Differential Editosome Protein Function between Life Cycle Stages of *Trypanosoma brucei*

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**Background:** The mechanism that regulates differential RNA editing between life cycle stages in *Trypanosoma brucei* is unknown.

**Results:** Mutations in specific editosome proteins differentially affect growth, editing and editosomes in bloodstream and procyclic stages.

**Conclusion:** Editosome functions differ between life cycle stages.

**Significance:** Certain editosome proteins likely function in differential editing which discriminates among mRNAs.

**ABSTRACT**
Uridine insertion and deletion RNA editing generates functional mitochondrial mRNAs in *Trypanosoma brucei*. The mRNAs are differentially edited in bloodstream form (BF) and procyclic (PF) life cycle stages and this correlates with the differential utilization of glycolysis and oxidative-phosphorylation between the stages. The mechanism that controls this differential editing is unknown. Editing is catalyzed by multiprotein ~20S editosomes that contain endonuclease, TUTase, exonuclease and ligase activities. These editosomes also contain KREPB5 and KREPA3 proteins which have no functional catalytic motifs, but they are essential for parasite viability, editing, and editosome integrity in BF cells. We show here that repression of KREPB5 or KREPA3 is also lethal in PF, but the effects on editosome structure differ from those in BF. In addition, we found that point mutations in KREPB5 or KREPA3 differentially affect cell growth, editosome integrity and RNA editing between BF and PF stages. These results indicate that the functions of KREPB5 and KREPA3 editosome proteins are adjusted between the life cycle stages. This implies that these proteins are involved in the processes that control differential editing and that the 20S editosomes differ between the life cycle stages.

**INTRODUCTION**
The life cycle of *Trypanosoma brucei*, the etiologic agent of African trypanosomiasis, involves stage-specific adaptations in the bloodstream of the mammalian host and in various compartments of the tsetse fly, its insect vector. These adaptations include changes in cell surface composition, metabolism and particularly in mitochondrial function (1-3). During infection of the vertebrate host, the bloodstream form (BF) parasites primarily satisfy their energy requirements by glycolysis. The mitochondrion in BF is a single long tubule that is functionally repressed in that it lacks cytochromes and citric acid cycle enzymes. In contrast, the insect vector procyclic forms (PF) primarily generate energy by oxidative phosphorylation, and they have a larger single branching mitochondrion that contains a fully functional respiratory chain (4,5). Multiple components of the respiratory chain are encoded in the mitochondrial DNA that is termed kinetoplast DNA (kDNA).

The kDNA in *T. brucei* is comprised of a network of ~50 identical maxicircles that are intercatenated with thousands of heterogeneous minicircles (6,7). The ~22 kb maxicircles encode
two ribosomal RNAs (rRNAs) and mRNAs for 18 mitochondrial proteins including components of the respiratory complexes. Translatable messages are generated for 12 of these mRNAs by post-transcriptional RNA editing which precisely inserts and deletes uridines (Us) at hundreds and tens of editing sites (ESs) respectively. The minicircles encode numerous diverse ~60 nucleotide guide RNAs (gRNAs) which specify edited sequence (8-10). Editing progresses generally, but not precisely, 3’ to 5’ with respect to the mRNA, each gRNA specifies the editing of multiple ESs, and multiple gRNAs are required for complete editing of most transcripts.

*T. brucei* differentially edits mitochondrial mRNAs between the BF and PF stages which results in the differential presence of respiratory complexes during the life cycle. The mRNAs that encode the ND7, ND8, and ND9 subunits of NADH dehydrogenase (respiratory complex I) are primarily edited in BF (11-13). In contrast the mRNAs that encode cytochrome b (CYb) and cytochrome oxidase subunit II (COII) (respiratory complexes III and IV, respectively) are primarily edited in PF (14,15). ATPase subunit 6 (A6) mRNA is edited in both PF and BF (16) despite mitochondrial repression and absence of oxidative phosphorylation in BF which reflects the essentiality of the ATP synthase complex for ATP synthesis in PF and for maintenance of mitochondrial membrane potential in BF (17). The underlying mechanism that controls differential editing is not known, but it is not due to differential gRNA abundance (18,19). Differential gRNA utilization has been suggested to play a role in the developmental regulation of editing. The different temperatures between BF and PF environments might alter mRNA structure and targeting by gRNAs (18-20). The gRNAs may differentially associate with or be used by the editing machinery in the two life cycle stages by some unknown mechanism (21,22). Overall, how differential editing is controlled between the life cycle stages in *T. brucei* is unresolved.

