SUPPLEMENTAL METHODS

Generation of cell lines

To construct an RNA interference (RNAi) vector for MRB7260, a 714-bp portion of the open reading frame was PCR amplified with the addition of 5' BamHI and 3' HindIII restriction sites using the primers "For PhyH RNAi BamHI" (5'-ATA GAG GAT CCG TTC TTG CGA GGT CAG TTG C-3') and "Rev PhyH RNAi HindIII" (5'-ATG AAA AGC TTT CCA CCG TAA CGG GTG G-3'). This PCR product was cloned into the BamHI and HindIII sites of the p2T7-177 RNAi vector (1) yielding p2T7-177-MRB7260. For transfection into PF 29-13 or BF single-marker cells, 10 µg of NotI linearized p2T7-177-MRB7260 was electroporated into cells and transformants were selected with 2.5 µg/ml (PF) or 1 µg/ml (BF) phleomycin. Clones were isolated by limiting dilution to produce cell lines expressing inducible MRB7260 RNAi. Cell growth was monitored for 10 days after RNAi was induced by addition of 4 µg/ml tet to the growth medium.

To create a constitutive endogenous His-TEV-myc tagged TbRGG2, first the PTP-tag between the KpnI and EcoRI restriction sites was removed from the pC-PTP-PURO vector (2) and replaced with a multiple cloning site containing the HindIII MfeI XhoI BamHI and EcoRI restriction sites to create pC-PURO. Next, the TEV-2x-myc tag was amplified from the pLEW100-TEV2xmyc vector (3) using the primers "FWD pLEW100" (5'-GCT GCA CGC GCC TTC GAG-3') and "pLEW Transfer EcoRI" (5'-CGC GAA TCC CTG CTG TGC CAT CAG ATT ACT CC-3'), digested with BamHI and EcoRI, and cloned into a similarly digested pC-PURO resulting in pC-TEV-2xmyc-PURO. The entire TbRGG2 open reading frame was then amplified with primers described previously (4) and cloned into pC-TEV-2xmyc-PURO to create the plasmid pC-TEV-2xmyc-TbRGG2. In addition, an 8x-histidine tag was inserted following the TbRGG2 amino acid sequence using site-directed mutagenesis with the primers "QC 8x His TbRGG2 TEV myc" (5'-CTG AAA ATA CAA ATT CTC GTG ATG GTG ATG GTG GTG ATG ATG TCT AGA CAC CTT CTG-3') and "rQC 8x His TbRGG2 TEV myc" (5'-CAG AAG GTG TCT AGA CAT CAT CAC CAC CAT CAC CAT CAC GAG AAT TTG TAT TTT CAG-3') creating the pC-TbRGG2-HTM (His-TEV-myc) plasmid. This plasmid was

then linearized using BamHI and transfected into PF MRB7260 RNAi (phleomycin resistant) cells described above. Transformants containing both the pC-TbRGG2-HTM and the p2T7-177-MRB7260 constructs were selected on 1 μg/ml puromycin and 2.5 μg/ml phleomycin. The pC-PTP3010 (5) vector was also transformed into PF MRB7260 RNAi cells and selected as above.

To generate a cell line expressing exogenously expressed tagged MRB7260, first the myc-His-TAP (MHT) tag was PCR amplified from pLEW79-MHT (6) using the primers "FWD pLEW100" (5'-GCT GCA CGC GCC TTC GAG-3') and "pLEW Transfer BclI" (5'-CGC TGA TCA CTG CTG TGC CAT CAG ATT ACT CC-3'), digested with HindIII and BclI, and cloned into the pHD1034 (7) creating pHD1034-MHT. The MRB7260 open reading frame was then amplified using the primers "PhyH for HindIII" and "PhyH Rev BamHI". The HindIII and BamHI digested PCR product was then cloned into a similarly digested pHD1034-MHT resulting in the pHD1034-MHT-7260 plasmid. This plasmid was then linearized with NotI and transfected into PF *T. brucei* 29-13, followed by selection for puromycin resistance as stated above. All primers used in this study are listed in Supplemental Table S1.

