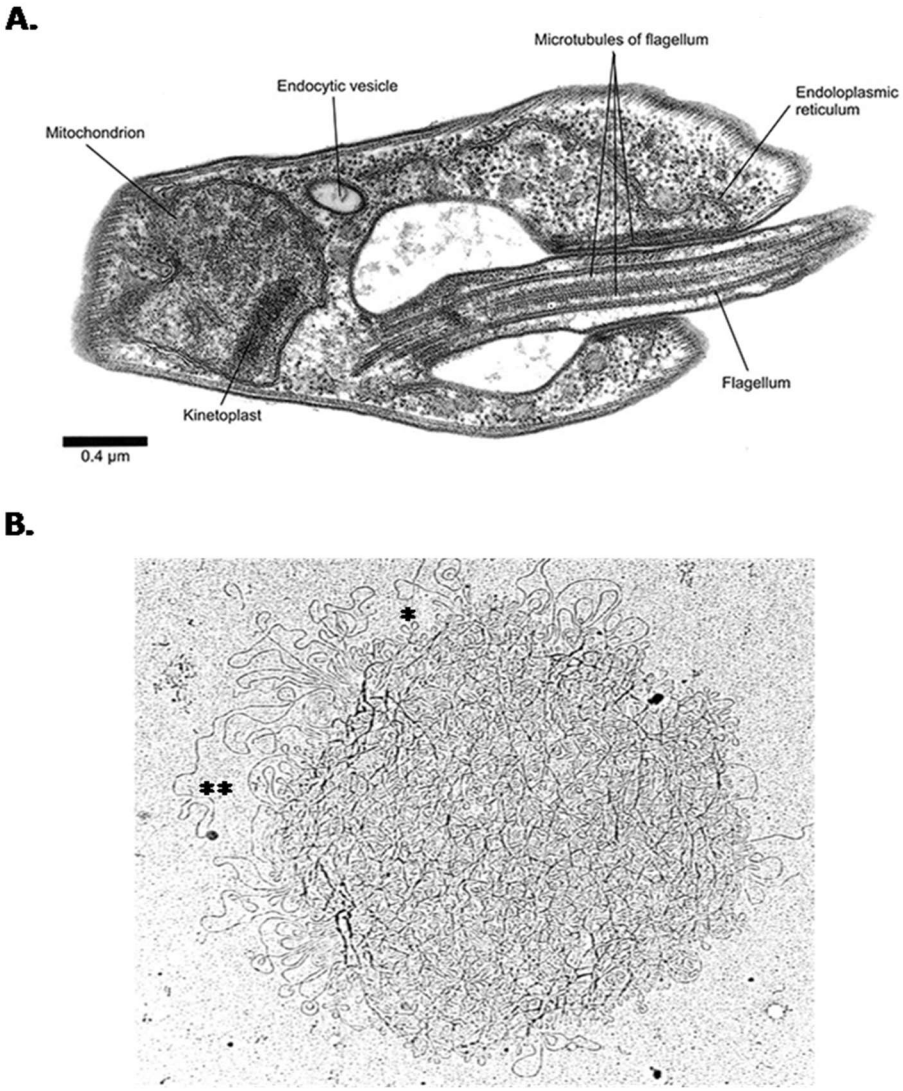

Figure 3. Electron micrographs of kDNA from *T. brucei*. (A) Section through the flagellar pocket region of the *T. brucei*. Mitochondria shown with the highly compact structure called the kinetoplast. (B) Purified kDNA showing the massive network of minicircles (*) and maxicircle (**). (Used with permission, A. from (70) and B. from (71)).

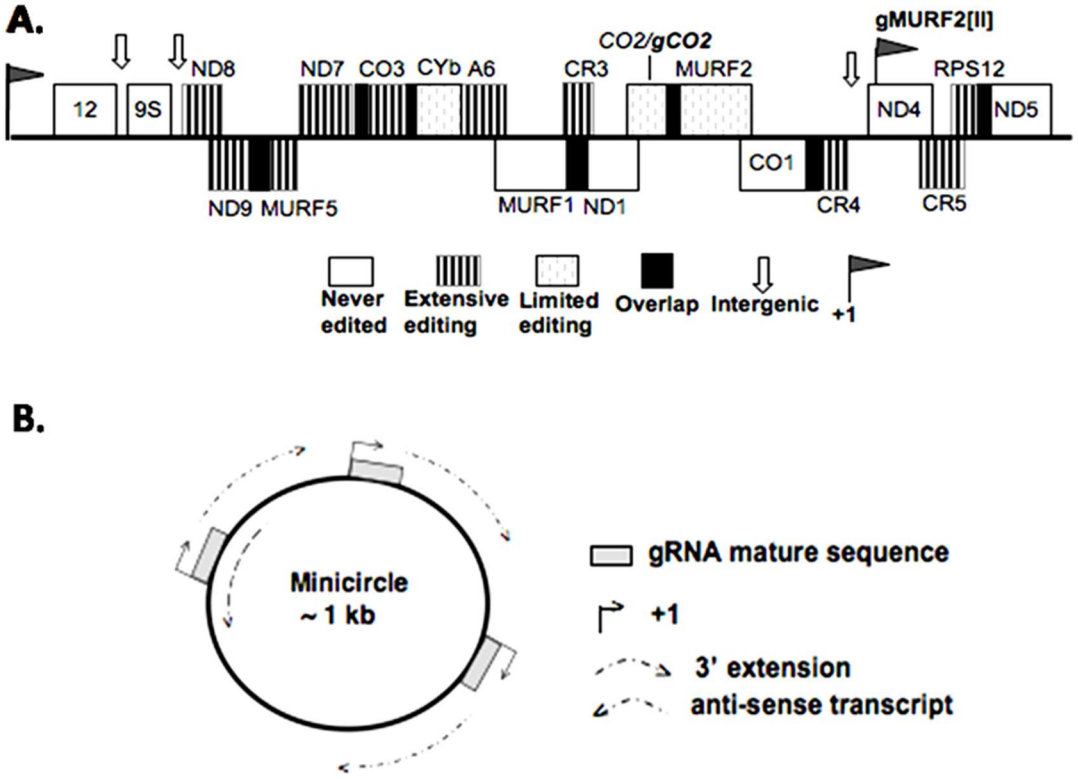

Figure 4. Gene arrangement on maxicircle (A) and minicircle (B). (A) Abbreviation: A6, ATP synthase subunit 6; CO, cytochrome *c* oxidase subunit; CYb, cytochrome *b*; CR, C-rich region; MURF, maxicircle unidentified reading frame; ND, NADH dehydrogenase subunit; RPS12, 40S ribosomal protein S12. Flag represents the transcription initiation site. The only known gRNA encoded by maxicircle is gMURF2[II] that has its own promoter (Flag). (B) +1 position marks the mature 5' end of gRNA. Clockwise dotted arrows represent the sense gRNA and anti-clock dotted arrow signifies the anti-sense gRNA. (Courtesy of Jorge Cruz-Reyes).

Transcription

The mechanisms of kDNA transcription in *T. brucei* are unknown. Yet, a mitochondrial RNA polymerase (mtRNAP) transcribes both maxicircle and minicircle genes. Most transcripts in maxicircles and minicircles are polycistronically precursors (72–75) that require further processing to generate functional monocistrons. Generation of mature mRNAs require 3' polyuridylation and polyadenylation (76–78). Whereas, generation of mature gRNAs was recently described to require 3' exonucleolytic trimming that involve anti-sense ncRNA (79).

The majority of the mRNAs if not all, have poly (A) tail that provides stability to the transcripts. The polyadenylation process is shown to be catalyzed by a kinetoplast poly (A) polymerase 1(KPAP1) enzyme (80). Pre-edited and partially edited mRNA only contains a shorter A-tail, whereas fully edited and never edited mRNA can possess both short and long A-tail, suggesting a correlation between extent of editing with length of the A-tail. Short A-tails are crucial for the stability of never-edited and fully edited mRNA transcripts unlike pre-edited mRNAs. In addition, arbitrary heteropolymers of A/U are added to the short A tails of mRNAs after the editing process, which requires a terminal uridylyl transferase RET1 (RNA editing TUTase 1) (81).

Interesting features of gRNA include a 5'- end triphosphate and a 3' oligo (U) tail (67, 82). The presence of 5'-triphosphate suggests that gRNAs are not processed at the 5'-end but are present as primary transcripts in mitochondria. However, 3' post-transcriptional gRNA processing was recently described to involve a complex of proteins termed mitochondrial 3' processome (MPsome) that includes RET1, DSS1 (a

RNase-II like 3'-5'exonuclease) and three mitochondrial processome subunits (MPSS1, MPSS2 and MPSS3) (79). This complex is proposed in 3' processing of gRNAs acting through uridylation of gRNA precursors by RET1, 3'-5' processive degradation by DSS1 generating 40-60nt long mature gRNAs, and a subsequent second event of uridylation. In addition to transcribing sense gRNAs, minicircles also transcribe anti-sense gRNA precursors (79, 81). The 3'-5' exonucleolytic activity of DSS1 appears to be obstructed by hybridization of these sense and anti-sense gRNA precursors that leads to pausing of the MPsome. This process appears to determine the size of the mature gRNA (79). Additionally, a mitochondrial RNA precursor processing endonuclease 1 (mRPN1) was proposed to participate in biogenesis of the gRNA in PCF trypanosome, while a mitochondrial exoribonuclease RNase D (TbRND) is involves in the gRNA metabolism (83–85). However, mRPN1 is not required in BSF trypanosomes and further research is required to fully understand the role of this factor (86).

Most mitochondrial mRNA transcripts (16 out of 18) are transcribed in a precursor form (pre-mRNA) that requires the unique form of RNA editing in kinetoplastids. The edited mRNAs are the mature protein coding form and, gRNAs contain the information that directs specific editing of the pre-mRNAs.

RNA editing

The term "RNA editing" was first described by Rob Benne and co-workers in year 1986, when they first demonstrated the post-transcriptional insertion of four uridines into the pre-mRNA encoding the cytochrome c oxidase subunit 2 (COII) mRNA in *Crithidia* (87). Later in 1988, another report by Stuart and co-workers showed

extensive modification of cytochrome oxidase subunit III (COIII) in *T. brucei* through massive insertion and deletion of uridines (88). These discoveries in kinetoplastids encouraged the researchers to focus on apparent inconsistency between DNA and RNA sequences in other biological systems (89). This led to the discovery of diverse and unrelated editing mechanisms that includes insertion and deletion of nucleotides other than uridines, substitution of A-to-I or C-to-U through base deamination, transfer RNA (tRNA) editing and others (90). Thus, usage of term "RNA editing" is not just confined to explaining the phenomenon of insertion and deletions of uridines in kinetoplastids but has extended to describe a post-transcriptional change in the sequence of the RNA transcript that differs from the sequence of the DNA template.

Types of RNA editing

The different types of editing are summarized in table 2.

Table 2 Types of RNA editing

Types	Organism	Substrate	Mechanism
tRNA editing	Plants, mammals and Archaea	tRNA	Insertion/deletion, and or deamination
C-to-U	Higher plants, Mammals	mRNA	Deamination
U-to-C	Lower plants, Higher plants (rarely)	mRNA	Not known
A-to-I	Mammals	mRNA	Deamination
Insertion/deletion	kinetoplastids	mRNA	Endonuclease, and or TUTase/exonuclease cleavage and ligation

tRNA editing

tRNA editing is found in lower (*Acanthamoeba, Physarum, Spicellomyces, Trypanosomes*) and higher level eukaryotes like plants (Arabidopsis) and animal (Metazoa) (91–93). Furthermore, tRNA editing was assumed to be limited to eukaryotes solely but this process was recently reported in Archaea (94). Before tRNA is involved in translation, it goes through post-transcriptional modifications that generate the functionally active mature tRNA. In addition to insertion/deletion of extra sequence at 5' and 3' ends, it also involves a number of specific base modifications. More than a hundred distinct base modifications in tRNA have been reported so far and functional relevance of most of these modifications still remains unknown (95). However, few of the reported cases suggest that modifications are crucial in generating secondary and tertiary structural elements for a fully functional tRNA (96–98).

C-to-U editing

C-to-U RNA editing has been studied extensively in the mRNA encoding the human apolipoprotein B (APOB100), which is responsible for the elimination of low-density lipoproteins. The post-transcriptional C-to-U editing event in APOB100 mRNA generates an in-frame stop codon. The newly edited sequence encodes for a truncated altered protein ApoB48 that plays completely distinct role than the unedited mRNA (99, 100). Another case of C-to-U editing in mammals involves alteration in the sequence of neurofibromatosis type 1 (NF1) mRNA through deamination. This process also creates a stop codon in the open reading frame (ORF) of NF1, encoding a shorter protein neurofibromin; causing a loss of a crucial domain for GTPase activation. However, there

are no reports showing that this truncated version of protein is ever translated (101). Another target for C-to-U editing is NAT1, this protein is homologous to eIF4G which acts as a repressor during translation process and undergoes C-to-U editing events resulting in stop codons that lowers the protein abundance (102). In addition, the APOBEC family of cytidine deaminases play an important role in innate immune response against virus including HTLV-1 and HIV-1 by altering the genetic information through hypermutations (103). One of the examples is the APOBEC3A protein that is able inhibit viral infections upon deamination in the viral DNA genome or its RNA transcripts (104).

In plants, C-to-U RNA editing event was first observed in mitochondria in 1989 and then in chloroplast (105–108). RNA editing seems to be more extensive in mitochondria with 300-500 editing sites, than in chloroplast transcripts targeting only 30-50 editing sites. Editing occurs most commonly in the coding region of mRNA transcript, targeting first or second codon that changes the nature of the encoded amino acid (109–112).

U-to-C editing

U-to-C RNA editing was first described in the Wilms Tumor-1 (WT-1) transcript in 1994 (113). U-to-C editing has been observed frequently in the early diverging plants, like ferns, mosses, and Lycopodiaceae but rarely in advanced land plants. U-to-C editing at nearly 1000 sites have been reported in the chloroplast of hornwort *Anthoceros formosae* (109, 114, 115). The mechanism for U-to-C is still not fully

understood yet but the presence of a transaminase enzyme has been proposed for this type of RNA editing event in mitochondria of plants (112).

A-to-I editing

A-to-I editing is the most prevalent form of RNA editing in mammals. The adenosine deaminase acting on RNA (ADARs) family of enzymes plays a crucial role in this type of RNA editing events (116). The ADARs selectively interacts with the double stranded region of the RNA, resulting in conversion of adenosines to inosine through deamination process (117). A well-studied case for A-to-I editing occurs in neural serotonin receptor HTR2C gene transcript at five editing sites that results in 28 mRNA transcripts 20 different protein isoforms (118–120). A-to-I editing can change the encoded information in mRNA due to coding of inosine as guanosine by the translation machinery which may alter the coding capacity (121).

U-insertion/deletion editing

U-insertion/ deletion editing was the very first example for RNA editing in mitochondrial mRNA of *T. brucei* (87). This parasite's complex life cycle in general and kinetoplastid RNA editing in particular have been topics of great interest in Biology. However, critical questions regarding the basic mechanism of operation and control remains poorly understood.

Maxicircle comprises of 18 protein-coding genes that encode traditional mitochondrial genes including ribosomal RNAs (rRNAs), as well as subunits of respiratory complexes. Of the 18 protein-coding genes, twelve of them undergo dramatic

remodeling by specific uridine (U) insertion/deletion post-transcriptional RNA editing in order to correct encoded frameshifts in the open reading frames (ORF) thereby rendering the mRNAs translatable (122, 123). However, the amount of editing can differ significantly, such as ND7 mRNA with the most extensive editing (553/89 U's inserted/deleted) and COII with the least amount of editing (4/0 U's inserted/deleted) (Table 3) (124). Extensive editing through most of the mRNA transcript substrate is also termed pan editing. This process is essential for viability in both the PCFs and BSFs of *T. brucei* (123, 125, 126).

Table 3 Extent of RNA editing in *T. brucei*.

Mitochondrial Transcript	Respiratory Complex/function	Number of U-insertion/deletion	Length of edited mRNA (nt)
ND1	Complex I	Never edited	-
ND3	Complex I	210/13	452
ND4	Complex I	Never edited	-
ND5	Complex I	Never edited	-
ND7	Complex I	553/89	1238
ND8	Complex I	259/46	574
ND9	Complex I	345/20	649
Cyb	Complex III	34/none	1151
COI	Complex IV	Never edited	-
COII	Complex IV	4/none	663
COIII	Complex IV	547/41	969
A6	Complex V	447/28	811
S12	Ribosomal protein S12	132/28	325
MURF1	Unknown Function	Never edited	-
MURF2	Unknown Function	26/4	1111
MURF5	Unknown Function	Never edited	-
CR3	Unknown Function	148/13	299
CR4	Unknown Function	325/40	567
9S rRNA	SSU ribosomal RNA	3'-oligo uridylation	-
12S rRNA	LSU ribosomal RNA	3'-oligo uridylation	-

Abbreviations: ND, NADH dehydrogenase subunits; Cyb, cytochrome *b*; CO, cytochrome *c* oxidase subunits; A6, ATP synthase subunit 6; S12, small subunit ribosomal protein 12; MURF, maxicircle unidentified reading frame; CR, C-rich genes. (Adapted from (124)).

Evolution of RNA editing

RNA editing was speculated to be a relic of primitive RNA world (127). Similar U-insertion/insertion RNA editing was observed in *Trypanoplasma borreli*, a parasitic bonodid that was thought to have diverged –from trypanosomatids 700 million years ago supporting the primitiveness of the RNA editing process (122, 128, 129). Diplonemids that are known to use trans-splicing, are also observed to utilize RNA editing process to alter their mitochondrial RNAs (122).

Although, the evolutionary origin of RNA editing still remains enigmatic, different scenarios have been proposed based on the complex life cycle of kinetoplastids and the extensive selection pressure that these organisms experience. One hypothesis is that the RNA editing process emerged as a counter measure to correct mutations in the mitochondrial genome and thus ensure its gene expression (130). When the parasite is living anaerobically, it may not utilize its kDNA. This may cause deleterious mutations in the kDNA that may spread through genetic drift and eventually may result in loss of genes (131). In other words, editing process may have surfaced to counter the loss of genes that are either not expressed or not crucial in the BSFs.

Another explanation to the evolution of RNA editing is protein diversification. Hundreds of gRNAs are transcribed in *T. brucei* including some that may create alternative edited mRNAs and potentially a large repertoire of proteins than originally envisioned (69, 132–135). One well known example is an alternatively edited protein 1(AEP-1) that is a variant derivative of COIII gene (136, 137). In other words, RNA

editing may contribute by extending the coding capacity of the mitochondrial genome through translation of alternative proteins from a single gene (54, 90, 138).

Mechanism of U-insertion/ deletion RNA editing

The RNA editing process is directed by small *trans*-acting guide RNAs (gRNAs) that are primarily encoded in the minicircles of the mitochondrial genome. The enzymatic activities required for RNA editing process, resides within RNA editing core complex (RECC), and the editing progresses through an "enzyme cascade" described in Figure 5 (123, 139, 140). Editing of the mRNAs proceeds in small blocks from the 3' to 5' direction, each cognate gRNA targets a specific pre-mRNA and forms an anchor duplex immediately downstream to the edited site. gRNA acts as a quasi-template to guide the editing process by catalytic subunits of the RECC enzyme. Each cycle of editing includes 3 basic events. First is endonuclease cleavage of the mRNA at the editing location directed by the gRNA. Second, a specific number of U's are inserted or deleted by a terminal uridylyltransferase (TUTase) or exonuclease activities in the RECC enzyme, respectively. In the final step, the mRNA cleaved and U-remodeled halves are re-united by RNA ligation. A few mRNAs are never edited or are only minimally edited; however, the majority of mRNAs undergoes extensive editing, and requires dozens of gRNA to act sequentially in order to produce translatable mRNAs (122, 123, 125).

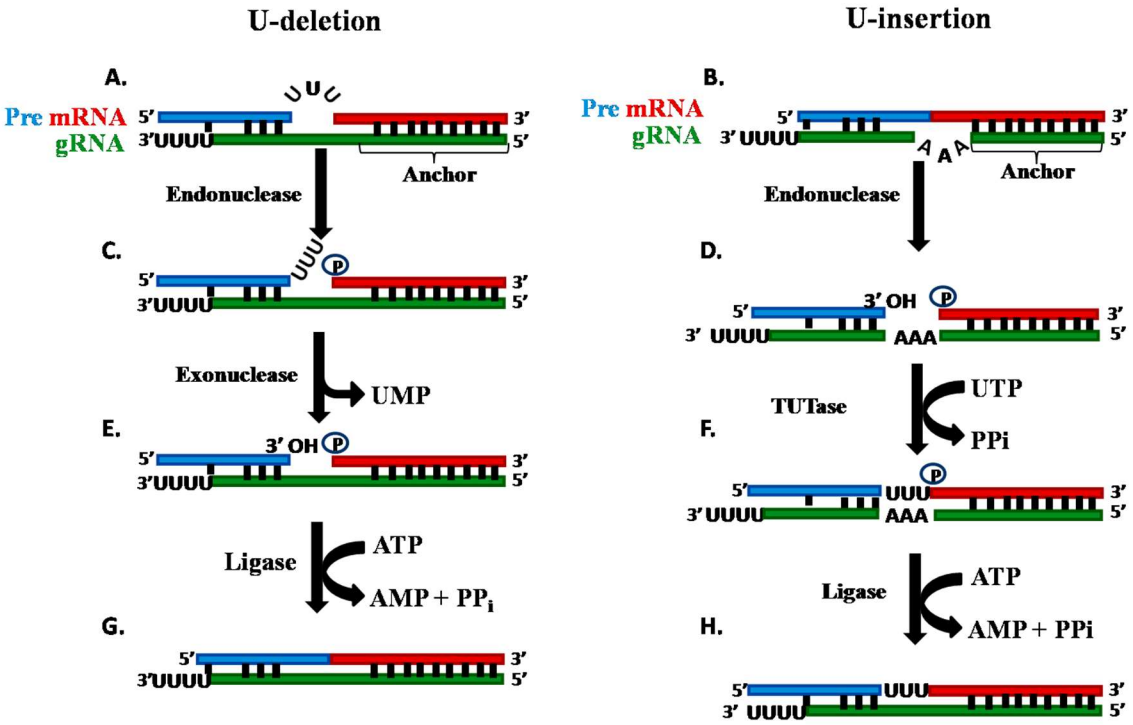

Figure 5. Mechanism of U-insertion/deletion RNA editing. (A, B) 1st step includes gRNA anneals to the mRNA through sequence complementary to 3' region of mRNA know as "anchor". (C, D) 2nd step involves the recognition of the first mismatch in the duplex by an endonuclease that cleaves the mRNA in the middle. gRNA consists of 3' poly-U tail that plays a role in stabilizing the duplex formation between mRNA and gRNA. After the mRNA has been excised this poly-U tail helps in holding 2 halves of the mRNA together, which would be lost otherwise after endonuclease cleavage. (E, F) 3rd step involves the deletion or insertion of uridines by exonuclease or terminal uridyltransferase (TUTase). (G, H) 4th and the last step involve a role of RNA ligase that joins the 5'-half and 3'-half of the mRNA. (Adapted from ref. (141)).

RNA editing core complex (RECC)

Catalytic protein subunits in the RECC enzyme are responsible for the three basic steps of editing. The composition and activity of multi-protein RECC enzyme, which is also known as the 20S editosome, has been extensively studied by several labs (Table 4) (142–152). In fact, some observations indicate that there may be three variant forms of RECC: one deletion subcomplex and two insertion subcomplexes with one being specific for COII. These three forms of RECC have twelve proteins in common that includes two RNA ligases (KREL1 and KREL2), TUTase (KRET2), U-specific exoribonuclease (KREX2), six interaction proteins (KREPA 1-6) and two proteins with RNase III domains (KREPB4 and KREPB5) (140). Specific protein components distinguish the three RECC variants. Each RECC variant has one of the three RNAse III-type endonuclease homologs, termed KREN1, KREN2 or KREN3. Each KREN nuclease is also accompanied by either KREPB8, KREPB7 or KREPB6 respectively (145, 153).

Table 4. RECC components of *T. brucei*.

Alternative names from proteins	Gene ID	Proposed function
REL1/KREL1	Tb927.9.4360	RNA ligase
REL2/KREL2	Tb927.1.3030	RNA ligase
RET2/KRET2	Tb927.7.1550	TUTase
REX1/KREX1	Tb927.7.1070	U-specific exonuclease
REX2/KREX2	Tb927.10.3570	U-specific exonuclease
MP81/KREPA1	Tb927.2.2470	Structural, U-insertion subdomain organizer
MP63/KREPA2	Tb927.10.8210	Structural, U-deletion subdomain organizer
MP42/KREPA3	Tb927.8.620	Structural
MP24/KREPA4	Tb927.10.5110	Structural, RNA binding
MP19/KREPA5	Tb927.8.680	Structural
MP18/KREPA6	Tb927.10.5120	Structural, RNA binding
REN1/KREN1	Tb927.1.1690	Insertion site specific endonuclease
REN2/KREN2	Tb927.10.5440	Deletion site specific endonuclease
REN3/KREN3	Tb927.10.5320	Cis-editing site specific endonuclease
MP46/KREPB4	Tb927.11.2990	Structural, heterodimer with endonuclease
MP44/KREPB5	Tb927.11.940	Structural, endonuclease
MP49/KREPB6	Tb927.3.3990	Structural, part of KREN3 module
MP47/KREPB7	Tb927.9.5630	Structural, part of KREN2 module
MP41/KREPB8	Tb927.9.5630	Structural, part of KREN1 module

Based on current literature, two different nomenclatures are used for RECC components (123, 139, 141). (Gene ID was acquired from http://tritrypdb.org/tritrypdb/).

Core interactive components of RECC

The subunits of RECC called kinetoplastid RNA editing proteins (KREP) are cataloged in subgroups. The "A"-subgroup (KREPA) included structural proteins that are essential for the stability of the RECC enzyme. Disruption of these proteins results in the loss of editing function and stability of RECC. This includes a specific decrease in endonuclease cleavage, subsequent editing steps and reduced sedimentation of the complex (154–160). KREPA1-3 consists of two zinc finger (ZnF) motifs in the N-terminus, used for RNA binding; and all the proteins of this family contain oligonucleotide/oligosaccharide binding domain (OB-fold) in the C-terminus responsible for protein-protein interactions. Mutational analyses reports of these domains in KREPA2 (ZnF) and KREPA3 (ZnF and OB-fold) suggests that these domain are crucial for the maintaining the integrity of RECC and also, for a fully functional editosome (160, 161). In recent studies, a more comprehensive structure of editing enzyme has been reported that elucidates to the protein interactions within RECC (Figure 6) (140, 162).

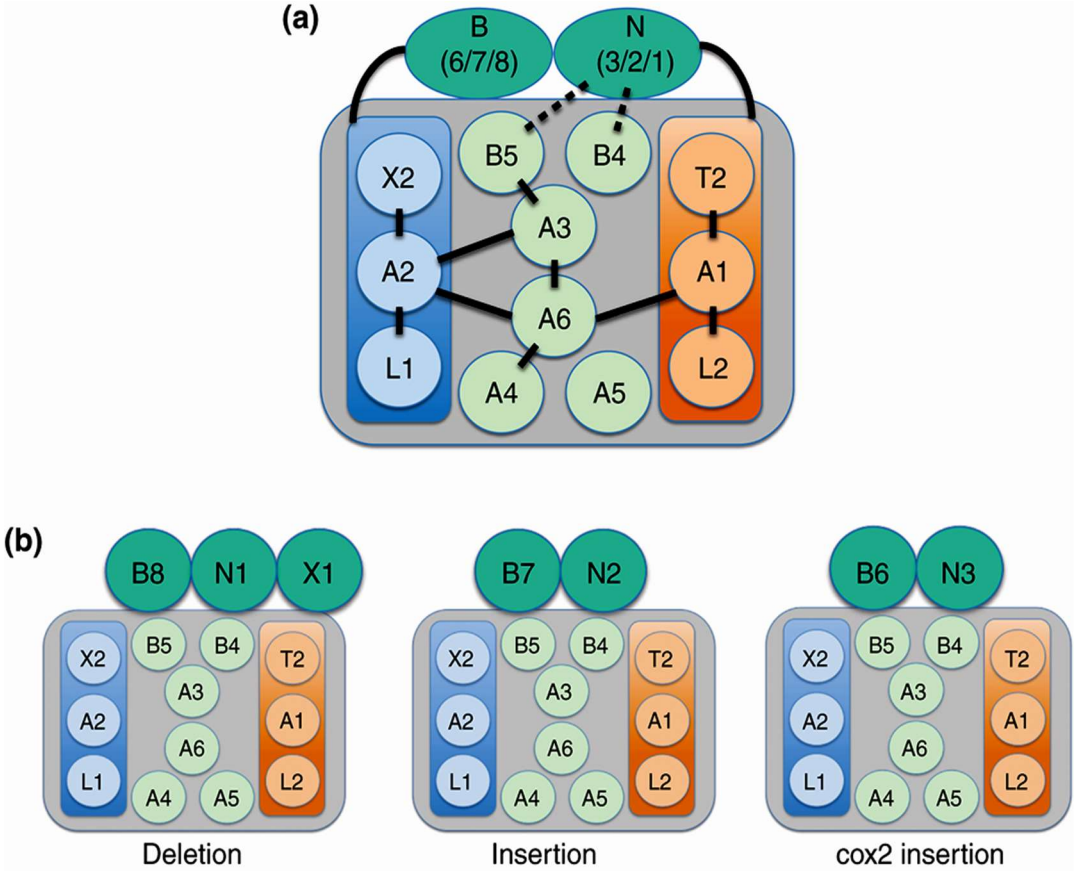

Figure 6. Interactions of protein components within RECC. (a) Out of 12 common RECC proteins, three (X2, A2, and L1) are specific to deletion subcomplex and three (T2, A1, and L2) are associated with insertion subcomplex. A3, A6, A4, and A5 are structural proteins. B4 and B5 interacts with endonucleases N1, N2, and N3 and their respective partners B8, B7, and B6. (b) RECC is classified in three different subcomplexes; one deletion subcomplex that includes N1/X1/B8, and two insertion subcomplexes that includes N2/B7 or N3/B6. N3/B6 containing insertion subcomplex is specific for cox2 mRNA. Solid line shows strong interactions and dotted line shows weak interactions. Abbreviations: L, RNA ligases (KRELs); T2, TUTase (KRET2); X, U-specific exonucleases (KREXs); A, kinetoplastid RNA editing protein A (KREPA); N, kinetoplastid RNA editing endonucleases (KRENs); B, kinetoplastid RNA editing protein B (KREPB) (Used with permission from (140)).

Endonucleases and their accessory proteins

Kinetoplastid RNA editing endonucleases (KRENs) are the catalytic subunits of RECC that catalyze the first step of RNA editing by cleavage of the mRNA. KRENs interact with their respective kinetoplastid RNA editing B proteins (KREPBs) to create three editosome variants. Each editosome is responsible for distinct RNA editing event: KREN1/KREPB8/KREX1 for U-deletion, KREN2/KREPB7 for U-insertion and KREN3/KREPB6 for cox2 specific insertion (Figure 6). All KREN proteins contain, a U1-like zinc finger domain (ZnF) in the N-terminus, RNase III domain in the middle, followed by double-stranded RNA binding domain (dsRBD) in their N-terminus (123, 163). In knockdown studies of KREN1 and KREN3 (former names KREPB1 and KREPB2 respectively), reduction in deletion editing was observed but in KREN3 case, this repression of deletion editing was only confined to cox2 transcripts that suggests a specific role of KREN3 in cox2 editing (145, 147). On the other hand, RNAi of KREN2 (former name KREPB3) showed reduced growth and inhibition of insertion editing in *T. brucei* (146).

KREPB proteins are considered to be accessory proteins since these proteins interact with different KRENs and provide distinct functional properties to the RECC (164). Presence of a U1-like zinc-finger domain in the N-terminus of all KREPB family members, suggests a role in RNA/protein binding (123, 165). Although, like KREN proteins, KREPB4 and KREPB5 also contains RNase III domain but this domain lacks enzymatic activity. Both these candidates are known for the maintaining the integrity of the RECC and are two of the twelve common proteins (Figure 6).

ExoUases

During deletion editing, uridyl-specific exonuclease catalyzes the excision of mismatched uridines from the pre-mRNA that do not base pair with cognate gRNA. Two known exonucleases, KREX1 and KREX2 are known to have 3'-5' ExoUase activities (166–168). RNAi of both these proteins shows significant reduction in U-deletion during editing event. However, other than suppression of U-deletion, they have different effects upon knockdown. KREX1 ablation results in growth phenotype and significant reduction in endogenous levels of KREN1, which suggests that KREN1 is unstable outside the core editosome in the absence of KREX1. Conversely, KREX2 knockdown shows no growth phenotype in either of the life stage of *T. brucei* but causes change in the editosome sedimentation and slight reduction in insertion/deletion editing (166–169).

Terminal uridyl-transferase (TUTase)

Two RNA editing TUTase 1 and 2 (RET1 and RET2) are known in the editosome of *T. brucei* that are involved in RNA editing. RNA editing TUTase 2 (RET2) is reported to catalyze the addition of U's to the 5' cleavage fragment of the mRNA transcript subsequent to the endonuclease cleavage step in U-insertion RNA editing event (148, 170). RET1 is known to play a role in 3'-end polyuridylation of gRNA transcripts and acts as stability factor. Knockdown studies of RET2 revealed a loss of insertion editing without altering the deletion editing and significant growth reduction in *T. brucei*. In addition, RNAi of RET2 resulted in reduction of RNA editing ligase 2 (REL2) and KREPA1 (or MP81) levels in the cells (170). Upon knockdown of RET1, gRNA species with shorter U-tails was observed but showed only a modest reduction of

in vitro U-insertion editing, opposed to complete inhibition on ablation of RET2. This suggests indirect effects of RET1 on insertion editing perhaps because RET1 controls the poly-U tail of the gRNA which directs the editing process (170–172). Both RET1 and RET2 contain a poly-(A) polymerase and a nucleotidyl transferase domain but they both have distinct functional properties (123, 170).

RNA editing ligases

The last enzymatic step in RNA editing process involves the ligation of 3' and 5' cleavage fragments of the mRNA by RNA editing ligases (RELs) generating the mature translatable mRNA (Figure 5). REL1 and REL2 are the two ligases known for this function in the editosomes of *T. brucei*. These two proteins share the same structural architecture, which includes the signature ligase motif and unique C-terminal domains that are responsible for their catalytic activity and protein-protein interactions respectively (123, 125, 162, 173, 174). In addition, RELs in *T. brucei* are close relatives to the nucleotidyl transferases superfamily. Based on structural similarity, RELs are most closely related to T4 RNA ligase (Rnl2) with highest sequence conservation in nucleotidyl transferase motif I, III, IIIa, IV and V. Based on mutational analyses, motif IV and V are crucial for the ligase activity (175, 176). Compared to other ligases, the lack of an oligonucleotide/oligosaccharide binding domain (OB-fold) makes REL1 and REL2 unique. However, it is proposed that OB-fold is offered *in trans* to RELs by the other interactive proteins in the editosomes (163, 173, 177).