RNA editing occurs by a series of coordinated catalytic steps: cleavage of the mRNA by endonuclease, addition of Us by 3’ terminal uridylyl-transferase (TUTase) or removal of Us by U-specific 3’ exonuclease (exoUase) at insertion and deletion ESs, respectively, followed by rejoining of the mRNA fragments by RNA ligase. The enzymes that catalyze RNA editing are in ~20S multiprotein editosome complexes that also contain proteins that have no known catalytic functions (8,23-33). Mass spectrometry of editosomes isolated from BF or PF cells indicates that the same set of proteins is present in both life cycle stages (28). Three similar, but distinct versions of these ~20S complexes exist, with each containing a different endonuclease and specific partner protein (27-30,32,34). These distinct ~20S editosomes differ in their ES cleavage specificity (29,30,32). One complex contains the KREN1/KREPB8 protein pair and the KREX1 3’ exonuclease, and cleaves deletion ESs. The other two complexes contain the KREN2/KREPB7 or KREN3/KREPB6 protein pairs and cleave insertion sites, albeit with different preferences.

KREPB5 is one of 12 proteins common to all ~20S editosomes, and contains a U1-like zinc finger (ZnF) motif, a PUF motif, and a degenerate noncatalytic RNase III domain (35,36). Mutation of KREPB5 residues that are universally conserved in all known catalytic RNase III domains has no effect on editing or *in vitro* cleavage of ESs (36), while equivalent mutations in the RNase III domains of the KREN1, KREN2 or KREN3 endonucleases eliminate *in vivo* editing and *in vitro* cleavage of ESs (27). We have hypothesized that the noncatalytic RNase III domain of KREPB5 forms a heterodimeric RNase III active site with the editing endonucleases. In BF, KREPB5 is essential for editing, and the absence of KREPB5 results in the complete loss of editosomes and their components in BF (37).

KREPA3 also belongs to the common set of 12 editosome proteins, and is one of six related proteins, KREPA1-KREPA6, that have no recognizable catalytic motifs, but contain oligonucleotide/oligosaccharide binding (OB)-fold motifs. KREPA1, KREPA2 and KREPA3 also have two ZnF motifs (35). These proteins interact with each other and with other proteins in the complex (38-40) (McDermott et al., unpublished). Knockdown of KREPA3 in conditional null (CN) BF cells results in complete loss of editosomes, while RNAi knockdown in PF results in partially disrupted editosomes that retain TUTase, exoUase and RNA ligase activities but lack endonuclease activity *in vitro* (41,42). Recombinant KREPA3 was reported to have U-specific endo- and exonuclease activity *in vitro* (43-45), but the
biological relevance of these results is unclear (41,42,46). Mutational analyses of KREPA3 in BF showed that the OB-fold domain is necessary for editosome integrity, and that the ZnFs are essential for editing progression in vivo (42,47). Thus, KREPA3 may function via interactions with other editing proteins and perhaps RNA, and affect the activities of the editing endonucleases either directly or indirectly.

In this work we generated PF KREPB5 and KREPA3 CN cell lines in order to further analyze the function of these proteins in PF. These are cell lines in which both alleles of KREPB5 or KREPA3 have been replaced by drug resistance cassettes, and that also contain a tetracycline (tet)-inducible wild-type (WT) ectopic copy of KREPB5 or KREPA3. We demonstrate that both KREPB5 and KREPA3 are essential in PF. We used CN cells for comparative mutational analysis of KREPB5 and KREPA3 in BF and PF cells. We show that single amino acid changes within editosome proteins differentially affect cell growth, editosome integrity and RNA editing between BF and PF cells. Depending upon the specific mutation, editosome function is disrupted in BF but not PF, in PF but not BF, or in both life cycle stages. The results indicate that KREPB5 and KREPA3 are involved in the processes that control differential editing between life cycle stages, although the mechanisms underlying these processes are unknown. The results also indicate that editosomes functionally differ between life cycle stages perhaps due to differences in protein modification, conformation, internal interactions, or interactions with other proteins or complexes.

