Trypanosome REH1 is an RNA helicase involved with the 3′–5′ polarity of multiple gRNA-guided uridine insertion/deletion RNA editing

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Uridine insertion/deletion RNA editing in kinetoplastid mitochondria corrects encoded framenshifts in mRNAs. The genetic information for editing resides in small guide RNAs (gRNAs), which form anchor duplexes just downstream of an editing site and mediate editing within a single editing “block.” Many mRNAs require multiple gRNAs; the observed overall 3′ to 5′ polarity of editing is determined by the formation of upstream mRNA anchors by downstream editing. Hel61, a mitochondrial DEAD-box protein, was previously shown to be involved in RNA editing, but the functional role was not clear. Here we report that down-regulation of Hel61 (renamed REH1 RNA editing helicase 1) expression in Trypanosoma brucei selectively affects editing mediated by two or more overlapping gRNAs but has no effect on editing within a single block. Down-regulation produces an increased abundance of the gRNAedited mRNA duplex for the first editing block of the A6 mRNA. Recombinant REH1 has an ATP-dependent double strand RNA unwinding activity in vitro with a model gRNA-mRNA duplex. These data indicate that REH1 is involved in gRNA displacement either directly by unwinding the gRNAedited mRNA duplex or indirectly, to allow the 5′ adjacent upstream gRNA to form an anchor duplex with the edited mRNA to initiate another block of editing. Purified tagged REH1 is associated with the RNA editing core complex by RNA linkers and a colocalization of REH1, REL1, and two kinetoplast ribosomal proteins with the kinetoplast DNA was observed by immunofluorescence, suggesting that editing, transcription, and translation may be functionally linked.

Leishmania | RNAi | tandem affinity purification | streptavidin binding and protein A purification

RNA editing has a 3′→5′ polarity (3). A single gRNA encodes the information for several adjacent editing sites; this constitutes an “editing block.” RNA editing has a 3′→5′ polarity within a single block (3) due to the fact that the gRNA first forms a duplex anchor just downstream of an editing site. Insertion and deletion of uridylyl residues occurs at the first gRNA/mRNA mismatch. This extends the mRNA-gRNA duplex in a 5′ direction and editing is then reinitiated at the next upstream editing site. However, “misdited” sequences occur that are due in some cases to the hybridization of the incorrect gRNA and in other cases apparently to stochastic errors in the editing mechanism. Alternative editing has been identified in several mRNAs that contain multiple gRNA-mediated editing domains (17). This mechanism would greatly increase the repertoire of proteins, but confirmation of this phenomenon and its generality remain to be examined.

The editing of most mRNAs is mediated by multiple overlapping gRNAs. Editing utilizing the adjacent upstream gRNA cannot proceed until the first block is completely edited because the gRNA can only form an anchor duplex with edited sequence to initiate the second editing block. This is responsible for the observed overall 3′ to 5′ polarity (18), but the mechanism of displacement of adjacent gRNAs is unknown. An RNA helicase has been suggested to be involved in this process by displacing the initial gRNA, thereby allowing the adjacent upstream gRNA to form an anchor duplex with the edited sequence (19). Two trypanosome mitochondrial DExD/H-box proteins have been identified (14, 16, 20, 21) and shown to be involved in RNA editing. RNA editing helicase 2 or REH2 is a component of GRBC or MRB1 complexes and is involved in gRNA biogenesis (13, 14, 16). Hel61, another DEAD-box protein, was shown to be involved in RNA editing by a gene disruption experiment in T. brucei, which produced a slow growth phenotype and affected editing efficiency, and ectopic reexpression of Hel61 rescued a partially restored editing phenotype (21). However, gene disruption had no effect on either an experimentally observed mito-

chondrial RNA unwinding activity (20) or on full cycle in vitro editing reactions (21). Furthermore, the observed RNA unwinding activity did not cosediment with Hel61 (21). The functional role of Hel61 and the mechanism of gRNA displacement were not clear.

In this paper we present evidence that Hel61 is involved in the displacement of gRNAs to allow the 5′ adjacent gRNA to form an anchor duplex with the edited sequence. We also show that recombinant REH1 has ATP-dependent duplex RNA unwinding activity. We have therefore reevaluated Hel61 with the functional name, REH1 (RNA editing helicase 1).

Results

Effect of Down-Regulation of REH1 Expression in T. brucei on Relative Abundance of mRNAs Edited in Block 1 Versus Those Edited in Two or More Blocks. Down-regulation of expression of REH1 by conditional RNAi in T. brucei procyclic cells produced a slow growth phenotype (Fig. 1A). An 80% decrease in the abundance of REH1 mRNA by day 3 was demonstrated by RT-PCR and real-time RT-PCR (Fig. 1B and D) and REH1 protein was not detectable by Western blot analysis (Fig. 1B). Repression of REH1 expression showed no effect on the stability or length of the gRNA 3′ oligo U tails (Fig. 1C), as was reported for the trypanosome REH2 mitochondrial RNA helicase (14, 16).