REL1 is essential for cell viability; on the contrary REL2 is not, suggesting that REL1 can compensate for the function of REL2. Based on their distinct RNA specificity, RELs are associated with different ligation activities. REL1 has been shown to play role in U-deletion and REL2 in U-insertion editing events (152, 166, 174, 178–181). In addition, RELs have different affinities for ATP and phosphate, with REL1 having a higher affinity for ATP in comparison to REL2 (151, 152, 182).

Accessory complexes in RNA editing of *T. brucei*

The RNA editing core complex (RECC) or 20S editosome, has been extensively studied (183–185), but many central questions remain unanswered. For example, how are the substrates recruited, and how are editing initiation and progression controlled? This is particularly puzzling because the purified RECC enzyme lacks the editing processivity found *in vivo* (186–188) and early studies showed that it does not contain endogenous gRNA or mRNA (142). A substantial number of non-RECC proteins that control editing have been reported.

Several reports have suggested the presence of gRNA binding proteins in the mitochondria of Trypanosomatids (189–193). Two proteins among them are, MRP1 and MRP2 (mitochondrial RNA binding protein 1 and 2) formerly named as gBP21 and gBP25 (gRNA binding protein 21 and 25) respectively (194). MRP1, an arginine rich protein, was first observed in *T. brucei* via gRNA crosslinking studies (195), followed by the discovery of MRP2. MRP1 and MRP2 proteins form a MRP1/2 complex and are shown to facilitate RNA annealing activity *in vitro* and may promote gRNA-mRNA duplex formation (196–198). RNAi studies shows that MRPs are essential for the PCFs

viability. Also, upon knockdown of MRPs, relative levels of several mitochondrial edited and never edited transcripts were affected but not all, which is confusing because purified editosomes perform full round of U-insertion/deletion editing even in the absence of MRPs. However, it is likely that these proteins only effect the editing of specific mRNAs possibly by effecting gRNA utilization (199).

RBP16 is another accessory protein with similar functional properties as MRPs, such as, gRNA binding and gRNA-mRNA annealing activity (200, 201). RBP16 protein contains a cold shock domain in its N-terminus and its C-terminus is comprised of an RG-rich domain. Methylation of arginines in the RG-rich domain is shown to perturb the RNA binding function of RBP16 and conversely increasing its macromolecular interactions (202, 203). Upon knockdown RBP16 is shown to be crucial for the viability and editing of selective Cyb mRNA. In addition, reduced steady-state levels of never-edited COI and ND4 mRNAs is observed, suggesting its role in RNA stability as well (204).

Another accessory protein known as RNA editing associated protein 1(REAP1), is reported to associate with REL and TUTase in a large ribonucleoprotein (RNP) complex (205). This protein is shown to be involved in the recognition and binding of preedited mRNA substrates and is capable of differentiating between preedited and never edited mRNAs (206). An additional proposed function of REAP1 is to deliver preedited mRNA substrates to the editing machinery (193). Null mutants of this protein were viable, however growth rate was reduced. In addition, several mitochondrial RNA levels in these mutants shows a significant increase suggesting that REAP1 may play a

role in RNA stability, presumably through increased RNA turnover (206). There are additional non-RECC proteins that stably bind a complex termed mitochondrial RNA binding complex 1(MRB1).

MRB1 aka GRBC complex

The mitochondrial RNA-binding complex 1 (MRB1) also known as gRNA binding complex (GRBC) is essential for RNA editing, MRB1 is a large and dynamic macromolecular complex with at least 30 proteins but the number of subunits are still not precisely defined. This complex has been studied by different labs including ours (207–209). As, it is further described below, studies by me and others in our lab recently discovered that instead of a single MRB1 complex, there are two gRNA-bound variants that we initially termed MRBs (1, 2). Before discussing our studies in detail, below is a description of prior studies of MRB1. The original purifications of MRB1 involved tagging of gRNA associated proteins 1 and 2 (GAP1 and GAP2 aka: GRBC1 and GRBC2) (210–212). MRB1 has an imprecisely defined composition because the initial and subsequent MRB1 purifications identified both common and distinct components (211, 213). Several direct interactions of MRB1 proteins were found in yeast two-hybrid studies (208). The yeast two-hybrid contacts and several prior studies together proposed a core of six proteins in MRB1. The proposed core proteins are GAP1/2, MRB8620, MRB5390, MRB11870 and MRB3010 (Also termed as GRBC1-6 respectively) (89, 208, 214, 215).

GAP1 and GAP2 proteins were first reported in *L. tarentolae* (196). Orthologs of these proteins were then reported in *T. brucei*. Those studies also showed that MRB1 proteins were associated via RNA-dependent contacts with an mRNA polyadenylation complex, which will be discussed later in this section (216). GAP1 and GAP 2 are known to form α2β2-type heterotetramer and are linked to direct binding of the gRNAs. RNAi studies of GAP1 and GAP2 reveal that both these proteins are essential for the viability of *T. brucei*, and directly impact RNA editing. Depletion of GAP1 and GAP2 had no effect on the integrity of RECC or on mRNA maturation. However, the GAP proteins bind gRNA directly and are essential for the general stability of the gRNA pool (212, 217).

MRB3010, one of the core proteins of MRB1 complex, is known to be crucial for the survival of both PCFs and BSFs (213). Upon MRB3010 ablation, a significant reduction in RNA editing is observed with extensively edited mRNAs being affected more than minimally edited transcripts. Unlike GAP1 and GAP2 proteins, MRB3010 has no effect on gRNA abundance but is observed to be involved in initiating steps of RNA editing process presumably through gRNA/mRNA utilization (213, 218). MRB11870, another core member of the MRB1 complex is known to have similar functional properties as MRB3010. Such as, its requirement for the viability of both forms of *T. brucei*, its impact on early steps of RNA editing process without perturbing the steady-state levels of mitochondrial gRNAs. However, unlike MRB3010, MRB11870 is essential for protein interactions within MRB1 complex (214). Knockdown studies of other core proteins including MRB5390 and MRB8620, shows modest reduction of

growth in the PCFs of *T. brucei*. Both these proteins show reduced RNA editing upon knockdown but not to the same extent as MRB3010. However, a recent report shows that MRB8620 is required for the integrity of MRB1 complex (218, 219). Recently, another protein MRB0880 (aka: GRBC7) was reported that copurifies with MRB5390. However, its function still remains to be determined (89, 208, 215).

Subcomplexes within MRB1 complex

As explained earlier, MRB1 complex is highly dynamic and consists of subcomplexes that associate with the core of MRB1 through RNA-protein or protein-protein interactions. These subcomplexes are the RNA editing mediator complex (REMC) and polyadenylation mediator complex (PAMC). GRBC together with REMC and PAMC are proposed to form a tripartite complex, also termed as RNA editing substrate binding complex (RESC) (215, 220).

RNA editing mediator complex (REMC), contains seven proteins (REMC1-6) including extensively studied protein RGG2 (215, 220). RGG2 is comprised of a glycine-rich RGG domain in its N-terminus and an RNA recognition motif (RRM) in its C-terminus. RGG2 is shown to be essential for the survival of both life stages in *T. brucei* and appears to have direct impact on RNA editing of pan-edited mRNAs (219, 221). In addition, RGG2 plays a role in initiation and 3’ to 5’ progression of the editing process (222). The remaining six proteins still remain to be functionally characterized. However, knockdown studies of these proteins clearly demonstrate that all the REMCs except for REMC4 are essential for the survival of the PCFs. In terms of RNA editing,

REMC1 is a crucial player in editing for all the mRNAs, REMC4 impacts selectively few mRNAs, whereas, REMC5 only affects the editing of single mRNA (ND7) (215).

Polyadenylation mediator complex (PAMC) consists of three proteins excluding the REMC1 protein (termed PAMC1 in this complex) that is a common protein in both REMC and PAMC complex, and is studied extensively as a PAMC component. Role of PAMC complex is linked to managing the completion of editing process and 3' A/U tail addition. The reason for in depth study of PAMC1 is that it co-purifies with proteins that are functionally associated with polyadenylation/uridylation of fully edited mRNAs (215, 220). These proteins include KPAP1, RET1, KPAF1 and KPAF2 and are discussed in detail later in this chapter.

Similar to other biochemical process in biology that involve ribunucleoprotein (RNP) complexes it is possible that kinetoplastid RNA editing process is extremely dynamic. It is likely that the proteins and RNA molecules that are involved in this process, go through structural changes during the editing machinery's recruitment and departure (223). Conformational changes of the mRNA, the gRNA or their hybrids may be needed to make the RNA substrate(s) accessible to the RECC enzyme. These dynamic steps may involve auxiliary protein factors that are not part of RECC enzyme. This thesis focuses on a DExH-box RNA Editing helicase 2 (REH2) which is an auxiliary factor in editing and may promote essential changes in RNA conformation and in assembly of the RNP subcomplexes of the two MRB studied in our lab.

DExH/D family of helicases

There are six super families of helicases. Of these, superfamily 2 (SF2) seems to include most eukaryotic RNA helicases (224). SF2 proteins include the well-studied DEAD-box and DEAH-box protein families. These designations refer to conserved amino acid in motif II: D (Asp), E (Glu), A (Ala), D (Asp) in DEAD-box proteins and D (Asp), E (Glu), A (Ala), H (His) in DEAH-box proteins (225). A subgroup of DEAH-box proteins that include the kinetoplastid REH2 helicase is known as DEAH/RHA subfamily, which is described in more detail below. RHA subfamily helicases are involved in a number of essential processes that require RNA unwinding or change in RNP assembly. These processes include: transcription, translation, nuclear transport, RNA turnover and ribosome biogenesis (226, 227).

The catalytic core of these helicases is formed by two RecA-like domains that include several conserved motifs in DEAD-box helicases (Q, I, Ia, Ib, and II–VI) and in DEAH-box helicases (I, Ia, Ib, II, III, IV, V and VI) (Figure 7) (228–232). Motif II is reported to be responsible for the binding and hydrolysis of ATP and other motifs play a role in RNA binding. These DExH/D helicases are found in all organisms, including trypanosomatids.

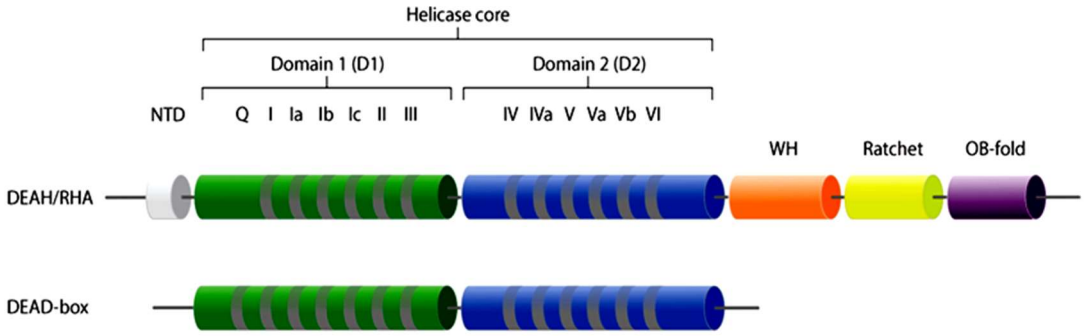

Figure 7. Structural architecture of domains in DEAH/RHA and DEAD-box helicases. DEAH/RHA and DEAD box family domain arrangement shown based on Prp43 and Mss116 respectively. Conserved motifs of the helicase core are shown in dark gray. NTD and CTD (WH, Ratchet and OB-fold) are missing in DEAD- box family. Abbreviations: NTD (N-terminal domain), WH (Winged helix), OB-fold (oligonucleotide/oligosaccharide-binding fold). (Used with permission from (233)).

Biological functions of RNA helicases

In transcription

Well studied RNA helicases in transcription are DEAD-box proteins p68 and p72 that are responsible for recruiting transcriptional regulators like tumor suppressor p53 to RNA polymerase II in humans (234). Dbp2, a homolog of these proteins in *S. cerevisiae,* is reported to remove cryptic unstable transcripts from chromatin through cotrancriptional RNA remodeling and facilitates assembly of the RNA decay factors (235). Additionally, Dbp2 has also been reported to promote binding of the export factors on the native transcripts inside the nucleus and is crucial for the RNP complex formation (236).

In nuclear export

Several RNA helicases are reported to be indispensable for RNP remodeling events during the nucleus export of mRNA. One of the examples include Dbp5 that is present on the cytoplasmic side of nuclear pore complex during mRNA export and is responsible for dislodging the export factors from the mRNA. There are several regulatory factors involved in this process, which regulates the ATP hydrolysis of Dbp5; these include Gle1 and IP6 that function as activator and coactivator respectively. After ATP hydrolysis, Dbp5 is recycled back into the nucleus with the assistance of the Nup159 protein (237–239). In addition, DDX3, DDX1 and RNA helicase A (RHA) in humans are reported to be hijacked by viruses to export viral RNAs (240).

In translation

Due to the complexity of the eukaryotic translation, several helicases are required for this process. During initial steps of translation, preinitiation complex (PIC) is recruited through eIF4F complex followed by scanning through 5' UTR of mRNA for start codon. While scanning the 5' UTR of the mRNA, PIC faces the structural obstacles that need to be cleared for PIC to continue the scanning process. eIF4A, Ded1, and DHX29 are proposed to play role in this process by melting the mRNA structures (241). DHX29, a DEAH/RHA helicase also plays similar role and additionally, is required for efficient and overall translation (242, 243). In addition, another DEAD/RHA RNA helicase A (RHA) is known to play role in the translation through selectively recognition of the 5'-terminal posttranscriptional elements and even translation of complex mRNAs (244, 245).

In ribosomal biogenesis

In *E.coli*, four DEAD-box helicases; DbpA, SrmB, CsdA, and RhlE, are reported to play a role in ribosome biogenesis. DbpA is shown to remodel the RNA structure of 23S rRNA through ATP hydrolysis, which appears to be facilitated by a small hairpin structure within 23S rRNA called hairpin 92 (hp92) (234, 246–248). SrmB is also proposed to play a role in 23S rRNA remodeling and prevention of 5S rRNA annealing to 23S rRNA (249) . On the other hand, CsdA and Rh1E depletion is linked with accumulation of 50S precursors (250). In eukaryotes, RNA helicases are involved in remodeling the small nucleolar RNAs (snoRNAs) that are responsible for guiding the site-specific cleavage, methylation, pseudouridylation and proper folding of pre-rRNA (251). Out of 75 snoRNAs in yeast, U3, U14 and snR30 are essential for guiding cleavage events. Dismissal of these snoRNAs from pre-rRNA requires three RNA helicases; Has1 dissociates U3 and U14. Conversely, dissociation of snR30 requires Rok1 and Dbp4 assists Has1 in U14 dissociation (251–254).

In remodeling the RNP complexes

Studies showing the role of RNA helicases in unwinding duplexes or melting the RNA structure are profound; however, their role in remodeling assembled protein surfaced more than a decade ago. For example, a viral helicase NPH-II is capable of stripping proteins off RNAs through ATP-hydrolysis, perhaps gliding through the RNA strand without disrupting the duplex (255–257). Another helicase in yeast called Ded1, is known to dislodge proteins like U1A from the RNA before performing the unwinding of the duplex that has a U1A binding site (256, 258).

In RNA editing

Even though the editing core machinery that alters the sequence of the RNA through U-insertion/deletion is extensively studied, there are only few a examples of RNA helicases studied in the mitochondria of *T. brucei*. RNA editing helicase 1 (REH1 aka Hel61), was the first helicase characterized in kinetoplastid mitochondria; Yet, REH1 is not a part of MRB1. RNA editing progression requires overlapping gRNAs. After one of mRNA is edited, the responsible gRNA must partially dissociate to allow the annealing of the incoming gRNA for editing of the next block. This process continues with all needed consecutive gRNAs. REH1 has been proposed to be involved in the succession of editing from one editing block to the next by either melting the secondary/tertiary structure of mRNA or displacement of the first gRNA enabling the annealing of the second gRNA(217, 259).

RNA editing helicase 2 (REH2)

RNA editing helicase 2 (REH2), a 2167 amino acids long polypeptide (241kDa), is a component of GRBC or MRB1 complexes (208, 209). REH2 interacts with RECC and with both MRBs studied in our lab in a RNA-dependent manner (1–3). Figure 8 shows the structural architecture of REH2 that is typically associated with DEAH/RHA subfamily of helicases (224). The C-terminus of these proteins includes: tandem RecA-like domains (DExHc and HELICc) as well as HA2 and OB fold domains. A few members of RHA-type proteins also include at least one double-strand RNA binding domain (dsRBD) at its N-terminal. In the specific case of the helicase REH2 in *T. brucei*, genetic downregulation via RNAi targeting inhibited RNA editing and growth of

procyclic trypanosomes in culture (209). Our laboratory showed that REH2 is responsible for the unwinding activity in MRB1, and that its activity and its co-purification with gRNA require the normal function of the REH2 dsRBD and its catalytic core. Pull-down experiments using a TAP-tagged version of REH2 showed that association of this helicase with the RECC enzyme is RNA mediated. Conversely, REH2 was also detected in immunoprecipitation (IP) assays using antibodies against several RECC subunits (209).

A dsRBD domain is typically ~70 amino acids long (Figure 8A). The dsRBD in REH2 adopts the α1-β1- β2- β3- α2 fold (Figure 8B) which is the normal configuration of this domain in many protein, including in Rntp1, vaccinia virus E3 protein, ADAR2 and protein kinase PKR (260–264). Mutation of conserved lysine and alanine amino acids in α2 helices has been reported to severely affect the RNA binding by the dsRBD (260, 262). The DExH-box in REH2, which defines the SF2 RNA helicase family, consists of six motifs including the motif II signature amino acids DExH, where "X" is any residue. Four such conserved motifs (motif I, II, III and VI shown in Figure 8C) are evident in REH2. Conserved amino acids in motif I was shown to be essential for RNA unwinding and RNA binding activity of REH2 (209). As mentioned above, we reported that the C-terminus of REH2 includes an oligonucleotide/oligosaccharide binding (OB)-fold (Figure 8D) (3). The OB fold has been found to promote ATPase activity and RNA substrate binding by DEAH/RHA helicases (265). However, the function of this domain still needs to be investigated in REH2 helicase.

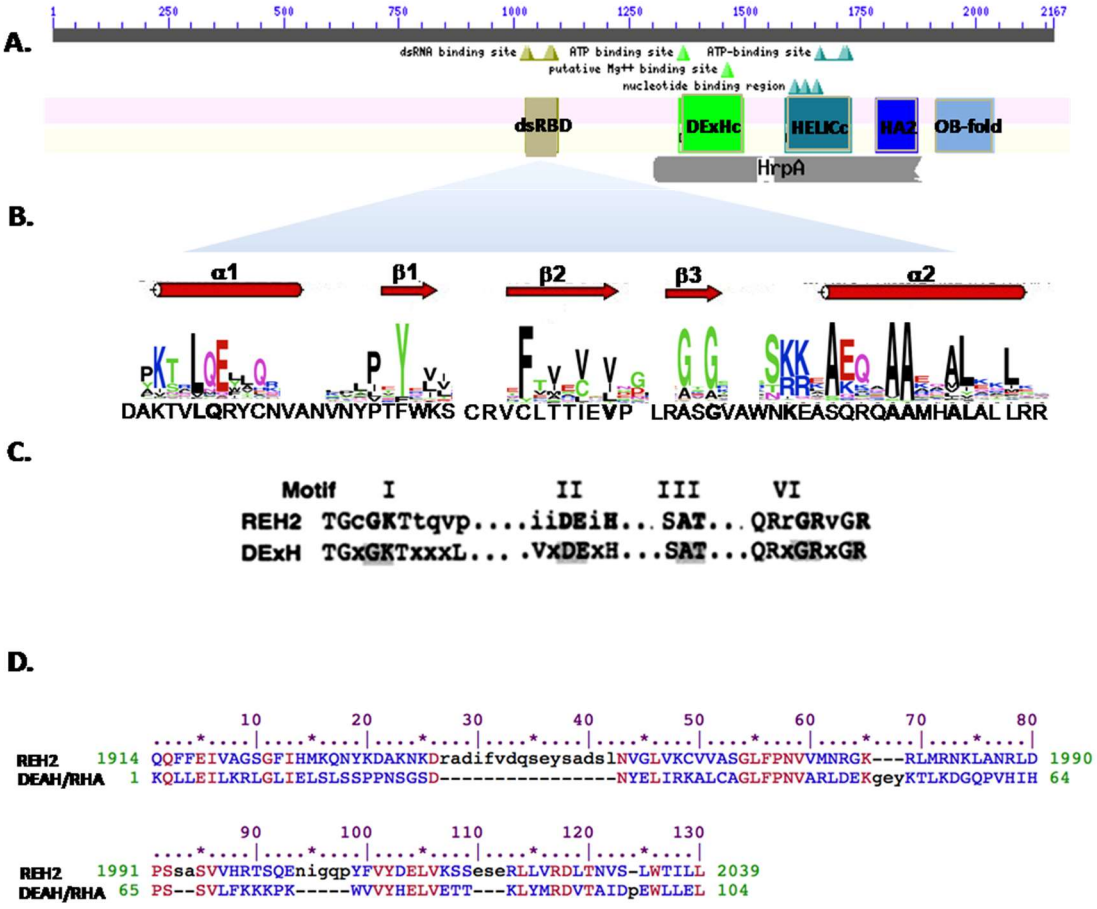

Figure 8. REH2 gene organization with highlighted dsRBD and helicase motif**.** (A) *T.brucei* REH2 has 2167 amino acids, including a conserved mitochondrial import signal (Mito), dsRBD, and domains typically associated with DExH-box helicases. (B) dsRBD, a double-stranded RNA-binding domain, most conserved residues are shown in *bold,* downward arrows shows the mutated residues**.** (C) DExH, which defines this protein family, where "X" is any residue. Four such motifs are evident in REH2 (I, II, III and VI) and shown with the most conserved residues in *gray*. (D) REH2 C-terminal resembles what is known to be an OB-fold domain, which usually associated with HA2 in DExH RNA helicases. This figure shows the conserved residues in this OB-fold (shown in *red*) at C-terminal of REH2.

OB-fold in helicases

OB-fold was first described in four distinct proteins of *E. coli*, to bind oligonucleotide or oligosaccharides (266). Later, it was discovered that the role of a OB-fold domain in a protein is more diverse in terms of mediating its interaction with DNA, RNA or other proteins (267–269). Structurally, the OB-fold consists of five anti-parallel β sheets forming a β barrel, which is capped at one end with an α helix. The OB-fold structure seems to maintain a binding face which adjusts according to the binding molecule. Distinct proteins with OB-fold utilize this binding face to bind RNA, DNA or proteins (267). The role of the OB-fold domain has been characterized in several proteins; however there are only a few examples that show its role in RNA helicases.

RNA helicase A (RHA), a DExH box protein, is known to play diverse role in the human cells that includes transcription, translation and association with protein complexes like nuclear pore complex and RNAi machinery. RHA is observed to consist of OB-fold domain in its C-terminus and is linked to transcriptional activation in HIV-1 infection and splicing of viral RNAs (270). Another example of a DEAH/RHA helicase is Prp43p, a well-studied helicase in *Saccharomyces cerevisiae*, and is known for its role in splicing and ribosomes biogenesis. Prp43p is also shown to have an OB-fold domain in its C-terminus that has been observed to be crucial for RNA binding and ATPase activity of this helicase (265). In addition, the OB-fold domain in Prp43p appears to act as a binding site for the RNA and regulatory proteins containing G-patch domains. Prp43p interacts with distinct G-patch proteins through its OB-fold domain depending on the process. It associates with Ntr1 and Ntr2 while functional in splicing and interacts

with Pfa1 when active in ribosomal biogenesis (271). Our laboratory recently discovered a similar G-rich protein in RNA-independent association with REH2 we termed ^{H2}F2. This protein does not appear to have an obvious G-patch domain but this protein may be a diverged member in the family of G-patch proteins that may play a role in regulation of REH2 helicase.

Associated proteins (cofactors) of RNA helicases

RNA helicases are usually found in association with other proteins in a biological environment. At the present time, research is more focused on discovering the associating proteins (co-factors) and understanding their impact on the RNA helicase function. Several cofactors have been reported that stimulates RNA helicase function through enhancing the unwinding or ATPase activity. Conversely, some co-factors are shown to inhibit helicase activity or ATPase hydrolysis. An example for a stimulatory cofactor is Dbp5p that is known to function in mRNA export and, is activated by Gle1p protein (238). This same protein Dbp5 can be inhibited by the cytoplasmic nucleoporin NUP214, which obstructs the binding site for the RNA in Dbp5p and consequently inhibiting its RNA binding and ATPase activity (272).

G-patch containing co-factors

G-patch domain is a conserved motif containing 45-50 amino acids with a consensus hhx(3)Gax(2)GxGhGx(4)G sequence; ‘h’ denotes a hydrophobic residue, ‘x’ denotes number of nonconserved amino acid and, ‘a’ denoted an aromatic residue (273). G-patch proteins are shown to engage in protein-protein as well as protein-nucleic acid

interaction. G-patch proteins are profoundly studied in context of their role in stimulating DEAH/RHA helicase. Six such G-patch proteins are reported that includes Spp2, Ntr1, Pfa1, Gno1, RBM5 and GPATCH2 (274–277). Spp2 is the first G-patch protein that was observed in regulation of Prp2 helicase in pre-mRNA splicing. Spp2 association with Prp2 is shown to be crucial for the activation of Prp2 function. Another example is TFIP11, a homolog for Ntr1 in humans, is shown to colocalize with human RNA helicase DHX15. Knockout of Prp43 is known to impair the function of DHX15 in spliceosome disassembly (271).

Zinc finger containing co-factors

The first zinc finger (ZnF) protein cofactor to be functionally studied was TFIIIA in *xenopus* oocytes (278). Subsequently, numerous proteins with ZnFs that differed in structure and function were identified. Classical Cys_2His_2 (C_2H_2) ZnF is the well characterized and most common among the eukaryotes transcription factors (279, 280). ZnF proteins are shown to be capable of binding DNA, RNA and proteins as well. Not much is known about ZnF proteins in terms of regulating DEAH/RHA helicase. However, one such example is MEP1, a ZnF motif containing protein that is known to regulate a DEAH box protein; MOG4, a homolog of Prp2 in *Caenorhabditis elegans*. MEP1 is shown to interact with MOG proteins including MOG4 and is further linked to the repression of fem-3 (sex determining gene) mRNA through its 3'UTR (281, 282). In our recent studies, we discovered a ZnF protein that associates with our REH2 in an RNA-independent manner and is previously shown to have a major impact on RNA editing. It will be intriguing to study its impact on REH2 regulation and function.

Maturation of RNA and translation in mitochondria

In mitochondrial RNAs, ribosomal binding sites are absent in their short 5' UTRs and they contain 3' poly(A) tail (283). 3' polyadenylation is performed by kinetoplast poly(A) polymerase 1 (KPAP1) that inserts 20-30 A's at the tail (80). In the case of fully edited transcripts, this A-tail is further extended by addition of long A/U heteropolymer via KPAP1 and RET1 terminal uridylyl transferase with assistance of kinetoplast polyadenylation/uridylation factors (KPAF1 and KPAF2) (126). The 3' short A-tail is shown to be essential for the stability of the partial edited and fully edited mRNA, which will otherwise decay (284, 285). On the other hand, ribosomes only appear to bind mRNAs with A/U-tail, suggesting that A/U-tails is essential for mRNA recognition by small ribosomal subunits (126, 286, 287).

With regards to replication, preservation of mitochondrial DNA (kDNA), transcription, RNA editing and RNA processing, *T. brucei* mitochondria fits in among the well-studied organelles (122). However, translation in mitochondria still remains a mystery, since 10% of the cytosolic translation in *T. brucei* is cycloheximide resistant, which makes it even more difficult to study mitochondrial translation (288, 289). So, far translation has only been studied in the PCFs.

The mitochondrial ribosome is made up of 30S small subunits (SSUs) and 50S large subunits (LSUs) including the associated maxicircle encoded rRNA 9S and 12S respectively (290, 291). However, currently no report is available in terms of functional analysis of these protein components (292). Surprisingly, another 45S SSU ribosome related complex has been reported with 9S rRNA. This 45S complex is further linked

with the translation of selective mRNAs such as COI and Cyb (287, 290). Additionally, several other proteins is reported to associate with the ribosome known as pentatricopeptide repeat (PPR) proteins that are shown to be required for rRNAs stability (293, 294). In *T. brucei* genome-wide searches, 39 of these PPR proteins were identified and the one associating with the ribosomes are termed kinetoplast ribosomal PPR proteins (KRIPPs) (126, 295).

Hypothesis

Based on my studies, I hypothesize that REH2C (1) is an mRNP (2) it hybridizes with the gRNA-bound subcomplexes (gRNP) through specific base pairing of mRNA-gRNA, and (3) is required for the recruitment of the RECC enzyme, ultimately completing the editing holoenzyme or holo-editosome. This stepwise assembly pathway may involve specific checkpoints that are controlled by the REH2 and its cofactor ^{H2}F1.

Dissertation overview

This dissertation is focused on characterizing the function of DEAH/RHA helicase REH2 and its cofactors in RNA editing of *T. brucei.* This involves the identification of the *in vivo* helicase associated mRNA and gRNA molecules, illustrating helicase function in multiple steps of RNA editing and also, discovery of the REH2 helicase co-factors and their role in RNA editing. Based on the results from our research, we propose a model to explain how distinct RNP's may assemble through base pairing of pre-mRNA substrates and gRNAs. This stepwise assembly brings together functional

mRNA-gRNA hybrids and the RECC enzyme, ultimately forming a complete catalytic holoenzyme. These results are explained in chapter II-IV.

Chapter II describes the first illumina libraries of gRNA composition in the two native MRBs, currently being characterized in our laboratory (termed H2-MRB and 3010-MRB). We present the first experimental evidence that mRNAs in editing (both substrates and products) are associated to these MRBs *in vivo*. Our study of the associated gRNAs shows distinct composition among these native MRBs. In addition, we show examples of predicted alternative editing at mRNA's 3' end and gRNA divergence in *T. brucei*. We propose a model in which these MRBs serve as scaffolds for the assembly of substrates and the RECC enzyme.

Chapter III describes the proposed specialized functions of MRBs in *T. brucei* RNA editing. We show experimental evidence that the 3010-MRB may be the major scaffold for RNA editing. This chapter also shows that the REH2 helicase, the defining protein component of the second MRB termed REH2-MRB, has the capacity to affect in *trans* the 3010-MRB. Our studies also show that REH2 participates in multiple steps of RNA editing that include editing in first block and in subsequent blocks during editing progression. We found that REH2 associates with its own MRB through RNA linkers. Moreover, REH2 fails to associate with both RNA and proteins in its MRB upon mutation of its dsRBD and helicase core domains. Finally, we show in our crosslinking experiments that REH2 associates with a novel RNA binding protein and propose that REH2 and this proteins are part of a novel REH2-associated subcomplex (in the high-order REH2-MRB).

In chapter IV, we focus on defining the composition (protein and RNA components) and function of the novel REH2C subcomplex. In this study, we discovered two cofactors of REH2 that we termed ^{H2}F1 and ^{H2}F2. We show that the association of REH2C subcomplex also includes mRNA (substrate and products) and therefore it represents an mRNA-bound ribonucleoprotein complex (mRNP). This mRNP is stable in transgenic cell lines that are depleted of gRNA. Thus, the helicase-associated mRNP is independent of gRNA or its native gRNA-associated subcomplexes in mitochondria. We showed that ^{H2}F1 impacts RNA editing but ^{H2}F2 has no effect on this process. In addition, ^{H2}F1 is required in *cis* for the assembly of REH2 helicase with its own MRB components (namely gRNA and gRNA-associated proteins) and with the RECC enzyme. Furthermore, ^{H2}F1 is required in *trans* for the association of 3010-MRB (which lacks REH2) with RECC enzyme. Our updated model makes predictions for the assembly of mRNP and gRNP modules in REH2-MRB and the transient functional interaction of the RECC enzyme with both REH2-MRB and 3010-MRB. In this model, the OB-fold domains in REH2 and the zinc fingers of its ^{H2}F1 cofactor are proposed to participate in surveillance mechanisms during substrate recruitment and mRNA-gRNA hybrid quality. These checkpoints may be pre-requisite for efficient RECC binding and catalysis.

Chapter V includes the conclusion based on studies explained in chapter II-IV and possible future directions.

CHAPTER II

NATIVE MITOCHONDRIAL RNA-BINDING COMPLEXES IN KINETOPLASTID RNA EDITING DIFFER IN GUIDE RNA COMPOSITION*

Summary

Mitochondrial mRNAs in kinetoplastids require extensive U-insertion/deletion editing that progresses 3′-to-5′ in small blocks, each directed by a guide RNA (gRNA), and exhibits substrate and developmental stage-specificity by unsolved mechanisms. Here, we address compositionally related factors, collectively known as the mitochondrial RNA-binding complex 1 (MRB1) or gRNA binding complex (GRBC), that contain gRNA, have a dynamic protein composition, and transiently associate with several mitochondrial factors including RNA editing core complexes (RECC) and ribosomes. MRB1 controls editing by still unknown mechanisms. We performed the first next-generation sequencing study of native subcomplexes of MRB1, immunoselected via either RNA helicase 2 (REH2), that binds RNA and associates with unwinding activity, or MRB3010, that affects an early editing step. The particles contain either REH2 or MRB3010 but share the core GAP1 and other proteins detected by RNA photo-crosslinking. Analyses of the first editing blocks indicate an enrichment of several

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initiating gRNAs in the MRB3010-purified complex. Our data also indicate fast evolution of mRNA 3′ ends and strain-specific alternative 3′ editing within 3′ UTR or C-terminal protein-coding sequence that could impact mitochondrial physiology. Moreover, we found robust specific copurification of edited and pre-edited mRNAs, suggesting that these particles may bind both mRNA and gRNA editing substrates. We propose that multiple subcomplexes of MRB1 with different RNA/protein composition serve as a scaffold for specific assembly of editing substrates and RECC, thereby forming the editing holoenzyme. The MRB3010-subcomplex may promote early editing through its preferential recruitment of initiating gRNAs.

Introduction

Kinetoplastid protozoa include medically relevant species of *Trypanosoma* and *Leishmania* with life cycle stages that experience dramatic adaptations to host and changes in energy metabolism (296). A unique mitochondrial process of RNA editing by uridylate insertion and deletion is directed by guide RNAs (gRNAs) (67, 82) and controlled in mRNA-specific and stage-specific manners by mechanisms that remain fundamentally unresolved (297). The mitochondrial genome of *Trypanosoma brucei* (kinetoplast or kDNA) consists of several copies of a maxi-circle (∼23 kb) and thousands of minicircles (∼1 kb). Twelve out of 18 mRNAs encoded by the maxi-circle are edited. Minicircles encode most gRNAs, estimated at ∼1200 on their "sense" strand (69, 298). gRNAs are primary transcripts with three regions: a short 5′ anchor that anneals to pre-edited or edited mRNA next to the sequence to be edited, a guide

sequence with complementarity to edited mRNA (editing block), and a ~10–15U 3′ tail added post-transcriptionally. An mRNA editing domain usually spans a set of juxtaposing and often overlapping editing blocks.