EXPERIMENTAL PROCEDURES

Growth of cells in vitro-BF cells were grown in HMI-9 with 10% FBS at 37°C, 5% CO₂. PF cells were grown in SDM-79 with 10% FBS at 27°C. For selection with ganciclovir, PF cells were grown in MEM-Pros (48) with 10% FBS at 27°C. For growth curve analysis, cell density was measured by Coulter Counter. BF cultures were reseeded at 2 x 10⁵ cells/mL in 10 mL every day, whilst PF cultures were reseeded at 2 x 10⁶ cells/mL in 10 mL every two days. The mean doubling times of three independent replicates for each cell line were calculated between days 1 and 6 (BF), and days 1 and 8 (PF).

Plasmid constructs and cell lines-BF KREPB5 CN cells were described in Wang et al., 2003 (37). PF KREPB5 and KREPA3 CN cells, and all BF and PF exclusive expression cell lines, were generated in this study. Transfections of BF cell lines with the Amaxa Nucleofector (Lonza), and of PF cell lines with the BTX transfection device (Harvard Apparatus, Inc.), were carried out as described in Merritt and Stuart, 2013 (49).

Concentrations of drugs used for selection and tetracycline-regulated expression of transgenes in this study are as follows. For BF: 2.5 μg/mL G418, 5 μg/mL hygromycin, 2.5 μg/mL phleomycin, 0.5 μg/mL tetracycline, 0.1 μg/mL puromycin. For PF: 15 μg/mL G418, 25 μg/mL hygromycin, 2.5 μg/mL phleomycin, 0.5 μg/mL tetracycline, 1 μg/mL puromycin, 10 μg/mL blasticidin, 25 μg/mL ganciclovir.

Generation of PF KREPB5 conditional null cell lines-Wild-type KREPB5 (minus the stop codon) flanked by in-frame attB Gateway recombination sites was PCR amplified using primers described in Supplemental Table 1. The PCR product was included in a BP reaction with pDONR221 (Life Technologies). The resulting entry vector, pENTR-KREPB5(-stop) was then used in a LR reaction with the destination vector pLEW100V5(BLE)GW-CtermTAP which contains the phleomycin resistance (BLE) selectable marker and allows for tetracycline regulatable expression of C-terminal TAP-tagged KREPB5 in the ribosomal DNA spacer region. The resulting pLEW100v5(BLE)-KREPB5-CtermTAP plasmid was linearized with NotI and transfected into PF 29.13 cells. Transgenic lines were selected by phleomycin resistance and tetracycline-dependent expression of TAP-tagged KREPB5 confirmed by Western blot. DNA constructs for endogenous KREPB5 allele knockouts were generated by PCR amplification of loxP-flanked drug selection cassettes from modified pyr-FEKO vectors (pSM07 and pSM06) (49) containing blasticidin (BSD) or puromycin (PAC) drug resistance genes, a Ty1 epitope tag, and the Herpes Simplex Virus Thymidine Kinase (HSVTK) gene. Differential allele targeting sequences homologous to ‘outer’ and ‘inner’ sequences flanking endogenous KREPB5 alleles were also amplified using primers described in Supplemental Table 1 and combined with drug selection cassettes in fusion PCR reactions as...
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previously described (49). The products of these fusion PCR reactions were transfected stepwise into the regulatable KREPB5-TAP cell line. Transgenic lines were selected by blasticidin and puromycin resistance and correct insertion of the knockout cassettes assessed by PCR and by Western blotting for expression of the Ty1 tag. The resulting conditional null cell lines were finally transfected with undigested pLEW100Cre_del_tetO (Addgene plasmid 24019; George Cross) for the transient expression of Cre recombinase and excision of the loxP-flanked knockout cassettes, allowing reuse of drug resistance selectable markers (50, 51). Cell lines that had lost the HSVTK gene were selected by resistance to ganciclovir (GCV) (Invivogen).