Real-time RT-PCR was also used to quantitate the effect of REH1 down-regulation on the relative abundance of several preedited and mature edited mRNAs (Fig. 1D and Table S1). The abundances of edited CR3 and A6 mRNAs were significantly reduced with down-regulation of REH1, but the effects on edited mRNAs for Cyb, ND7, CO3, and ND9 were small and probably not significant because a similar decrease was observed for the CO2 edited mRNA, which is mediated by a single in cis gRNA and does not require an overlapping gRNA. Interestingly, the abundances of preedited mRNAs for CO2 and ND9 were increased 30–40%, raising the possibility that REH1 has an effect on RNA turnover of some mRNAs. Two never-edited RNAs, ND4 and COI, were examined as controls: Neither showed a significant change in abundance. The changes in the abundances of the A6, CR3, Cyb, ND7, and CO3 preedited mRNAs were not statistically significant.

One possible mechanism for the inhibition of editing by REH1 down-regulation is that REH1 is involved in displacement of the gRNA from the edited mRNA/gRNA duplex. To test this hypothesis, we quantitatively compared the effect of down-regulation of REH1 expression on the relative abundance of mRNAs edited in block 1 versus those edited in block 1 and the adjacent upstream blocks. The experimental protocol is diagrammed in Fig. 2A (also see Table S1). The only T. brucei genes for which putative gRNAs are known for the 3′-most editing blocks are A6 and Cyb (Figs. S1 and S2) (22, 23). Real-time RT-PCR was used to measure the relative abundances of T. brucei partially edited A6 and Cyb mRNAs after 3 d of REH1 RNAi compared to these mRNAs in cells without induction of RNAi. Downregulation caused an increase of around 20% in the A6 mRNAs completely edited in block 1 (Fig. 2A). Real-time RT-PCR was also used to measure the relative abundances of T. brucei partially edited A6 and Cyb mRNAs after 3 d of REH1 RNAi compared to these mRNAs in cells without induction of RNAi. Downregulation caused an increase of around 20% in the A6 mRNAs in both blocks 1 and 2 and a decrease of around 40% in A6 mRNAs edited in blocks 1, 2, and 3 (Fig. 2B). It was noted that the primers for edited A6 mRNA in Fig. 1D are located near the 5′ end of A6 gene. Repression of REH1 expression caused an increase of around 20–30% in both the preedited Cyb mRNA and Cyb mRNA completely edited in block 1 and a decrease of around 20% in the mature Cyb mRNA edited in both blocks 1 and 2 (there are only two editing blocks in Cyb) (Fig. 2C). Thus, down-regulation of REH1 expression in T. brucei selectively affects editing that is mediated by two or more overlapping gRNAs but does not affect editing occurring within a single gRNA-mediated editing block. This result is consistent with a role in gRNA displacement.

Effect of Down-Regulation of REH1 Expression on Relative Abundance of Block 1 Edited mRNA/gRNA Duplex. Another prediction is that REH1 down-regulation would produce an increased abundance of a block 1-fully edited mRNA/gRNA duplex by inhibiting the progression of editing from block 1 to block 2. The relative abundance of this duplex was assayed by an RNase protection experiment in which poisoned primer extension using a primer complementary to fully edited block 1 mRNA was used to measure the abundance of the RNase-protected duplex RNA. The experimental protocol is diagrammed in Fig. 3. As shown in Fig. 3A, lanes 2 and 4, there is a significant increase in the relative abundance of the RNase-protected block 1 duplex after down-regulation of REH1 expression. Although the primer covers two U-deletion and two U-insertion sites, there is no significant decrease in the no RNAse control band in lanes 1 and 3, indicating that down-regulation of REH1 has no effect on intrablock editing. We conclude that REH1 is required for releasing gRNA from A6 mRNA that is completely edited in block 1.

Recombinant Streptavidin-Binding Motif (SBP)-tagged REH1 has RNA-dependent ATPase and Double Strand RNA Unwinding Activities. REH1 has conserved motifs found in DEAD-box proteins.
the results in this experiment are due to the use of an upstream primer near the 5′ end of the mRNA in Fig. 1D instead of the primer used in this assay, which is close to the 3′ end.

(Fig. 4F and Fig. S3), but there is no evidence in the literature for REH1 having RNA helicase activity. To address this question, we expressed tagged *Leishmania major* (Lm) REH1 (Fig. 4B) in S9 cells and analyzed the activity. A modified tandem affinity purification (TAP) tag (24), the streptavidin binding and protein purification (SAP) tag (Fig. 4B), was used in which the calmodulin binding peptide motif was substituted with a high affinity streptavidin binding peptide (SBP) motif. The rREH1 was purified to homogeneity by IgG-Sepharose binding, MonoS ion-exchange chromatography, and streptavidin affinity chromatography (Fig. 4C, lane 1). The expressed band was confirmed to be SBP-tagged REH1 by Western analysis using anti-REH1 or HRP-conjugated streptavidin (SA-HRP) (Fig. 4C, lanes 2, 3). His6x-tagged REH1 was also expressed in *Escherichia coli* and purified to homogeneity for antibody generation.