While the basics of the editing reaction catalyzed by RNA editing core complexes (RECCs) is well characterized, the regulatory aspects remain largely unknown (183–185, 299). Editing domains in mRNAs mature from 3′ to 5′ in small blocks, each directed by a gRNA. Most mRNAs are extensively edited, while a few require limited editing or are never edited (297). How a functional cognate substrate reaches the RECC is currently unknown. Accessory factors may facilitate selective binding of editing substrates, annealing, unwinding, and chaperone activities to stabilize precise base-pairing between the target mRNA and the gRNAs directing the U-insertions and U-deletions. Consistent with this idea, purified RECC was reported to be RNA-free and lacks the processivity and substrate specificity of editing *in vivo* (142, 186–188, 300). Many non-RECC proteins impact editing (65, 183, 207, 300). Some proteins preferentially affect a few mRNAs, while others affect a broader range. In a few cases, effects at initiation or during progression have been proposed, and RNA binding, annealing, and unwinding have been observed with recombinant protein or purified complexes (209, 222, 301). So, while much progress has been made, central long-standing questions remain perplexing, including the mechanisms of substrate recruitment, regulation of editing activity, and editing integration into mitochondrial RNA metabolism. The answer may involve a group of ribonucleoprotein particles of surprising complexity, known as the mitochondrial RNA-binding complex 1 (MRB1) (or

gRNA binding complex, GRBC), which contain gRNA and a dynamic protein composition (209–213). Subcomplexes of MRB1 (called here MRBs for simplicity) transiently associate via RNA linkers with several factors including RECCs, proteins that may affect processing and stability, and ribosomes. Our lab and others have proposed that these complexes may serve as "organizers" in the control of editing and its integration in mitochondria (183, 207, 209, 302). For example, the MRB3010 subunit (3010) affects an early editing step, whereas TbRGG2 impacts progression between blocks (213, 222). We reported a helicase REH2 (H2) in an MRB that contains 3′-5′ unwinding activity. Copurification of REH2 with gRNA and unwinding activity is inhibited by mutation of its helicase or RNA binding domains (209). Importantly, RNAi-based repression of REH2 inhibits cell growth and RNA editing in trypanosomes (83, 209, 217).

We considered that MRBs of different protein composition could exhibit differences in their associated RNAs. We tested this hypothesis by performing the first next-generation sequencing (NGS) study of two native MRBs that contain either H2 or 3010. Analyses of the first editing blocks showed that the 3010-associated MRB is enriched in initiating gRNAs. We also found specific copurification of MRBs with mRNAs that undergo editing. Interestingly, we identified differences in gRNA expression, including alternative editing of mRNA 3′ ends, between the Lister strain 427 used here, EATRO 164 cells in a recent NGS study of total gRNA in procyclics (303), and currently annotated sequences. This adds a level of complexity to the potential of alternative editing first observed by the Hajduk lab (138). Based on cumulative

observations in our lab and others, we propose a model whereby subcomplexes of MRB1 with distinct RNA/protein composition serve as scaffolds that recruit editing substrates and route them into transiently associated RECCs. A dynamic higher-order MRB1 complex, formed by several subcomplexes with specialized roles, may provide the necessary context for concerted substrate selection or usage during editing initiation and progression.

Methods

Cell culture

T. brucei Lister strain 427 29-13 procyclic "PF" (tryps.rockefeller.edu) was grown axenically in log phase in SDM79 and harvested at a cell density of 1–3 × 10^7cells/mL. A derived cell line expressing tetracycline-inducible TAP-REH2 was used as described (209).

Protein purification

Native MRB1 subcomplexes were immunoprecipitated from freshly prepared mitochondrial extracts (304) using affinity-purified peptide antibodies raised in rabbit against REH2, MRB3010, and cytochrome oxidase 2 (mock) in *T. brucei* (Bethyl Laboratories, Inc), as we reported, with some changes (209). Briefly, specific antibodies of identical quality and concentration were conjugated to Protein A-Dynabeads (Life Technologies) that were pretreated with 5% BSA. Approximately 2 mg of mitochondrial extract was supplemented with 1× cOmplete Protease Inhibitor cocktail (Roche), SUPERase.In RNase inhibitor (Life Technologies), and precleared by passage over

Protein A-Sepharose beads (GE Healthcare) before loading onto antibody-conjugated beads. Ectopically expressed TAP-REH2 was specifically immunopurified using IgG-Dynabeads (Life Technologies) as reported (209). All washes were performed at 200 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$ and 25 mM Tris, pH 8.0.

Western analyses and radioactive assays

Western blots were examined with rabbit affinity-purified anti-REH2 (209) and anti-MRB3010 polysera, or rabbit anti-GAP1 prebleed polyserum. Anti-MRB3010 antibodies were raised against the C-terminal peptide CPPLYQLYISRGSTPQA (Bethyl Laboratories, Inc), which is uniquely aligned to this protein in a Blast search of *T. brucei*. Monoclonal antibodies against REL1 ligase (a RECC subunit) were used as reported (125). A 61-nt fragment of pre-edited A6 mRNA bearing a photo-reactive 4thio-U and ^{32}P at the first editing site was mixed with immunopurified MRBs and subjected to 365-nm UV irradiation on ice, as previously described (305, 306). Radioactive capping of gRNA and adenylation of RECC ligases were performed as reported (82, 307). Northern blots of RNA extracted from IPs from whole-cell lysate with 5′ labeled probes for gA6 B1.alt [1357]: CCACTGTAAAACTGATTTCGTCATTGGAG (Tm 57.9) and gND7 3′ domain B1 [1358]: CTTATACATGAAGTCACTGTAGGATTG (Tm 53.3) were performed in ULTRAhyb solution (Life Technologies) and 2× SSC washes at 40°C.

Illumina libraries of gRNA from purified MRBs and procyclic parasites

Identical immunoprecipitations were performed in parallel using REH2, 3010, and mock affinity-purified antibodies. The final washed beads were treated with proteinase K and SDS, and the associated RNA was treated with a DNA-free DNase kit and recovered by acid phenol-chloroform extraction and ethanol precipitation. RNA samples were treated with the ScriptCap capping system (CELLSCRIPT) and [α-^{32}P]-GTP, and resolved on 15% UREA-PAGE to concentrate the gRNA in a narrow band. Size markers of gRNA mobility (~40–60 nt) were prepared with synthetic RNA fragments. Capped gRNA from IPs and total mitochondrial RNA were resolved in parallel with identical uncapped (unlabeled) samples (Figure 16). The labeled samples served as controls to adjust the amount of unlabeled gRNA used for library construction. We note that the total RNA sample was treated with Terminator 5′ Phosphate-Dependent Exonuclease (Epicentre) that degrades rRNA but not gRNA, as these transcripts carry a 5′ triphosphate (82). This step was not applied to the IPs because much nuclear rRNA and cytosolic mRNA were selected out in those samples (Figure 12A). Terminator-treated RNA was diluted to generate comparable amounts of labeled gRNA in the IP and total RNA lanes on the gel for concurrent excision of identical cuts, elution, and adapter ligations. We used an Illumina small RNA protocol with modifications. The IP and total gRNA samples were ligated to different barcoded 3′ adapters. At this point, all samples were mixed, and the subsequent steps were performed in a single reaction: phosphatase/PNK treatment to add a single phosphate, 5′ adapter ligation, cDNA synthesis, and PCR amplification. Analysis in an Agilent Bioanalyzer showed a narrow

distribution of the libraries, with an average size of ~150 bp, reflecting the average size of gRNA plus adapters. The multiplexed libraries were sequenced on Illumina GAIIx (single-end, 75-base run), producing ~40 million total reads.

Computational analyses and read preprocessing

All base calls in the sequencing reads with a Phred score below 20 were converted into N's and runs of trailing N's were removed from the reads. Reads in which more than 10 N's remained were discarded. Sequencing adapters were identified by requiring at least the first five bases of the adapter to be contained in the read and the sequence identity between the known adapter sequence and the part of the read containing the adapter to be at least 75%. Reads in which no adapter could be identified and reads with less than 10 bases after adapter removal were discarded. The bases immediately preceding the adapter were compared with the barcodes ATAC, ACCAA, CGAGA, and ATAGC, allowing at most one mismatch to distinguish reads originating from the procyclic, 3010, H2, and cytochrome oxidase 2 (mock) libraries, respectively, and the barcodes were removed. All reads with more than one mismatch to one of the four barcodes at the 3′ end were discarded.

Alignment to edited mRNAs

Reads from each library were separately aligned to the known edited mRNA sequences deposited at the KISS bioinformatics site (http://splicer.unibe.ch/kiss/). To this end, all reads with three or more consecutive N's were discarded. Gapless semilocal alignments with a match score (including GU base-pairing) of 1 and a mismatch score of

−2 were used to identify regions of local similarity between the reads and edited mRNA sequences. Reads with a local similarity score of at least 20 were parsed into a 5′ unmatched region, the matching region, a 3′ unmatched region, and a poly-U tail. These reads were post-filtered in order to only retain reads with at most two mismatches, a minimum length of the matching region of 25 bases, and a poly-U tail of at least three U's. For RPS12, we also required that the length of the 5′ unmatched region was, at most, 20 bases. For the remaining reads, we tallied the number of bases in matching regions covering each position in the edited mRNA.

Quantitative real-time PCR (qPCR)

RNA from the IPs and procyclic cells used in the preparation of Illumina libraries was used for cDNA synthesis, after treatment with a DNA-free DNase kit, in reactions with random hexamers in the iScript cDNA kit (Bio-Rad). Twenty microliters qPCR reactions were performed with reported primers specific for edited or pre-edited mRNAs, never-edited, and two reference transcripts, 18S rRNA and tubulin (184), in a SsoFast EvaGreen Supermix (Bio-Rad). Diluted samples to score test transcripts (1:7) and reference transcripts (1:50) produced a single amplicon during linear amplification. All end-point amplicons described here were gel-isolated, cloned, and confirmed by sequencing. Fold-enrichment of mitochondrial and reference transcripts in the sample IPs, relative to the mock IP, was calculated as follows: Fold = $2^{(-ddCq)}$, where ddCq = Cq test IP − Cq mock IP. Steady-state mRNAs in mitochondrial extract relative to background tubulin and nuclear 18s rRNA (reference transcripts) were scored as

follows: $dCq = 2^{(\text{Target Cq} - \text{Ref Cq})}$. Cq duplicates of each sample (STDEV ≤ 0.1) were averaged, and dilutions were adjusted to 100%.

Results

Native subcomplexes of MRB1 containing either REH2 or MRB3010

MRB1 ribonucleoprotein complexes were previously isolated from procyclic trypanosomes expressing tagged copies of REH2 or MRB3010 (3010), and their protein composition examined in mass spectrometric studies (209, 213), but the RNA composition of these complexes is not known. Furthermore, the large tag in the constructs or ectopic expression could induce unintended effects on the protein and RNA composition of MRBs. The current study characterized immunoprecipitated (IP) native MRBs from mitochondrial extracts using affinity-purified peptide polysera against H2 or 3010 (Figure 9A; Figure 14). Helicase H2 is often partially fragmented in extracts (209), whereas 3010 migrates slightly below IgG on SDS-PAGE. Interestingly, 3010 was not detected in Western analyses of native H2 IPs, whereas H2 is not detected or is barely visible in native 3010 IPs. We reproduced this observation multiple times using mitochondrial extract or whole-cell lysate in the IPs (e.g., Figure 15). Consistent with our analysis of native MRBs, H2 was not detected in a previous affinity-purification of tagged MRB3010 (213). So, loss of H2 in that study was not caused by the large tag in 3010 but instead is an intrinsic feature of native 3010-MRB.

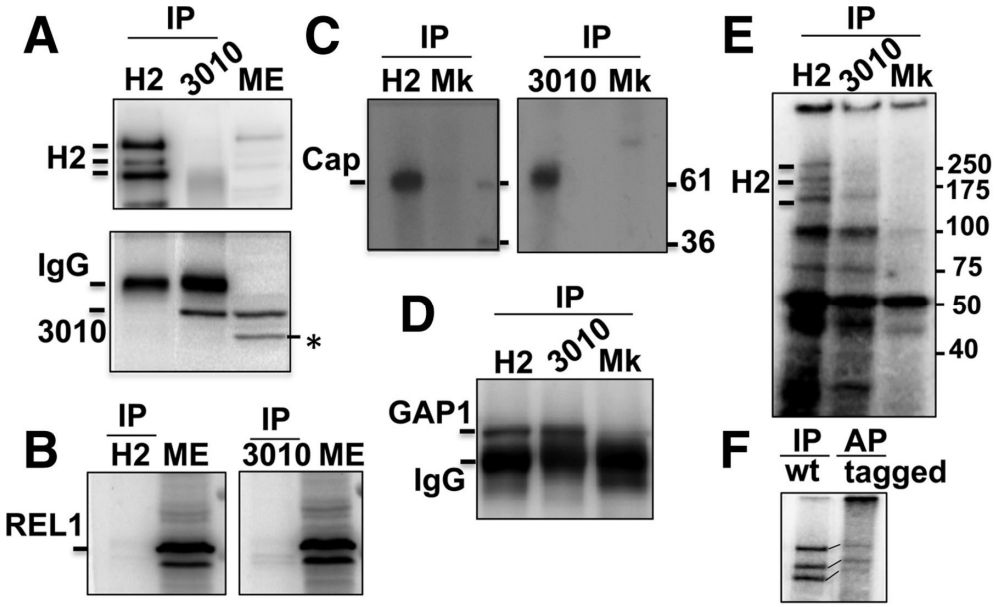

Figure 9. Native immunopurified subcomplexes of MRB1 with either H2 or 3010 subunits. (*A*) Western analysis of H2 and 3010 in IPs by the indicated antibodies, and in mitochondrial extract (ME). H2 (~240 kDa) is often fragmented, and 3010 (57.5 kDa) migrates slightly *below* IgG in the IPs. A fainter band *below* 3010 is a breakage product of this protein in ME (*). The blot was split into halves treated with either anti-H2 or anti-3010 antibodies. (*B*) Western analysis of the RECC subunit REL1 ligase in IPs and ME. (*C*) [^{32}P]G-capping of gRNA 5′ triphosphate with guanylyltransferase on 15% UREA-PAGE to concentrate gRNA in a discrete band. (*D*) Western analysis of the MRB1 subunit GAP1 in test and mock IPs. Mock IPs (Mk) used an irrelevant affinity-purified antibody. (*E*) Site-specific crosslinking of H2 and 3010 IPs with a pre-edited mRNA construct whose first editing site contains ^{32}P in its phosphodiester bond and 4-thioU in the flanking 5′ nucleotide. After RNase trimming, the protein-RNA adducts were resolved on 10% SDS-PAGE. (*F*) Crosslinks as in *E*, but on a high-resolution 6% SDS-PAGE. Native (wt) and ectopic (tagged) H2 differ in mobility due to a ~20 kDa tag. Tagged H2 was affinity-purified (AP). Intact and fragmented H2 are marked. Molecular markers are in kDa. All IPs (200 mM KCl, 5% BSA) used precleared extract.

A transient association between RECC and H2-purified MRB was previously observed (209). RECC subunits were almost undetectable with different antibodies in the current native H2 and 3010 purifications (Figure 9B; data not shown). However, editing ligases can be radiolabeled by a sensitive auto-adenylation assay indicating substoichiometric levels of RECC (data not shown;(209)). As expected, both natively purified MRBs contain gRNA (Figure 9C) and the proposed core GAP1 subunit (Figure 9D), consistent with previous purifications of REH2 and MRB3010 (209, 213).

Only endogenous gRNA has been reported to bind purified MRB1 complexes; however, several subunits have domains that suggest a role in RNA biology (211, 212). For example, our lab reported that H2 crosslinks with an mRNA editing site bearing a photo-reactive group (209). Importantly, immunopurified H2 and 3010 MRBs produced similar crosslinking patterns with this substrate (Figure 9E), except for the crosslinks induced by H2 itself. The H2 crosslinks were confirmed by their mobility shift in a direct comparison of tagged vs. native H2 (Figure 9F). This is the first time purified MRBs are directly compared by crosslinking, and the analysis suggests that H2 and 3010 MRBs share several RNA-binding proteins. Most of these crosslinks were not detected in the mock IP. Together, these data describe two native subcomplexes of MRB1 that stably copurify with gRNA, the core GAP1 subunit, and several common RNA-crosslinking proteins. However, these MRBs were readily distinguished by the presence of H2 or 3010. This result prompted us to investigate the RNA composition of our purified samples.

Native H2-MRB and 3010-MRB subcomplexes differ in gRNA composition

To address the possibility that MRBs of different protein composition may also differ in their gRNA complement, we compared the gRNA pools from H2, 3010, and mock IPs, and from procyclic (PF) parasites using next-generation sequencing. Barcoded Illumina libraries made in parallel with comparable amounts of gRNA scored in a capping assay (see Figure 16 and the Materials and methods section) were multiplexed, and reads were aligned to edited mRNA sequences deposited at the KISS bioinformatics site (http://splicer.unibe.ch/kiss/) (69). We filtered out fragmented RNA by scoring only gRNAs bearing a 3′ U-tail. Furthermore, because 3010 may impact editing at an early step (213), we focused on the first editing blocks of mRNAs (Figure 10). In line with a recent characterization of the gRNA pool in PF cells (303), we found that initiating gRNAs for several mRNAs occur in relatively low abundance. Because our libraries used limiting gRNA amounts extracted from the IPs, we only found initiating gRNAs for six mRNAs. The following analyses compare cumulative nucleotide frequency plots (NFPs) of edited mRNA coverage (Figure 10) and individual frequency values and the ratio of major gRNAs in editing blocks 1 and 2 (B1 and B2, respectively) (Table 5). Some gRNAs predicted alternative editing sequences to those currently annotated (termed B1.alt or B2.alt) and are further discussed in Figure 11. Negligible gRNA levels in the mock IP library confirmed the specificity of our MRB libraries (Table 5).

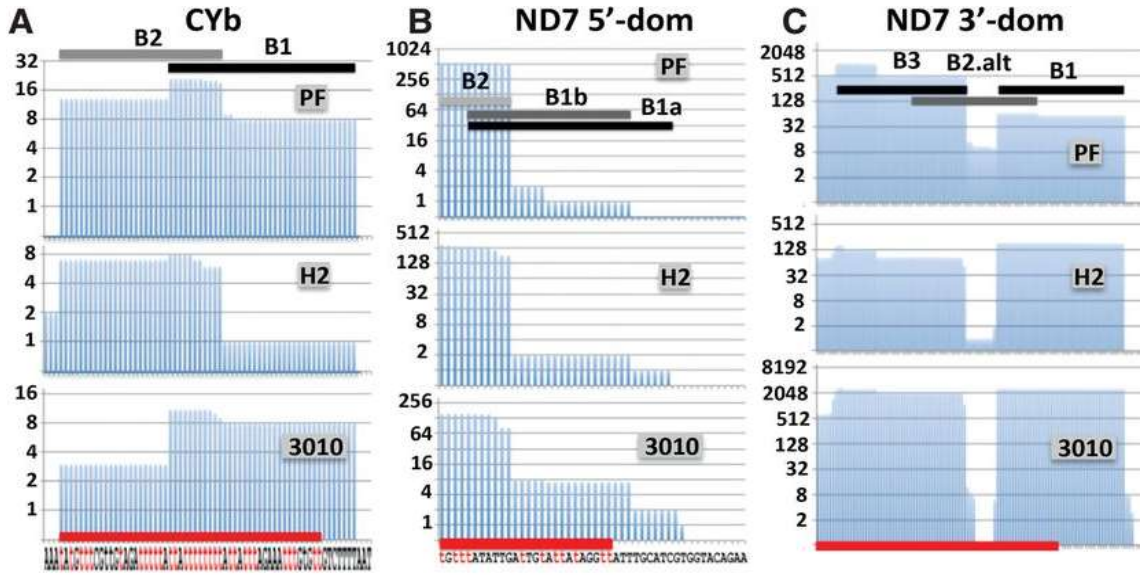

Figure 10. Analysis of edited mRNA coverage by gRNAs in illumina libraries. (*A–G*) Steady-state gRNA from PF parasites and gRNA in immunopurified MRBs are annotated in blocks of edited sequence, each directed by a gRNA. Cumulative standard and G·U pairs between edited mRNA and gRNAs are scored in nucleotide frequency plots (NFPs Log_2), including initiating gRNAs (i.e., block 1 or B1) and major upstream gRNAs (B2 or B3) in our libraries. Some gRNAs may guide alternative editing (e.g., B1.alt and B2.alt), and the ND7 5′ domain uses at least two similar initiating gRNAs (B1a and B1b). The entire editing domain in cytochrome B (CYb) and the 3′ terminus of other domains are plotted. The sequence of CYb (including all 34 U-insertions as lowercase t's) and the ND7 5′ domain are shown. Equal protein loads were applied to the IPs, and the gRNA was gel-isolated and extracted. gRNA from IPs and total RNA were adjusted to apply comparable amounts in the libraries (e.g., Figure 16; see Materials and Methods). gRNA from the IPs was directly ligated to the adapters, whereas total gRNA from PF parasites was first treated with 5′ monophosphate specific Terminator exonuclease, which degrades rRNA but not 5′ triphosphate gRNA ends. (*H*,*I*) Northern blots of select initiating gRNAs from IPs and PF cells. The blot was hybridized with the A6 gRNA probe, stripped, and re-used with the ND7 gRNA probe. 15% UREA-PAGE was run as in Figure 9C.

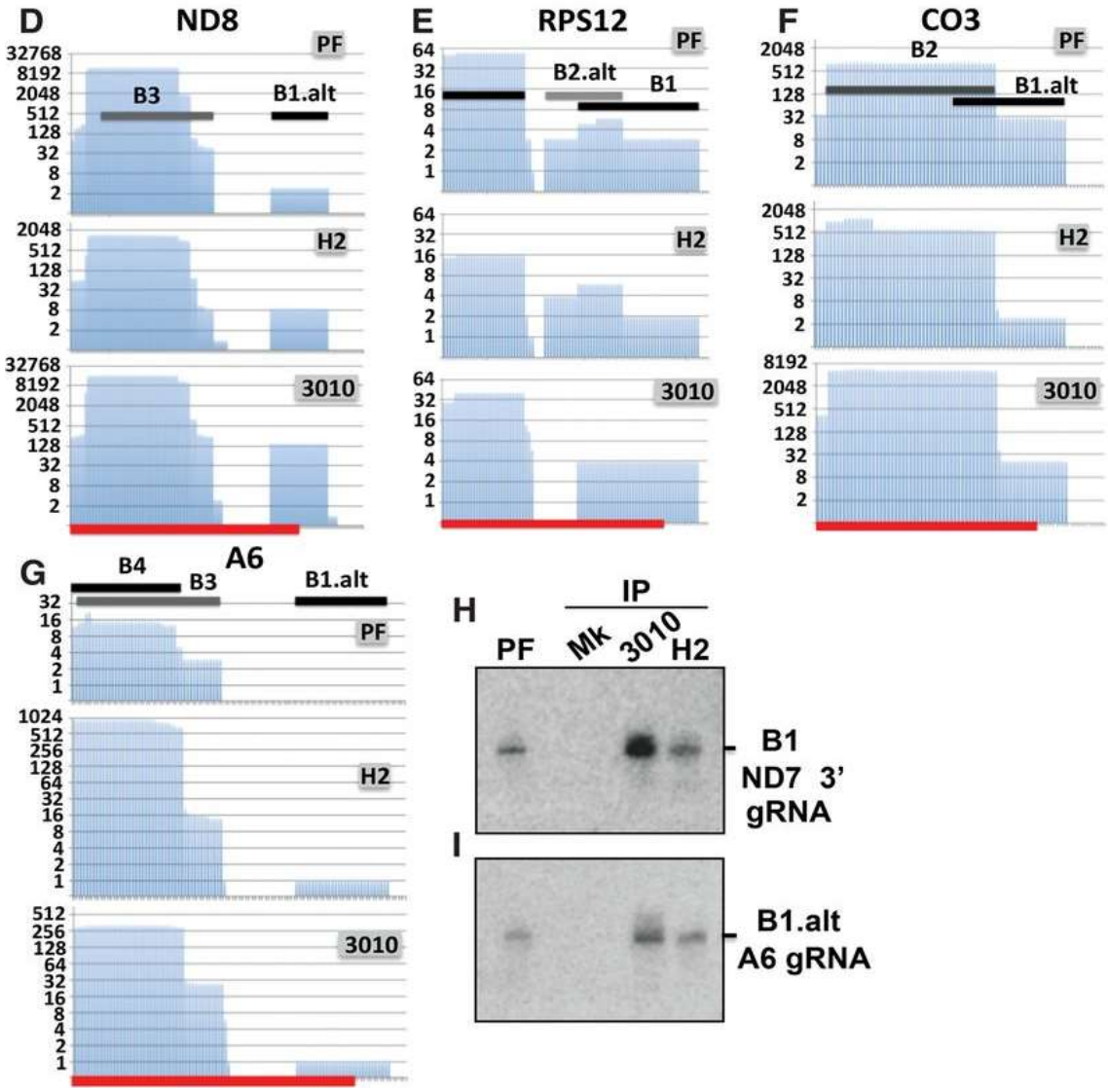

Figure 10. Continued.

Table 5. Relative amounts of B1 and B2 gRNAs

	B1				B2				B1/B2	
	PF	H2-IP	3010-IP	Mock-IP	PF	H2-IP	3010-IP	Mock-IP	H2-IP	3010-IP
CYb	8	1	8	nd	10	5	3	1	0.2	2.7
ND7 5′ dom	1**	1**	5**	nd	494	180	82	8	0.006	0.06
ND7 3′ dom	60	179	2592	9	8	1	nd	nd	179	≥2592
ND8	3	9	147	nd	nd	nd	nd	nd	≥9	≥147
RPS12	3	2	4	nd	3	4	nd	3	0.5	≥4
CO3	28	3	21	nd	801	565	5208	22	0.005	0.004
A6	0	1	1	nd	nd	nd	nd	nd	≥1	≥1
ND7 5′ dom	0*	1	1*	nd						

Frequency of a gRNA is defined as the value of the dominant 5′ end in a block. The 5′ end of guide sequences is generally more conserved than the 3′ end. (*) and (**) distinguish two analog initiating gRNAs (B1a and B1b, respectively) with a different 5′ end but the same 3′ end. A block may include one gRNA or multiple redundant gRNAs of equivalent guide sequence with conserved nucleotide polymorphisms. Redundant gRNAs may reflect genetic drift of a minicircle or homolog genes in different minicircles. (nd) Not detected. Calculations of B1/B2 assume "nd" to be ≤1.

Cytochrome B (CYb)

mRNA CYb has a small editing domain (~50 nt) with only two blocks (Figure 10A). Notably, the coverage of the B1 and B2 blocks was similar in the parasites but different between MRBs. Relative to the B2 gRNA in each library, the initiating B1 gRNA appears enriched in 3010-MRB. A higher coverage of the B2 editing block in the H2-MRB libraries relative to the B1 block also suggests intrinsic differences between native MRBs. Table 5 shows the actual values and ratio of major gRNAs in these blocks (B1/B2), which is a simple parameter to evaluate relative B1 enrichment in MRBs independent of loading.

NADH dehydrogenase subunits 7 and 8 (ND7 and ND8)

mRNA ND7 has two separate editing domains with initiating gRNAs that were enriched in 3010-MRB (Figure 10B,C). Interestingly, the 5′ domain has two possible initiating gRNAs, whose guide sequences have different 5′ ends but the same 3′ end: B1a

and B1b (Figure 10B). The relative values of B1b and B2 gRNAs suggested a preferential binding of B1b gRNA in 3010-MRB. In contrast, the B1a gRNA was too low in the MRB libraries and absent in the PF library (Figure 10B; Table 5). As in mRNA CYb, the H2-MRB library exhibited a higher coverage of the B2 gRNA for the 5′ domain. In the ND7 3′ domain (Figure 10C), the B1 gRNA was dramatically increased in 3010-MRB. In contrast, the B2 gRNA in this domain was too low to quantify in the MRB libraries. In mRNA ND8 (Figure 10D), we found an initiating gRNA but not a B2 gRNA in the libraries. However, a relatively high value of the initiating gRNA in 3010-MRB when compared to upstream gRNAs also suggested a specific increased abundance in this complex. B1/B2 determinations assuming a value of ≤1 for nondetected gRNAs indicated B1 enrichment in 3010-MRB for ND8 and both ND7 domains, especially in the 3′ domain.

Ribosomal protein subunit 12 (RPS12), cytochrome oxidase 3 (CO3), and ATPase subunit 6 (A6)

Our libraries contain possible initiating gRNA for all three mRNAs RPS12, CO3, and A6 (Figure 10E–G), whereas a B2 gRNA was missing for A6. In mRNA CO3, both B1 and B2 gRNAs were increased in 3010-MRB. However, the B1/B2 ratio for mRNA RPS12 suggested a preferential increase of B1 gRNA in 3010-MRB (Table 5). In A6 mRNA, we found an initiating gRNA in the MRB libraries but no B2 gRNA in any of the libraries. Nevertheless, the B1 gRNA was too low in our libraries to be examined quantitatively.

As a complementary approach to our above NGS studies, we performed Northern blots of B1 gRNAs for the ND7 3′ domain and A6 mRNA from direct IP samples of MRBs (Figure 10H,I). Consistent with our findings in the libraries, the level of B1 gRNA in the ND7 3′ domain was much higher in the 3010 IP than in the H2 IP. The B1.alt gRNA in A6 mRNA was slightly higher in the 3010 than in the H2 IP, possibly reflecting a moderate but reproducible difference in capping signal in our unadjusted samples from direct IPs (see Figure 16 and the preparation of libraries in the Materials and Methods section).

Collectively, these data showed important differences between our MRB libraries in coverage NFPs of the first editing blocks. Relative comparisons of gRNAs (B1/B2) in the same library were consistent with an enhanced association of initiating gRNAs for mRNAs CYb, ND7 (both domains), ND8, and RPS12 in 3010-MRB. Furthermore, evidently different patterns in the coverage NFPs between procyclic and MRB libraries also indicated differential gRNA binding by MRBs. An increased selectivity of B1 gRNA by 3010-MRB implies that this complex may be more active in editing initiation than H2-MRB. Interestingly, the mRNAs ND7 (3′ domain) and ND8 exhibited the largest relative accumulation of initiating gRNA.

Alternative editing at mRNA 3' ends and gRNA divergence in *T. brucei strains*

Some gRNAs in Figure 10 suggest alternative editing patterns compared to annotated edited mRNAs from studies in the early 1990s. All cases of alternative editing discussed here significantly extend the length and quality of the predicted duplex between guide sequence and edited mRNA. Importantly, the observed mismatches with

annotated sequence were outside the gRNA anchor. Some guide sequences were well conserved between Lister 427 studied here and EATRO 164 cells in a recent study by Koslowsky et al. (Figure 11), but others exhibited important strain-specific differences, consistent with a rapid evolution of minicircles. This section compares annotated and predicted alternative editing directed by B1 and B2 gRNAs in our libraries and examines the conservation of guide sequences between strains. Some gRNAs with very similar or identical guide sequences in both strains often exhibit highly diverged nonguiding termini.

CYb editing domain

The B1 and B2 gRNAs in Lister 427 were identical to those reported by Koslowsky et al., including a 5′ terminal run of As (Figure 11A). As indicated by that lab, an initiating gRNA, gCYb[560A] from an earlier study in EATRO 164 cells (308) has the same guide sequence but a very different 5′ end. This gRNA was not present in their recent library from EATRO 164 cells or ours reported here.

ND7 3′ editing domain

The guide sequence of the B2.alt gRNA formed one mismatch (Figure 11B[1]). However, simple alternative editing (one U-insertion and one U-deletion) would allow annealing with most of the gRNA (Figure 11B[2]). Notably, this would also substitute two of the three C-terminal amino acids without changing the coding frame. The guiding sequence of B2.alt is nearly identical in Lister 427 and EATRO 164 cells (Figure 11B[3]).

ND7 5′ editing domain

As mentioned above, this domain had two initiating gRNAs (B1a and B1b) (Figure 11C[1]). The B1a gRNA was not found in the EATRO 164 library, but the B1b gRNA was the same in Lister 427 and EATRO 164 cells. An additional initiating gRNA, gND7(147–199), in EATRO 164 cells was not present in our libraries. These three gRNAs shared significant homology (Figure 11C[2]) including a 7-nt identity at their 5′ termini. Based on this 5′ conservation, Koslowsky et al. suggests that gND7(147–199) could form an alternative anchor duplex (i.e., the same anchor by the B1a gRNA). However, the resulting alternative editing would introduce two A:C mismatches, decreasing the quality of the duplex (303).

CO3 editing domain

The B1.alt gRNA in our libraries (Figure 11D[1]) was not seen in the EATRO 164 library, and vice versa, we did not find the initiating gRNA in Koslowsky's study. Alternative editing by the B1.alt gRNA would substantially modify the 3′ UTR sequence (Figure 11D[2]). Coincidently, both the B1.alt gRNA and both CYb gRNAs contain a 5′ A-run. The B2 gRNA, gCO3(935–977), was the same in both strains.

ND8 editing domain

The B1.alt gRNA formed a relatively short continuous duplex with annotated edited sequence (Figure 11E[1]). However, alternative editing by a single U-insertion in the 3′ UTR extended the duplex dramatically (Figure 11E[2]). This gRNA was similar to gND8(554–598) in EATRO 164 cells, except that the B1.alt gRNA in Lister 427 cells

had one additional internal guiding “A” (see arrow, Figure 11E[3]) that causes the alternative editing.

RPS12 editing domain

The guide sequence of the B1 gRNA (Figure 11F[1]) was the same in Lister 427 and EATRO 164 cells. Alternative editing by the B2.alt gRNA would shorten the encoded C terminus and substitute its last four amino acids (Figure 11F[2]). The B2.alt gRNA was not seen in the EATRO 164 library, and we did not find the gRPS12(267–322) reported in the study by (303). However, these two gRNAs exhibit partial homology and have identical 5′ anchor sequences (Figure 11F[3]). Interestingly, gRPS12(267–322) in EATRO 164 introduces an internal A:C mismatch that does not predict alternative edits (this “A” is noted by an arrow).

A6 editing domain

The B1.alt gRNA, gA6(774–822), formed two mismatches with annotated edited mRNA (Figure 11G[1]) and is present in EATRO 164 cells. Alternative editing of the 3′ UTR by this gRNA (Figure 11G[2]), as proposed by Koslowsky et al. (303), allowed almost full annealing of the gRNA. This gRNA was almost identical in both strains (Figure 11G[3]). Koslowsky et al. reported a second initiating gRNA gA6(770–822), a low abundance transcript not detected in our libraries.