Generation of PF KREPA3 conditional null cell lines—DNA constructs for endogenous KREPA3 allele knockouts were pLEW13-A3-KO1 and pLEW90-A3-KO2 (42). The 10% T7 promoter that drives expression of the Tet Repressor protein (TetR) in pLEW90-A3-KO2 was converted to a wild-type 100% T7 promoter by digestion with XhoI and BglII and ligation of annealed oligonucleotides 7108 and 7109. Myc-tagged KREPA3 ORF was amplified from pLEW79-A3-TAP (42) using primers 6727 and 6125 and cloned into pLEW79 after digestion with XhoI and BamHI. This generated pLEW79-A3-myc that allowed for tetracycline regulatable expression of myc-tagged KREPA3. Wild-type PF 427 cells were transfected with NotI digested plasmids in a stepwise manner; first with pLEW13-A3-KO1, followed by selection with G418 to replace one KREPA3 allele, then with pLEW79-KREPA3-myc and selection with phleomycin to insert the tetracycline-regulatable KREPA3-myc into the ribosomal DNA spacer region, and finally with pLEW90-A3-KO2 and selection with hygromycin in addition to tetracycline to replace the second KREPA3 endogenous allele and induce ectopic KREPA3-myc expression. Correct integration of each plasmid was confirmed by PCR, and tetracycline-dependent expression of myc-tagged KREPA3 confirmed by Western blot.

Generation of exclusive expression cell lines—pENTR-KREPB5(-stop) was used in a LR reaction with the destination vector pHD1344tub(PAC)-KREPB5-Cterm3V5, which contains the puromycin resistance (PAC) selectable marker and allows for constitutive expression of C-terminal 3xV5 tagged KREPB5 in the β-tubulin locus. The resulting pHD1344tub(PAC)-KREPB5-Cterm3V5 plasmid was used as a template for site-directed mutagenesis (QuikChange II kit; Agilent) using forward and reverse primers listed in Supplemental Table 1. Wild-type KREPA3 and zinc-finger mutants were PCR amplified from pLEW79-A3-TAP (wild-type), pLEW79-A3-ZFm1-TAP (C53A/C56A) and pLEW79-A3-ZFm2-TAP (C183A/C186A) (47) using primers 6125 and 6360, and cloned into pHD1344tub(PAC) after digestion with XhoI and BamHI. NotI digested plasmids were transfected into the relevant BF (37) and PF KREPB5 or PF KREPA3 conditional null cell line. Transgenic lines were selected by puromycin resistance and constitutive expression of KREPB5-3xV5 or KREPA3 was confirmed by Western blot.

Fractionation on glycerol gradients—Glycerol gradient fractionation was carried out on crude mitochondria preparations from 2 × 10^9 PF or BF cells in the presence or absence of 0.5 μg/ml tetracycline. Following lysis in 1 mL lysis buffer (10 mM Tris-HCl [pH 7.2], 10 mM MgCl2, 100 mM KCl, 1% Triton X-100) and centrifugation (13,000 rpm, 10 min, 4 °C), cleared lysates were loaded onto 11 mL 10-30% glycerol gradients and centrifuged at 38,000 rpm for 5 h or 9 h at 4°C in a SW40Ti rotor (Beckman). 24 fractions of 500 μL were collected from top to bottom, flash frozen in liquid nitrogen and stored at -80°C. Where indicated, gradient fractions were concentrated using StrataClean Resin (Agilent).

Immunoprecipitation—Cleared lysate was prepared by lysis of 2 × 10^8 cells in 1 mL IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) with 1% Triton X-100, followed by centrifugation at 10,000 ×g at 4 °C. For each immunoprecipitation, 0.5 mL cleared lysate (1 × 10^8 cells) was incubated overnight with 1 μL of rabbit antibody (Rockland Immunochemicals; Table 1) specific for the V5 epitope tag. 12.5 μL of Protein G Mag Sepharose Xtra (GE Healthcare) were washed twice with 1 mL of PBS with 0.1% BSA and once with 1 mL IPP150. Beads were then incubated for 4 h rotating at 4 °C with cleared lysate/antibody. After incubation, the supernatant was removed, and beads were washed four times with 1 mL of IPP150. Complexes bound to beads...
were eluted with 100 μL of 2 × SDS sample buffer and heated for 5 min at 95 °C.