The purified SBP-tagged recombinant REH1 protein showed a robust poly U–stimulated ATPase activity. This activity was destroyed by RNase treatment (Fig. 4D). As a control for ATPase contamination from the S9 cells, SAP-tagged RET2 RECC protein was expressed and purified using the same procedure. The tagged RET2 did not show ATPase activity (Fig. 4D). The REH1 ATPase activity requires ATP or dATP (Fig. S4D and E). The optimal pH is around 8.3, the *K*_m for the reaction is 620 μM ATP, and the *K*_cat is 82.3 min⁻¹ (Fig. S4A–C).

Recombinant REH1 showed an ATP-dependent double strand RNA unwinding activity using a partially edited model gRNA edited mRNA duplex (Fig. 4E and Table S2). The reaction required ATP and showed a protein dose response (Fig. 4E and F). We also tested the unwinding of shorter RNA duplexes with 5′ or 3′ overhangs or no overhang (Fig. 4F–H). The latter were unwound more efficiently probably due to their lower stability. It has been shown that helicase activity in vitro shows an inverse relationship between duplex stability and unwinding (25). As a control, a mutation of K143A in motif I, which is the conserved ATP binding domain of REH1, was introduced and the mutant protein lost ATPase and unwinding activities (Fig. S5). These data indicate that REH1 is an ATP-dependent RNA helicase. The REH1 helicase apparently differs from most other DEAD-box RNA helicases that exhibit very poor in vitro unwinding activity and require a 5′ or 3′ single-stranded RNA region for unwinding activity (25).

### REH1, REL1, and the Kinetoplast Ribosome Proteins, L3 and S17, Show Concentration in the Kinetoplast Region of the Mitochondrion.

Immunolocalization of SAP-tagged REH1 within the single mitochondrion of transfected *L. tarentolae* cells was performed using an antibody against the C-terminal FLAG epitope (Fig. S4). This antibody does not have cross-reactivity with any endogenous protein (Fig. S6D). The cells were stained with MitoTracker Red to visualize the single mitochondrion and stained with DAPI to detect the nuclear and kinetoplast DNA, and then treated with anti-FLAG antibody and secondary anti-IgG antibody conjugated with Alexa Fluor 488 to visualize SAP-tagged REH1. The REH1 protein colocalized with the MitoTracker mitochondrial image. Interestingly, in addition to a dispersed localization throughout the tubular mitochondrion there is an apparent concentration of REH1 protein in the kinetoplast DNA-containing region. Lower resolution images of entire fields that show that the selected fields in Fig. 5A are representative are shown in Fig. S6A. As a control, cells were analyzed for the localization of glutamate dehydrogenase, a soluble mitochondrial protein not involved in RNA editing (Fig. S6C). These cells showed a somewhat punctuate distribution of immunofluorescence throughout the mitochondrion without the level of concentration in the kinetoplast region observed in the REH1 immunofluorescence, indicating that the observed kinetoplast concentration of REH1 is probably not artifactual (Fig. S6).

The localization of TAP-tagged REL1 RNA ligase, a RECC core component, and two TAP-tagged kinetoplast ribosome proteins (Fig. S6E), S17 and L3, were also analyzed by indirect immunofluorescence. REL1 and the two ribosome proteins showed a similar kinetoplast concentration as the SAP-tagged REH1 (Fig. 5B–D and Fig. S6B).

**REH1 Is Associated with the RECC by RNA Linkers.** To investigate possible interactions between REH1 and the RECC, SAP-tagged Lm REH1 with a mitochondrial target signal was expressed in *L. tarentolae*. The tagged REH1 was found only in the whole cell
and mitochondrial fractions and not in the cytosol fraction (Fig. S7A). Cell lysate was allowed to bind to IgG-Sepharose, and the bound material was released by digestion with tobacco etch virus (TEV) protease. Half of the eluted material was treated with RNase A prior to fractionation on a glycerol gradient. The unwinding reaction required ATP and showed a dose response with the amount of protein. (Fig. S7B) The diagram of the SAP-tagged Lm REH1. The unwinding reaction required ATP and showed a dose response with the amount of protein. (Fig. S7C) The diagram of the SAP-tagged Lm REH1. The unwinding reaction required ATP and showed a dose response with the amount of protein. (Fig. S7D) The diagram of the SAP-tagged Lm REH1. The unwinding reaction required ATP and showed a dose response with the amount of protein.
stained with Sypro (Invitrogen), or blotted and probed with anti-REH1 or anti-MRP1/2. Note that the labeled REL1/REL2 and the MRP1/2 proteins were detected only prior to RNase treatment. The three major stained bands were subjected to mass spectrometry (data shown in Table S3).

Possibility of regulation of helicase activity in vivo. In fact, the evidence that the largest effect of REH1 could be to unwind the mRNA/gRNA duplex formed by the editing of one gRNA-mediated block as editing proceeds from site to site or (ii) to be involved in gRNA displacement between adjacent editing blocks. Model 1 is ruled out by the observed lack of effect of REH1 down-regulation on intrablock editing. Model 2 is supported by the significant decrease in the relative abundance of mRNA edited in two or more adjacent blocks by REH1 down-regulation as compared to those edited in a single block, and by the accumulation of the gRNA/block 1 edited A6 mRNA duplex. These data suggest that REH1 is involved with the progression of editing from one gRNA-mediated editing block to the next adjacent upstream block.