Pulldowns

Immunoaffinity purification of MRB7260 was carried out using 4 x 10^{10} PF cells containing the MRB7260-MHT construct. Cells were lysed in 40 ml of N150 buffer (50 mM Tris [pH 8], 150 mM NaCl, 0.1 % (v/v) NP-40 and 5 mM β-ME) with 1% (v/v) Triton X-100 in the presence of Complete protease inhibitor cocktail (Roche) taking cells to 1 x 10^9 cells/ml. Lysate was then divided into two 20 ml fractions with one 20 ml fraction was incubated with 60 U of RNase inhibitor (Applied Biosystems) and DNase 1 (1 μg/ml). In addition to DNase1, the other 20 ml portion was also treated with a nuclease cocktail containing 8 μg RNase A (Thermo Scientific), 2500U RNase T1 (Ambion), 28U RNase H (Invitrogen), and 2040U micrococcal nuclease (Thermo Scientific) for 1 hour on ice. Nuclease-inhibited (RNA+) and nuclease-treated (RNA-) lysates were then incubated with IgG Sepharose 6 Fast Flow beads (GE Healthcare) for 2 hours at 4°C. Beads were washed with N150 and TEV cleavage buffer (10 mM Tris [pH 8], 150 mM

NaCl, 0.1 % (v/v) NP-40, 0.5 mM EDTA, and 1 mM DTT) and incubated with 100U of AcTEV Protease (Invitrogen) at 4°C overnight. TEV elutions from tandem affinity purifications of MRB7260-MHT were analyzed by Western blot using anti-c-Myc antibodies (Invitrogen) to detect MRB7260. This procedure was also employed with cell lysates containing TbRGG2-HTM or MRB3010-PTP from cells grown the presence or absence of 4 µg/ml tetracycline for 3 days to knockdown expression of MRB7260. TEV elutions from these purifications were also analyzed by Western blot using anti-c-Myc antibodies (Invitrogen) for the TbRGG2-HTM immunoaffinity purification or anti-Protein C antibodies (ICL) for the MRB3010-PTP purification.

Quantitative RT-PCR

Total RNA was extracted from PF and BF *T. brucei* MRB7260 RNAi cells grown in the presence or absence of 4 µg/ml tetracycline for 3 days using TRIzol reagent (Ambion). RNA was then DNase treated with a DNA-free DNase kit (Ambion) and reverse transcribed to cDNA using random hexamer primers and the Taq-Man reverse transcription kit (Applied Biosciences). Quantitative reverse transcription PCR (qRT-PCR) reactions were performed using established primers specific to the pre-edited, edited, and precursor mitochondrial transcripts from *T. brucei* (8, 9). The 3' end of the MRB7260 open reading frame was amplified using the following primers: "qPCR For PhyH" (5'ATG GTA ATC GCT CAT TGG AT-3') and "qPCR Rev PhyH" (5'-CGC CTG GAA TTT AGT CGT AT-3'). The qRT-PCR (n = 6-15) reactions were performed in a final volume of 25 µl and cDNA was amplified using a CFX Connect Real-Time System (Bio-Rad). Results were analyzed using the Bio-Rad CFX Manager 3.1 software, and the RNA levels were normalized to levels of 18S rRNA using the standard-curve method.

Recombinant protein expression

Recombinant glutathione *S*-transferase (GST)-tagged TbRGG2 and p22 were purified as described previously (8, 10, 11). To generate the N-terminal MBP and C-terminal histidine tagged MRB7260, the MRB7260 open reading frame was PCR amplified using the following primers: "PhyH new for BamHI" (5'-TAA CAG GAT CCA TGC GAT CCG