**SDS-PAGE and Western blotting**

SDS-PAGE loading buffer was added to samples containing purified protein complexes and resolved on 10% SDS-PAGE gels (Criterion Tris-HCl, Bio-Rad). For Western analysis, resolved proteins were transferred to Immobilon-P PVDF membranes (Millipore), and probed using monoclonal antibodies against KREPA1, KREPA2, KREL1, KREPA3, or mitochondrial Hsp70 as previously described (23) (Table 1). Blots were sequentially stripped and reprobed using mouse monoclonal primary antibody against the V5 epitope tag (Life Technologies) at 1:5000 with goat-anti-mouse conjugated HRP secondary antibody at 1:5000, and reprobed using Peroxidase Anti-Peroxidase (PAP) Soluble Complex Antibody against the TAP-tag at 1:2000 (Table 1). Blots were developed with an enhanced chemiluminescence kit (Thermo Scientific) per the manufacturer’s instructions, and imaged using the FluorChem E system (ProteinSimple) or using x-ray film (Kodak). Positive control 20S samples from purified PF mitochondria (IsTaR 1.7a strain) were generated as previously described (29,52).

**RNA isolation and Fluidigm BioMark RT-qPCR Analysis**

Total RNA was harvested from BF and PF cell lines using TRIzol reagent and treated with TURBO DNase (both Life Technologies) according to manufacturer’s instructions. RNA integrity was confirmed using a RNA nanochip on a BioAnalyzer (Agilent Technologies). 2 μg of total RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents and MultiScribe Reverse Transcriptase (Life Technologies). Reference (53), never-edited, pre-edited and edited (29,30) transcript cDNAs were then pre-amplified in multiplex Specific Target Amplification (STA) reactions using TaqMan PreAmp Master Mix (Life Technologies). STA reactions were performed with the following thermocycling conditions: 1 cycle at 95°C for 10 min; 14 cycles of 95°C for 15 sec and 60°C for 4 min. Pre-amplified cDNA was treated with Exonuclease I (NEB), and diluted 10-fold. High-throughput real-time PCR was then conducted on the Fluidigm BioMark HD system, using Ssofast Evagreen Supermix with Low ROX (Bio-Rad) and Fluidigm 48.48 Dynamic Array Integrated Fluidic Circuits (IFCs). Primers were described in Supplemental Table 1. The same primers were used for both STA and real-time PCR reactions. Processsing of the IFCs and operation of the instruments were performed according to the manufacturer’s procedures. PCR was performed using the thermal protocol GE Fast 96 × 96 PCR + Melt v2.pcl. Data was analyzed using Fluidigm Real-Time PCR Analysis software using the Linear (Derivative) Baseline Correction Method and the Auto (Global) Ct Threshold Method. The Ct values determined were exported to Excel for further processing. Calculation of the fold change in mRNA levels in samples following tet withdrawal relative to the presence of tetracycline was done using the 2 [-ΔΔC(T)] method (54) using TERT as an internal control. Four technical replicates of each cDNA sample were assayed for each target and internal reference RNA per experiment, and Ct data were averaged before performing the 2 [-ΔΔC(T)] calculation.

**RT-PCR analysis**

RT-PCR analysis was used to amplify all pre-edited and edited MURF2 mRNAs. Total RNA isolation, DNase treatment, and reverse transcription were as described in the Materials and Methods. PCR amplification of MURF2 cDNAs was carried out using primers 10711 and 10712 (Supplemental Table 1). PCR products of MURF2 mRNAs were analyzed on nondenaturing 10% polyacrylamide gels.

**Homology modeling**

The comparative models of the RNase III domain of KREPB5 were generated using HHpred (55) and Modeller (56), and by ModPipe (57). Swiss-model (58) was also used as a further tool to independently verify models created by ModPipe and Modeller.

**RESULTS**

**KREPB5 is essential in both BF and PF T. brucei**

Knockdown of KREPB5 expression resulted in the cessation of growth of both BF and PF CN cells (Figure 1A). These are cells in which both endogenous alleles were deleted, and into which a tet-regulatable WT KREPB5 allele had been inserted into the **RRNA** locus. The KREPB5 allele in the **RRNA** locus contained a C-terminal TAP-tag in the PF cell line. Western blots of PF KREPB5 CN total cell lysates taken every day following tet withdrawal showed that WT KREPB5-TAP is not detectable after four days of growth in the absence of tet (Figure 1B). RT-qPCR analysis using the high-throughput Fluidigm BioMark system showed that KREPB5 mRNA
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coding (KREPB5 ORF) and UTR sequences (KREPB5 Reg) of the tet-regulatable KREPB5 allele in the RNA locus were reduced by >95% upon withdrawal of tet from these CN cells (Figure 1C and Table 3). The knockdown in both BF and PF also resulted in a dramatic reduction in the relative levels of all of the edited mRNAs analyzed and an increase in the relative levels of pre-edited mRNAs, but had no effect on the never-edited mRNAs COI and ND4. Edited mRNAs in BF were reduced by 88% or more, and the levels of edited CYb and COIII were below detection. In PF they were reduced by between 67% (ND7) and 98% (A6) and edited MURF2 was not detected. The relative levels of never edited ND4 and COI mRNAs in repressed cells were unaffected, and the levels of pre-edited mRNAs were generally unchanged or increased by various amounts (Figure 1C and Table 3).