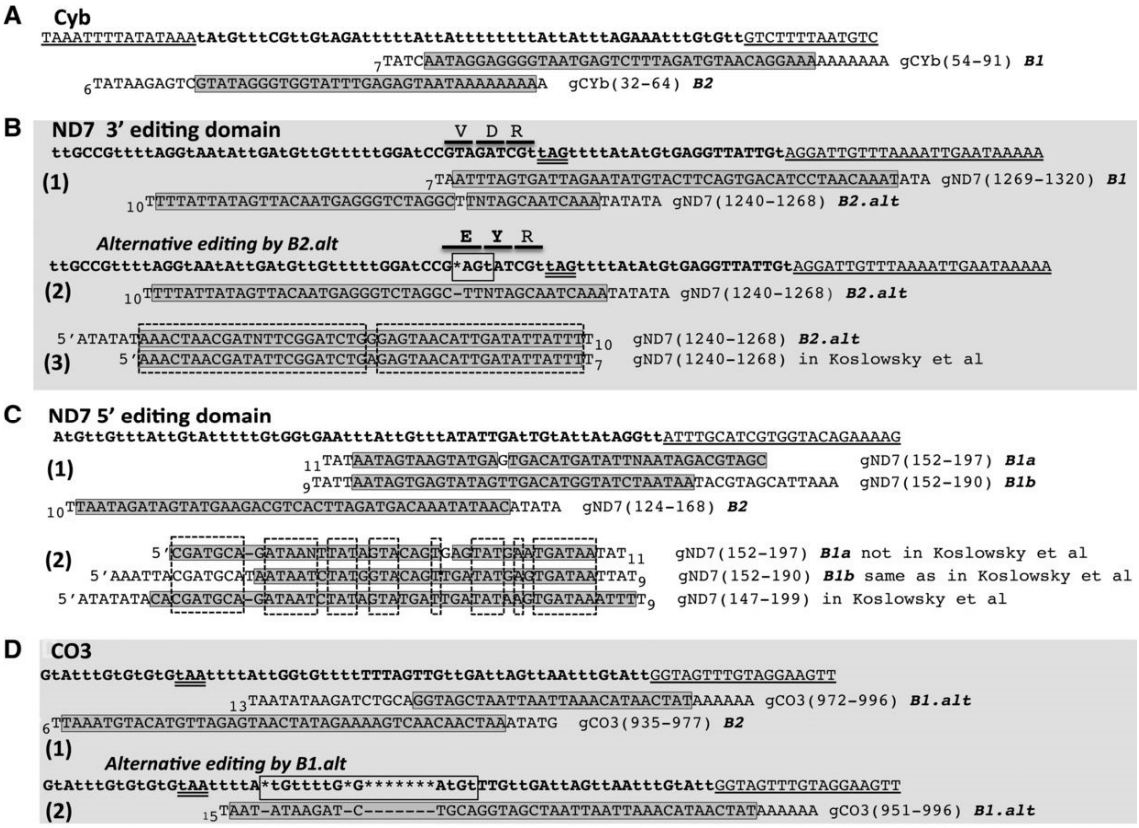

Figure 11. gRNA alignments at editing blocks B1 and B2 with currently annotated edited mRNAs, and predicted alternative editing patterns. Base-paired gRNA sequences are in 3′→5′ orientation. Regions of interest are identified as follows: editing domain (bold), mature mRNA sequence (underlined), gRNA guide domain (gray box), length of the 3′ U tail (subscript), and stop codon (double line). Proposed alternative U-insertions and U-deletions ("t" and "⋆", respectively, in a box) result in higher quality duplexes, i.e., longer guide domains and changes in encoded C-terminal amino acids (in ND7 3′ domain [*B*] and RPS12 [*F*]) or 3′ UTR (in CO3 [*D*], ND8 [*E*], and A6 [*G*]). No alternative editing is predicted for CyB (*A*) and ND7 5′ domain (*C*). Homology alignments of gRNAs are given in 5′-3′ orientation with identities (dotted boxes) and guide domains (gray boxes). Aligned gRNAs from the study in EATRO 164 cells by Koslowsky et al. (2013) are indicated. The gRNA numbering uses standard nomenclature indicating paired nucleotide positions in edited mRNAs. The arrow in ND8 indicates an extra guiding "A" in B1.alt. The arrow in RPS12 indicates a position in the guide domain of gRPS12(267–322) that forms an A·C mismatch with mRNA.

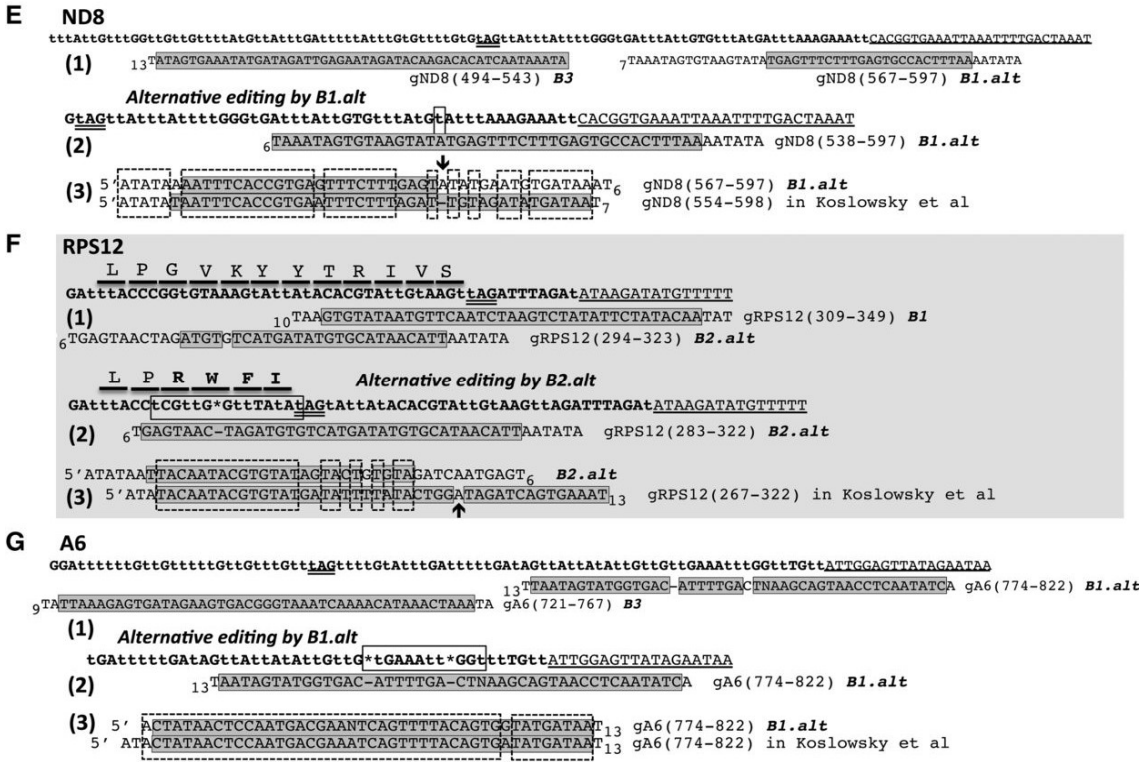

Figure 11. Continued.

Native H2 and 3010 MRBs specifically copurify with pre-edited and edited mRNAs

MRB1 complexes are only known to associate with gRNA (209, 212, 217). Because protein subunits of our immunopurified MRB samples including H2 photo-crosslinked with a synthetic mRNA (Figure 9E and 9F), we considered that H2-MRB, but possibly also 3010-MRB, purifications could contain endogenous mRNAs. Mitochondrial mRNAs may associate with MRBs or with transiently bound factors including RECC or mitoribosomes (209, 212, 302). Initial primer extension assays of purified H2-MRB and 3010-MRB detected some mRNA ND7 but no other mRNAs (data not shown). Subsequent qPCR assays of purified MRBs and a mock IP revealed a

robust enrichment of edited and pre-edited mRNAs (ND7, CYb, A6, and Murf2) relative to never-edited mRNA (COI), nuclear 18S rRNA, and cytosolic tubulin (Figure 12A). Housekeeping transcripts, usually carried over during purification, served as a reference in our assays. Because editing progresses in a 3′-5′ direction and the amplicons (~50–100 bp) score 5′ edited (or pre-edited) targets, the corresponding downstream sequences are presumed to be correctly edited. Interestingly, both the relative abundance and ratios of edited and pre-edited targets differ substantially between MRBs. Also, while all tested edited and pre-edited mRNAs were significantly more enriched than COI, the trend appeared somewhat more consistent in edited transcripts. Relative to the mock IP, edited ND7 was enriched several thousandfold, while edited CYb was enriched only about a hundredfold. In contrast, COI (never-edited) and CYb and A6 mRNAs (edited and pre-edited) exhibit comparable steady-state levels (Figure 12B). Although, edited ND7 was about one-thousandfold more abundant than other mRNAs at steady-state (i.e., it exhibits the lowest dCq), this difference was still several times smaller than the enrichment fold of edited ND7 in the 3010-MRB sample. Should the mRNAs described above be largely bound to mitoribosomes contaminating our samples, never-edited mRNAs would be enriched, and pre-edited mRNAs should not be present (302). Altogether, these data suggest a selective copurification of MRBs with mRNAs that undergo editing, relative to never-edited COI and housekeeping transcripts.

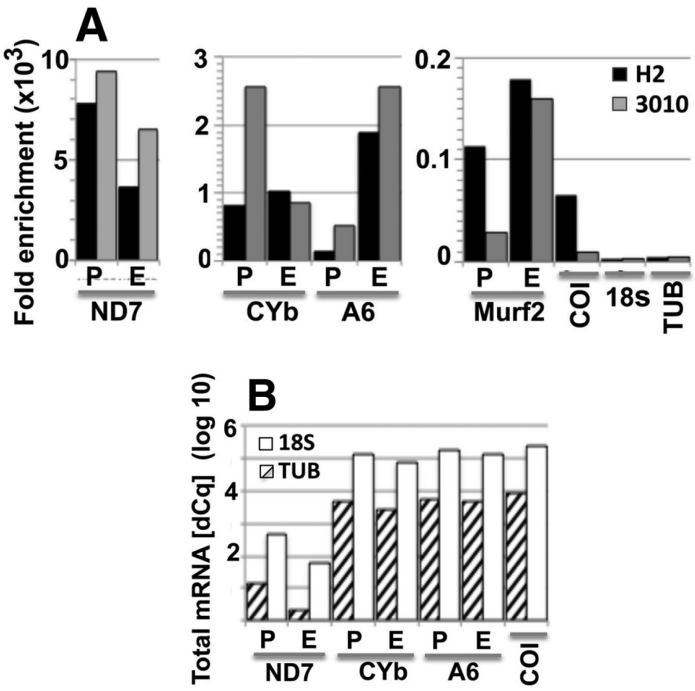

Figure 12. H2-MRB and 3010-MRB copurify with pre-edited and edited mRNAs. (*A*) Fold-enrichment in IPs of the samples normalized to the mock IP in qPCR assays. (E) Edited, (P) pre-edited (P), (COI) never-edited COI mRNA, two reference transcripts: (TUB) tubulin mRNA and (18S) nuclear 18S rRNA (Fold = 2^{-ddCq}, where ddCq = Cq test IP−Cq mock IP). (*B*) dCq of steady-state mRNAs in mitochondrial extract relative to background tubulin mRNA or nuclear 18S rRNA used as reference (dCq = $2^{(\text{Target Cq}-\text{Ref Cq})}$). For example, edited ND7 is more abundant than other edited mRNAs (i.e., has a lower Cq). All end-point amplicons in this study were single products during linear amplification, gel-purified and sequenced. Cq duplicates (STDEV ≤ 0.1) were averaged, and dilutions were adjusted to 100%.

Discussion

MRB1 complexes, isolated via epitope tags and specific antibodies, exhibit overlapping protein composition that has been discussed (183, 207–209, 213). However, the RNA component of these particles was unknown. Our current study of native MRBs with different protein composition indicated that these particles also differ in RNA composition. We distinguished two MRBs that contained either REH2 or MRB3010 subunits. Based on this work and our previous study (209), we propose that REH2 forms an MRB subcomplex that contains gRNA, unwinding activity, and several subunits that can be photo-crosslinked to RNA. REH2-associated unwinding may remodel RNA-protein interactions needed for efficient editing substrate association with REH2-MRB ((209); V Kumar, B Madina, and J Cruz-Reyes, unpubl.). Whether or not this activity affects editing substrate binding to other MRBs needs to be examined. REH2-dependent unwinding could also control global intra-strand mRNA structure or gRNA exchange during editing progression, as it was proposed for TbRGG2 and REH1, respectively (222, 259). It was proposed that RNAi-repression of REH2 reduces gRNA stability (217). We also saw some gRNA reduction at Day 6 of REH2 repression (209). However, this may be an indirect result of the late time point or a small but specific impact on metabolically stable gRNA. Revised analyses by us and by another lab indicate that robust REH2 depletion at Day 4 of RNAi does not significantly impact the gRNA steady-state level (Figure 17; R Aphasizhev, pers. comm.). MRB3010 may not bind gRNA but affects an unidentified early editing step (213). The REH2 and 3010 subcomplexes examined here contain the core GAP1 subunit. GAP1/2 homologs are

known to bind and stabilize gRNA (212). Interestingly, native REH2-MRB and 3010-MRB may share multiple RNA crosslinking subunits, suggesting a common RNA-binding core. So, REH2-MRB, 3010-MRB, and other MRB subcomplexes may transiently associate in higher-order MRB1 complexes that assemble editing substrates and RECCs creating an editing holoenzyme (Figure 13) (139, 183, 207), and dynamic contacts between the individual holoenzyme components may modulate the editing process, as well as productive interactions with mitoribosomes and other mitochondrial factors.

This study introduces NFPs (nucleotide frequency plots) of gRNAs, a simple but powerful tool that allows direct quantitative comparisons of gRNA content in purified complexes and total mitochondrial RNA. These NFP analyses provide (1) annotation of specific gRNAs at base resolution within editing domains, and (2) a measure of cumulative guide RNA potential at single and overlapping editing blocks in the parasite and purified complexes. This information is important because multiple redundant gRNA genes from polymorphic or entirely different minicircles often contribute to editing of the same block. Consistent with a role of the MRB3010 subunit in editing initiation, our NGS study of the first editing blocks in several mRNAs indicated a higher accumulation of initiating gRNAs in 3010-MRB than in H2-MRB. We note that similar amounts of gRNA (scored by capping of 5′ triphosphate ends) from IPs of REH2 and MRB3010 were used in the construction of Illumina libraries. This was largely confirmed given that counts go up in some gRNAs and down in others between the libraries. It is possible that MRB3010 promotes specific recruitment or retention of

accumulated initiating gRNAs in its subcomplex. A selective accumulation of initiating gRNA was especially clear for ND7 and ND8 but was also clear for other mRNAs. A partitioning of initiating gRNAs between MRBs, together with the observed low steady-state level of several initiating gRNAs in the parasite, both in our study and a recent report (303), suggest that recruitment or usage of these particular gRNAs is controlled *in vivo*. Currently, we are working to determine the impact of initiating gRNA enrichment on the target mRNAs. Interestingly, the study by Koslowsky did not find one or more gRNAs for half the mRNA substrates, implying that rare gRNAs may stall editing progression.

Purified MRBs have only been reported to contain gRNA; however, we found between ten- and several thousand-fold specific enrichment of edited and pre-edited mRNAs in immunoselected MRBs relative to a mock IP. Housekeeping 18S rRNA and tubulin mRNA were not enriched, and the never-edited mRNA COI may be slightly increased in the H2-MRB sample. MRB subcomplexes may specifically bind edited and pre-edited mRNAs in addition to gRNA, because RECCs are substoichiometric in purifications of MRB1 (Figure 9B; (209, 212, 221)), and natively purified RECC was reported to be largely devoid of RNA (142). In contrast to our purifications of native MRBs, mitoribosomes associate with never-edited, but not with pre-edited, mRNAs (302). So, assembled MRB1 complexes may store and route edited mRNAs from RECCs into ribosomes (Figure 13).

REH2 and the GAP1/2 homologs are the only known RNA-crosslinking subunits in the context of purified MRBs, but other RNA-binding proteins and their cognate RNA

targets *in vivo* need to be identified. The mRNA probe in our crosslinking assays may or may not reflect the cognate target's specificity (209), and REH2 may potentially bind gRNA, mRNA, or both. Recombinant versions of TbRGG2 and the paralogs MRB8170 and MRB4160 were reported to preferentially crosslink *in vitro* with synthetic sequences resembling cognate mitochondrial mRNA (221, 301). Strong GAP1/2-dependent stability of gRNA *in vivo* and preferential crosslinking of a synthetic gRNA with GAPs in their purified MRB point to these transcripts as their natural target (212). The common ~100-kDa crosslink in native H2-MRB and 3010-MRB in our assays (Figure 10A) may be induced by MRB8170, its paralog MRB4160, or both.

Several of the gRNAs, aligned to annotated mRNAs from early studies, predict alternative 3′ editing patterns within 3′ UTR or ORF regions that could impact mRNA stability, translation efficiency, or the encoded C-terminal amino acid sequence. Notably, our study in Lister 427 procyclic cells and recent analyses in a different strain (EATRO 164 cells) indicate important differences in minicircle content or expression, including strain-specific gRNAs, e.g., gRNAs detected in our study but not in Koslowsky's. Continuing analysis of mRNA 3′ ends in our lab is consistent with our proposed alternative 3′ editing between strains (B Madina, V Kumar, and J Cruz-Reyes, unpubl.). The library of total gRNA examined by these authors achieved significantly higher depth than ours, as our preparations were limited by the gRNA amount extracted from purified MRBs. A high evolution rate of minicircles may create some variability in mitochondrial function among strains and thereby add adaptive potential in *T. brucei*. However, essential edits may be under strong selection pressure, as is illustrated by CYb

gRNA genes in the same EATRO 164 cells used recently and in the early 1990s (303, 308) that have identical guide sequence but highly diverged nonguiding termini.

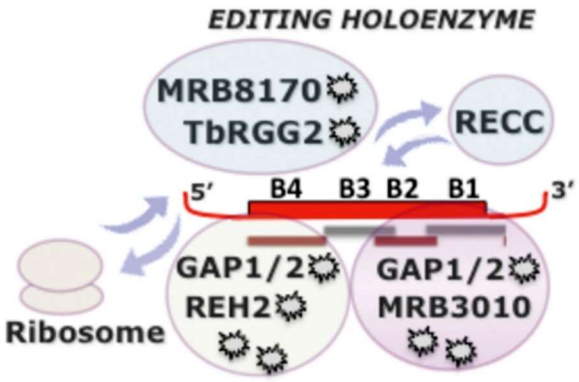

Figure 13. Working model. Dynamic higher-order MRB1 "organizers" composed of several specialized subcomplexes (MRBs) with different RNA/protein composition may coordinate recruitment of editing substrates (pre-edited mRNA and gRNA) and catalytic RECCs forming an editing holoenzyme. MRBs may bind edited mRNAs and route them into ribosomes by undefined "hand-over" mechanisms. Native helicase REH2 binds RNA, presumably gRNA, mRNA, or both, and forms a stable MRB with editing substrates, unwinding activity, and several subunits including other RNA-binding proteins (stars). REH2-associated unwinding seems to promote stable assembly of editing substrates with REH2-MRB but could also affect other MRBs and editing progression. The MRB3010 subunit affects an early editing step and may not bind RNA directly but forms a separate stable MRB. The current study showed that the MRB3010-subcomplex associates with editing substrates and edited mRNA and suggests that it promotes early editing through its preferential recruitment of initiating gRNAs. Both purified REH2-MRB and MRB3010-MRB contain GAP1/2 subunits that bind and stabilize gRNA. A common RNA-binding core in MRBs may include GAP proteins and several RNA crosslinking subunits that we detected but need to be identified. Dynamic interactions between these MRBs and additional factors (MRBs and non-MRBs not shown in the cartoon) may control substrate recruitment and interactions during initiation and progression between blocks (e.g., B1-to-B4). A proposed TbRGG2 MRB-like subcomplex does not contain REH2, MRB3010, or GAP proteins (83, 208). Distinct RNA/protein composition of MRBs may also impact transient associations with RECCs, mitoribosomes, and other mitochondrial factors. Recombinant proteins known to crosslink with synthetic RNA (stars) include the following: GAPs with gRNA, and the 4160/8160 paralogs and TbRGG2 preferentially with mRNA-like fragments. The initiation gRNA anchor anneals just 3′ of the editing domain. Later anchors often need edited sequence. Whether gRNAs remain annealed to edited mRNA is unknown.

Supporting information

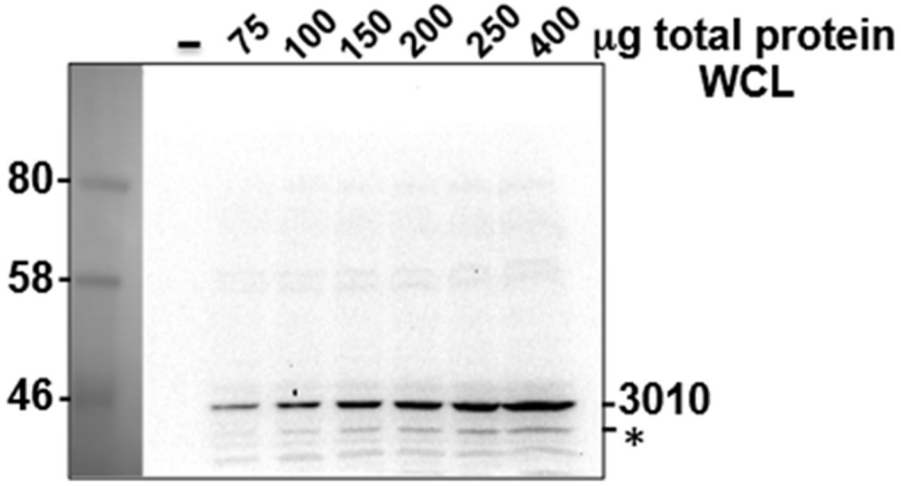

Figure 14. Specificity of affinity-purified anti-MRB3010 polyserum. Western blot of native MRB3010 at increasing concentrations of whole cell lysate (WCL) from *T. brucei* 29:13 cells. A Blast search of the peptide sequence used as antigen in rabbit produces a unique alignment to this protein in *T. brucei* (data not shown). A crossreacting band below MRB3010 represents some breakage of MRB3010 in the lysate (*).

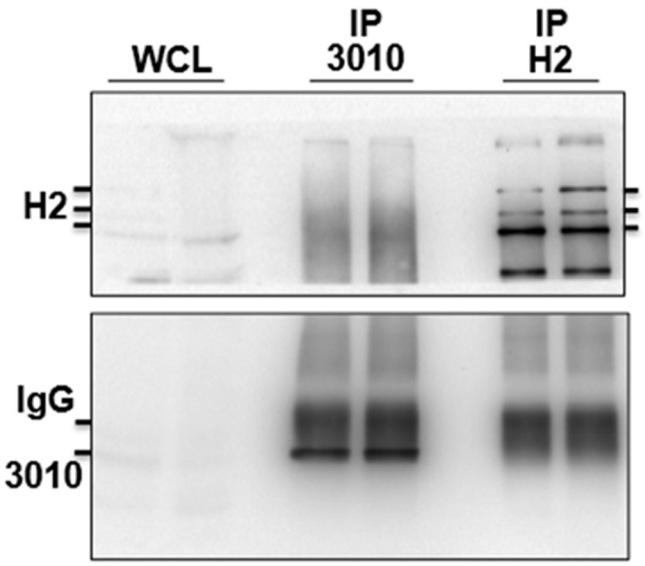

Figure 15. Native immunopurified subcomplexes of MRB1 that contain either REH2 (H2) or MRB 3010 (3010). Western analysis of H2 and 3010 in IP replicates with anti-REH2 and anti-MRB3010 antibodies from a whole cell lysate (WCL) from *T. brucei* 29:13 cells. H2 (~240kDa) is often significantly fragmented in WCL, and 3010 (57.5 kDa) migrates slightly below IgG in the IPs, which is used as a loading control. The blot shown was split into two halves. Each half was treated separately with the indicated antibodies.

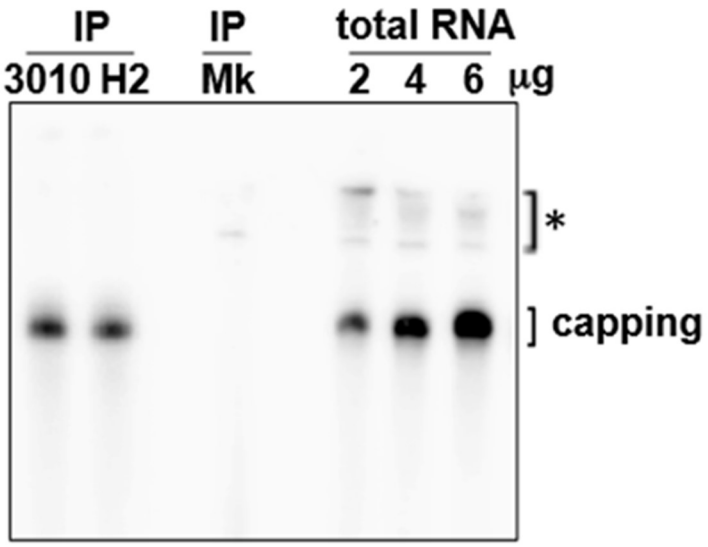

Figure 16. Radiolabeled capping of gRNA in immunoprecipitations of REH2 and MRB3010 and titration of total RNA in mitochondrial extract. IPs from ~2mg of the pre-cleared mitochondrial extract were performed as described in the methods section. Radiolabeled samples in direct IPs and total RNA dilutions were used as control to adjust identical uncapped samples and used comparable gRNA amounts in the Illumina libraries. Capped and uncapped samples were prepared in parallel and identical cuts of the latter were obtained. Eluted RNAs were ligated to 3' barcoded adaptors, and multiplexed prior to phosphatase/PNK treatment to add a single phosphate, 5' adaptor ligation, and RT-PCR. The asterisk indicates non-specific capping products usually detected in total RNA and mock IP samples but not in REH2 and MRB3010 IPs.

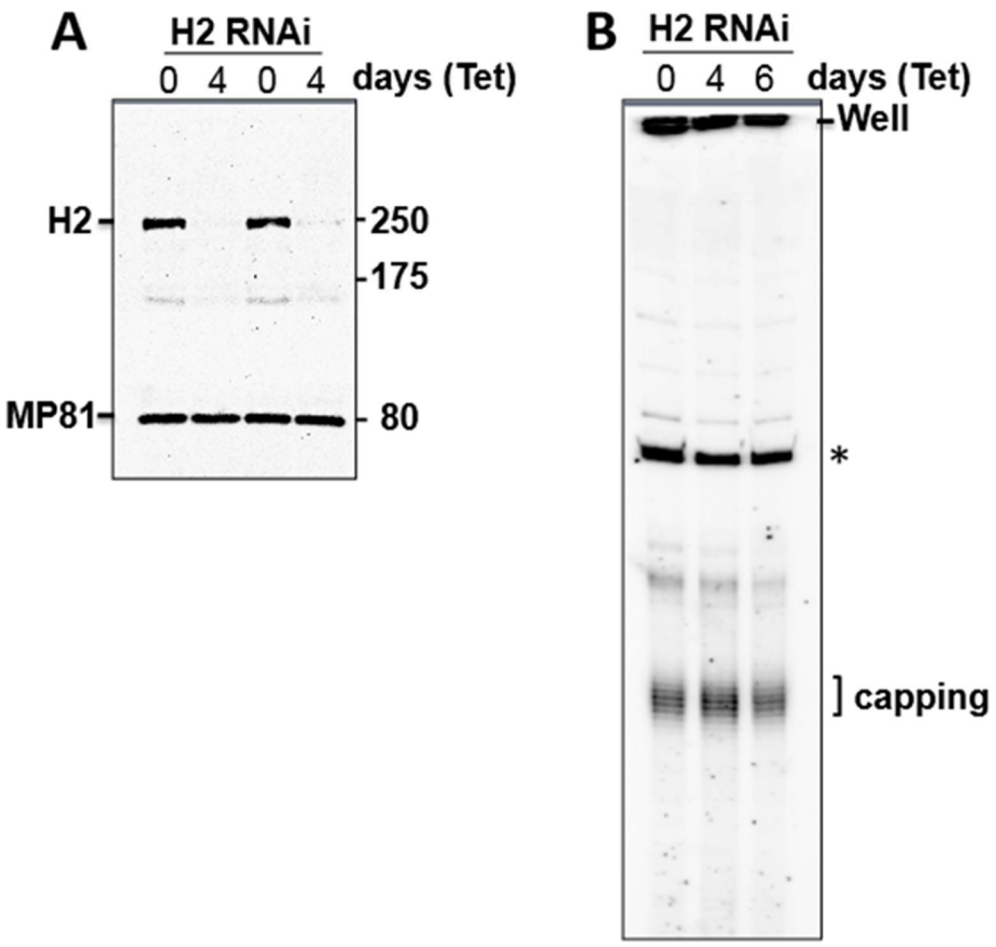

Figure 17. RNAi-repression of REH2 has no major effect on gRNA steady-state at day 4 post-induction.(A) Western blot of REH2 in whole-cell lysates upon REH2-RNAi repression to 0 and 4 days post-induction with tetracycline (Tet). The mitochondrial MP81 subunit of the RECC complex examined with specific antibodies is a control for sample loading and RNAi specificity. (B) [^{32}P]G-capping of gRNA 5' triphosphate with guanylyltransferase in whole-cell lysates upon REH2-RNAi repression at 0, 4 and 6 days post-induction. An asterisk denotes a non-specific labeled product typically seen in crude lysates and extracts that serves as loading control. The gRNA was resolved in 10% UREA-PAGE.

CHAPTER III

NATIVE VARIANTS OF THE MRB1 COMPLEX EXHIBIT SPECIALIZED FUNCTIONS IN KINETOPLASTID RNA EDITING*

Summary

Adaptation and survival of *Trypanosoma brucei* requires editing of mitochondrial mRNA by uridylate (U) insertion and deletion. Hundreds of small guide RNAs (gRNAs) direct the mRNA editing at over 3,000 sites. RNA editing is controlled during the life cycle but the regulation of substrate and stage specificity remains unknown. Editing progresses in the 3' to 5' direction along the pre-mRNA in blocks, each targeted by a unique gRNA. A critical editing factor is the mitochondrial RNA binding complex 1 (MRB1) that binds gRNA and transiently interacts with the catalytic RNA editing core complex (RECC). MRB1 is a large and dynamic complex that appears to be comprised of distinct but related subcomplexes (termed here MRBs). MRBs seem to share a 'core' complex of proteins but differ in the composition of the 'variable' proteins. Since some proteins associate transiently, the MRBs remain imprecisely defined. MRB1 controls editing by unknown mechanisms and the functional relevance of the different MRBs is unclear. We previously identified two distinct MRBs,

*Reprinted with permission from "Native variants of the MRB1 complex exhibit specialized functions in kinetoplastid RNA editing" by Bhaskar R. Madina, Vikas Kumar, Blaine H. M. Mooers, Jorge Cruz-Reyes. PLoS ONE. 2015. 10(4): e0123441.

and showed that they carry mRNAs that undergo editing. We proposed that editing takes place in the MRBs because MRBs stably associate with mRNA and gRNA but only transiently interact with RECC, which is RNA free. Here, we identify the first specialized functions in MRBs: 1) 3010-MRB is a major scaffold for RNA editing, and 2) REH2-MRB contains a critical trans-acting RNA helicase (REH2) that affects multiple steps of editing function in 3010-MRB. These *trans* effects of the REH2 include loading of unedited mRNA and editing in the first block and in subsequent blocks as editing progresses. REH2 binds its own MRB via RNA, and conserved domains in REH2 were critical for REH2 to associate with the RNA and protein components of its MRB. Importantly, REH2 associates with a ~30 kDa RNA-binding protein in a novel ~15S subcomplex in RNA-depleted mitochondria. We use these new results to update our model of MRB function and organization.

Introduction

Trypanosoma brucei and other kinetoplastid protozoa have a single mitochondrion with an unusual mitochondrial genome (kDNA) consisting of many copies of an identical "maxicircle DNA" (~23 kb) and several hundred different types of "minicircle DNAs" (~1 kb). Maxicircle DNA encode 18 mRNAs, most of which require maturation through a remarkable form of RNA editing that changes the length of the mRNA by insertion or deletion of uridylate (U) nucleotides. In contrast, each minicircle DNA encodes 3–4 genes for small guide RNAs (gRNAs) that bind the pre-mRNA in *trans* and direct editing at more than 3000 sites (69, 298). gRNAs (~45–60 nt) are primary transcripts with a 3' tail of ~10–15 Us added post-transcriptionally. Each gRNA

has base-sequence complementary to the fully edited mRNA sequence (308). RNA editing progresses from 3' to 5' along the mRNA through sets of overlapping sequence blocks; the editing of each block is directed by a different gRNA. This process also exhibits substrate specificity during the life cycle, but the mechanisms involved remain unknown (183). Most mRNAs are extensively edited, while a few require limited editing or are never edited (130). This process often creates start or stop codons, and often generates most of the open reading frame. A mistaken insertion or deletion of a single U creates a frameshift in the mRNA and could create a nonfunctional protein during translation. Surprisingly, mitochondria contain many partially edited mRNA transcripts. Partial editing is always located in a junction in the RNA between the 5' unedited and 3' fully edited sections (309, 310).

The editing enzyme, known as the RNA editing core complexes "RECC" or editosomes, has been extensively studied (183–185), but many central questions remain unanswered. For example, how are the substrates recruited, and how are editing initiation and progression controlled? This is particularly puzzling because the purified RECC enzyme lacks the editing processivity found *in vivo* (186–188) and early studies showed that it does not contain endogenous gRNA or mRNA (142). A substantial number of non-RECC proteins that control editing have been reported (65, 183, 207). Several of these latter proteins are components of a large accessory complex, MRB1, which binds and stabilizes gRNA, and transiently interacts with the RECC (212, 221). Most proposed MRB1 subunits lack recognizable sequence motifs, reflecting the early phylogenetic divergence of kinetoplastids (311). Some proteins may be part of a putative

core in MRB1, while others appear to be variable components. The functional relevance of the variation in the composition of the MRBs remains unclear (209, 213). Inducible RNAi of select MRB1 proteins have suggested effects in editing at an early stage or during progression (209, 213, 222). Certain proteins primarily affect specific mRNAs, while others have broader effects (221, 301), implying substrate preferences. We have reported two native MRB1 variants (termed here "MRBs"), REH2-MRB and 3010-MRB, with clear differences in protein and RNA composition. Importantly, these MRBs carry unedited and fully edited mRNAs, in addition to gRNA. Our findings suggested that these complexes assemble mRNA-gRNA hybrids and raised the possibility that different MRBs exhibit specialized functions (1). A more recent report from another lab confirmed that MRB1 contains mRNAs (215). REH2-MRB and 3010-MRB have a differential partition of several initiating gRNAs and of the RNA helicase REH2 and MRB3010. RNAi knockdown of either protein inhibits editing and the latter was proposed to be important in early editing (209, 213). Notably, deep sequencing studies in total mtRNA in the Koslowsky's lab and in purified MRBs in our lab found that most initiating gRNAs are rare (1, 303). These results suggest that the initiation of editing may be regulated.

In our model of MRB1 function and organization (1), that we further test here, MRB1 variants serve as scaffolds for the assembly of mRNA-gRNA hybrids and the RECC enzyme, and these variants can be linked to specific roles in RNA editing. Specifically, we hypothesized that 3010-MRB supports efficient editing initiation and that REH2 is a trans-acting factor of 3010-MRB. Our findings indicate that 3010-MRB

is relatively enriched with mRNAs that are edited at the first block directed by the initiating gRNA and that REH2 affects multiple editing steps in mRNAs associated with 3010-MRB. Furthermore, REH2 binds its native MRB via RNA, and point mutations in conserved motifs of the helicase inhibit its association with RNA and protein components of the REH2-MRB. Finding functionally distinct MRBs that include regulatory proteins and all mRNAs involved in editing, i.e., unedited, partially edited and fully edited transcripts, raises a number of important mechanistic questions that can now be directly addressed in the RNA editing of early-branched kinetoplastids.