GTC GCA AAC TCG GTT GTT TT-3') and “PhyH rev add His stop XbaI” (5'-GTG TTC TAG ATT AGT GGT GAT GGT GAT GAT GTG CTC CAG CGC CAC CCG CCT CCT CTT CGG ATG G-3'). PCR amplification using the above reverse primer places 6 histidine residues prior to the stop codon. The resulting PCR product was digested with BamHI and XbaI and ligated into pMal-C2 (NEB). The resulting pMal-MRB7260-His construct was then transformed into *Escherichia coli* Rosetta cells (Novagen) for expression of the recombinant protein. Cells were grown to an optical density (OD) of ~0.6, followed by addition of 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) to induce protein expression overnight at 18°C. Recombinant protein was first purified using a standard MBP purification method using amylose resin (NEB). Bio-Gel P-6DG polyacrylamide beads (Bio-Rad) were then used to remove the 1 mM EDTA from the MBP purification. The desalted MRB7260 was then incubated with ProBond™ Resin (Novex) in the presence of 5 mM imidazole. The beads were then washed with a buffer containing 20 mM imidazole and protein was then eluted from the beads with 200 mM imidazole. The resulting MBP-MRB7260-His fractions were then pooled and dialyzed in 20 mM Tris [pH 8], 150 mM NaCl, and 5 mM β-ME. To generate the MRB7260-His protein, 2 µg of MBP-MRB7260-His was incubated with 1 µg of Factor Xa (NEB) at 23°C for two hours to remove the MBP tag. For circular dichroism (CD), 585 nM MBP-MRB7260-His is PBS (pH 7.4) was placed in a fluorimeter cell (Starna Cells, Inc.) with a 10-mm path length at 25°C. Far UV spectra (201-260 nm) were collected for the protein with a Jasco J-815 CD spectrometer. Data were processed using the Jasco software and exported to Microsoft Office Excel 2010.

***In vivo* RNA crosslinking**

To determine RNA binding *in vivo*, 5 x 10^8 PF cells containing the TbRGG2-HTM construct, MRB7260-MHT construct, or the parental cell line 29-13 were suspended in phosphate-buffered saline containing 0.1 % (w/v) glucose and cross-linked with either 200 mJ/cm^2 or 400 mJ/cm^2 UV light with a Stratalinker 2400 (Stratagene) or left untreated. Mitochondria were then enriched as described previously (12). Enriched mitochondria were lysed in lysis buffer (50 mM Tris [pH 8], 150 mM NaCl, 0.7 % (v/v) NP-40) plus Complete protease inhibitor cocktail (Roche) and 10U of RNaseOUT

(Invitrogen) with shaking at room temperature for 5 minutes. Lysates were cleared by centrifugation and treated with 3U of TURBO DNase (Ambion) and 15U RNaseI (Invitrogen) in the presence of 1 mM CaCl at 37°C for 3 minutes followed by 3 minutes on ice. Nuclease treated lysates were then pre-cleared for 45 minutes at 4°C with Superdex 200 (Amersham) under denaturing conditions (50 mM Tris [pH 8], 300 mM NaCl, 0.1 % (v/v) NP-40, 5 mM imidazole, and 7 M urea). Cleared lysates were then incubated with ProBond™ Resin (Novex) for 2 hours at 4°C. Beads were washed with 50 mM Tris [pH 8], 500 mM NaCl, 0.1 % (v/v) NP-40, and 10 mM imidazole followed by washing with PNK buffer (50 mM Tris [pH 8], 10 mM $MgCl_2$, 0.1 % (v/v) NP-40, and 5 mM β-ME). Protein bound RNA was then labeled with 50µCi of [γ ^{32}P]-ATP (3000Ci/mmol) using 50U of T4 polynucleotide kinase (NEB) for 30 minutes at 37°C. Beads were washed 4 times using PNK buffer and protein was eluted in SDS-dye for 10 minutes at 95°C. Protein was visualized using Western blot with anti-c-Myc antibodies (Invitrogen). Nitrocellulose membranes were exposed to a phosphor storage screen, and analyzed using IMAGEQUANT software (GE Healthcare).