KREPB5 knockdown had a differential effect on editosome integrity between BF and PF. Western analysis was performed on total cell lysates from BF and PF CN cells that were grown in the presence or absence of tet. Blots were probed using a mixture of monoclonal antibodies for four ~20S complex proteins (KREPA1, KREPA2, KREL1, and KREPA3). KREPB5 repression resulted in a loss of editosome components that was greater in BF than in PF cells and with a somewhat different pattern of loss and retention (Figure 1D). Mitochondrial Hsp70 levels were unchanged in the absence of tet in both BF and PF, indicating that this protein loss is specific to components of the editing machinery. Western analysis of fractions from glycerol gradients of enriched mitochondrial lysates revealed a dramatic loss in BF of complexes in the ~20S fraction as we previously reported (37) (Figure 1E). In contrast, complexes were retained in PF but they were shifted to smaller S values. A considerable proportion of KREPA3 and to a lesser extent KREPA1, were shifted to the top fractions of the gradient. This shift in KREPA3 upon loss of KREPB5 is consistent with our previous yeast two-hybrid studies that indicated that KREPB5 and KREPA3 can directly interact (38) and indicates that they interact in vivo. KREPB5 mRNA and the tagged KREPB5 were undetectable in PF cells grown in the absence of tet which indicates that the greater retention of editosomes in PF than in BF is not due to a greater KREPB5 knockdown in BF than in PF. The differential effect between life cycle stages implies intrinsic differences between BF and PF editosomes.

Analysis of a putative functional PUF motif in KREPB5-We explored the functions of the single PUF motif in KREPB5 that we had also identified in KREPB4, KREN1, KREN2, and KREN3 (35,36). Proteins that contain PUF motifs (e.g. Pumilio and fem-3 binding factor) typically contain multiples of these ~40 amino acid RNA-binding motifs and they can function in pre-rRNA processing, mRNA localization, mRNA decay, or translation repression or activation (59-64). PUF domains bind RNA via three key amino acid side chains in each motif that contact a single specific nucleotide. With amino acid numbering relative to the PUF domain, two ‘edge-on’ residues at motif positions 12 and 16 make hydrogen bonds and Van der Waals contacts with the Watson-Crick edge of the RNA base and the residue at position 13 forms planar stacking interactions with the aromatic ring of the base together resulting in base specific binding (Figure 2A) (61-63,65-67). Using MUSCLE (68), we compared 109 PUF motif sequences of KREPB5, KREPB4, KREN1, KREN2, and KREN3 from a range of kinetoplastid species and 17 proteins with multiple repeats from human, Drosophila melanogaster, Saccharomyces cerevisiae, and Caenorhabiditis elegans proteins (Supplemental Figure 1). We found that the PUF motif in editosome proteins conserved the aromatic residue at position 13 which might form stacking interactions with RNA (Figure 2A) and hence contribute to base specific interactions with RNA during editing.