Materials and methods

Cell culture

T. brucei Lister strain 427 29–13 procyclic "PF" (tryps.rockefeller.edu) was grown axenically in log phase in SDM79 medium (312) and harvested at a cell density of 1-3x10^7 cells/ml. Cell lines expressing TAP-REH2 variants or a construct for REH2 down-regulation were induced with tetracycline at 1 μg/ml.

REH2 plasmid constructs

We introduced point mutations K1078A and A1086D in the double-stranded RNA binding domain (dsRBD) of REH2 by PCR-based site-directed mutagenesis of a pLew79 TAP-REH2 construct (209) using a proofreading thermostable polymerase mix (AccuTaq, Sigma) and oligonucleotides described in Table 6. We made an RNAi construct by PCR amplification of a 344-bp fragment from the REH2 3'UTR region using oligonucleotides described in Table 6, and we cloned this fragment into the XhoI

and BamHI sites of p2T7-177 (313). All constructs were confirmed by DNA sequencing, linearized with NotI, and transfected in procyclic 29–13 trypanosomes (312).

Protein and RNA purification from the pulldowns

We performed immunoprecipitation of REH2, MRB3010, and cytochrome oxidase 2 (mock) using affinity-purified peptide antibodies as described (1). Briefly, specific antibodies were conjugated to Dynabeads Protein A (Life Technologies) that were pre-treated with 5% BSA. Approximately 2 mg of mitochondrial extract was supplemented with 1X Complete Protease Inhibitor cocktail (Roche) and SUPERase·In RNase inhibitor (Life Technologies). The extract was pre-cleared by passage over Protein A-Sepharose beads (GE Healthcare) before it was loaded onto antibody-conjugated beads. Ectopically expressed TAP-REH2 was specifically immunopurified using Dynabeads IgG (Life Technologies), as reported elsewhere (209). All washes were performed with 200 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, and 25 mM Tris, pH 8. Protein was extracted with 1X SDS loading buffer at 95°C for 2 min. RNA was extracted by treating the beads with 0.8U proteinase K (NEB) for 30 min at 55°C, followed by phenol extraction and ethanol precipitation.

Glycerol gradients, western and northern blots, and radioactive assays

Sedimentation fractions were obtained from freshly made mitochondrial extracts in 10–30% glycerol gradients (209). Western blots of REH2, MRB3010 (3010), GAP1, and MP63 (a RECC subunit used as a ~20S marker) were performed as reported (209, 314). Crosslinking assays used gRNA gA6 B1.alt (1) as a model initiating gRNA

bearing a photo-reactive 4thio-U and ^{32}P that was mixed with immunopurified MRBs and subjected to 365 nm UV irradiation on ice as previously described (305, 306). The photo-reactive gRNA was prepared by a splint ligation with the oligonucleotides described in Table SI (305). Radioactive capping of gRNA used RNA extracted from the Dynabeads protein A pulldowns (209). Northern blots of total mitochondrial RNA or IPs from mitochondrial lysate, extracted using TRizol LS reagent (Life Technologies) and proteinase K (NEB), respectively, used 5' labeled probes for the initiating gRNAs gND7 3' domain B1, gCyB B1 (1, 67), and for tRNA-Cys (Table 6). These procedures were performed in ULTRAhyb solution (Life Technologies) with 2X SSC washes at 40°C.

Quantitative RT-qPCR and endpoint RT-PCR of mRNAs

RNA from pulldowns was treated with RNase-free DNase (Thermo) and used in the preparation of cDNA as described elsewhere (1). RT-qPCR assays normalized using the delta delta CT "ddCT" (Livak) method (184, 315) were performed in 20 microliter reactions with the primers reported to be specific for unedited mRNAs, edited at a 5' distal block, and reference transcripts (184), or primers designed by us in the present study to analyze early 3' editing (Table 6) in a SYBR Green PCR Master Mix (Bio-Rad). RT-PCR of RPS12, A6, and ND7 fragments was performed using PerfeCTa SYBR Green FastMix (Quanta) with specific oligonucleotides with unedited or never-edited sequences (Table 6), and analyzed on 8% native acrylamide gels. All amplicons in this study were verified by cloning and manual sequencing. cDNA at different dilutions and no-RT controls were tested to confirm the linearity and specificity of the amplifications.

Cloning and sequencing of mRNA block 1 sequence

RNA extracted from REH2 and 3010 antibody pulldowns was C-tailed with Poly A Polymerase (NEB) and CTP. The C-tailed RNAs were used for cDNA synthesis with Superscript III reverse transcriptase (Invitrogen) and a 3' polyG-ended RT primer (Table 6). This cDNA was amplified with the RT primer and a transcript-specific oligonucleotide using Taq polymerase (NEB). PCR products were isolated from a 10% native PAGE and re-amplified by nested PCR (Table 6). The final amplicons were analyzed on 10% native acrylamide gel or were gel isolated, cloned (StrataClone PCR Cloning Kit, Agilent), and sequenced.

Structural analyses of REH2

Domain annotations in REH2 including the previously unidentified OB-fold domain were performed using the Conserved Domain Search tool (CD-Search) at NCBI (316). The mot I mutations were modeled using a homology model of the helicase portion (residues 1308 to 1846) of *T. brucei* REH2. The coordinates of the ADP were from the crystal structure of a yeast Prp43p/ADP complex (2XAU) (313) after superposition of the crystal structure of the complex onto the homology model of REH2 which had been made with Phyre2 (317). The mutations were made using the backbone dependent rotamer library (318) in PyMOL. The selected glutamine rotamer was the only rotamer that lacked steric clashes and that had favorable interactions with the surrounding atoms. The distances are in ångstroms.

Results

3010-MRB associates with mRNAs that exhibit efficient editing at block 1 directed by the initiating gRNA

We tested whether or not 3010-MRB and REH2-MRB are functionally different because they have distinct protein compositions and carry unedited and fully edited RNAs, and because 3010-MRB is enriched in initiating gRNAs (1). We began by comparing the efficiency of editing directed by the initiating gRNA in mRNAs associated with 3010 and REH2 MRBs. To this end, we established quantitative RT-PCR (RT-qPCR) assays for block 1 in mRNAs A6 (ATPase subunit 6) and ND7 (NADH dehydrogenase subunit 7) or the first few blocks in mRNA RPS12 (ribosomal protein subunit 12) (Figure 18). Our assays were based on confirmed 3' edited sequence described below (Figure 19 and Figure 29, and additional data not shown) and the recently identified gRNAs (1, 303).

The results from these assays showed a greater concentration of block 1 edited mRNA in 3010-MRB than in REH2-MRB (Figure 18A). We also scored unedited transcripts and found that the three tested mRNAs were enriched in 3010-MRB (Figure 18B), consistent with our previous study (1). Interestingly, the A6 edited pattern in block 1, which is located in the mRNA 3' UTR, matched nearly the entire initiating gRNA that was recently identified in two strains of procyclic trypanosomes (Figure 18C) (1, 303). Both edited block 1 and the initiating gRNA differed substantially from those originally reported in the 1990s (308), indicating a rapid evolution of the responsible minicircle(s). The sequence of the 3' edited mRNA ND7 was as reported earlier, except for one

residue (Figure 29). The first few blocks in mRNA RPS12 that we examined were also as originally reported. The edited sequence for block 2 in mRNA RPS12 was surprising because the guiding potential of a major gRNA in the Lister strain predicted an alternative editing pattern (1). Thus, the tested block 1 edited mRNAs and their unedited substrates are relatively more abundant in 3010-MRB than in REH2-MRB.

To determine if 3010 and REH2 MRB complexes carry mRNA intermediates with partial editing directed by the initiating gRNA, we sequenced the editing block 1 in A6 and ND7 mRNAs isolated from the complexes or from total mitochondrial mRNA (mtRNA) (Figure 19A–19C, and Figure 29). Briefly, we amplified the cDNAs with primers containing unedited sequences just 5' of block 1 and never-edited sequences just 3' of the first editing site. This sequencing strategy allowed us to survey the first few editing sites for editing efficiency. Although we analyzed only a few clones, it was clear that both 3010 and REH2 MRBs carry RNAs with partial editing at block 1. Interestingly, the examined transcripts from 3010-MRB exhibited more complete editing in the first sites than transcripts from REH2-MRB and the mtRNA population. A count of the edited sites in the clones analyzed above is shown in Figure 19D. Interestingly, editing in ND7 was not as extensive as it was in mRNA A6, suggesting a substrate preference. In fact, the sequenced ND7 transcripts from the REH2-MRB complex were unedited, consistent with the reported ratio of unedited to edited mRNA ND7 in this complex (1). Importantly, purified 3010 and REH2 MRBs in our experiments associate with comparable amounts of RECC (Figure 19E). Thus, both MRB complexes associate with the editing enzyme and contain all mRNAs involved in editing, i.e., substrates,

intermediates and products, indicating that both 3010 and REH2 MRB complexes are competent editing scaffolds. However, the above data together with our previous report (1) also indicate that the 3010-MRB complex is enriched in mRNAs with efficient editing at block 1 and the corresponding initiating gRNAs and pre-mRNA substrates.

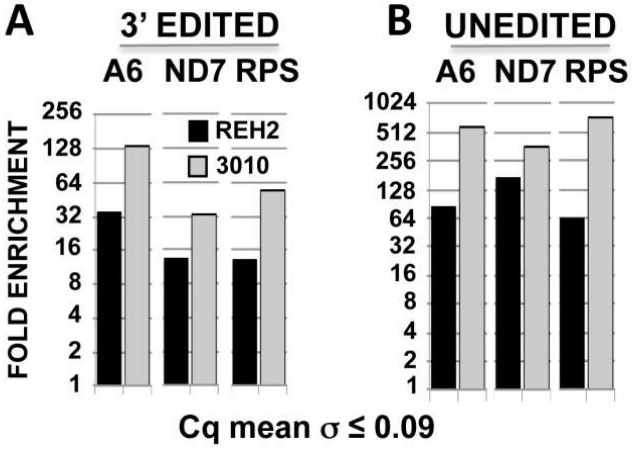

Figure 18. Quantitative analysis of early 3' edited and unedited mRNAs. (A) Enrichment of edited block 1 in mRNAs A6 and ND7, or the first few blocks in RPS12, in 3010 and REH2 IPs relative to a mock IP set at 1. RT-qPCR values of test IPs were normalized to input values and to a low nuclear 18S rRNA carryover in the beads as loading control. [Fold = 2^{ddCq}, in which *ddCq = IP dCq – Input dCq*, and *dCq = test Cq – 18S Cq*. Cq is the quantification cycle.]. Standard deviation of the average value of Cq duplicates is shown. All end-point amplicons were single products during linear amplification, and sequenced. (B) Relative fold enrichment of unedited pre-mRNA calculated as in panel B. (C) Amplified edited sequence in panel A. Edited 3' sequence in mRNAs A6, ND7, and RPS12 is aligned with a major initiating gRNA (in 3'→5' orientation) (1, 303). Regions of interest are identified as follows: mRNA editing domain (bold), never-edited sequence (underlined), gRNA guide domain (gray box), and length of the 3' U tail (subscript). PCR primers (arrows) were designed based on the sequenced 3' end of cloned cDNA fragments (Figure 19 and Figure 29, and data not shown). The U-insertions ("t") and deletions (not shown) allow high-quality duplexes with the guide domain of initiating gRNAs. All amplicons were cloned and sequenced. A previously annotated encoded T at position 1269 in block 1 of mRNA ND7 is not included because it was missing in the sequenced unedited transcripts from the Lister strain in this study (Figure 29).

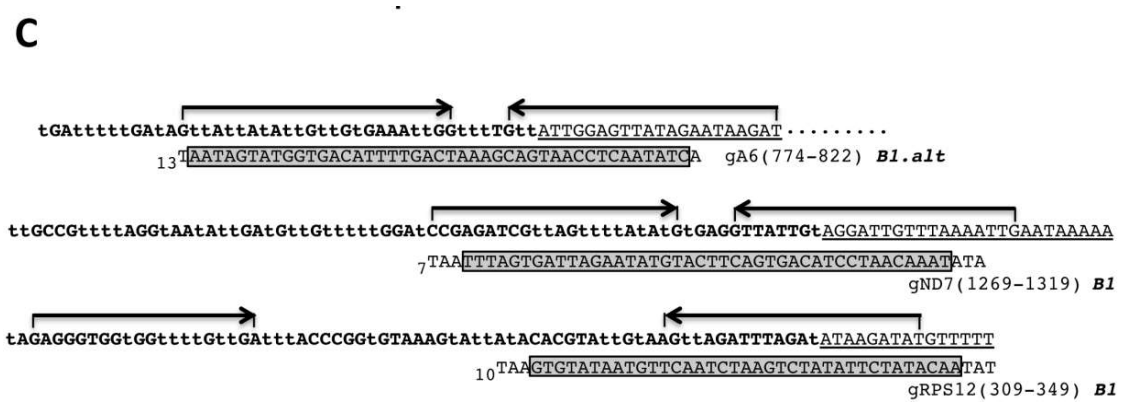

Figure 18. Continued.

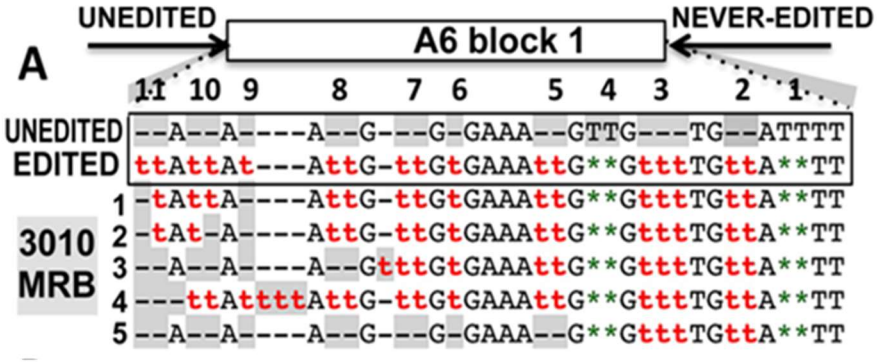

Figure 19. Efficient editing at block 1 in mRNAs bound to 3010-MRB. Block 1 sequence of a few mA6 transcripts amplified from (A) 3010 and (B) REH2 IPs or (C) total mtRNA from the mitochondrial extract loaded in the IPs. PCR primers (arrows) target 5' unedited and 3' never-edited sequence. The sequences in a box show sites 1-to-11 either unedited (gray) or fully edited with insertions 't' and deletions '★'. In clones 1-to-5, misediting (in number or site) is also shown in gray. (D) Number of correctly edited sites in the randomly selected clones of mRNAs A6 and ND7 (11 and 8 sites, respectively) purified from the IPs in panels A and B above and Figure 29 each bar is one site. The 3'-5' direction of editing (arrow) in block 1 (box) is indicated. (E) Radioactive autoadenylation of ligases REL1 and 2 in the RECC enzyme detected in REH2 and 3010 IPs. An asterisk marks a contaminant cytosolic ligase in mitochondrial extract input (IN). Pre-charged REL2 with endogenous ATP is poorly radiolabeled (152).

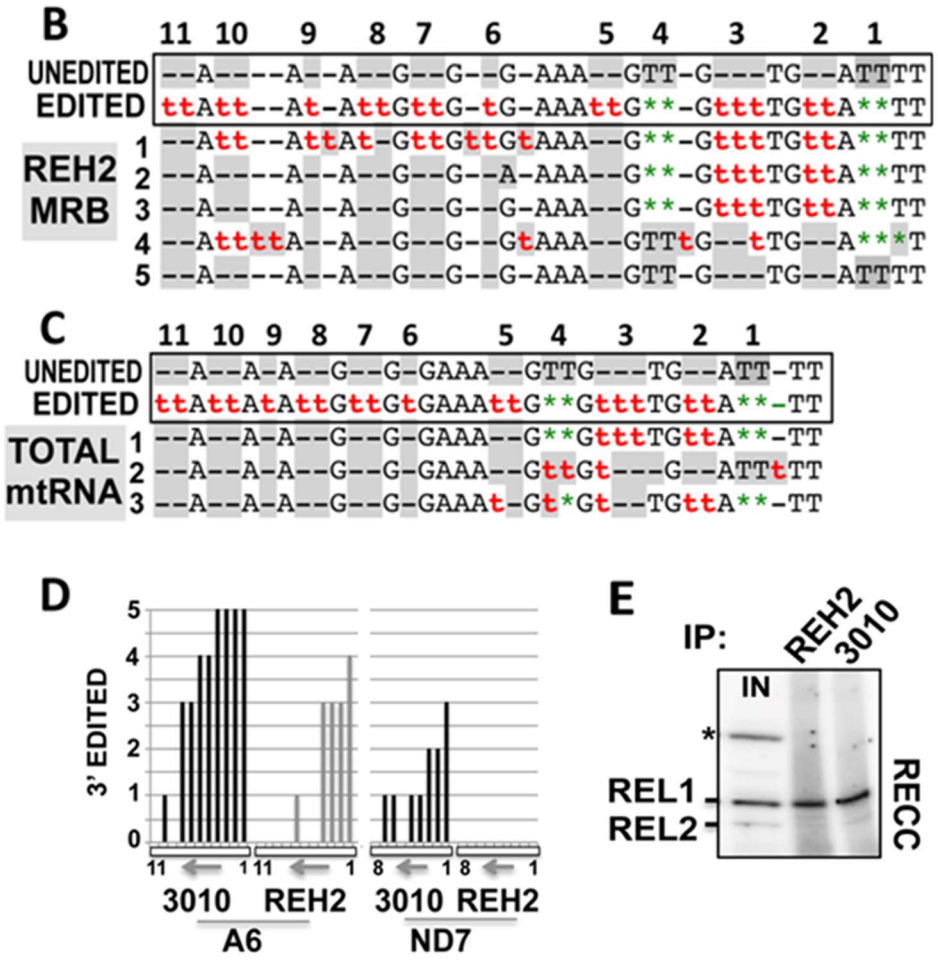

Figure 19. Continued.

REH2 affects the level of unedited pre-mRNA, and editing at block 1 and at upstream blocks in 3010-MRB

Although REH2 and 3010 are subunits of different MRBs, we asked if the REH2 helicase affects in *trans* the function or composition of the 3010-MRB complex. For example, REH2 may impact the initiation or progression stage of editing in 3010-MRB, or REH2 may impact the association of the substrate pre-mRNAs with this complex. Inducible RNAi of REH2 inhibited editing at early 3' sites on pre-mRNA substrates in total mtRNA (Figure 20A and 20B), but this REH2 depletion did not affect the cellular level of 3010 or the copurification of 3010 with the core GAP1 subunit, with RGG2 (another common protein in MRB purifications), total gRNA, or the RECC enzyme (Figure 20C). Northern blot analyses showed that the level of initiating gRNA gND7 in the total mtRNA and in the 3010-MRB complex was not substantially affected by depletion of REH2 (Figure 20D). A somewhat decreased level of the initiating gRNA (gCYb B1) in the complex is puzzling. A recent study suggested that gRNA recycling during editing leads to accumulation of gRNAs upon editing inhibition (215). In summary, these results indicate that the editing directed by the initiating gRNA requires REH2.

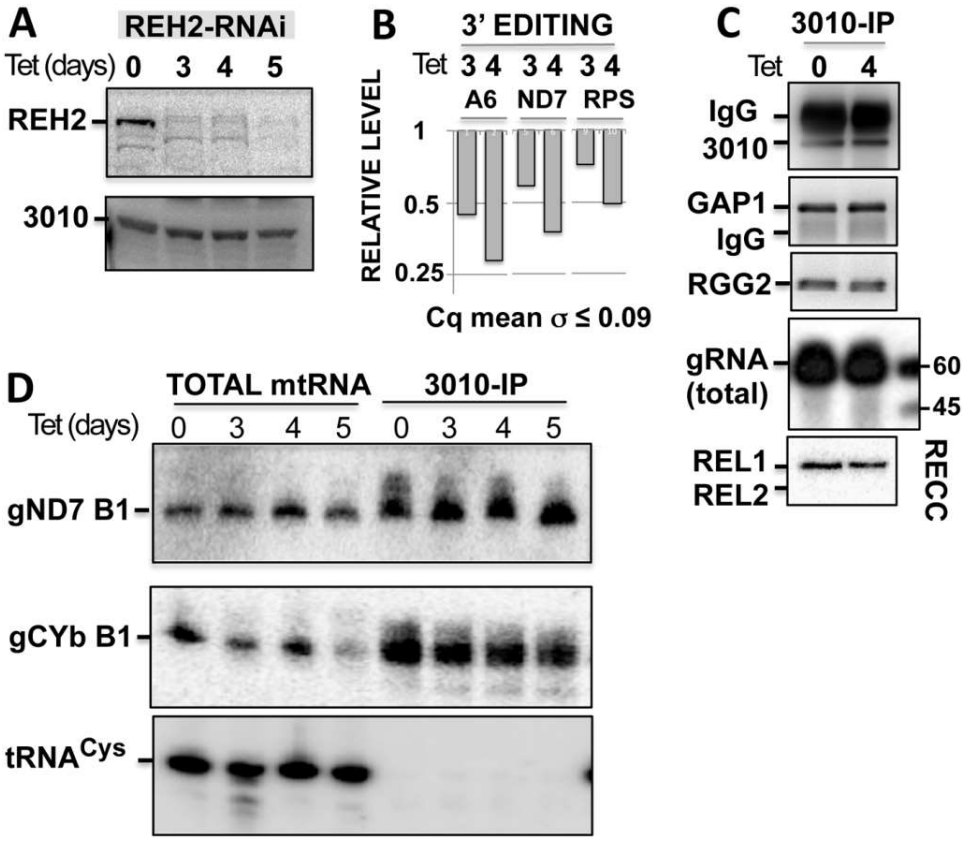

Figure 20. REH2 knockdown affects editing by the initiating gRNA in 3010-MRB but not 3010 association with common MRB1 proteins, gRNA or RECC. (A) Western blots of REH2 and 3010 in lysates of induced cells with tetracycline (Tet). Full length and proteolytic fragments of REH2 are detected in all figures in this study. (B) Relative level of early 3' editing in block 1 of mRNAs A6, ND7, and the first few blocks in RPS12 (RPS). RT-qPCR assays were performed in cell lysates using tubulin as the reference. Uninduced samples are set at 1. (C) 3010-IPs of mitochondrial extracts at 0 or 4 days post-induction in western blots of 3010 and GAP1, or radiolabeled capping (gRNA) and autoadenylation (editing ligases REL1/2). (D) Northern blots of initiating gRNAs for block 1 (B1), gND7 (1269–1319), and gCYb gCYb (54–91) in total mtRNA and 3010-IPs at multiple time points of RNAi induction. The blots were stripped and reprobed for tRNA-Cys, showing that the pulldowns are specific for gRNA.

Further analyses in mtRNA compared editing at early 3' sites and at a distal block sequence (late 5' sites) and showed greater inhibition of editing at the late sites (Figure 21). This suggests that editing progression across multiple blocks also requires REH2. In contrast, the levels of the unedited and never-edited mRNAs, and mitochondrial rRNA 9S remained relatively constant. Thus, REH2 depletion did not significantly affect unedited pre-mRNA at steady state. REH2 could, however, affect the level of unedited pre-mRNA in the 3010-MRB complex. To assess RNA association with the complex, we determined the ratio of mRNA in the 3010-IP and mitochondrial lysate input, i.e., IP/input (Figure 22). That is, regardless of the steady state level of pre-mRNA substrate (rather stable) and edited transcripts (decreased) upon REH2 depletion, we asked whether or not the proportion of molecules in the 3010-MRB complex and in the total mtRNA population is maintained. Notably, REH2 ablation decreased the ratio of unedited substrates in the 3010-MRB complex between 7 and over 10 fold at different time points of induction, particularly with RPS12. This change in the level of unedited pre-mRNA in the complex was confirmed by gel analysis of semi-quantitative RT-PCR amplicons (data not shown). In contrast, the ratio of edited RNA in the complex that we scored at early 3' sites or at a distal 5' block was maintained during the REH2 knockdown (Figure 22). Thus, while REH2 depletion decreased significantly the total amount of edited mRNA in the parasite, it did not significantly affect the ratio of associated edited molecules with the complex. This effect was consistently observed at multiple time points of the REH2 RNAi induction. Moreover, all observations can be made at the shortest time point (day 3) when the growth phenotype is first detected (not

shown). We controlled for secondary effects by showing normal steady state levels of MRB1 markers (e.g., 3010 and gRNA) and several mitochondrial transcripts in the total mtRNA pool (unedited and never edited mRNA, gRNAs, tRNA and 9S rRNA) at all time points of induction in these studies (Figure 20 and 21).

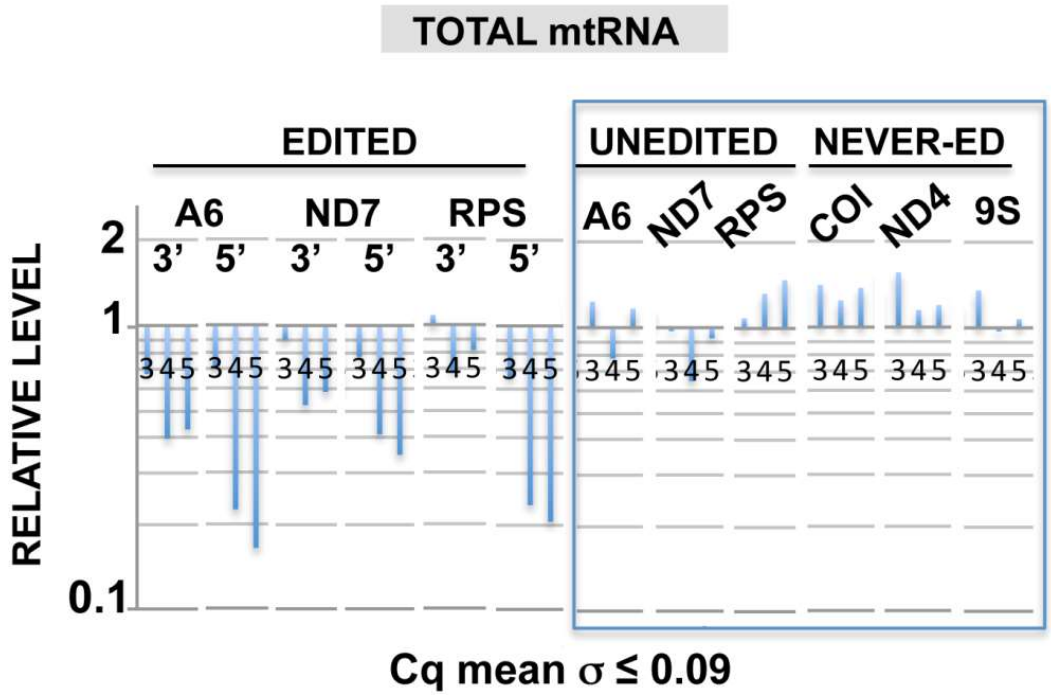

Figure 21. REH2 knockdown affects editing progression**.** (A) RT-qPCR of steady-state edited mRNA at block 1 in mRNAs A6 and ND7, and the first few blocks in RPS12 (3' sites) or a distal block (5' sites), unedited or never-edited RNAs at days 3, 4, and 5 of REH2 RNAi. Uninduced samples are set at 1.

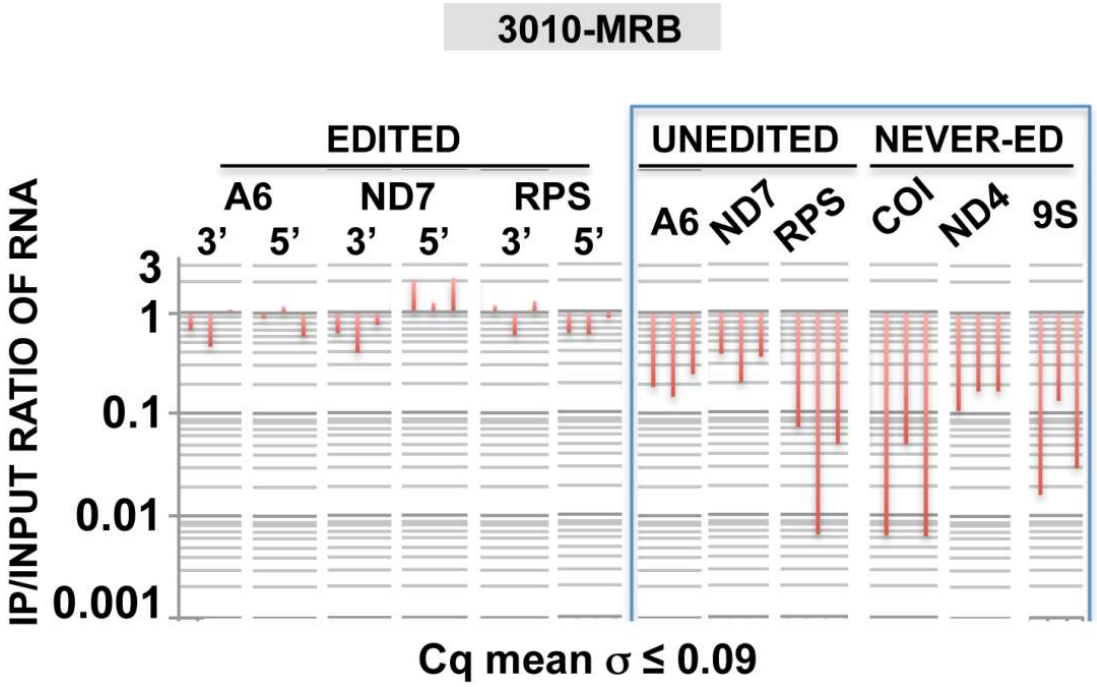

Figure 22. REH2 knockdown decreases the content of unedited pre-mRNAs in 3010-MRB. The ratio of select transcripts in IP samples and mitochondrial extract input (IP/input) was determined. Each RNA transcript was scored by RT-qPCR at days 3, 4 and 5 of REH2 RNAi. Uninduced ratios are set at 1. Standard deviation of the average value of Cq duplicates is shown. All amplicons were validated by cloning and sequencing. 18S rRNA in lysates and beads was used as reference.

Interestingly, REH2 depletion also caused a substantial loss of never-edited mRNA and 9S rRNA in the 3010 pulldowns indicating a decreased association of the purified 3010-MRB with mitoribosomes (1). Never-edited mRNA is thought to directly bind ribosomes (302). Thus, depletion of REH2 helicase appears to affect the substrate content of the 3010-MRB as well as the association of this complex with mitoribosomes.

The pool of transcripts in the mitochondrion includes unedited, fully edited, and partially edited RNAs of different sizes. To further analyze the REH2 effects, we amplified the entire ~200 nt mRNA RPS12 using primers targeting never-edited

sequences at the termini of the transcript, and we visualized the products in a gel (Figure 23A and 23B). In total mtRNA from REH2 knockdown cells, the amount of fully edited RNA decreased, while the amount of unedited RNA remained fairly constant (Figure 23A). However, in the purified 3010-MRB, the amount of unedited RNA decreased (Figure 23B). Furthermore, the complex accumulated partially edited molecules of various sizes in a pattern suggesting preferential sites for pausing. These findings support the idea that REH2 acts at multiple editing steps in pre-mRNAs associated with 3010-MRB. Analysis of mRNAs ND7 and A6 yielded similar results (Figure 23C, 23D, and 23E, 23F, respectively, and additional data not shown). However, these effects were particularly clear with RPS12 consistent with our data in Figure 22. Also, because the ND7 and A6 substrates are much longer, we only examined a 3' fragment (~200 nt), including the first editing blocks, to analyze unedited and partially edited sequences. In summary, our data indicate that REH2 has multiple editing roles including in substrate loading, editing by the initiating gRNA and editing progression in upstream blocks.

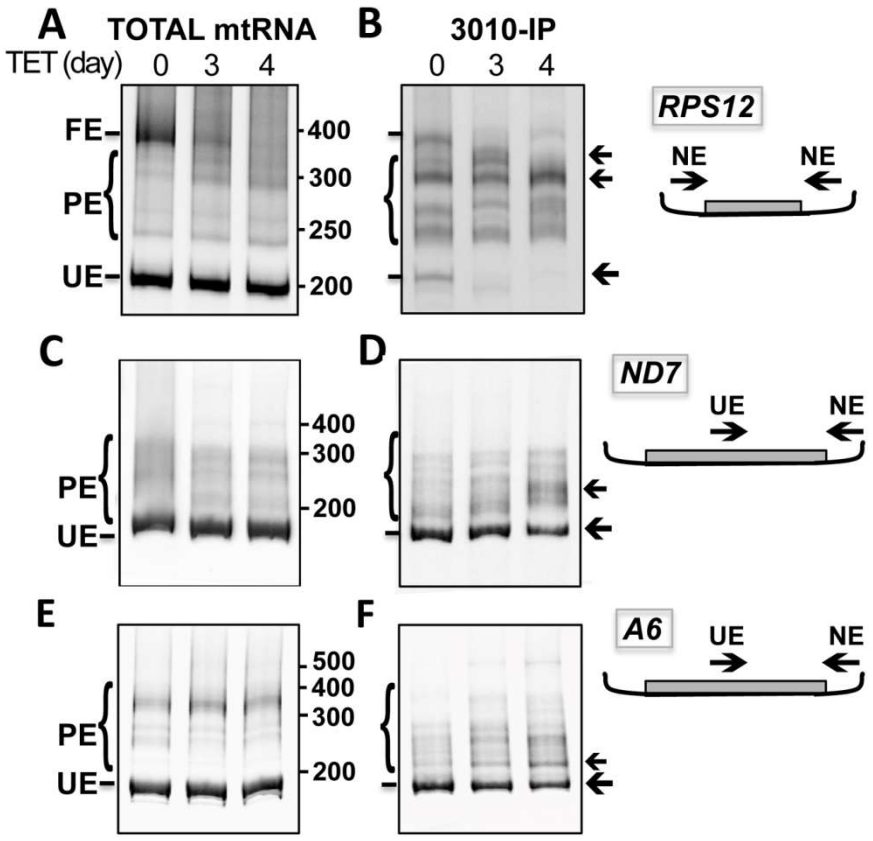

Figure 23. REH2 knockdown decreased substrate loading and increased pausing during editing in 3010-MRB**.** Endpoint RT-PCR products in total mtRNA and 3010-IPs at 0, 3, and 4 days of REH2 RNAi. mRNAs RPS12 (A and B), ND7 (C and D) or A6 (E and F). The full editing domain in RPS12 or a 3' fragment of similar size in ND7 and A6 was amplified. Products of varying sizes are unedited (UE), partially edited (PE), or fully edited (FE), in the case of RPS12. Arrows point to regions of apparent major pausing induced by REH2 depletion. The primers target 5' unedited (UE) or 3' never-edited (NE) sequences, as depicted in the cartoons.

REH2 binds its native MRB via RNA, and can be purified in a novel ~15S RNA-free subcomplex

Although native REH2 and 3010 are found in large ribonucleoprotein MRBs, the nature of the stable association of REH2 with other MRB1 proteins is unclear. A previous purification of REH2 contained core GAP subunits (GAP1/2), 3010, and other

known MRB1 proteins (209). Conversely, purifications of GAP subunits contained REH2 (209, 212). Some of these purifications included an extensive RNase treatment in attempts to remove RNA-mediated associations in the complexes. Interestingly, a yeast two-hybrid screen of several MRB1 proteins did not detect interactions with a REH2 fusion (208). We further examined the REH2 interaction with GAP1 in the native REH2-MRB. Because RNA-mediated associations in MRBs may partially resist RNase attack, we stopped RNA production in mitochondria by knocking down its single RNA polymerase (RNAP) in a procyclic cell line (319).