The mechanism of this involvement, however, is not clear. The fact that recombinant REH1 protein has gRNA/mRNA duplex unwinding activity in vitro may suggest an in vivo role in directly unwinding the mRNA/gRNA duplex formed by the editing of all the sites mediated by a single gRNA and liberating an edited mRNA single strand available for hybridization of the adjacent gRNA. However, in the case of REH1, the evidence does not distinguish between a direct effect on unwinding of the gRNA/mRNA duplex or an indirect effect, such as described for eIF4AIIII, a core component of the exon junction complex, and some putative RNA helicases that are involved in snRNA release from preribosomes (27–31). Another potential function of REH1 could be to unwind cis elements within the preedited mRNA. A dominant-negative mutation of REH1 and ectopic reexpression of wild-type REH1 might indeed help to explore the role of REH1 in vivo. In fact, the evidence that the largest effect of REH1 down-regulation on editing is on the pan-edited A6 and CR3 mRNAs suggests that the in vivo activity of REH1 is substrate-specific and perhaps is regulated by transient binding of cofactors. Along this line, no detectable in vitro unwinding activity had been observed with T. brucei gradient fractions that showed a peak of Hel61 (REH1) by blot analysis (21) nor in the Lm REH1 SAP pull-down (Fig. S7B). These data suggest the possibility of regulation of helicase activity in vivo.

There have been conflicting reports on the association of REH1 with the RECC. Tb REH1 was detected by mass spectrometry analysis in an MP63-immunoprecipitated sample and also in a RECC preparation isolated by ion-exchange chromatography (32–34). However, REH1 was not detected in REN1-, REN2-, or REN3-TAP pull-downs from T. brucei (32, 35), nor in REL1-TAP and MP44-TAP pull-downs from L. tarentolae (7, 11). These results could be explained by our finding that REH1 is associated with the RECC by RNA linkers, as are several other editing-associated complexes (6, 11), and that this linkage is easily disrupted during the isolation.

Immunofluorescence of REH1 showed a concentration in the kinetoplast DNA (kDNA) region of the mitochondrion. Interestingly, the REL1 RNA ligase, a component of the RECC, and the S17 and L3 kinetoplast ribosome proteins also exhibited a concentration in the kDNA region of the mitochondrion, suggesting the intriguing possibility of a physical linkage of kDNA transcription, translation, and editing pathways, but this remains to be investigated. Our localization results differ somewhat from several previous studies that showed that editing proteins are distributed throughout the mitochondrion with no apparent concentration (36, 37) and that the RNA-linked editing proteins, GAPI and GAPII, are localized in discrete particles throughout the mitochondrion (14). These differences could be species-dependent or could be due to technical details.

More work is required to fully understand the precise role of the REH1 helicase in RNA editing, but it is clear from the results in this paper that REH1 is involved in the 3′ to 5′ polarity of editing in a multi-gRNA-mediated editing domain.

Discussion

REH1 is essential as shown by the growth phenotype caused by down-regulation of REH1 expression. The partial growth inhibition could be due to incomplete down-regulation, a slow turnover of the protein or an enzyme redundancy. Down-regulation also causes a decrease in abundance of edited mRNAs for the six genes assayed, with editing of the A6 and CR3 mRNAs being the most affected. Possible roles of the REH1 helicase in RNA editing are (i) to directly assist in the processive editing reaction itself within a single gRNA-mediated block as editing proceeds from site to site or (ii) to be involved in gRNA displacement between adjacent editing blocks. Model 1 is ruled out by the observed lack of effect of REH1 down-regulation on intrablock editing. Model 2 is supported by the significant decrease in the relative abundance of mRNAs edited in two or more adjacent blocks by REH1 down-regulation as compared to those edited in a single block, and by the accumulation of the gRNA/block 1 edited A6 mRNA duplex. These data suggest that REH1 is involved with the progression of editing from one gRNA-mediated editing block to the next adjacent upstream block.

Materials and Methods

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Supporting Information

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SI Materials and Methods.

Construction of Plasmids. For conditional RNAi, a 500-bp fragment from the 5′ end of Tb REH1 was amplified with the primers shown in Table S1. The fragments were inserted into the pLew-HX-100-GFP vector using the HindIII, Xbal and NdeI, BamHI sites to form a head-to-head RNAi plasmid under poly (ADP-ribose) polymerase promoter control.

The REH1 gene was amplified from *Leishmania major* (Lm) genomic DNA with LmH1-F and LmH1-R primers. The full-length gene was cloned into pCR2.1-TOPO (Invitrogen) with EcoRI. HT-tagged REH1 was purified by metal affinity chromatography on Talon resin (BD Biosciences) according to the manufacturer’s protocol. One microgram of pX-REH1-TAP vector was digested with BamHI and NotI. Expression of proteins in baculovirus transfected Sf9 cells was inserted into pET28a (Novagen) vector with NdeI and BamHI sites to form a head-to-head RNAi plasmid under poly (ADP-ribose) polymerase promoter control.