We probed the function of the putative stacking residue in KREPB5 by substituting the conserved histidine with alanine (H233A), which is predicted to disrupt the putative stacking function of this residue, or with tryptophan (H233W) which is predicted to conserve this function (66,69,70). We also substituted the putative edge-on interacting C232 cysteine and E236 glutamic acid with alanine, and also generated a ‘PUF-triple’ allele in which all three potential RNA-binding residues were substituted with alanine. WT and mutant KREPB5 alleles were inserted into the β-tubulin locus of BF and PF KREPB5 CN cells, where they are constitutively expressed (Figure 2B). These alleles were tagged with a C-terminal V5-epitope tag in
order to discriminate them from the tet-regulated WT allele which is TAP-tagged in PF parental CN cells. All BF and PF cells grew at the same rate as the parental BF or PF CN cells in the presence of tet regardless of which allele was in the \( \beta \)-tubulin locus, showing that none of the mutations had a dominant negative effect (Table 2). Removal of tet led to exclusive expression from the \( \beta \)-tubulin locus of the WT or mutant allele as was confirmed by Western analysis that probed for the V5-tag and TAP-tag (Figure 2B). Exclusive expression of WT or any mutant KREPB5 resulted in normal growth in BF (Figures 3A, 3C, and Table 2) as was also the case for exclusive expression of C232A and E236A in PF (data not shown). However, exclusive expression of the PUF-triple mutant and H233A mutant alleles resulted in a small but reproducible growth defect after four days in PF that was not observed by substitution of the aromatic residue with tryptophan in the H233W allele (Figure 3B-D, and Table 2). This growth defect was not as severe as was observed upon repression of KREPB5 in PF CN cells (Figure 1A, Figure 3B-D, and Table 2) and did not lead to complete cessation of cell growth. Loss of tet regulation after \(-8\) days lead to re-expression of the WT tet-regulatable KREPB5 allele in the \( RRNA \) locus, which may explain the observed resumption of normal growth (Figure 3E).

The effects of mutant allele exclusive expression on RNA editing in vivo were assessed by RT-qPCR. The abundances of pre-edited, edited and never-edited mitochondrial mRNAs, and KREPB5 mRNAs in BF and PF CN cells that were exclusively expressing WT or mutant alleles were calculated relative to the corresponding cells in which the WT tet-regulatable KREPB5 allele was also expressed. Parallel to the effects on cell growth, exclusive expression of WT KREPB5 rescued in vivo editing defects observed in the parental BF and PF CN cells as did exclusive expression of alleles with C232A, E236A and H233W mutations (Figure 4, Table 3 and data not shown). Exclusive expression of alleles with H233A and PUF-triple mutations resulted in a reduced abundance of edited transcripts in PF. The amount of reduction in edited mRNAs in PF H233A and PUF-triple mutants are not as great as compared to the complete absence of KREPB5 in PF CN cells. The edited transcript levels that remain in the H233A and PUF-triple mutants are sufficient for growth, resulting in more modest growth inhibition (Figure 3B-D, and Table 2) than in the absence of KREPB5 (Figure 1A and Table 2). The edited transcripts were not significantly reduced in BF cells that exclusively express these mutant alleles, with the exception that COII mRNA was relatively reduced by \(50\%\) in BF cells that exclusively express the PUF-triple mutant. Similar to the effects on growth, the aromatic residue replacement H233W, did not result in a significant relative reduction in edited mRNA in BF or PF. Parallel RT-PCR analysis of RNA from these cells using primers spanning the entire edited regions of MURF2 mRNA and that amplify both unedited and edited sequences also revealed partial depletion of fully-edited and a corresponding accumulation of pre-edited products in PF only (Figure 4B).

Life cycle-dependent effects of KREPB5 PUF motif mutations were also observed by Western analysis of immunoprecipitated editosomes. V5-tagged WT and mutant KREPB5 protein was incorporated into editosomes in vivo in both BF and PF cells that exclusively express these proteins (Figure 5A). Tagged KREPB5 co-immunoprecipitated with the KREPA1, KREPA2, KREL1 and KREPA3 editosome proteins as shown by Western analysis using a mixture of anti-V5 antibody and monoclonal antibodies for the four editosome proteins. Analysis of input total cell lysates showed equivalent amounts of these editosome components in all PF cells lines and in all BF cell lines (Figure 5A and data not shown). Western analysis of fractions from \(10-30\%\) glycerol gradients showed that the exclusively expressed WT V5-tagged KREPB5 co-sedimented with editosomes in both BF and PF (Figure 5B). The sedimentation profiles of editosomes from BF and PF cells that exclusively expressed WT KREPB5 were essentially the same as that when the regulatable WT allele was also expressed from the \( RRNA \) locus. However, the H233A mutant KREPB5 which has the aromatic histidine residue substituted by alanine resulted in KREPA3 and KREL1 sedimenting at smaller \( S \) values in PF. This effect was not observed in BF, nor was it
observed in PF if the histidine was substituted by tryptophan. Substitution of three residues in the PUF-triple mutant had a similar effect to the H233A mutation in PF. In BF, the PUF-triple mutant did not affect sedimentation of editosomes when it was expressed either in the presence or absence of WT tet-regulatable KREPB5. However, the V5-tagged PUF-triple mutant did not primarily co-sediment with editosomes when WT KREPB5 was also expressed from the *rrna* locus but did co-sediment with editosomes when it was exclusively expressed. This implies that WT KREPB5 was preferentially incorporated into editosomes over PUF-triple mutant KREPB5 in BF. When the PUF-triple mutant is exclusively expressed in BF it is incorporated into editosomes and can function with minimal effects on the editing of the majority of mRNAs.