UV cross-linking assays

Guide RNA gA6[14] and mRNA A6U5 pre-mRNA (79 nt) were transcribed from plasmids as described previously (8, 13). Internally [α ^{32}P]-UTP (800 Ci/mmol) labeled RNA was synthesized with the T7 Maxiscript Kit (Ambion) and purified on 8% (w/v) acrylamide/7M urea gel. UV cross-linking reactions containing 5 fmol (0.25nM) of radiolabeled RNA and 2 µg of protein (4.5µM p22, 1.6µM GST-TbRGG2, 4.5µM MBP, 1.2µM MBP-MRB7260-His) were incubated in a mixture containing 6 mM HEPES [pH 7.5], 2.1 mM $MgCl_2$, 0.5 mM DTT, 1.5 mM ATP, 5 mM creatine phosphate, 0.1 mM EDTA, 10 µg/ml torula (*Candida utilis*) yeast RNA, and 6% (v/v) glycerol for 20 minutes at room temperature. Reaction mixtures were UV cross-linked using a Stratalinker 2400 (Stratagene) for 10 minutes on ice, followed by treatment with RNase A for 15 minutes at 37°C. Reactions were stopped by the addition of SDS-PAGE loading buffer and analyzed using 10% (w/v) SDS-PAGE. The gels were dried, exposed to a phosphor storage screen, and analyzed using IMAGEQUANT software (GE Healthcare).

Yeast two-hybrid analysis

The open reading frame of MRB7260 was PCR amplified from PF *T. brucei* strain 29-13 genomic DNA using the primers: "For PhyH NdeI" (5'-TAA TCT CAT ATG CGA TCC GGT CGC AAA C-3') and "Rev PhyH BamHI" (5'-TAC ATA GGA TCC TTA CGC CTC CTC TTC GGA TGG-3'). The PCR product was cloned into the yeast two-hybrid Gal4 activation domain (AD) vector pGADT7 and the Gal4-binding domain (BD) vector pGBKT7 (Clontech). All other RESC protein pGADT7 and pGBKT7 vectors were created was described previously (4). Plasmids were transformed into *Saccharomyces cerevisiae* strain PJ69-4A and protein-protein interactions were selected on plates supplemented with 1 mM 3-amino-1,2,4-triazole (3-AT) as described previously (4, 10).

Guanylyltransferase assays

Guanylyltransferase labeling was performed with total RNA from the *T. brucei* MRB7260 RNAi cell line grown for three days in the presence or absence of 4 μg/ml tetracycline. RNA was purified from cells using TRIzol (Ambion). Guanylyltransferase reactions were performed as described previously (14, 15).

Bioinformatic analysis

In brief, ESs were only considered EPSs when the increase in sequence abundance at that ES was statistically significant ($p<0.05$ and $q<0.05$) in both biological replicates compared to the uninduced controls. To examine junction length, the number of sequences generated at each editing stop site with a junction length of 0, 1-10, 11-50, and greater than 50 ES long was determined. The average number of sequences was determined for the uninduced and induced samples, and the values were plotted in R. To examine junction sequences, the major junction sequences found within gRNA-2 were determined from the database of all sequences found in all samples across both biological replicates by limiting analysis to those whose editing stop site was within gRNA-2 direct block of RPS12 mRNA (editing stop sites 22 to 40). The average number of times this sequence was retrieved was determined by taking the sum of the normalized counts for each unique sequence across all samples of that type divided by the sample number ($n=2$ for induced RNAi samples; $n=10$ for all uninduced samples).

RIP Analysis

Fold enrichment compared to the mock RIP was calculated for each gene of interest (GOI) using the ΔΔCt method comparing the tet uninduced sample to that of the mock IP. Fold change to mock IP = $2^{(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct^{GOI\text{-}Mock} - Ct^{18S\text{-}Mock}) - (Ct^{GOI\text{-}Uninduced} - Ct^{18S\text{-}Uninduced})$. To determine the fold change in the RNA associated when MRB7260 was depleted, the RNA detected in the tet-induced sample was compared to the uninduced IP using the ΔΔCt method. Fold change with MRB7260 knockdown = $2^{(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct^{GOI\text{-}Uninduced} - Ct^{18S\text{-}Uninduced}) - (Ct^{GOI\text{-}Induced} - Ct^{18S\text{-}Induced})$.

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