Native MRBs are heterodispersed in sedimentation gradients (Figure 24A). However, depletion of most mitochondrial RNA (not shown) (319) significantly reduced the sedimentation peak of REH2 and 3010 to fractions slightly lighter than RECC at ~20S (142), which we estimate to be near 15S (Figure 24B). The unaffected migration of RECC, which seems largely RNA-free in its purified form (142), served as a cell quality control. REH2 and 3010 IPs from mitochondrial lysates of the RNAP knockdown cells were examined for the presence of the GAP1 core protein. Notably, REH2 copurification with GAP1 was nearly lost (Figure 25), and RNase-treatment of the IP sample rendered the interaction undetectable. In contrast, 3010 copurified with GAP1 in all tested conditions. Thus, REH2 binds GAP1 via RNA.

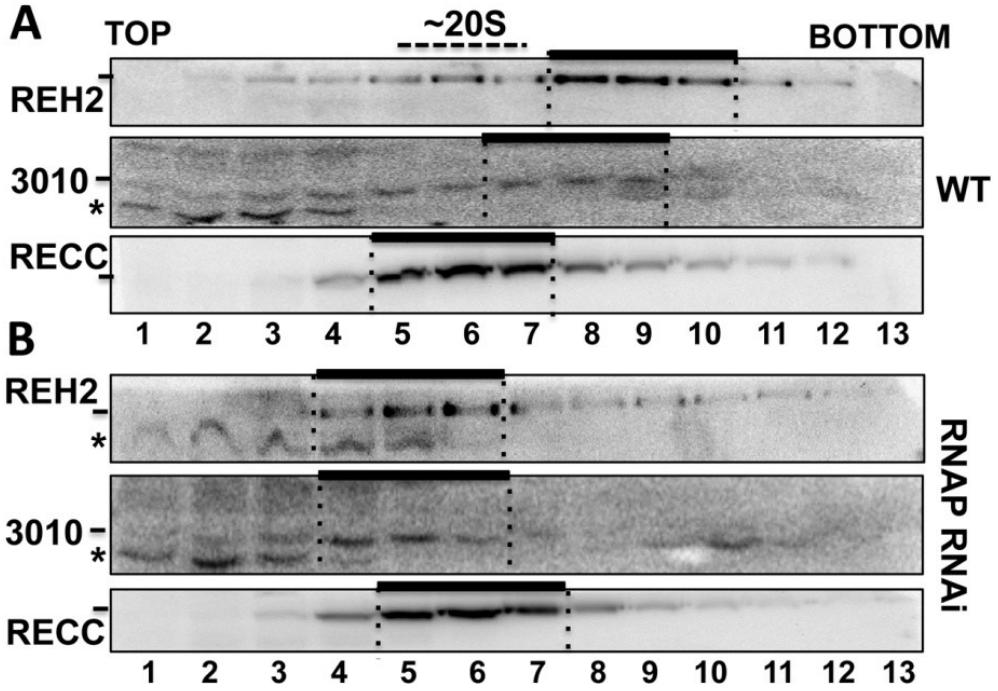

Figure 24. Sedimentation of MRBs. REH2 and 3010 MRBs in 10–30% glycerol gradients of mitochondrial extract from (A) wild-type (WT) or (B) RNAP knockdown cells at day 3 post-induction. The RECC complex (MB63 subunit) is at ~20S in panels A and B. Some proteolysis (*) occurs in the top fractions. Bars mark major peaks of REH2, 3010, and RECC examined in western blots.

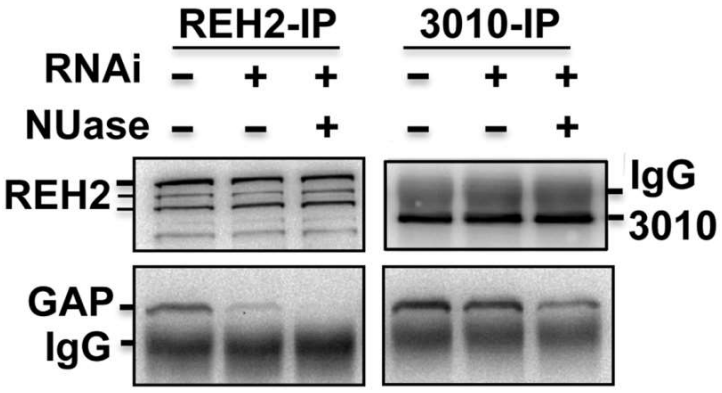

Figure 25. REH2 associates with GAP1 via RNA. Western blots of REH2, 3010, and GAP1 in IPs from RNAP knockdown cells, with or without a cocktail of nucleases (NUase): RNases A, and T1 and micrococcal nuclease. Untreated mitochondrial extract was used as a control.

We previously showed that REH2 and other presumed components of REH2-MRB in normal cells were able to photocrosslink with a 3' fragment of A6 mRNA (209). In the present study, we compared IP samples from the peak sedimentation fractions 4–6 (Figure 24B) from RNAP knockdown extracts in a photocrosslinking assay with a model initiating A6 gRNA (Figure 26A). Notably, we detected distinct crosslinks in the isolated REH2 and 3010 MRBs, which were absent in a mock purification. The REH2-associated crosslinks included a crosslink at ~30 kDa and weaker crosslinks at ~250 kDa, most likely involving REH2 itself because these crosslinks comigrate with REH2 in western blots (Figure 26B). Also, all other known protein subunits of MRB1 are much smaller than REH2. Recombinant GAP1 is known to bind a synthetic gRNA (212) but it is unclear if it photocrosslinks with our RNA probes. The crosslinks that we detected were stably bound because the samples in the beads had been treated with RNases and washed with 200 mM KCl. Together, our previous studies (1) and these new data indicate that native REH2 stably associates with its MRB via RNA, binds to both model mRNA and gRNA transcripts, and can be further purified together with a ~30 kDa RNA-binding cofactor in a novel ~15S "RNA-free" particle.

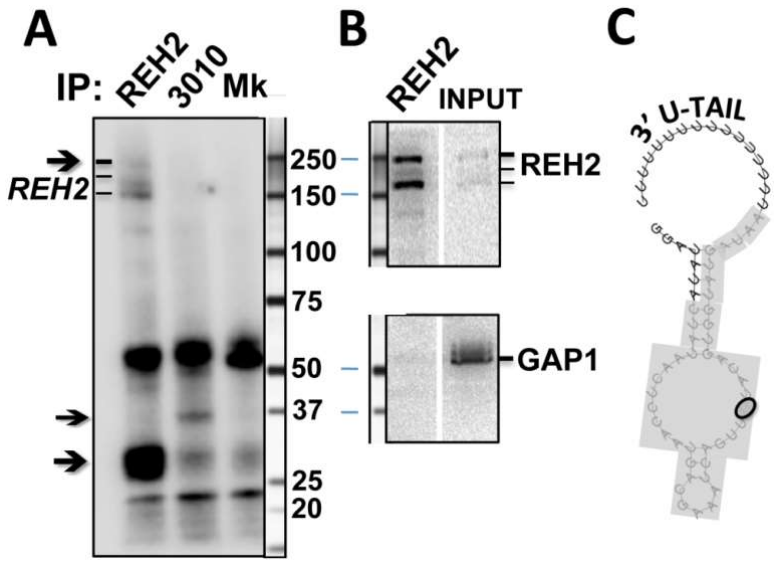

Figure 26. Photo-crosslinks in REH2 and 3010 pulldowns with an initiating gRNA. Antibody pulldowns of sedimentation fractions 4-to-6 in mitochondrial extracts from RNAP knockdown cells (Figure 24B) analyzed by (A) Site-specific crosslinking (365-nm UV) with a model initiating gRNA for mRNA A6 that includes a photo-reactive thio-U and ^{32}P in a single phosphodiester bond, or (B) Western blots of REH2 or GAP1 with size markers in the REH2 IP or the 15S input fractions 4-to-6. (C) Mfold prediction (320) of the secondary structure of the gRNA in panel A. The photo-reactive base is circled and guide sequence is inclosed in the gray box. Arrows in A indicate crosslinks in the REH2 or 3010 IP, including at ~30 kDa and, apparently, REH2 itself in the REH2 IP. A mock control (Mk) used an irrelevant affinity-purified antibody.

Site-directed mutagenesis of conserved REH2 domains prevents the association of REH2 with GAP1 and editing substrates

Because REH2 binds its native MRB via RNA, we examined the importance of the REH2 structure in these interactions. REH2 is a large (2,167 residues), non-ring-forming helicase (Figure 27A) that belongs to the RHA subfamily of the superfamily 2 (SF2) DEAH/RHA RNA helicases (233, 321). SF1 and SF2 helicases have a catalytic core of tandem RecA-like domains with characteristic motifs (I-VI) that participate in

ATP-binding (Figure 30) and hydrolysis and in RNA binding and unwinding. Accessory domains flanking the catalytic core determine their diverse functions by interacting with specific RNAs and proteins that modulate their activity (265, 322). RHA subfamily members, including REH2, have a unique conserved C-terminal region following the helicase core that contains an oligonucleotide-binding domain (OB-fold domain). A few subfamily members, including REH2, contain a ~70 residue double-stranded RNA-binding domain (dsRBD). The REH2 domain organization is conserved in kinetoplastids, including species of *Trypanosoma and Leishmania* (data not shown). Using expressed tagged constructs, we had shown that mutant REH2 proteins with either the dsRBD domain deleted or with two residue changes, G1365A/K1366Q, in the helicase motif I (mot I) were unable to copurify with gRNA (209). We then tested the effect of mot I, and alanine substitutions of two highly conserved residues (K1078, D1086) (262) in the dsRBD on the normal interactions of REH2 (*cis* effects) in its MRB. The mot I and dsRBD point mutations inhibited REH2 copurification with editing substrates (gRNA and unedited mRNAs) and edited mRNAs at block 1 or at 5' distal blocks (Figure 27B and 27C), and GAP1 (Figure 27D). Notably, homology modeling of the motif I or P loop using for the template the closest RHA subfamily member that has a published crystal structure with ADP bound (265) indicates that the mot I mutation removed a salt bridge (a H-bond plus ion-ion interaction) between the beta phosphate of the adenosine nucleotide and K1366 of REH2 (Figure 30). This mutation would weaken the binding energy of ADP with the motif I through the loss of the counter ion and add the large energetic penalty of burying a negative charge without a counter ion. The beta

phosphate would be left with four H-bonds. The alpha phosphate lacks a stabilizing salt bridge with a positively charged side-chain, so the protein will have difficulty compensating for the loss of the positive charge at site 1366 while continuing to counter the two negative charges of the ADP. The alpha phosphate may form two H-bonds (one with the backbone amide of T1386 and one with the side-chain hydroxyl of T1386), so the alpha phosphate contributes much less to the binding by ADP.

Overall, the above observations indicate that the integrity of native REH2-MRB, which includes GAP1 but not 3010, requires functional catalytic and RNA binding domains of REH2. These findings further suggest that the "RNA linkers" in REH2-MRB are in fact mRNA, gRNA, or mRNA-gRNA complexes.

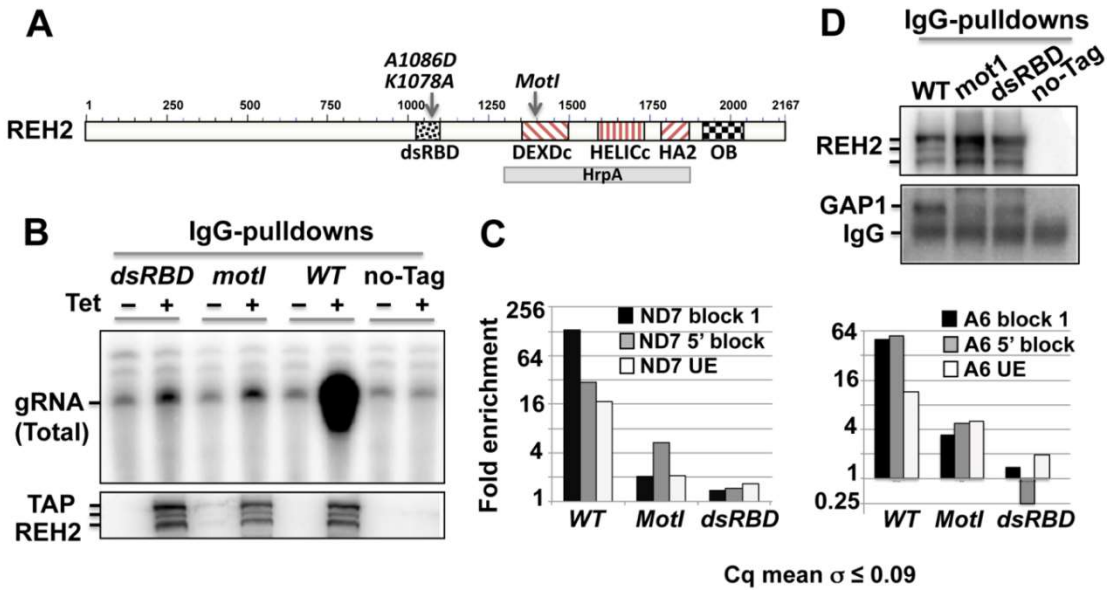

Figure 27. Association of REH2 with components of its MRB requires normal conserved domains. (A) REH2 (2,167 amino acids) includes the double-stranded RNA binding (dsRBD) and DExH-helicase domains, and an OB-fold domain not previously identified. Expressed TAP-tagged REH2 variants in Dynabead IgG pulldowns were tested for (B) gRNA in radiolabeled capping assays of the 5' triphosphate, (C) fold enrichment of edited mRNA at block 1 or at a distal block, and unedited pre-mRNA. RT-qPCR assays were normalized to values in the untagged REH2 pulldown set at 1. Tubulin and 18S rRNA carryover were used as reference, (D) GAP1 and REH2 in western blots. Cells-/+ Tet induction of wild-type (WT), mutants, or untagged REH2 (no-Tag) were compared. Standard deviation of the average value in Cq duplicates is shown.

Discussion

MRB1 is a large and dynamic ribonucleoprotein complex that binds gRNA and is critical in the control of kinetoplastid RNA editing. However, MRB1's specific molecular mechanisms of action and the rationale for MRB1's dynamic composition are unclear. Our previous report (1) supported by the current study offer a novel conceptual framework proposing that editing is controlled and regulated in the context of at least two substrate-loaded MRB1 variants with specialized functions: 3010-MRB and REH2-MRB complexes. So far, the dissection of the function of specific editing proteins almost exclusively relied on RNAi knockdowns of the protein, followed by the analyses of several editing substrates. Also, early studies showed that RECC enzyme does not contain editing substrates (142). So, long-standing questions in the field include: how does RECC access the editing substrates and how is this enzyme controlled? Our studies offer a path to systematically address the physical and functional interplay between the RECC editing enzyme, editing substrates and accessory MRB1 complexes.

We previously showed that the MRB1 variants 3010-MRB and REH2-MRB, with differing protein and gRNA composition, bind the mRNA substrates and products of editing (1). In that study, we also proposed that these complexes serve as scaffolds for the assembly of gRNA-mRNA hybrids and transient but productive contacts with the RECC enzyme. The current studies showed that: (*i*) these MRB1 variants are tied to distinct editing functions, and (*ii*) specific *cis* and *trans* effects by the regulatory RNA helicase REH2 on substrate loading, complex integrity, and editing can be directly

studied in the context of MRB1 complexes. Based on our previous report and current new data, we propose an updated model of MRB1 organization and function (Figure 28).

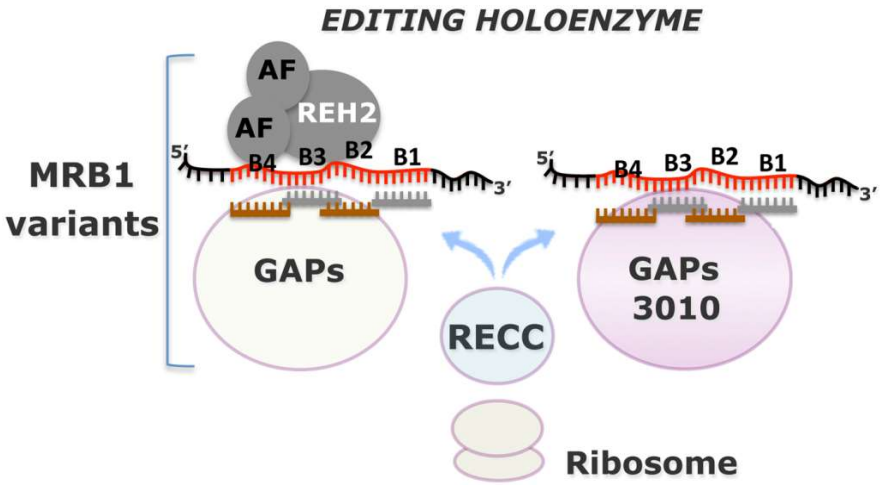

Figure 28. Model of MRB1 function and organization**.** MRB1 complexes carry editing substrates (pre-mRNA and gRNA), partially-edited intermediates and fully-edited mRNA, core GAP1/GAP2 proteins (GAPs), and other common and variable proteins. Addition of the RECC enzyme to MRB1 scaffolds forms the editing holoenzyme. Two MRB1 variants, REH2-MRB and 3010-MRB are structurally and functionally distinct. 3010-MRB appears to be a more active editing scaffold than REH2-MRB. REH2-MRB includes the regulatory REH2 helicase that affects 3010-MRB at multiple steps: mRNA substrate loading, and maturation at block 1 and subsequent blocks during editing progression. REH2 binds RNA and REH2's association with other MRB1 components is mediated by bridging RNA and the helicase catalytic and RNA-binding domains. REH2 can be further isolated in a "RNA-free" ~15S subcomplex with a ~30 kDA RNA binding cofactor and, potentially, with other associated cofactors (AFs). The editing domain in mRNAs typically spans multiple blocks (e.g., B1-to-B4), each directed by a gRNA. Transient contacts between RECC and MRB complexes imply that catalysis involves multiple rounds of enzyme association and dissociation with the MRB scaffolds. MRB complexes may also control passage of the edited mRNAs to the mitoribosomes.

Both 3010-MRB and REH2 MRB complexes contain all types of mRNA molecules expected to be present during editing, including intermediate transcripts, gRNA and critical subunits such as GAP1/2 proteins, indicating that both complexes are competent editing scaffolds. However, the 3010-MRB complex that we originally purified by immunoprecipitation of native MRB3010 (1) appears particularly active in editing. Interestingly, inducible knockdown of MRB3010 was proposed to inhibit early editing (213). The hypothesis that the 3010-purified MRB is a major scaffold of the editing machinery is based on our observations that this complex contains increased levels of both initiating gRNAs and mRNAs with increased editing directed by the initiation gRNA. The observed REH2 effects on the RNA profile in 3010-MRB and the integrity of REH2-MRB illustrate how other regulatory proteins may contribute to the editing process. Overall, these data represent the first examples of specific *cis* and *trans* effects by a regulatory helicase on the higher-order RNA editing holoenzyme or "editosome". REH2, the lone confirmed subunit of MRB1 with a conserved helicase domain, may be a chaperon or remodeling factor that impacts multiple aspects of RNA editing.

We anticipate the characterization of additional MRB1 variants that carry gRNA, GAP proteins, and mRNAs that require editing. Distinct MRB1 variants may control substrate specificity (either loading or stability), and may control different steps during editing or post-editing, including the association of mitoribosomes with the editosome (e.g., as in Figure 22). Specialized or preferential roles of distinct MRB1 variants may depend on the associated "variable" protein subunits, such as REH2, specific protein-

RNA interactions, stoichiometric differences of "core" proteins, or combinations of some of these differences. The stoichiometric differences of "core" proteins most likely include MRB3010 ("3010"), which was found to interact with GAP1, but not GAP2, in a yeast two-hybrid screen (208). Thus, it is possible that 3010 is underrepresented, if not missing, in the protein core of the REH2-MRB. We expect (1) that MRB1 variants that carry gRNA will also contain GAP proteins and (2) that 3010 is necessary for the efficient editing of the associated mRNAs.

Some of the ancillary proteins of the editosome may include known MRB1 components, but not gRNA or GAP proteins. An example is the proposed ~15S REH2 subcomplex including a 30 kDa RNA-binding protein that we described (Figures 24–26). TbRGG2, and the paralogs MRB4160 and MRB8170, that have been detected in most purifications of MRB1, were also found in a separate subcomplex (83). Interestingly, MRB4160 and MRB8170 interacted with each other in a yeast two-hybrid screen. However, their association with each other *in vivo* is critically dependent on RNA (208, 301). This underscores the importance of stabilizing RNA-protein interactions in the function and regulation of MRB1.

The association of REH2 with an MRB1 variant that contains reduced or no 3010 is puzzling. The stable association of REH2 with GAP proteins and, presumably, other common components of its MRB likely occur via mRNA, gRNA, or both. In addition, the observed transient functional contacts of the REH2 helicase with 3010-MRB may be bridged by mRNA-gRNA hybrids. Both types of REH2 interaction may rely on a coordinated action of the catalytic and accessory domains in this protein. Notably,

although depletion of REH2 decreases the loading of the mRNA substrate, it does not dissociate the mRNA already engaged in editing. Nevertheless, additional transient REH2 interactions with 3010-MRB occur during block 1 editing and editing progression through upstream blocks. Putative cofactors of REH2 in the proposed ~15S REH2 subcomplex may influence the interaction and function of conserved domains in REH2 with both the RNA and proteins partners during editing. Interestingly, a putative helicase (Tb927.4.3020) with the same domain organization as REH2 was previously identified among several RNase-sensitive proteins that copurified with REH2 (209, 321). However, a knockdown construct of this protein did not induce an evident editing phenotype, suggesting at least a partial functional redundancy with REH2 (data not shown). A recent study by the Aphasizhev lab confirmed our report that MRB1 complexes contain mRNA editing substrates and products, and also proposed the interesting concept that the subunits of MRB1 complexes, related to 3010-MRB in our studies, may be functionally partitioned in subgroups based on their role in editing or polyadenylation (215). Several central aspects of RNA editing in kinetoplastids need to be studied including the control of gRNA loading and the transient assembly and activity of RECC complexes in the MRB scaffolds. It seems that editing progression may involve multiple rounds of transient contacts of the RECC enzyme with the MRB1 scaffolds, rather than a stable processive interaction. Also, the presence of fully edited mRNA in MRB1 complexes suggests that these complexes mediate the handoff of translatable mRNA into mitoribosomes. Overall, our current observations offer a

conceptual framework to undertake systematic studies of the regulation of RNA editing by MRB1 complexes.

Supporting information

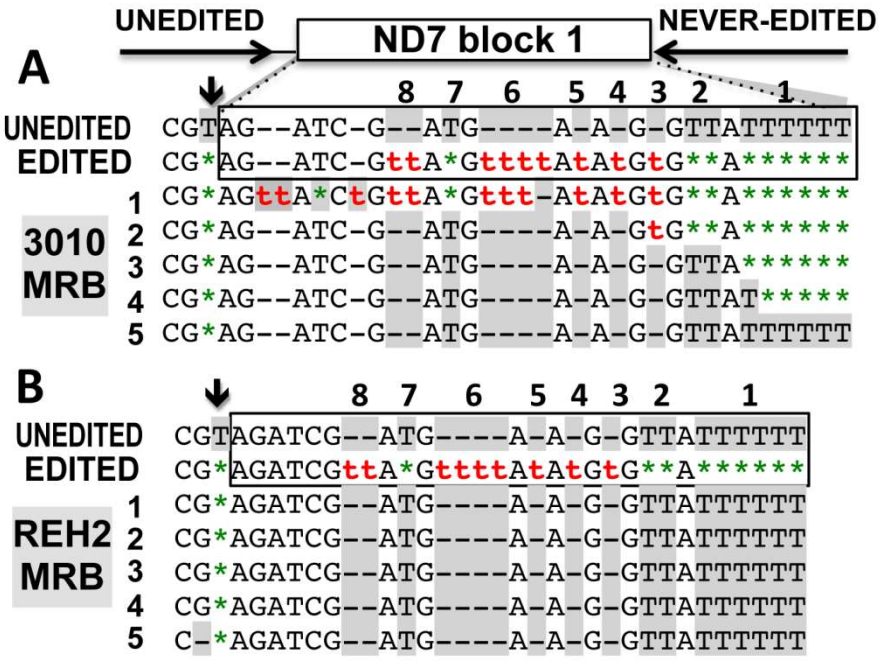

Figure 29. Editing initiation of mRNA ND7 in native 3010-MRB and REH2-MRB. cDNA sequence of block 1 in mRNA ND7 3' domain amplified from (A) 3010 and (B) REH2 IPs. PCR primers (arrows) flanking the first block target 5' unedited and 3' never-edited sequence. Precursor unedited or edited mRNA (boxed) with editing sites 1-to-8: unedited (gray) or with deletions '★' and insertions 't'. Miss-edits (in number or site) are also shown in gray. Edited sequence in block 1 is consistent with the guide domain of gRNA gND7 B1 [gND7(1269–1319)] identified in recent reports in procyclic strains Lister 427 and EATRO 164. A previously annotated encoded T in the ND7 gene (arrow) was missing in all 10 cDNA clones examined here from the Lister 427 strain.

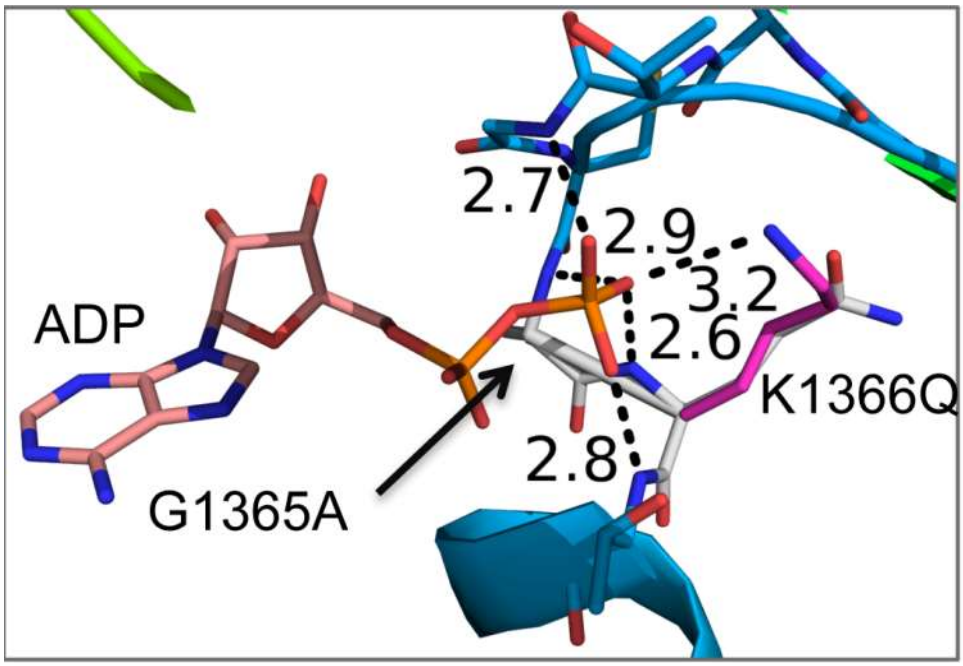

Figure 30. Homology model of motif I mutations. The mutated sites G1365A/K1366Q are shown with the carbons colored white. These mutations in the P loop or motif I (atoms of motif I are shown as sticks) remove one H-bond between beta phosphate of the ADP and REH2. Four H-bonds remain after the mutations.

Primers

Manual sequencing of block 1 nested amplicons

First PCR

A6 For (1287)	5' GAAGAAAGAGCAGGAAAGGTTAG
RT primer (1345) *	5' TAATACGACTCACTATAGGGG
ND7 For (1289)	5' GCAGAAGGCTTTCTGAGGAAAG

Nested PCR

A6 For (1302)	5' GTTAGGGGGAGGAGAGAAG
A6 Rev [RT primer (1345)]	same as above
ND7 For (1343)	5' TTTCTGAGGAAAGAGGGGAC
ND7 Rev (1344)	5' CAATTTTAAACAATCCTCAATAACC

RT-PCR

Entire [RPS12] or first ~200 bp of editing domain [A6, ND7]

A6 For (1417)	5' ACG GCG GTT TTG AAA ACA C
A6 Rev (951)	5' AAT AAC TTA TTT GAT CTT ATT CTA TAA CTC C
ND7 For (1414)	5' GGA ATT CAG CTT TGT GGA AC
ND7 Rev (1344)	5' CAA TTT TAA ACA ATC CTC AAT AAC C
RPS12 For (952)	5' CTA ATA CAC TTT TGA TAA CAA AC
RPS12 Rev (953)	5' AAA AAC ATA TCT TAT ATC TAA A

RT-qPCR

Block 1 [A6 and ND7], Block 1-3 [RPS12]

A6 For (1379)	5' GTTATTATATTGTTGTGAAATTGG
A6 Rev (1380)	5' ATCTTATTCTATAACACCAATAAC
ND7 For (1376)	5' CCGTAGATCGTTAGTTTTATATG
ND7 Rev (1378)	5' CAATTTTAAACAATCCTACAATAAC
RPS12 For (1360)	5' GAGGGTGGTGGTTTTGTTG
RPS12 Rev (1361)	5' CATATCTTATATCTAAATCTAAC

3' UTR RNAi

REH2 For BamHI (987)	GGATTCATGTGGCCAAAGATGAGTGGGTCGAGCTCA
REH2 Rev XhoI (988)	CTCGAGCCAGCAGACAGAACACAGGAGGGGGC

Northern blots

gND7 B1 (1358)	5' CTTATACATGAAGTCACTGTAGGATTG
gCYb B1 (1388)	5' CTGTTACATCTAAAGACTCATTACCCCTC
tRNA.Cys (976)	5' GGGACCATTCGGACTGCAGCCG

Photo-reactive gA6 B1.alt

5' half RNA (1428)	5' ggauauaCUAUAACUCCAAUGACGAAAUCAGUU
3' half RNA (1427)	5' $\mathbf{U}^{s}$UACAGUGGUAUGAUAAUUUUUUUUUUUUUUUU
Bridge oligo (1390)	5' ATCATACCACTGTAAAACTGATTTCGTCAT

REH2 constructs

dsRBD point mutations (underlined codon)

K1078A For (1045)	5' GGCGTAGCGTGGAATGCAGAGGCCTCGCAACGC
K1078A Rev (1046)	5' GCGTTGCGAGGCCTCTGCATTCCACGCTACGCC
A1086D For (1047)	5' GCAACGCCAGGCGGACATGCACGCC
A1086D Rev (1048)	5' GGCGTGCATGTCCGCCTGGCGTTGC

Amplification of the full gene

WT For Xho (1156)	CTCGAGATGCGGGCCATACGACTAACTGTTGC
WT Rev BglII (1264)	AGATCTGGCCGAGTCTCCACCAGCCTCAGCACTTGTGGTGG

* This primer was used for 1st strand cDNA synthesis of C-tailed RNA as described in the methods section

Table 6. Oligonucleotide primers designed in this study. We designed the indicated primers to perform RT-qPCR of 3' early editing sites, manual sequencing of block 1 sites, RT-PCR of the first ~200 bp of editing domain (A6 and ND7), RNA interference (RNAi), point mutations and generation of photo-reactive gA6 B1.alt. RT-PCR primers to amplify the entire RPS12 were as in (213).

CHAPTER IV

REH2C HELICASE AND GRBC SUBCOMPLEXES MAY BASE PAIR THROUGH MESSENGER RNA AND SMALL GUIDE RNA IN KINETOPLASTID EDITOSOMES*

Summary

Mitochondrial mRNAs in *Trypanosoma brucei* undergo extensive insertion and deletion of uridylates that are catalyzed by the RNA editing core complex (RECC) and directed by hundreds of small guide RNAs (gRNAs) that base pair with mRNA. RECC is largely RNA-free and accessory mitochondrial RNA-binding complex 1 (MRB1) variants serve as scaffolds for assembly of mRNA-gRNA hybrids and RECC. Yet, the molecular steps that create higher-order holoenzymes ("editosomes") are unknown. Previously, we identified an RNA editing helicase 2-associated subcomplex (REH2C) and showed that REH2 binds RNA. Here, we showed that REH2C is an mRNA-bound protein subcomplex (mRNP) with editing substrates, intermediates and products. We isolated this mRNP from mitochondria lacking gRNA-bound subcomplexes (gRNPs), and identified ^{H2}F1 and ^{H2}F2 (REH2-associated cofactors 1 and 2). ^{H2}F1 is an octa-zinc finger protein required for mRNP-gRNP docking,

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pre-mRNA and RECC loading, and RNP formation with a short synthetic RNA duplex. REH2 and other eukaryotic DEAH/RHA-type helicases share a conserved regulatory C-terminal domain cluster that includes an OB-fold. Recombinant REH2 and ^{H2}F1 constructs associate in a purified complex *in vitro*. We propose a model of stepwise editosome assembly that entails (a) controlled docking of mRNP and gRNP modules via specific base pairing between their respective mRNA and gRNA cargo, and (b) regulatory REH2 and ^{H2}F1 subunits of the novel mRNP that may control specificity checkpoints in the editing pathway.

Methods

Cell culture

T. brucei Lister strain 427 29-13 procyclic "PF" (tryps.rockefeller.edu) was grown axenically in log phase in SDM79 medium (312) and harvested at a cell density of 1-3x10^7 cells/ml. Cell lines expressing constructs for RNAi-based genetic downregulation were induced with tetracycline at 1 μg/ml.

DNA constructs

We made inducible RNAi constructs for ^{H2}F1 and GAP1 as described (212, 219). The RNAi construct for REH2 was reported in our previous study (209). RNAi and TAP-tagged constructs for ^{H2}F2 were both prepared by PCR amplification of a 804-bp fragment from the ^{H2}F2 entire open reading frame using 5'-CTCGAGATGTTCCGCTGGTCG-3' 5'- AGATCTGGTTAAGGACGCAGAAAC-3', as forward and reverse primers, respectively. The amplified fragments were cloned into

the XhoI and BamHI sites of p2T7-177 or pLEW79-TAP (209, 313). All constructs were confirmed by DNA sequencing, linearized with NotI, and transfected in procyclic 29-13 trypanosomes (312).

Protein and RNA sample purification

Immunoprecipitation of REH2, MRB3010 (3010), and cytochrome oxidase 2 (mock) from freshly made mitochondrial extracts was performed using affinity-purified peptide antibodies as described (1). Affinity-purified antibodies against ^{H2}F1 and ^{H2}F2 were produced (Bethyl Laboratories, Inc.) in rabbits using the peptides CKRKKTTEVSEVTS and KVSAESYVDYLQNSDRELPA as antigens, respectively. As in our previous studies (1, 2), specific antibodies were conjugated to Dynabeads Protein A (Life Technologies) that were pretreated with 5% BSA. Approximately 2 mg of mitochondrial extract was supplemented with 1X Complete Protease Inhibitor cocktail (Roche) and SUPERase·In RNase inhibitor (Life Technologies). The extract was pre-cleared by passage over Protein A-Sepharose beads (GE Healthcare) before it was loaded onto antibody-conjugated beads. All washes were performed with 200 mM NaCl, 1 mM EDTA, 10 mM MgCl2, and 25 mM Tris pH 8. Protein was extracted with 1X SDS loading buffer at 95ºC for 2 min. RNA was extracted by treating the beads with 0.8U proteinase K (NEB) for 30 min at 55ºC, followed by phenol extraction and ethanol precipitation. For mass spectrometry analyses the antibodies were crosslinked to the beads with 25 mM DMP (dimethylpimelimidate) in 0.2 M triethanolamine, pH 8.2. Samples from tandem affinity purified complexes were prepared for protein identification as in prior studies (83, 209).