The REH1 gene was amplified from *Leishmania tarentolae* with a streptavidin-binding motif (SBP) tag, the REH1 gene was excised with BamHI and Xbal and then cloned into the same sites of pX-derived px-RELI-TAP vector. To replace the calmodulin binding peptide tag in the vector, the SBP tag (1) was amplified from the pBEn-SBPSET1 plasmid (Stratagene) with SBP-F and SBP-R primers and the product was ligated recombinant plasmids expressing S17-TAP and L3-TAP into the pFastBac1 vector. To replace the calmodulin binding peptide tag, the REH1 gene was excised with BamHI and Xbal and cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer’s protocol. Polyclonal antibodies were raised against the purified *E. coli* expressed recombinant REH1 (Covance).

The coding regions of mitochondrial ribosomal proteins S17 from *L. major* and L3 from *L. tarentolae* were amplified using oligonucleotide primers derived from the corresponding *L. major* sequences (S17: LmjF35.3850 and L3: LmjF29.0030). The S17 primers were DM6845, 5′-GGGGATCCATGTTGTCGAGATGTTTCGTCAGTGCGTCTTGACGCGCTCGGCGTCTCTGCAGCTCA (BamHI site italicized), and DM6846, 5′-CATCTTAGAGGCGGACTTCTTCAAGTTTTTGAAGTCTCTAGATAC (Xbal site italicized). The L3 primers were: DM6847, 5′-GGGGATCCATGTCGAGATGTTTCGTCAGTGGGCGTCTCAGGTCTGCGC-GCCATATTTCAGAATCTTTG. The amplified DNA fragments were digested with BamHI and Xbal and cloned into pX-RELI-TAP vector in *E. coli* using standard procedures. The selected recombinant plasmids expressing S17-TAP and L3-TAP fusions were used to transflect *L. tarentolae*. Mitochondrial importation and cosedimentation of the TAP-tagged proteins with the 45S–50S ribosomal complexes was confirmed in both cases.

Real-Time PCR Analysis. Total RNA was isolated from cells grown in the presence or absence of tetracycline with Tri reagent as described by the manufacturer (MRC). Twelve micrograms of RNA was treated with DNase I (Ambion). The cDNA templates were reverse transcribed from 4.5 μg of RNA using random hexamers and Taqman reverse transcription reagents (Applied Biosystems) in a 30-μL reaction. The templates were diluted eightfold in water. For each PCR reaction, 2.5-μL cDNA template (or −RT control), 5 μL of 1.5 μM forward primer, 5 μL of 1.5 μM reverse primer, and 12.5 μL of SYBR Green Master Mix (Applied Biosystems) were combined in a well of a 96-well plate (Denville Inc.). The PCR amplification conditions were 95°C (10 min) and 45 cycles of 95°C (15 s), 55°C (30 s), and 63°C (30 s). The amplification was performed in triplicate. The sequences of primers for each preedited and edited mRNA can be found in Table S1.

RNase Protection and Poisoned Primer Extension. Total RNA was isolated with the Tri Reagent (MRC) as described in the manufacturer’s protocol, and contaminating DNA was eliminated with DNase (Ambion). About 35 μg RNA was treated with 0.5–1 U RNase One (Promega) in 100 μL of 10 mM Tris pH 7.5, 5 mM EDTA, 200 mM NaOAC buffer for 1 h at 10°C and SDS was added to a final concentration of 0.1% to deactivate RNase One. 5′-32P-end labeled oligonucleotides (0.25 pmol) were co-precipitated with residual RNA and dissolved in 10 μL of 30 mM Hepes pH 7.5, 100 mM KOAC, 0.1 mM EDTA for annealing. Primer extension was carried out as described previously (2).

Samples were heated for 2 min at 90°C and cooled down to 37°C for 1 h. Twenty microliters of extension solution prewarmed to 37°C were quickly added to the hybridization mix. The final reaction mixture contains 50 μm Tris-HCl pH 8.3, 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, 10 mM DTT, 200 μm dATP, dGTP, dTTP, and dUTP, and 5 μL of avian myeloblastosis virus reverse transcriptase (Promega). Upon extension at 37°C for 30 min, RNA was hydrolyzed by adding 1.5 μL of 0.5 M EDTA and 3.2 μL of freshly made 2 M NaOH, and incubating at 65°C for 30 min. Reaction mix was neutralized by 4 μL of freshly made 2 M HCl. Extension products were precipitated with 1 μg/reaction of glycogen (Ambion) and 3 V of ethanol, washed with 90% ethanol, and resuspended in 5 μL in 95% formamide, 10 mM EDTA, 0.05% xylene cyanol, 0.05% bromphenol blue loading buffer. Products were analyzed on 10% polyacrylamide/8M Urea gel.

Purification of SAP-Tagged Recombinant Lm REH1. Insect Sf9 cells (2 g) were resuspended in 12 mL TMK buffer (20 mM Tris-HCl, pH7.6, 60 mM KCl, 10 mM MgCl2) with 0.5% NP-40 and one tablet of Complete Protease Inhibitors (Roche). After lysis on ice for 30 min, the lysate was sonicated 3 times for 20 s. The lysate was cleared by centrifugation at 200,000 × g for 20 min. The supernatant was incubated with 0.5 mL IgG Sepharose FF (GE Healthcare) for 3 h in the cold room and transferred to a disposable 5-mL column. The beads were washed sequentially with 30 mL of TMK buffer plus 0.1% NP-40 and 10 mL of TEV-protease buffer (TMK buffer plus 0.1% NP-40 and 1 mM DTT). Four microliters of the same buffer with 100 U of TEV protease were added to the column and the column was incubated overnight with constant mixing. The IgG resin was drained and washed with 2 mL of TMK buffer plus 0.1% NP-40. The eluted material was pooled and applied to a Mono S 5/50 GL FPLC column (Amersham Biosciences) previously equilibrated with the same buffer. The column was eluted with a gradient of NaCl (0–0.6 M) in the same buffer. The fractions containing ATPase activity were pooled and incubated with 0.5 mL of streptavidin sepharose (GE Healthcare) for 1 h in the cold room and transferred to a disposable 5-mL column. The beads were washed with 20 mL of 20 mM Hepes-KOH pH 7.6, 60 mM KCl, 2.5 mM MgCl2, 1 mM DTT, and 0.1% NP-40, and then the bound proteins were eluted with 3 mL of 20 mM Hepes-KOH pH7.6, 60 mM KCl, 2.5 mM MgCl2, 1 mM DTT, 2 mM biotin, and 0.1% NP-40.
ATPase Activity Assay. ATPase reactions were carried out as described (3) with minor modifications. A typical ATPase assay was carried out in a 40-μL reaction volume containing 50 mM Hepes-KOH pH 8.2, 2.5 mM MgCl₂ and 2 mM ATP, 2 μg of appropriate RNA and 5 μL of recombinant REH1 helicase. The reaction mixture was incubated at 27°C for 30 min in a 96-well plate. Then the reaction was terminated by adding 160 μL of a malachite green/molybdate/polyvinyl alcohol acidic solution. The absorption at 630 nm was measured and the ATPase activity was calculated as the amount of Pi produced through hydrolysis of ATP using a standard curve constructed using known concentrations of potassium dihydrogen phosphate.

Unwinding Assay. In brief, 20 μL reaction mixture contained 10 mM Hepes pH 7.5, 60 mM KCl, 2.5 mM MgCl₂, 2 mM DTT, 2 mM ATP, 5% glycerol, 60 μg/mL BSA, 400 units/mL RNase guard, 2.5 mM dsRNA, and 2 μL SBP-tagged REH1. Reactions were incubated at 27°C for 60 min then stopped by adding 5 μL of a solution containing 50% glycerol, 2% SDS, 20 mM EDTA, 0.01% bromphenol blue, and xylene cyanol dyes. Samples were separated by 15% non-denaturing PAGE.

Immunofluorescent Localization of REH1, REL1, S17, L3, and Glutamate Dehydrogenase. L. tarentolae (2 × 10⁴) cells expressing TAP/SAP-tagged proteins were harvested and resuspended in Brain Heart Infusion Medium with 0.4 μM MitoTracker CMXRos (Invitrogen), and the cells were incubated at 27°C for 30 min and washed with PBS. Then the cells were fixed in 4% paraformaldehyde in PBS for 5 min and fixation was stopped by two 5-min washes in 0.1 M glycine in PBS. The cells were allowed to adhere to poly-L-lysine-coated glass slides for 30 min, followed by incubation for 10 min in 0.025% Triton X-100 in PBS. The slides were kept in methanol at -20°C overnight and rehydrated by three washes in PBS for 5 min each. The cells were then blocked in 20% goat serum in PBST (0.05% Tween-20 in PBS) at room temperature for 2 h, followed by a 60-min incubation with anti-FLAG monoclonal antibody at a 1 : 100 dilution (Trend Phara & Tech). After three times washing in PBST, cells were incubated further with F(ab)₂ fragment of goat anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen) at a 1:500 dilution for 60 min. The slides were washed again three times for 5 min each in PBST and were mounted by using ProLong Gold antifade reagent with DAPI (Invitrogen), which stains both the nucleus and the kinetoplast. For fluorescence microscopy, cells were imaged by using a Zeiss Axioskop II compound microscope with a 63× Plan-neofluor oil-immersion objective lens, and images were captured using a Zeiss Axioimaging digital camera and Zeiss Axiovision 4.5 software. Red, blue, green, and phase-contrast images of the same field were captured independently and were merged using Adobe Photoshop. The same procedure was used to detect immunofluorescence of the endogenous mitochondrial glutamate dehydrogenase using a polyclonal antibody against this protein.

Mitochondrial Importation of L3-TAP and S17-TAP Fusions. The transfected cell lines of L. tarentolae were propagated as 200-mL cultures to a density of 10⁸ cells/mL. Cells were completely ruptured using the Stanenz homogenizer and a crude mitochondrial pellet obtained as described previously (4). The pellet was resuspended in 2 mL of SHE (0.25 M sorbitol, 20 mM Hepes, and 2 mM EDTA, pH 7.5) buffer solution. The supernatant, representing the cytosolic fraction, was concentrated using the Amicon Ultra-15 Centrifugal Filter Unit to the volume of 2 mL. An equal volume representing each fraction and an equivalent amount of a total cell lysate were analyzed in a 8—16% polyacrylamide Tris-glycine SDS Novex® gel (Invitrogen). Western blots were probed for the TAP moiety present in the fusions with the PAP reagent (Sigma) followed by ECL detection (SuperSignal® West Pico substrate kit, Thermo Scientific).