Mass spectrometry

The immunopurified or tandem affinity purified protein complexes were reduced, alkylated and digested as described (323, 324). The peptide mixture was desalted, concentrated using C18-packed pipette tips (Thermo Fisher) and fractionated online using a 75 μM inner diameter fritted fused silica capillary column with a 5 μM pulled electrospray tip and packed in-house with 17 cm of Luna C18(2) 3 μM reversed phase particles. The gradient was delivered via an easy-nLC 1000 ultra-high pressure liquid chromatography (UHPLC) system (Thermo Fisher). MS/MS spectra were collected on a Q-Exactive mass spectrometer (Thermo Fisher) (325, 326). Data analysis was performed with ProLuCID and DTASelect2 implemented in the Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, Inc., San Diego, CA) (327–330). Protein and peptide identifications were filtered with DTASelect and required at least two unique peptides per protein with a peptide-level false positive rate of 5% as estimated by a decoy database approach (331). Normalized spectral abundance factor (NSAF) values were calculated as described (332) and multiplied by a factor of 105 for readability.

Western blots and radioactive assays

Western blots of REH2, 3010, GAP1, RGG2 and KREPA1 (also termed MP81, a RECC core subunit) were performed as reported (209, 222, 314). Western blots of MRB6070 (6070) and MRB8170 (8170) were performed as in (208). Western blots of ^{H2}F1 and ^{H2}F2 were performed with sera diluted to 1:2,000. RNA ligases in the RECC enzyme were radioactively labelled by selfadenylation directly on the beads (307).

Capping assays of gRNA used RNA extracted from the Dynabeads protein-A pulldowns (209). Unwinding assays of REH2 or ^{H2}F1 antibody pulldowns were performed as in our previous studies with a few modifications (209, 236). The RNA duplex substrate in the assays in this study was prepared by annealing 5'GUCUUACGGUGUCUAAAACAAAACAAA ACAAAACAAAG3' (38-nt) and complementary 5'GACACCGUAAGAC3' (13-nt). 5 picomoles of labeled 13 nt oligonucleotides were boiled at 95ºC for 2 min followed by pre-annealing for 30 min at room temperature in 10X Annealing buffer (100mM MOPS, 10mM EDTA pH6.5 and 0.5M KCl) and the hybrids were isolated from an native acrylamide gel, run in the cold room at 50V volts for 120 min (0.5X TBE buffer). After the IPs, 20 cps of hybrid were mixed with antibody-conjugated beads (5 µL of resin) and incubated for 30 min at 19ºC in 40mM Tris-Cl pH 8.0, 0.5mM MgCl2, 0.01% NP-40 and 2mM DTT. The reaction was stopped by addition of 5 µL of helicase reaction stop buffer (HRSB) (2X HRSB contains: 50mM EDTA, 1% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol and 20% glycerol) and kept on ice for 5 min. The beads were then collected in a magnetic stand and the released complexes in solution were loaded onto the native gel and resolved at 25V volts, 4ºC temperature for 120 min.

Quantitative qRT-PCR of mRNAs and densitometry

RNA from total mtRNA or pulldowns was treated with RNase-free DNase (Thermo) and used in the preparation of cDNA as described elsewhere (1). qRT-PCR assays normalized using the delta delta Cq "ΔΔCq" (Livak) method (184, 315) were performed in 20 microliter reactions with the primers reported to be specific for

unedited mRNAs, edited at a 5' distal block, and reference transcripts (184) in a SYBR Green PCR Master Mix (Bio-Rad). Editing phenotype in total mtRNA was analyzed as in prior studies (2) using two biologically distinct samples under similar induction conditions (days 3 and 4; in lieu of identical biological replicates). Measurements were normalized to uninduced cells (set at 1) and to tubulin and 18S rRNA references. Fold change determinations of bound mRNAs in purified complexes from RNAi cells, as in a prior study (2), is based on the ratio of each mRNA in the IPs and total mtRNA input at days 3 and 4 of induction. The ratio in induced samples is plotted relative to the ratio in uniduced samples (day 0 set at 1). qRT-PCR calculations were normalized to a mock IP and input values. As in the phenotypic analyses, two biologically distinct samples under similar induction conditions (days 3 and 4) were examined in parallel measurements. In all analyses, two technical replicates were obtained per Cq measurement. The maximum standard deviation of the average value in the Cq duplicates that was observed in the entire analysis is indicated in each plot. End-point RT-PCR of the entire RPS12 editing domain was performed using PerfeCTa SYBR Green FastMix (Quanta) with specific primers targeting 5' and 3' UTR sequence, and analyzed on 8% native acrylamide gels as in our previous studies (2). All amplicons in this and our previous studies were verified by cloning and manual sequencing. cDNA at different dilutions and no-RT controls have been tested to confirm the linearity and specificity of the amplifications. Quantitative densitometry was done using Quantity One 1-D analysis software. Measurements were

adjusted after subtraction of a background value determined from a blank lane in each blot.

Homology modeling and bioinformatic analysis

Domain annotations in REH2 including the dsRBD, DEXDc, HELICc, HA2 and the previously unidentified OB-fold domain were performed using the Conserved Domain Search tool (CD-Search) at NCBI (316). A homology model of the helicase portion (residues 1308 to 1846) of *T. brucei* REH2 was generated using the program Phyre2 (317). The model was refined with KoBaMIN (333). The coordinates of the ADP were from the crystal structure of a yeast Prp43p/ADP complex (PDB-ID 2XAU) (265) after superposition of the crystal structure of this complex onto the homology model of REH2. The high quality of the geometry around the ADP binding site suggests that this part of the homology model was reliable. Sequence analysis of the gene for ^{H2}F1 suggested the presence of eight zinc-finger domains of the C2H2 type. An initial homology model of ^{H2}F1 made with I-Tasser (333) failed to give all of the alpha helices expected in the predicted zinc fingers. Cys CB–His CG distance restraints were applied in a second run of I-Tasser. The homology model that was returned had geometry that lead to the automated identification of three zinc fingers with the zincs placed (gray balls by Zfn5, Znf7, and Znf8). In addition, a DNA ligand was automatically proposed by I-Tasser due to the similarity of the position of some of the zinc finger domains in the homology model with the crystal structure of a designed six-zinc finger DNA binding domain bound to DNA (PDB-ID 2i13, (334)). The

multiple-alignment sequence analysis of the ^{H2}F1 zinc finger domains was done with BOXSHADE version 3.21 http://www.ch.embnet.org/software/BOX_form.html

Recombinant proteins

6xHis-REH2 (residues 1261-2167) and MBP-TEVrs*-^{H2}F1 (TEVrs: TEV recognition site, full length ^{H2}F1) were amplified by PCR. The final products were cloned in the expression vector pET15b (for REH2) and pMALX-B (for ^{H2}F1) (335). The purified plasmids were transformed in *E. coli* Rosetta2 DE3 for protein expression. Two liters of culture for each cell line was grown at 37°C in Terrific Broth. At ~0.8 A600 the temperature was reduced to 22°C, and protein overexpression was induced with 0.5mM IPTG for 22 h with shaking at 100 rpm. The harvested cells for both recombinant proteins were mixed together and stored at -80°C for 60 hours. The combined cell pellets of 6xHis-REH2 and MBP-TEVrs-^{H2}F1 were resuspended in 50mM Tris-HCl pH 8.5, 1M NaCl, 20mM BME including 10 µg of RNase-free DNase and 15 mg of hen egg white lysozyme. Cells were lysed in an Emulsiflex hydraulic press. Cell debris was removed at 18K rpm for 30 minutes at 4°C. The supernatant with 6xHis-REH2 and MBP-TEVrs*-^{H2}F1 was batch attached to equilibrated Qiagen Ni-NTA Agarose at 4°C for 16 hrs. The complex was eluted with 50mM Tris- HCl pH 8.5, 1M NaCl, 20mM BME and 250mM imidazole. The first elution of the purification was tested in native gels, western blots, and antibody pulldowns for the presence of both recombinant proteins and of the complex.

Introduction

RNA editing by uridylate (U) insertion and deletion in *T. brucei* modifies over 3000 sites in mitochondrial mRNAs in a gradual process directed by hundreds of small guide RNAs (gRNAs) (140, 183, 220). The basic regulatory mechanisms of substrate specificity and developmental control in RNA editing remain unknown. The U-changes are catalyzed by the RECC enzyme from 3'-to-5' in discrete blocks. Each gRNA directs editing of one mRNA block. Surprisingly, RECC has little or no RNA and lacks the processivity found *in vivo*, as established in early purifications of this multiprotein enzyme (142, 336, 337). So, accessory components of the editing apparatus must facilitate substrate recruitment and editing catalysis. There are many non-RECC proteins that affect editing. Most of these proteins (>25 proteins) are components of the MRB1 complex in *T. brucei, also termed* gRNA-binding complex (GRBC) in *Leishmania,* that binds and stabilizes gRNA. MRB1 transiently interacts with the RECC enzyme and mitoribosomes (141, 183, 207). It was also found that MRB1 contains all three classes of mRNA in editing: unedited pre-mRNAs, partially edited intermediates and fully edited transcripts. This indicates that MRB1 complexes serve as scaffolds for the assembly of hybrid substrates and the RECC enzyme (1, 2, 215). Transient addition of RECC to these scaffolds would establish higher-order editing holoenzymes or editosomes. MRB1 was first considered a single dynamic complex but the reason of its variable composition was unclear. However, layers of organization in MRB1 are emerging. We found MRB1 variants or MRBs (REH2-MRB and 3010-MRB) via immunoprecipitation (IP) of RNA editing helicase 2 (REH2) and MRB3010 (3010),

respectively. These complexes have REH2 or 3010, but not both, and differ in the content of some initiating gRNAs, according to western blot and RNA-seq analyses, respectively (1). Recent studies and the current work show that these MRBs consist of several subcomplexes (summarized in Figure 31) (2, 215). 3010-MRB includes a gRNA-binding core subcomplex (GRBC) also called MRB1 core (208). REH2-MRB includes the REH2 subcomplex (REH2C) and a variant of GRBC (2). To distinguish these gRNA-bound variants, hereon we refer to GRBC and GRBC* found in IPs of 3010 and REH2, respectively. GRBC and GRBC* bind multiple gRNAs (1). Other proposed subcomplexes of MRBs are REMC (typified by RGG2) and PAMC. These subcomplexes take part in editing progression and post-editing mRNA 3' maturation, respectively (222, 302). As the physical organization of MRBs becomes clearer, specialized subcomplexes and critical domains in their subunits open a path to study editing control in substrate-bound molecular scaffolds. For example, the stable association of REH2 with mRNA and GRBC* requires the functional domains in the helicase for dsRNA binding and ATP binding/hydrolysis (2, 209). Also, REH2 helicase is a *trans* factor that promotes pre-mRNA association and editing progression in the 3010-MRB variant (2). Our reported findings represent the first identified *cis* and *trans* effects by the sole MRB1 protein with RNA helicase conservation in editosome assembly and function (2, 209). The current study provides new insights on assembly and regulation of higher-order editosomes, with REH2C playing a central role. Namely, REH2C is an mRNA-associated ribonucleoprotein subcomplex (mRNP) that includes REH2, two cofactors (^{H2}F1 and ^{H2}F2) and all mRNA types in editing: pre-mRNA

substrates, partially edited transcripts, and fully edited products. The integrity of this mRNP is independent of the GRBC variants. We propose that assembly of stable mRNA-gRNA hybrids in editing scaffolds requires docking of mRNP and gRNP modules by base pairing of mRNA and gRNA. The helicase mRNP binds stably with GRBC* but interacts with GRBC via transient contacts. Our homology modeling of REH2 revealed a conserved regulatory C-terminal domain cluster (CTD) in eukaryotic RHA-type helicases. This CTD includes winged helix, ratchet and oligonucleotide-binding (OB fold) domains. ^{H2}F1 has 8 putative Cys2His2 zinc fingers potentially involved in dsRNA or protein binding. ^{H2}F2 has a glycine-rich C-terminus. ^{H2}F1 depletion caused *cis* and *trans* effects. That is, ^{H2}F1 promotes: (i) GRBC* association with REH2, RECC and pre-mRNA (in *cis*), and (ii) GRBC association with RECC and pre-mRNA (in *trans*). ^{H2}F1 depletion had no major effect on REH2-dependent unwinding of a synthetic short RNA duplex. Yet, it inhibited RNP formation with this RNA duplex in *cis* and in *trans*. Purified recombinant REH2 and ^{H2}F1 form a complex *in vitro*. The regulatory REH2 helicase and its eight zinc finger ^{H2}F1 cofactor enable studies of mRNA-gRNA hybrid assembly and substrate access to RECC on molecular scaffolds. This stepwise process creates the catalytic center in editosomes and likely entails specificity checkpoints.

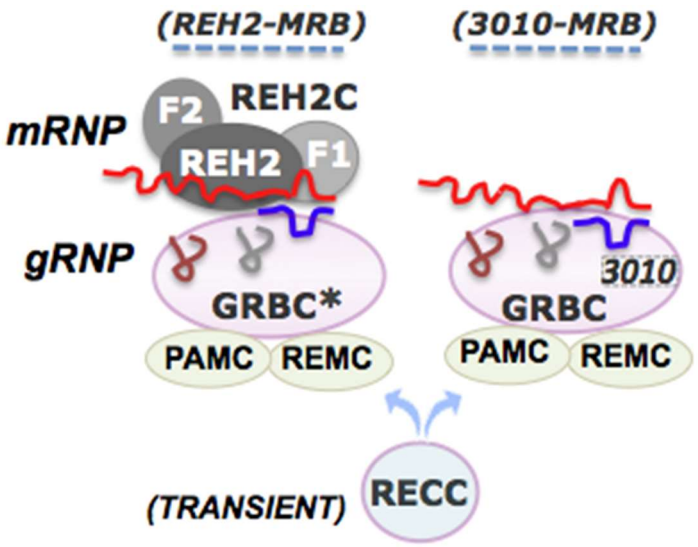

Figure 31. RNP subcomplexes with mRNA (*mRNP*) or gRNA (*gRNP*) in MRB1. Two stable forms of the MRB1 complex (termed MRBs) are known. Each MRB form consists of multiple subcomplexes. In this study, we characterize the REH2C subcomplex (with its protein subunits indicated as gray ovals). This subcomplex includes the REH2 helicase, two cofactors (^{H2}F1 and ^{H2}F2), and mRNA (pre-edited and edited). GRBC* and GRBC are variants of another subcomplex that contains several proteins and gRNAs. These gRNA-bound variants are distinguished by their content of a protein subunit (labeled 3010). Only GRBC* binds REH2C via stable RNA contacts. Two more subcomplexes, REMC and PAMC, participate in RNA editing and post-editing mRNA 3' maturation, respectively. Holo-editosomes are formed upon transient addition of the RECC enzyme to the higher-order complexes. The mRNAs are in red, and the gRNA transcripts are in various colors.

Results

Subunits of the novel REH2-associated subcomplex (REH2C)

We recently reported a ~15S REH2-associated subcomplex (REH2C) that contains a ~30 kDa RNA-binding protein and resists extensive RNase treatment (2). REH2C binds stably via RNA to the GRBC* subcomplex which contains GAP1 and gRNA but lacks 3010 (2). We distinguish GRBC* from the GRBC variant (also termed MRB1 core) by their content of 3010 and some gRNAs (Figures 32A-B) (1). Native REH2C-GRBC* and GRBC are isolated via immunopurification (IP) of REH2 and 3010, respectively (i.e., as part of mRNA-bound MRBs in prior work) (1, 2). These MRBs also associate with RGG2 and RECC (1, 2). RGG2 is the typifying subunit of another proposed subcomplex REMC or set of related subcomplexes containing RGG2 (83, 208, 215). Thus, the current studies examine the bipartite REH2C-GRBC* and GRBC. Both stable particles bind tightly to REMC (in MRBs) and weakly to the RECC enzyme (forming editosomes) (Figures 32A-B). To identify the subunits of REH2C, we performed a spectrometric analysis of a REH2-IP from RNA-depleted mitochondria in RNA polymerase knockdown cells as in our previous study (2). Removal of a C-terminal tag during affinity purification of this helicase was not efficient (209). Among a large number of proteins detected in the REH2 IP, a 28.8 kDa polypeptide (Tb927.6.2140) was prominent. This protein has not been studied but was observed in a purification of REH2 from normal mitochondrial extracts treated with RNase, and of TbRGG1 without RNase (209, 210). Affinity purification of TAP tagged Tb927.6.2140 showed REH2 and a 58 kDa polypeptide (Tb927.6.1680) with high

confidence scores. The latter protein was found in prior purifications of REH2 and other MRB1 proteins (209, 211, 212). We will refer to Tb927.6.1680 and Tb927.6.2140 as REH2-associated factors 1 and 2 (^{H2}F1 and ^{H2}F2), respectively. Because of the high molecular mass of REH2 (242 kDa) we expected a small number of proteins in the ~15S REH2C. Consistent with their stable association in REH2C, REH2, ^{H2}F1 and ^{H2}F2 were detected in western blots of REH2 IPs but not in 3010 IPs (Figure 32A). Also, REH2 co-purification with ^{H2}F1 and ^{H2}F2 was impervious to RNase treatment (Figure 32C). Some ^{H2}F2 was found in the unbound fraction from samples with or without RNase treatment (Figure 32C, and data not shown). This suggests that some ^{H2}F2 is not always associated with REH2 in mitochondria. Stable copurification of REH2C with GRBC* was disrupted by RNase treatment (Figure 32C). A reported RNAi-based knockdown showed that ^{H2}F1 downregulation inhibits editing (219). We confirmed that that ^{H2}F1 knockdown induces a robust editing phenotype (Figure 32D). It is common to find that accumulation of unedited pre-mRNA is not equivalent to the decrease in edited mRNA in RNAi knockdowns (2, 219). This may reflect differences in transcript stability. RNAi constructs directed to the open reading frame or the 3' UTR of ^{H2}F2 did not seem to affect *in vivo* editing of the tested substrates (Figure 32E, and data not shown). Thus, we identified two REH2 cofactors, ^{H2}F1 and ^{H2}F2. Yet only ^{H2}F1 was associated with editing of the tested substrates in procyclics.

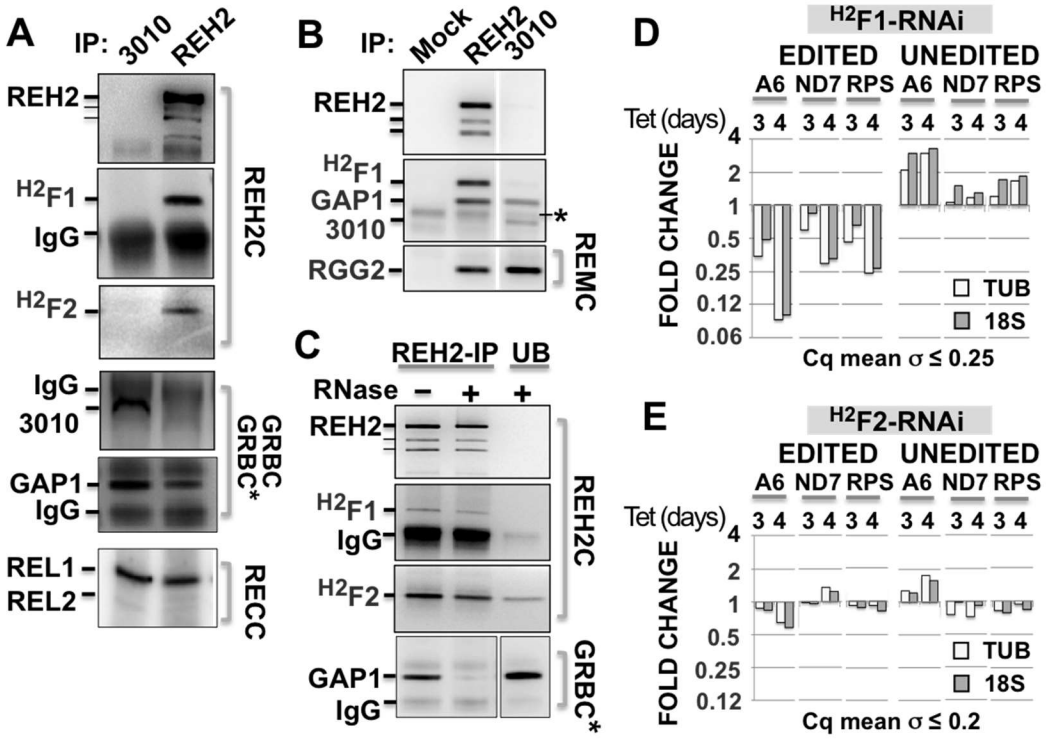

Figure 32. REH2 cofactors ^{H2}F1 and ^{H2}F2. *(A-B)* Western blots of REH2-IPs and 3010-IPs. Components of REH2C, GRBC*, GRBC, RECC and REMC. Editing ligases REL1 and REL2 were assayed by 32Pautoadenylation. REL2 was not detected in most gels due to pre-charging with endogenous ATP. IgG in IPs was often faint (*). *(C)* Western blots of REH2-IP or unbound material (UB) +/- RNase. *(D-E)* Fold change of mRNA transcript levels in ^{H2}F1 and ^{H2}F2 knockdowns after tetracycline (Tet) induction. Two biologically distinct samples with similar induction conditions (days 3 and 4) were examined in parallel measurements. qRT-PCR in ddCq calculations was normalized to uninduced cells (set at 1) and tubulin or 18S rRNA references. The standard deviation of the average value in Cq duplicates for each measurement is shown as the maximum deviation observed in the analysis.

Domain organization of REH2, ^{H2}F1 and ^{H2}F2

Crystallographic studies of monomeric superfamily 1 (SF1) and SF2 helicases revealed a catalytic core with tandem RecA-like domains (annotated in domain databases as DEXDc and HELICc) and characteristic motifs (I-VI) in ATP binding/ hydrolysis, RNA binding and unwinding (265). The first crystal structure in the DEAH/RHA family that comprises a number of nuclear and cytosolic proteins revealed a specific C-terminal cluster with winged helix (WH), ratchet and oligonucleotide-binding (OB fold) domains (265). The WH and ratchet domains are annotated as the 'helicase-associated domain' HA2. Our homology model of REH2 showed this domain cluster (Figure 33). The C-terminal OB-fold in REH2 was detected in a sequence analysis (321) but the domain cluster is only recognized in the three dimensional structure. So, this molecular design is found in a mitochondrial helicase in trypanosomes and pre-dates the evolutionary split of trypanosomes from other eukaryotes that occurred over 100 million years ago (311). This model was generated with part of *T. brucei* REH2 (residues 1308 to 1846) using as a template the crystal structure of a yeast Prp43p/ADP complex (PDB-ID 2XAU) (265). The coordinates of the ADP were found after superposition of the crystal structure of the complex onto the homology model of REH2. The high quality of the geometry around the ADP binding site suggests that this part of the homology model is reliable. REH2 and a few members in the RHA subfamily also contain a canonical double-stranded RNA (dsRNA)-binding domain (dsRBD) with a α1-β1-β2-β3-α2 fold that binds dsRNA (Figure 33) (338). ^{H2}F1 has 8 potential Cys2His2 zinc fingers (Znf1-8)

(Figure 33, and data not shown). A search for conserved domains (316) identified Znf5 as a dsRNA-binding domain (pfam12171; E-value 7.05e-03) originally reported in JAZ dsRNA-binding Znf proteins (339). The conserved domain database search predicted Znf6-8. Three zinc fingers (Zfn5, Znf7, and Znf8) were accurate enough in the homology model of ^{H2}F1 for the coordination of zinc atoms. Also, a DNA ligand was proposed due to the similarity of the position of some of the zinc finger domains in the homology model with the crystal structure of a designed six-zinc finger DNA binding domain bound to DNA (PDB-ID 2i13) (334) (data not shown). While we were able to fit a DNA double helix based on available domain databases, we expect specific ^{H2}F1 binding to helical RNA structures in the context of trypanosome editing. We identified Znf1-4 after visual inspection and a sequence alignment of all fingers in ^{H2}F1 (data not shown). ^{H2}F2 has a conserved hydrolase domain (cl11421) spanning most of its length. Also, the C-terminal half of this small polypeptide is rich in glycine. This feature of ^{H2}F2 is reminiscent of G-patch proteins including several helicase partners in the RHA family (271). G-patch proteins carry one or more copies of a glycine-rich domain (G-patch domain) defined by 7 conserved glycines. This motif is not obvious in ^{H2}F2 but this protein may be a diverged member in the family of G-patch proteins.

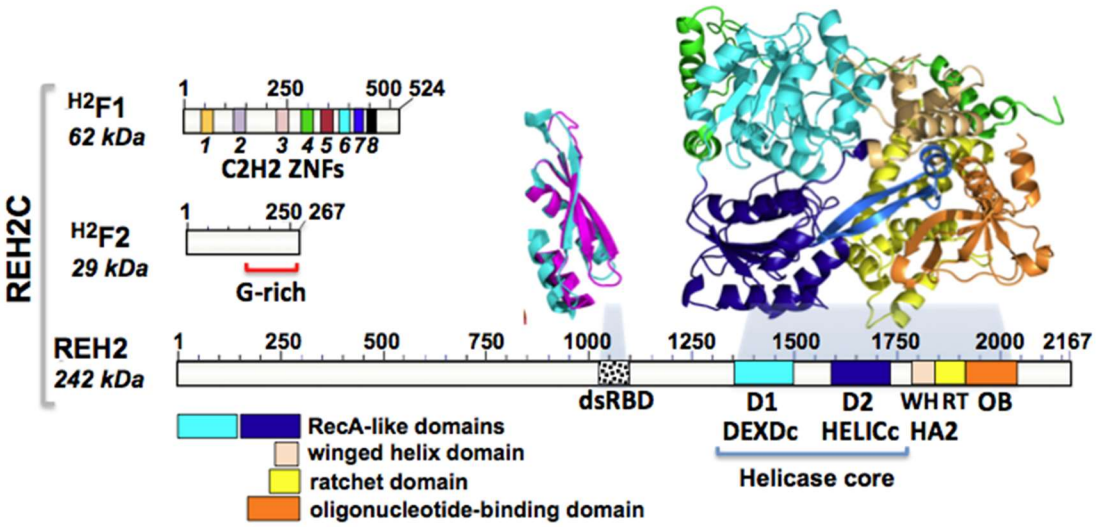

Figure 33. Domain organization of REH2, ^{H2}F1 and ^{H2}F2. Maps at scale of REH2 and its cofactors. Protein domains are color coded in the domain map and in the structure model of REH2 (right) and ^{H2}F1 (left). REH2 conserved features in a homology model made using ADP-bound Prp43p (PDB ID:2XAU) as a template. The conserved features include tandem RecA-like domains (DEXDc and HELICc in current domain databases) in the helicase core that are common to SF1 and SF2 helicase superfamilies. After the large helicase core there is a cluster of small domains that includes a winged helix and ratchet (annotated as 'helicase-associated domain' HA2 in domain databases) and an OB fold. This cluster is unique to RHA-type helicases. REH2-specific sequence or elements are depicted in green. A few RHAtype helicases including REH2 have a dsRBD. In our model of the REH2 dsRBD, the Tb protein is in magenta and the structure used as a template (PDB ID:1DI2) is in cyan.

REH2C and mRNA form a stable mRNP in absence of GAP1 and gRNA

We hypothesized that REH2C binds mRNA directly. This idea is consistent with the findings that REH2 binds RNA, inactivating point mutations in REH2 hinder mRNA association with native REH2C-GRBC* (in *cis*), and depletion of REH2 reduces pre-mRNA association with GRBC (in *trans*) (2, 209). Because GAP1 binds and stabilizes gRNA (212), we surmised that an mRNP with REH2C and mRNA could be isolated from GAP1-depleted mitochondria. As expected, RNAi-based GAP1 knockdown

removed most GAP1 and gRNA (GRBC*) in the REH2 pulldowns. Yet, loss of GRBC* did not affect the integrity of REH2C (Figures 34A-E; and data not shown) nor the steady state level of RECC and REMC core proteins in the parasites (Figures 34C and 34E, and data not shown). These data indicate that the protein integrity of REH2C is independent of gRNA-associated subcomplexes. REH2C retained substantial association with pre-mRNAs substrates, editing intermediates and fully edited transcripts in the GAP1 knockdown. Two complementary analyses applied in a previous study of isolated complexes showed this (2). First, end-point RT-PCR gels of mRNA RPS12, using primers directed to never-edited UTR sequence, showed a similar level of unedited pre-mRNA in all REH2 pulldowns (Figure 34A). The GAP1 knockdown caused a robust editing phenotype with gradual accumulation of partially edited RNA species and concurrent decrease of fully edited mRNA, when comparing 0, 3 and 4 days of induction. Second, qRT-PCR of unedited and fully edited transcripts in IPs, normalized to both a mock pulldown and input (loaded extract) values, showed REH2C retention (~50% or higher) of the tested transcripts in mitochondria depleted of GAP1 and gRNA (Figure 34F. This indicates that the REH2C subcomplex is a stable mRNP particle. In contrast, at least ~90% of unedited pre-mRNA and edited transcripts were lost in 3010 IPs from GAP1-depleted extracts Figure 34F). This was explained in part by a ~60% decrease in total 3010 in these extracts (Figures 34C and 34E). Despite a moderate reduction in 3010, the greater depletion of cellular GAP1 and gRNA (~80%; Figures 34C and 34E, and data not shown) matched more closely the dramatic loss of mRNA in 3010-IPs. This suggested that mRNA retention by GRBC requires gRNA. In

practice, two time points of RNAi induction (days 3 and 4) in the analyses provided biological replicates of the effects relative to uninduced cells. As noted above, the cellular level of RECC and REMC proteins was normal in the GAP1 knockdown. However, the amount of these complexes decreased in REH2-pulldowns from these cells (Figures 34A, 34B and 34D). Thus, normal association of REH2C with RECC enzyme and REMC requires GRBC* (or another subcomplex in the MRBs). The normal steady state level of RGG2 in cells depleted of either GAP1 (Figures 34C and 34E) (215) or REH2 (2) is consistent with the idea that REMC (typified by RGG2) is a separate subcomplex in the editing scaffolds. The presence of 9S rRNA and never-edited mRNA transcripts in IPs of REH2 and 3010 reflects transient association of mitoribosomes with native editing complexes (1, 2). These transcripts showed a moderate decrease in the IPs from GAP1-depleted extracts. This suggested an interaction of REH2C with at least some mitoribosomes in the absence of GRBC* (Figure 34F). It is possible that the remaining 3010-associated particles in these cells also retain some mitoribosomes. We have observed an accumulation of mitoribosomes in 3010-IPs that requires REH2 (2). Note that our plots indicate relative change in transcript abundance in the IPs (not total content). Induced samples are normalized to uninduced samples (see the figure legends and experimental procedures). Never-edited mRNAs occur at low level in 3010-IPs and REH2-IPs. Edited and unedited mRNAs are relatively enriched in the same samples (1). The above data showed that the REH2C subcomplex includes all mRNA types in editing, and that the integrity of this mRNP does not need GRBC* in *cis* or GRBC in *trans*. The data also imply that

the assembly of substrate-loaded editing scaffolds involves coupling of specialized RNPs: namely, an mRNP (REH2C) and gRNP variants (GRBC or GRBC*) via specific base pairing between their mRNA and gRNA cargo, respectively.

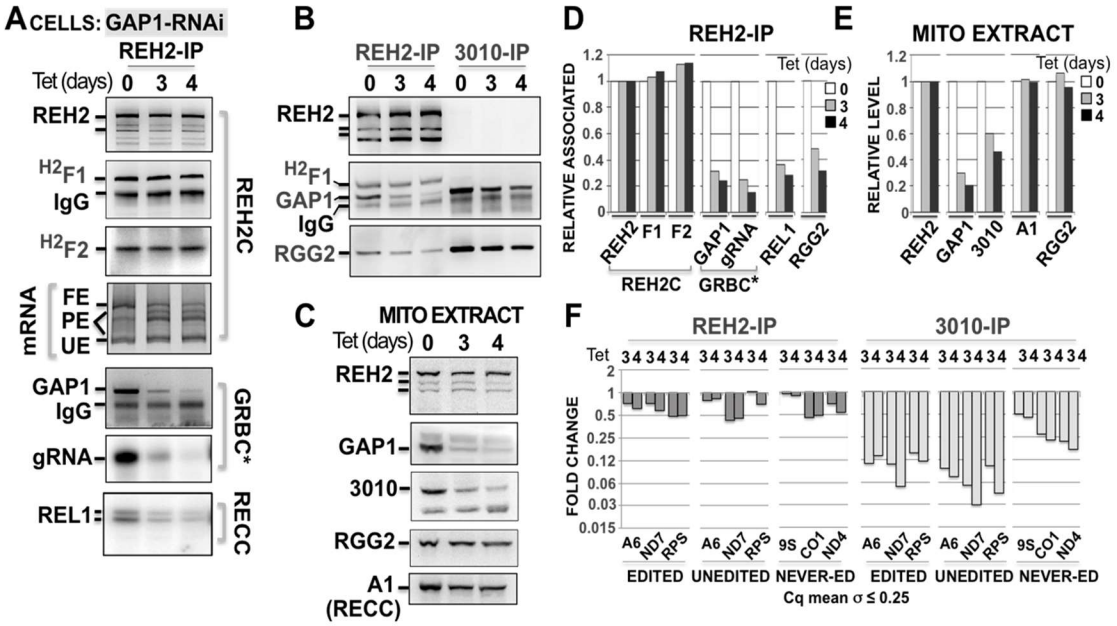

Figure 34. REH2C is an mRNP. Analyses as in Figure 32A of: *(A-B)* REH2-IPs and 3010-IPs from GAP1- RNAi extract, (*C)* total mitochondrial (mt) extract. KREPA1 (A1) is a core subunit of RECC. In Panel A, total gRNA and mRNA RPS12 (FE= fully edited; PE = partially edited; UE= unedited) were assayed by 32P-capping and by RT-PCR, respectively. Our cell line had two REL1 isoforms, which were clearly resolved in some gels. *(D)* Densitometry of proteins and gRNA in panel A. RGG2 was scored from panel B. Relative association was double normalized to recovered REH2 in IPs and each component at day 0 (set at 1). *(E)* Densitometry of proteins in panel C. Values were double normalized to recovered REH2 and to day 0. *(F)* Fold change in the levels of bound edited and unedited mRNAs in IPs in GAP1 RNAi cells. The ratio of mRNA in the IPs and total mtRNA input at days 3 and 4 of induction is compared to the same ratio in uninduced samples (day 0 set at 1). qRT-PCR calculations were normalized to a mock IP and input values. Standard deviation of the average value in Cq duplicates is the maximum deviation observed in the duplicates.