Sedimentation Analysis of Mitochondrial Ribosomal Complexes from L. tarentolae. The L. tarentolae cell lines expressing L3-TAP and S17-TAP fusions were used to isolate mitochondria, followed by velocity sedimentation of ribosomal complexes from a dodecyl maltoside lysate of mitochondrial in a 7—30% sucrose gradient as described previously (5). Gradient fractions were assayed for presence and relative quantity of the 9S and 12S ribosomal RNA (5), as well as for the TAP-tagged proteins by probing Western blots of electrophoretic gels with the PAP reagent.

Fig. S1. Real-time RT-PCR analysis of partially edited *Trypanosoma brucei* ATPase subunit 6 mRNA. (Upper) Diagram of A6 mRNA and gRNA sequences. The PCR primers are indicated by boxed sequences. The gRNAs 1–3 are indicated. :, Watson–Crick base pairs. :, G-U base pairs. (Lower) Sequences of the amplicons obtained. The editing blocks are indicated by color. Editing block 1 = primer 1 + primer 2. Editing blocks 1 + 2 = primer 1 + primer 3. Editing blocks 1 + 2 + 3 = primer 1 + primer 2 + primer 3.

**Pre mRNA**

```
AGUUGACAAAGUGGGCAAAUUAUUUAUAA
```

**Edited mRNA**

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

**gRNA**

```
A AG CA A
```

**Primer 1**

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

**Primer 2**

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

**Primer 3**

```
A AG CA A
```

**Amplicons**

```
1st block edited (Primer 1/Primer 2)
```

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

```
A AG CA A
```

**2nd blocks edited (Primer 1/Primer 3)**

```
CUUAGGACAAAGUGGGCAAAUUAUUUAUAA
```

```
CUUAGGACAAAGUGGGCAAAUUAUUUAUAA
```

```
A AG CA A
```

**Editing blocks 1 + 2 + 3**

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

```
GUUAGAAAGAAGGUGGCAAAUUAUUUAUAA
```

```
A AG CA A
```

Fig. S2. Real-Time RT-PCR analysis of partially edited *Trypanosoma brucei* cytochrome b mRNA. (Upper) Diagram of mRNA and gRNA sequences. The PCR primers are indicated by boxed sequences. The gRNA 1–3 are indicated. :, Watson–Crick base pairs. :, G-U base pairs. (Lower) Sequences of the amplicons obtained. The editing blocks are indicated by color. Editing block 1 = primer 1 + primer 2. Editing blocks 1 + 2 = primer 1 + primer 3. Editing blocks 1 + 2 + 3 = primer 1 + primer 2 + primer 3.