Cis and trans effects of ^{H2}F1 on native REH2- and 3010-purified editosomes, assembly of RNP modules, pre-mRNA loading and transient interaction with RECC

We examined ^{H2}F1 for possible *cis* and *trans* effects on the assembly of editing scaffolds with mRNA, and transient interaction with RECC enzyme. The depletion of ^{H2}F1 did not prevent formation of a ^{H2}F2-REH2 subcomplex but eliminated nearly 80% of its association with GRBC*, REMC, RECC, and pre-mRNA (in REH2-IPs; Figures 35A-C). Thus, ^{H2}F1 has strong *cis* effects on protein-RNA interactions in REH2-bound complexes. In *trans*, ^{H2}F1 depletion did not compromise the integrity of the GRBC subcomplex but clearly reduced its association with RECC and pre-mRNA (in 3010-IPs; Figures 35A-B and 35D). All proteins tested had a normal steady-state level in the ^{H2}F1-depleted cells. We confirmed this in replica IPs and in total mitochondrial extract, including a core subunit of RECC (data not shown). Also, pre-mRNAs in these cells were stable or accumulated slightly (Figure 32D) (212). Thus, a clear loss of RECC in both REH2-IPs and 3010-IPs from ^{H2}F1-depleted mitochondria, suggested that ^{H2}F1 promotes the addition of the RECC enzyme to editing scaffolds both in *cis* and in *trans*. In line with our GAP1- depletion experiments in Figure 34, loss of GRBC* and REMC in REH2-IPs, but normal retention of GRBC and REMC in 3010-IPs from the ^{H2}F1-knockdowns, show that the GRBC*-REMC and GRBC-REMC aggregates are tightly bound in scaffolds purified via REH2 or 3010, respectively. We note that the stability of REH2, but not ^{H2}F2, appeared slightly compromised after ^{H2}F1 depletion both in REH2-IPs and mitochondrial extract (Figure 35A, and data not shown). Still, the decrease in REH2 full-length is several fold less than the large loss of GAP1 and RECC in REH2-

IPs (Figure 35A, and data not shown). Thus, loss of ^{H2}F1 induced clear effects on editosome assembly and pre-mRNA association. In these experiments, we plotted the level of proteins normalized to REH2 full-length in the pulldowns (Figures 35B and 35F). As in Figure 34, two time points of RNAi induction (at days 3 and 4) enable analysis of biologically distinct samples under similar conditions. We note that the steady-state level of the tested subunits in GRBC, REMC and RECC appeared normal in the ^{H2}F1 knockdown. This was clear in 3010-purified GRBC and in mitochondrial extracts from ^{H2}F1 depleted cells (Figures 35A, and data not shown). This indicated that the cells were viable at the examined time points of RNAi induction. Downregulation of ^{H2}F2 did not prevent formation of a ^{H2}F1-REH2 subcomplex, and had no evident effects on REH2 stability or the assembly of ^{H2}F1-REH2 with GRBC*. Also, the integrity of GRBC was normal. A slight decrease in RECC was detected in both IPs of REH2 and 3010 (Figures 35E and 35F) but no evident editing phenotype was found in the tested substrates as shown above. The steady state level of RECC, examined in western blots of a core subunit was normal in the ^{H2}F2 knockdown (data not shown). Thus, ^{H2}F2 may affect the formation of higher order complexes in mitochondria that include the RECC enzyme but is not essential for editing. In summary, these data show that ^{H2}F1 affects the coupling of REH2C with GRBC* (in *cis*) and the association of RECC and pre-mRNA with the editing scaffolds (in *cis* and in *trans*). This agrees with a model in which stable or transient docking of mRNP and gRNP modules involves the specific base pairing of their mRNA and gRNA cargo.

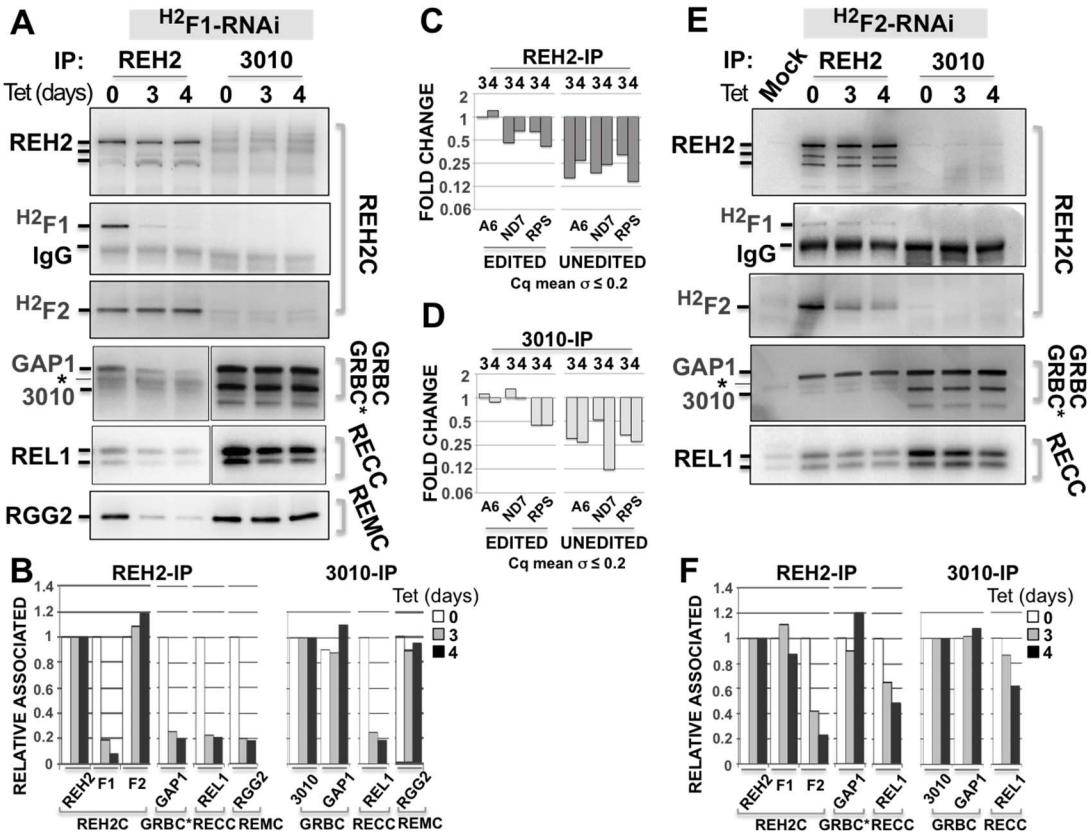

Figure 35. Effects of ^{H2}F1 or ^{H2}F2 knockdown on association between REH2C, GRBC*, GRBC, REMC and RECC. Analysis as above of IPs from RNAi extracts: *(A and E)* protein components. A mock IP was included in panel E. The IgG band in IPs was often faint (*). *(B and F)* Densitometry of panels A and E, respectively. *(C and D)* Fold change in the levels of bound edited and unedited mRNAs. The ratio of mRNA in the IPs and total mtRNA at days 3 and 4 of induction is compared to the same ratio in uninduced samples (day 0 set at 1). Standard deviation of the average value in Cq duplicates is the maximum deviation observed in the duplicates.

Depletion of REH2 helicase dissociates ^{H2}F1 from ^{H2}F2 but moderately affects the ^{H2}F1 association with other editosome components

We asked whether ^{H2}F1 associates with components of native REH2-purified editosomes in REH2-depleted mitochondria. ^{H2}F1-IPs from REH2-knockdown cells exhibited a comparable loss of REH2 and ^{H2}F2. This is consistent with the idea that REH2 directly associates with ^{H2}F2 (Figures 36A-B). Despite a large loss of REH2 and ^{H2}F2 in the ^{H2}F1-IPs from REH2 knockdown cells, there was a moderate decrease in ^{H2}F1 association with GRBC*, REMC and RECC. This suggested that ^{H2}F1 supports some interaction with GRBC* and RECC in these cells. It also implied added complexity in the network of interactions that control the assembly of editosomes. Native REH2-purifications exhibit a 3'-5' unwinding activity of short RNA duplexes that requires ATP as well as dsRBD and ATPase domains of REH2 (209). This REH2-dependent unwinding activity was observed in ^{H2}F1 pulldowns from normal but not REH2-depleted extracts Figure 36C. This is in line with the idea that REH2 is the major source of the RNA unwinding activity in our assays using short dsRNA and immunopurified native editing complexes. Thus, ^{H2}F1 may keep some interactions with components of native editosomes under conditions of REH2 downregulation. Yet, REH2 is necessary for optimal ^{H2}F1 stable association with GRBC*, and transient contact with the RECC enzyme.

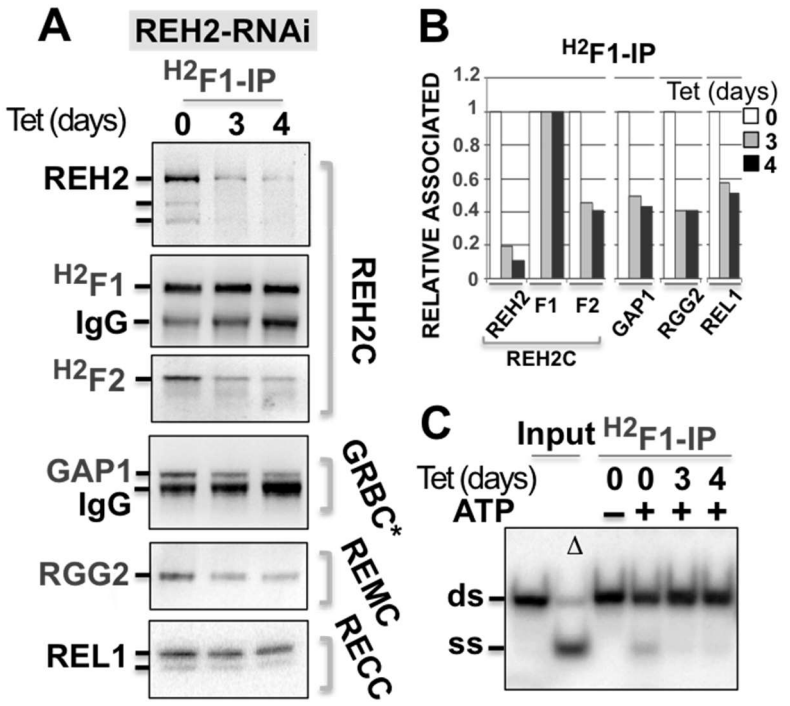

Figure 36. Effects of REH2 knockdown on the ^{H2}F1 association with ^{H2}F2, GRBC*, REMC and RECC. Analyses as above of ^{H2}F1-IPs from REH2-RNAi extracts: *(A)* protein components. *(B)* Densitometry of panel A. *(C)* Unwinding assays with a radiolabeled RNA duplex substrate in mixtures +/- exogenously added 2 mM ATP.

^{H2}F1 promotes, in cis and in trans, formation of stable RNPs with a short RNA duplex

As expected, we detected the unwinding of the short dsRNA described above in REH2-IPs but not in 3010 or mock IPs (Figure 37A). Interestingly, this dsRNA substrate in the unwinding assays also formed gel-retardation products in REH2-IPs and 3010-IPs. These products indicated stable association of the short dsRNA with the immunopurified native complexes. These RNPs were sensitive to proteinase K (data not shown) and co-migrated with RECC enzyme in identical IPs (Figure 37B). ^{H2}F1 depletion reduced the accumulation of RNPs containing REH2 or 3010 in *cis* and in *trans*, respectively, but

had little or no effect on the unwinding activity of the REH2-purified complex (Figure 37C). A GAP1 knockdown also decreased formation of the purified RNPs (Figure 37D). Thus, the RNPs detected with a short radiolabeled RNA duplex represent RNA-bound editosomes. In line with our model of editosome assembly, REH2 and ^{H2}F1 in the mRNP, and GAP1 in the gRNP variants, are required for docking of RNPs in higher-order editing complexes.

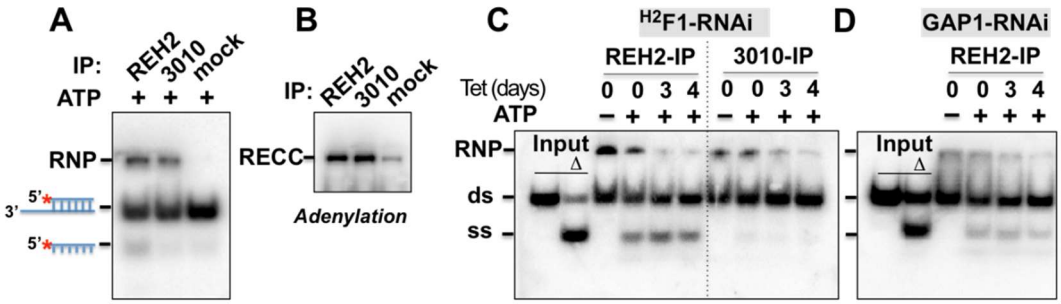

Figure 37. Analysis of REH2 and 3010 purified complexes for unwinding activity and formation of an RNP with a short radioactive RNA duplex. *(A)* Assays of unwinding activity and RNP formation in REH2, 3010 and mock IPs with 2 mM ATP. *(B)* Radioactive autoadenylation of editing ligases in complexes that co-migrate with the RNPs in panel A. *(C and D)* Assays as in *(A)* but from ^{H2}F1-RNAi or GAP1-RNAi extracts at 0, 3 and 4 days of induction +/- ATP.

Recombinant REH2 and ^{H2}F1 proteins associate with each other in a complex in vitro

We expressed 6xHis-REH2 (residues 1261-2167; (~100 kDa) and MBP-TEVrs*-^{H2}F1 (~100 kDa) in bacteria. To determine if these two recombinant proteins form a complex *in vitro*, we mixed bacterial extracts of each protein and purified 6xHis-REH2 from the mixture by affinity chromatography. In native gels, the eluted sample contained two or three main species that could represent free 6xHis-REH2 and complexes between 6xHis-REH2 and MBP-TEVrs*-^{H2}F1 (Figure 38A). We confirmed that both 6xHis-REH2 and MBP-TEVrs*-^{H2}F1 are present in the eluted sample (Figures 38B-C). IPs of ^{H2}F1 contained 6xHis- REH2, and IPs of REH2 contained MBP-TEVrs*-^{H2}F1 (Figures 38D-E). Finally, in IPs of REH2 or ^{H2}F1 the complex resisted RNase digestion (Figure 38F). Thus, these recombinant versions of REH2 and ^{H2}F1 form a stable complex. Direct association between these proteins in the REH2C subcomplex is consistent with the slight destabilization of REH2 that we observed in ^{H2}F1-depleted cells (Figure 35A, and data not shown).

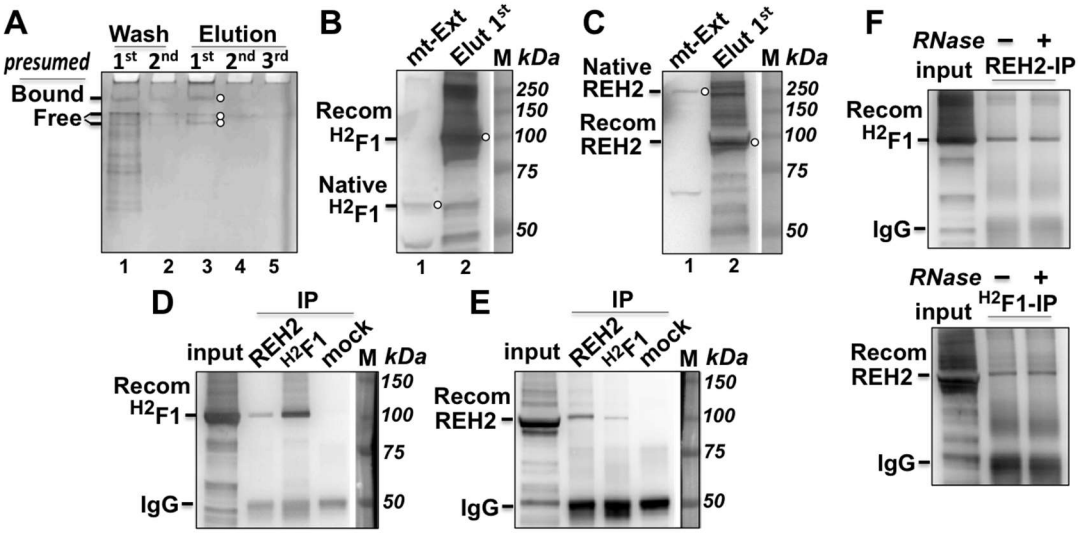

Figure 38. Recombinant REH2 and ^{H2}F1 proteins copurified in a complex. Silver stained native gel of a purification of 6xHis-REH2 from an extract mixture that also contained MBP-TEVrs*-^{H2}F1: *(A)* washes (lanes 1-2) and elutions (lanes 3-5). Presumed bound proteins in a complex or free are indicated. *(B-C)* SDS-PAGE western blots of IPs from mt-extract (lane 1) or 1st elution from panel A (lane 2). *(D-E)* SDSPAGE western blots of the 1st elution from panel A (input) or IPs of this fraction with anti-^{H2}F1, REH2, or irrelevant antibodies (mock). *(F)* RNase A/T1 treatment of the recombinant ^{H2}F1-REH2 complex while bound to the beads in REH2 or ^{H2}F1 IPs.

Discussion

These results support the hypothesis that the REH2C subcomplex controls the assembly and function of editosomes. A long-standing question is how RECC may reach editing substrates *in vivo*. Because MRB1 complexes contain pre-mRNA substrates and products of editing we and others proposed that these complexes serve as scaffolds for specific assembly of mRNA-gRNA hybrid substrates and their processing by RECC (1, 215). The current studies add several new insights to our model of assembly and regulation in higher-order editosomes. This model is based on a new conceptual framework in which MRB1 complexes include physically and functionally distinct RNPs (Figure 39): (a) the catalytic mRNP REH2C with mRNA, the helicase REH2, and specific cofactors, and (b) structural gRNP variants (GRBC and GRBC*) with multiple gRNAs and several proteins of MRB1 (data not shown) (2). GRBC including 3010 is also called MRB1 core (208).

REH2C is an mRNP with pre-RNA substrates, intermediates and products of editing

We proposed docking of mRNP and gRNP modules through specific base pairing of their respective mRNA and gRNA cargo, respectively. This model is consistent with our finding that REH2C retains mRNA editing substrates and products at near normal levels upon depletion of GAP1 and gRNA in mitochondria. Thus, the REH2C mRNP can be physically uncoupled from GRBC variants that carry and stabilize gRNA. Docking of the helicase mRNP with GRBC* is stable. In contrast, the interaction between mRNP and GRBC involves transient contacts. Either type of RNP association may stabilize hybrid substrates on the scaffolds that engage the RECC enzyme. Thus, in

the mitochondrial milieu, target search by small gRNAs is likely a protein-assisted process (Figure 39). This model is also consistent with our prior identification of *cis* and *trans* effects of REH2 (2): inactivating mutations in the helicase core or dsRBD motifs uncouple REH2 from GRBC* and mRNA substrates and products; RNAi-based depletion of REH2 reduce pre-mRNA content and editing progression of mRNAs bound to GRBC. Similar target search mechanisms by base pairing of guide RNA with target RNA or DNA occur in RNA silencing, mRNA splicing, and rRNA biogenesis in eukaryotes, and gene expression control by small regulatory RNAs (sRNAs) and CRISPR (clustered regulatory interspaced short palindromic repeats) RNAs (crRNAs) in prokaryotes (340).

Novel domain organization and participation of REH2 and ^{H2}F1 in RNA editing

REH2 is the first identified RHA-type protein that participates in a eukaryotic RNA editing system. The conserved C-terminal domain cluster (CTD) of REH2 is an ancient molecular design that pre-dates the origin of trypanosomes over 100 million years ago (311). It offers a unique opportunity to probe conserved features that may impact helicase function in REH2 and related eukaryotic proteins in transcription, mRNA splicing, silencing and innate immunity (233, 265). Specific CTD determinants may control different steps of the editing pathway, such as mRNA recruitment by REH2C, docking of mRNP and gRNP modules, and addition of RECC or editing progression in the molecular scaffolds. The OB-fold in other RHA type helicases interacts with helicase cofactors and RNA that often modulate ATPase and unwinding activities of the helicase (271). The CTD may control the REH2 binding with

^{H2}F1 that we found with recombinant proteins (Figure 38). The unique ~1000-residue long N-terminal region (NTR) lacks identifiable domains. The NTR could provide additional editing determinants or a docking surface for factors in other mitochondrial RNA processes, including mitoribosomes. In a prior study, we proposed the REH2-purified complexes could serve as "organizers" in mitochondrial RNA metabolism (209). Ongoing work in our lab is characterizing the conserved CTD and the unique NTR in REH2. ^{H2}F1 is related to a specialized class of Cys2His2 zinc finger (Znf) proteins that usually bind to dsRNAs (dsRBZFPs). Other known dsRBZFPs play important roles in cellular localization and apoptosis (339). dsRBZFPs have several domains, each containing zinc finger motifs and each capable of binding dsRNA. The mammalian JAZ protein has four Znf domains (341), the zinc finger protein ZFa has seven (342) and ^{H2}F1 potentially has eight (data not shown). While all Znf domains in ^{H2}F1 may contribute to dsRNA binding some Znf domains could also mediate direct protein interactions with REH2. This is currently being addressed in our lab.

Requirements for assembly of native higher-order editosomes

Genetic knockdown of ^{H2}F1 had differential *cis* and *trans* effects on native editosomes (Figure 35). For example, ^{H2}F1 depletion did not affect the formation of a ^{H2}F2-REH2 subcomplex but decreased its association with mRNA (particularly pre-mRNA), GRBC*, REMC and RECC (in *cis*). In contrast, the GRBC-REMC complex was normal in the ^{H2}F1 knockdown but it exhibited a decreased association with pre-mRNA and RECC (in *trans*). This result suggests that the RECC enzyme enters the pathway through interactions with pre-assembled mRNA-gRNA hybrids in the scaffolds,

rather than via mRNA in the mRNP or via gRNA in gRNPs before the RNPs interact. This model of RECC entry also agrees with the loss of copurification between REH2C and the RECC enzyme in a GAP1 knockdown. That is, in gRNA-depleted mitochondria, REH2C retained mRNA substrates and products but lost its interaction with the RECC enzyme (Figure 34). The requirement of ^{H2}F1 for normal editing and assembly of higher-order editosomes validated ^{H2}F1 as a *bona fide* regulatory cofactor of REH2. In summary, we propose that ^{H2}F1 controls the docking between mRNP and gRNP modules. The coupling of these RNPs seems to be an essential step in the assembly of fully competent editosomes. ^{H2}F1 may promote specific pre-mRNA recruitment by REH2C. The complete mRNP then joins gRNPs via specific base pairing (Figure 39) resulting in the assembly of stable of mRNA-gRNA hybrids. Finally, RECC is added directly to the substrate hybrids in the scaffolds. Our analysis of RGG2 in REMC is consistent with direct association of this subcomplex with GRBC and GRBC*. This implies that mitochondria contain at least two versions of the proposed tripartite GRBC/REMC/PAMC complex, also termed RESC (215). In summary, our recent report (2) and these studies indicate that REH2 and ^{H2}F1 subunits of REH2C are required to establish stable mRNA-gRNA hybrid substrates in molecular scaffolds for editing. In the proposed model of stepwise editosome assembly, specificity checkpoints may exist during (1) initial mRNA recruitment by REH2C, (2) subsequent target search and coupling of mRNP and gRNP via specific base pairing, and (3) quality control of pre-assembled mRNA-gRNA hybrids for RECC binding and catalysis. REH2

and ^{H2}F1 may act in synchrony, likely via the regulatory C-terminal and multiple zinc finger domains, respectively, during these specificity checkpoints (Figure 39, model).

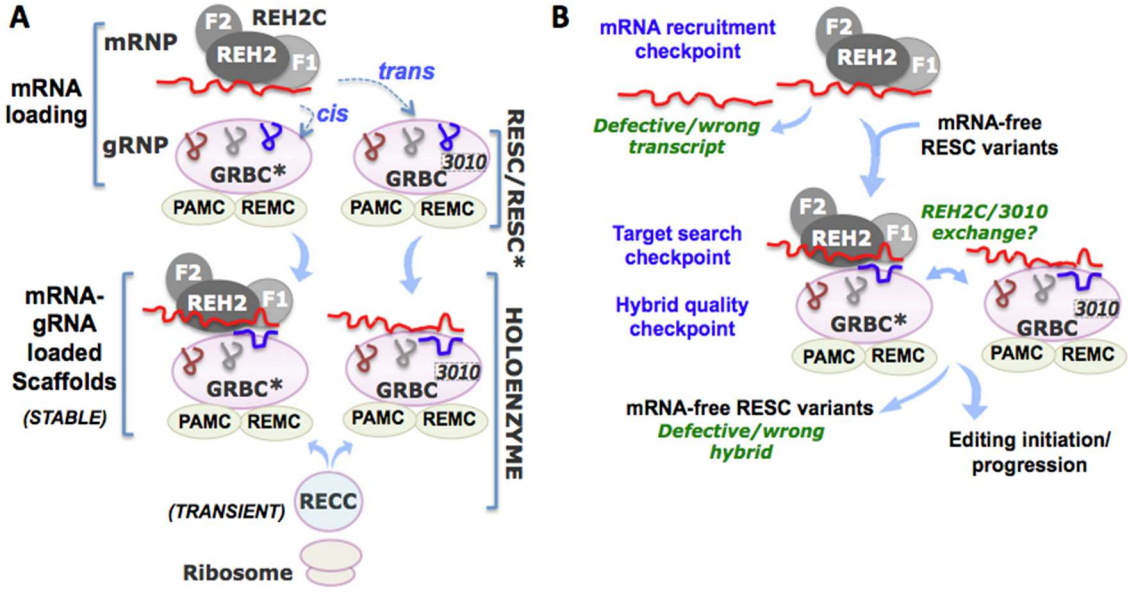

Figure 39. Model of mRNP and gRNP docking and specificity checkpoints in editosomes. *(A)* Assembly of mRNA-gRNA hybrid substrates on accessory editing scaffolds involves docking of mRNP and gRNP modules that hold mRNA and gRNA, respectively. In the proposed model, RNPs are joined via specific base pairing of mRNA and gRNA. REH2 and ^{H2}F1 subunits in the mRNP (termed REH2C) control mRNA substrate loading in *cis* or *trans* onto the gRNP variants GRBC and GRBC* (with and without 3010, respectively). The GRBC variants bind tightly to REMC and PAMC in tripartite aggregates labeled RESC and RESC*. The REH2C-RESC interaction is transient, but the REH2C-RESC* association is stable. Transient addition of RECC to mRNA-gRNA loaded scaffolds form editing holoenzymes or "editosomes". Mitoribosomes are also added in post-editing interactions. *(B)* Critical checkpoints in the editing pathway are expected during: mRNA recruitment, target search and scrutiny of hybrid quality in substrate-bound scaffolds. The regulatory C-terminus of REH2 and multiple zinc fingers of ^{H2}F1 may function in synergy at these checkpoints. REH2 helicase activity may remodel RNA-protein assemblies and resolve secondary structure during editing. Cycles of REH2C and 3010 exchange may create REH2C-RESC* and RESC complexes.

CHAPTER V

CONCLUSIONS

RNA editing is a unique post-transcriptional maturation of mitochondrial mRNAs that involves U-insertion/deletion at specific editing locations. This process requires a multiprotein enzyme known as RECC, which includes all the catalytic proteins required in the basic editing reaction. Small non-coding gRNA transcripts direct the insertion/deletion changes by RECC in the mRNA. Studies show that mRNA substrates or gRNAs are not found in isolated RECC indicating that enzyme-substrate interactions are transient. This raised the long-standing question: how are the substrates delivered to the RECC and what regulates the RNA editing process?

Our published study (1) in chapter II, revealed that native MRBs can vary in protein as well as RNA composition. In that study, we distinguished two MRBs that either carries REH2 or 3010 protein subunits. Our next generation sequence (NGS) analyses indicated that 3010-MRB carries a higher number of initiating gRNAs than REH2-MRB. This was consistent with a prior report suggesting that MRB3010 is important during early stages of editing. Purifications of MRB1 were only shown to contain gRNAs but our study showed that both MRBs studied in our lab are also enriched of mRNAs including both pre-edited and edited transcripts. In that study, we proposed for the first time that functional mRNA-gRNA hybrids are assembled in both REH2-MRB and 3010-MRB. Our model goes further to indicate that the resulting substrate-loaded “scaffolds” transiently associate with the RECC enzyme to establish complete higher-order holoenzymes.

In chapter III, our study defined functional differences between the MRBs characterized in our lab. Based on observations from chapter II and III, we have proposed that 3010-MRB may be the major scaffold for the RNA editing process. This complex has increased level of initiating gRNA and mRNA. Also, 3010-MRB shows increased editing in the first block (3' –most) directed by the initiating gRNA. We found that the REH2 helicase affects the REH2-MRB and 3010-MRB in *cis* and *trans*, respectively. For instance, in *cis* inactivating mutations in the dsRBD and the helicase catalytic core in REH2 prevented mRNA association with the helicase in *cis*. In line with our model, this loss of mRNA led to dissociation of the REH2 from the gRNP in its own MRB (i.e. from gRNA and gRNA-associated proteins). In *trans* effects, RNAi-induced depletion of REH2 caused loss of mRNA substrates in 3010-MRB without changing the gRNA levels in total mitochondrial RNA or in 3010-MRB. In addition, knockdown of REH2 effected multiple steps of editing function in 3010-MRB. This study also showed an RNA-dependent interaction between REH2 with its gRNP (gRNA and gRNA-associated proteins) in its own MRB. It also provided the first evidence that REH2 is part of a novel ~15S subcomplex that we termed REH2C. Based on this study we hypothesized that specific putative co-factors of REH2 in the REH2C subcomplex may control the functional interaction of REH2 with both the RNA and protein partners during editing.

In Chapter IV we discovered and characterized two REH2-associated cofactors (termed ^{H2}F1 and ^{H2}F2) which stably associate with REH2C in an RNA-independent manner. Our data indicate that REH2, ^{H2}F1 and ^{H2}F2 form an mRNP that contains all

mRNA types in editing (substrates, intermediates and products). This mRNP is stable upon the depletion of gRNPs. Knockdown studies of ^{H2}F1 showed severe reduction in editing of mRNA transcripts but ^{H2}F2 had no effect on *in vivo* editing. Furthermore, this study showed that ^{H2}F1 exhibits *cis* and *trans* effects on the assembly of the editing holoenzyme. In *cis* effect, depletion of ^{H2}F1 inhibited the REH2 interaction with pre-mRNA, the gRNP in its own MRB and with the RECC enzyme. Our results indicate that RECC is only recruited to establish the complete holoenzyme once mRNA-gRNA hybrids are pre-assembled. We propose that ^{H2}F1 controls the interactions between mRNP and gRNP components to form substrate-loaded scaffolds. In *trans*, the scaffolds engage in transient but protective interactions with the RECC enzyme.

Together, our data led to the model (Figure 39) proposing stepwise assembly of the editing holoenzyme: (1st step) specific recruitment of pre-mRNA substrate by REH2C in a process controlled by REH2 and ^{H2}F1, (2nd step) assembly of the mRNP with a gRNP through specific base-pairing of mRNA and gRNA, and (3rd step) recruitment of RECC on pre-assembled mRNA-gRNA hybrids in the MRB scaffolds. REH2 and ^{H2}F1 may function in synergy via interaction between their regulatory domains to control specificity checkpoints in the editing pathway.

In summary, the studies in this dissertation illustrate the essential function of REH2 and its cofactors in mitochondrial RNA editing of *T. brucei*. They provided the first detailed insights on a stepwise assembly process of the editing holoenzyme. The identification of specialized RNP subcomplexes in complete editosomes increased our understanding of RNA editing mechanism. These studies add to the exciting complexity

of the life cycle of kinetoplastids. This may also open venues towards the development of therapeutic agents against parasitic members in this incredible group of organisms.

Future directions

Characterizing the function of additional REH2 mutants

Mutations in the OB-fold at the C-terminus of REH2

Recently, we observed a C-terminal OB-fold in the sequence analysis of REH2. The OB-fold has been reported as a highly conserved regulatory domain in other DEAH/RHA family helicases that act as a platform for the binding of helicase co-factors and regulate helicase function (265). Therefore, it is important to study the relevance of the OB-fold domain in REH2. In order to study the significance of the OB-fold in REH2, one could generate a TAP-tagged version of an OB-fold mutant either through complete deletion of the OB-fold domain or an alternative approach could be to generate point mutations in conserved residues of the OB-fold. These mutants could be tested for their interactions with REH2 cofactors, MRB1 complex, RECC and the RNAs. Additionally, REH2 could be tested for RNA binding and unwinding activity *in vitro*.

Mutations in the N-terminus of REH2

Although, most of the conserved domains are present towards the C-terminus in REH2 including the core of helicase and dsRBD domain towards the middle. However, the sequence analysis of REH2 does not show any identifiable domain in the N-terminus. This unique ~1000 amino acid long N-terminus may provide binding for

additional editing proteins or other factors involved in different mitochondrial RNA processes such as, mitoribosomes. This makes it crucial to identify the significance of this long N-terminus without any defined domain. In order to so, one could create TAP-tagged N-terminus mutants with different length deletions. An approach similar to the one used to test OB-fold mutants can be used with N-terminus mutants. This includes testing the *in vivo* protein and RNA interactions and RNA binding and unwinding *in vitro*.

Identifying the function of zinc fingers in REH2 cofactor ^{H2}F1

Chapter IV describes that ^{H2}F1 is comprised of eight classical Cys2His2 zinc finger throughout the length of the protein. This class of zinc finger proteins is often seen in dsRNA binding. As described in chapter IV, the conserved domain database search predicted Znf6-8 but, Znf5, Znf7, and Znf8 were accurate enough in homology model of ^{H2}F1. However, we identified Znf1-4 upon visual inspection and sequence alignment with other zinc fingers in ^{H2}F1. Therefore, it will be interesting to identify the role of all these zinc fingers in RNA binding and its interaction with RNA helicase REH2. Since, classical Cys2His2 zinc fingers contain conserved Cys and His residue, which could be the target for the mutation in ^{H2}F1.

One could generate, TAP-tagged mutants targeting Cys or His or both residues in each Znf domains. Based on the observation in chapter IV, I propose that mutation of all zinc fingers domains may be lethal. Therefore, one could try mutating selective Znf domains in pairs. These mutants can be tested for its interaction with REH2, MRB1 complex, RECC and RNAs *in vivo*. Since, genetic knockdown of ^{H2}F1 showed very little

to no effect on RNA unwinding activity of REH2, an alternative approach could be applied. This approach may include recombinant expression of both REH2 and ^{H2}F1 (wildtype or mutants) as a complex and testing the RNA unwinding activity.

Null mutation of REH2 cofactor ^{H2}F2 through CRISPER/Cas9 system

In chapter IV we were not able to attain significant depletion of REH2 cofactor ^{H2}F2, and it appears that most the ^{H2}F2 does not always stay associated with REH2. It is possible that even minimal ^{H2}F2 is enough for its function. That could be the reason we did not see any evident editing phenotype. Therefore, it is important to test null mutants of ^{H2}F2 and its possible role in the mitochondrial REH2C. Currently, we are working on introducing CRISPER/Cas9 system in *T. brucei,* which is known to be effective in *Leishmania* (343). These null mutants of ^{H2}F2 can be tested for any editing phenotypes and its effect on the proteins and RNA interaction involved in RNA editing.

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