**Pre mRNA**

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

**Edited mRNA**

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

**gRNA**

```
A AG GAG AA
```

**Primer 1**

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

**Primer 2**

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

**Primer 3**

```
A AG GAG AA
```

**Amplicons**

```
1st block edited (Primer 1/Primer 2)
```

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

```
A AG GAG AA
```

**2nd blocks edited (Primer 1/Primer 3)**

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

```
A AG GAG AA
```

**Editing blocks 1 + 2 + 3**

```
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```

```
GCUUUGAGAAAAGUGGGCAAAUUAUUUAUAA
```

```
A AG GAG AA
```

Fig. S11. Real-time RT-PCR analysis of partially edited *T. brucei* cytochrome b mRNA. (Upper) Diagram of mRNA and gRNA sequences. The PCR primers are indicated by boxed sequences. The gRNAs 1–3 are indicated. :, Watson–Crick base pairs. :, G-U base pairs. (Lower) Sequences of the amplicons obtained. The editing blocks are indicated by color. Editing block 1 = primer 1 + primer 2. Editing blocks 1 + 2 = primer 1 + primer 3. Editing blocks 1 + 2 + 3 = primer 1 + primer 2 + primer 3.
Fig. S3. Alignment of conserved motifs of Lm REH1 and other RNA helicases. Sequences of the conserved motifs from Lm REH1 (Leishmania major), Tb REH1 (Trypanosoma brucei), Vasa (Drosophila Melanogaster), Ddx3x (Homo sapiens), MjDEAD (Methanocaldococcus jannaschii), eIF4A (Saccharomyces Cerevisiae), eIF4A III (Homo sapiens), Dhh1p (Saccharomyces Cerevisiae), DbpA (Bacillus subtilis), and DDX6 (Homo sapiens). Matches are shown in black highlight and conserved changes in gray. Performed using Vector NTI (Invitrogen). NTD is N-terminal domain. CTD is C-terminal domain.

Fig. S4. Biochemical characterization of recombinant REH1. (A) The optimal concentration of MgCl$_2$ was determined in 50 mM of Hepes-KOH (pH 8.2) with 2 mM ATP. (B) The optimal pH was determined with 2 mM ATP and 2.5 mM MgCl$_2$. (C) Kinetic analysis of ATPase activity of purified tagged Lm Hel61. The reaction was performed in 50 mM of Hepes-KOH pH 8.2, 2 mM MgCl$_2$, 2 μg of poly U, and various concentrations of ATP. The solid line represents a Michaelis-Menton nonlinear curve fit to the data points (Sigmaplot). Error bars indicate one standard deviation. (D). Extent of hydrolysis of NTPs and dNTPs assayed in 50 mM of Hepes-KOH (pH 8.2) with 2.5 mM MgCl$_2$, 2 μg of poly U, and 2 mM of each NTP or dNTP. Activity is represented as percentage of ATP-hydrolysis activity. (E). ADP and AMP and also adenosine methylene diphosphate (AMPCP), a nonhydrolysable ATP analog, were used as substrates with purified rREH1 under same conditions as in D.
with the PAP reagent, and ECL detection. The proteins from a total cell lysate (T) were analyzed in parallel. The expected size of L3-TAP is 76 kDa and that of S17-TAP is 58 kDa (including the 22-kDa TAP moiety). With L3, most of the expressed protein was found in the mitochondrial fraction, whereas only a smaller fraction of the tagged S17 protein was imported. In addition, the overall expression level of S17 was lower than that observed with L3. Whether or not this can be attributed to the use of a heterologous S17 remains unknown. The two right-most lanes of the panel represent a longer exposure of the same blot.

Fig. S6. Localization of mitochondrial proteins in *L. tarentolae*. (A) Immunolocalization of SAP-tagged Lm REH1 and (B) TAP-tagged Lt REL1 in late log phase *L. tarentolae* cells. Multiple layers of the same microscope field are shown. Green, cells stained with mouse anti-FLAG antibody and the F(ab) fragment of goat anti-mouse IgG conjugated with Alexa Fluor 488. Blue, cells stained with DAPI. Red, cells stained with MitoTracker CMXRos. The third panel in each set represents a merged image of the MitoTracker-stained cells and the antibody-stained cells. (C) Immunolocalization of Lt glutamate dehydrogenase in late log phase *L. tarentolae* cells. Multiple layers of the same microscope field are shown. Red, cells stained with rabbit antil glutamate dehydrogenase antibody and the F(ab) fragment of goat anti-rabbit IgG conjugated with Alexa Fluor 594. Blue, cells stained with DAPI. The last panel in each set represents a merged image of the DAPI-stained cells and antibody-stained cells. (D) Specificity of anti-FLAG antibody. Cell extract from different cell lines were analyzed by SDS gel and PAP reagent and anti-FLAG (Trend Pharma & Tech) monoclonal antibody were used to detect the tagged proteins. (E) Cosedimentation with mitochondrial ribosomal proteins of the L3-TAP and S17-TAP fusion proteins. (Upper) Cell lines of *L. tarentolae* expressing *L. tarentolae* L3-TAP (L3) and *L. major* S17-TAP (S17) were isotonically ruptured, and the cytosolic (C) and mitochondrial (M) fractions were separated by centrifugation. Proteins in each subcellular fraction were separated in a 8–16% polyacrylamide gel, followed by blotting, probing with the PAP reagent, and ECL detection. The proteins from a total cell lysate (T) were analyzed in parallel. The expected size of L3-TAP is 76 kDa and that of S17-TAP is 58 kDa (including the 22-kDa TAP moiety). With L3, most of the expressed protein was found in the mitochondrial fraction, whereas only a smaller fraction of the tagged S17 protein was imported. In addition, the overall expression level of S17 was lower than that observed with L3. Whether or not this can be attributed to the use of a heterologous S17 remains unknown. The two right-most lanes of the panel represent a longer exposure of the same blot. (Lower) Cosedimentation of TAP-tagged ribosomal proteins L3 and S17 with the mitochondrial ribosomal RNP complexes. (Upper) The typical relative quantity profile of the 9S small subunit and the 12S large subunit ribosomal RNAs in fractions of a sucrose gradient. In this case the profile represents the S17-TAP cell line. The 50S monosomes are detectable as a shoulder of a larger peak representing the small subunit-related 45S SSU* complex and a peak representing 40S large subunit (1–3). Sedimentation is from right to left. (Lower) Western blot analysis of proteins from fractions 2–38 of the sucrose gradients. Probing was done with the PAP reagent.

Fig. S7. Absence of unwinding activity of Lm REH1-SAP pull-down. (A) Expression of SAP-tagged Lm REH1 and mitochondrial importation. Equivalent amounts of whole cell, cytosol (Cyto) and mitochondrial extract (Mito) were separated in an 8–16% polyacrylamide SDS gel. The gel was blotted and probed with the peroxidase antiperoxidase (PAP) reagent (Sigma) to detect the SAP-tagged Lm REH1. (B) Model gRNA/mRNA substrate with a 22-bp duplex and a 15-nt 5’ overhang. The 32P-labeled end is indicated by * in the diagram. Concentration of SAP pull-down in lanes 1, 3, 4 was 10 nm. The REH1-SAP pull-down was obtained as described in *Purification of SAP-tagged recombinant Lm REH1* but without MonoS chromatography.

Table S1. DNA oligonucleotides

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Table S1. DNA oligonucleotides

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Table S2. RNA oligonucleotides

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*Primers were derived from ref. 1.

Table S3. Sequences derived from MS/MS and manual sequencing of peptides recovered via trypsin in-gel digestion of the protein bands in Fig. 3C

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