Together these data indicate that the PUF motif is important for full KREPB5 function in PF but not in BF. The rescue of growth and editing by substituting H233 with an aromatic residue instead of alanine suggests that the function of the motif entails stacking of H233 with RNA. The lack of an effect on BF editing or editosomes of the H233A mutation, and the relatively small effect of the PUF-triple mutation, suggests that KREPB5 function is associated with differences in editing between life cycle stages.

**Analysis of the RNase III domain of KREPB5**-Mutation of the degenerate RNase III domain of KREPB5 also had differential effects between life cycle stages, but in contrast to the PUF mutations these either affected the editosome and editing in BF but not PF, or affected both BF and PF. KREPB5 residue R77 at the RNase III domain N-terminal boundary is completely conserved in a MUSCLE alignment of sequences from kinetoplastid KREPB5, KREPB4, KREN1, KREN2, and KREN3 (36). G121 is also fully conserved in the same alignment. Mutation of G121 to arginine is thought to prevent function in BF by creating steric interference in an α-helix that is potentially important for dimerization of the RNase III domain with another (36). Exclusive expression of the G121R mutant KREPB5 resulted in growth inhibition in both BF and PF (Figure 6A and Table 4). The effect on growth was evident earlier in BF as we have observed in many other cases, which is consistent with the shorter generation time. Exclusive expression of mutant KREPB5 in which the charged R77 residue was replaced by a hydrophobic leucine residue resulted in a growth defect in BF only (Figure 6A and Table 4). RT-qPCR analysis revealed a substantial effect on *in vivo* editing of both mutations in BF. In PF there was strong inhibition of *in vivo* editing by the G121R mutation, while exclusive expression of R77L mutant KREPB5 had no effect on editing (Figure 6B).

The effects of these mutations on editosomes in BF and PF were also evaluated by immunoprecipitation of V5-tagged mutant KREPB5, and by glycerol gradient sedimentation of mitochondrial lysates. Immunoprecipitation with anti-V5 antibodies showed almost no co-immunoprecipitation of editosome components by both G121R and R77L mutants in BF. As in the BF CN cells, BF cells that exclusively express these mutant alleles have a much reduced level of editosome components compared to cells expressing WT KREPB5 (Figure 7A). In contrast, editosome proteins did co-precipitate in PF, albeit to a much reduced extent in the case of the G121R mutation (Figure 7A). PF cells that exclusively express either of these mutant alleles, including G121R, have the same levels of editosome components as cells expressing WT KREPB5 in lysates used as input for immunoprecipitation (Figure 7A). Glycerol gradient analysis revealed that, as observed for BF CN cells, editosome components and complexes were essentially absent in BF that exclusively express either mutant. In contrast, editosomes were present in PF although they were shifted to smaller S values by exclusive expression of the G121R mutation (Figure 7B). The shift caused by the G121R substitution in PF is greater than that caused by mutation of the PUF motif in PF, where the predominant effect is on KREPA3 and KREL1. In contrast, the G121R substitution in PF caused KREPA1, KREPA2, KREL1 and KREPA3 to shift to the upper fractions of the gradient. The sedimentation profile of the PF G121R mutant also differed from that observed upon loss of KREPB5 in the PF CN, indicating that the different KREPB5 substitutions resulted in specific effects on interactions with other editosome components. These data indicate that G121 is essential for KREPB5 function in both BF and PF, while mutation of R77 affects KREPB5 function in BF only. This is in contrast to
mutations of the KREPB5 PUF motif which is important for full KREPB5 function in PF but not in BF, and further supports that KREPB5 function is associated with differences in editing between life cycle stages.

**Functional analysis and differential function of KREPA3 zinc fingers (ZnFs) in BF and PF**—We previously found that knockdown of KREPA3 in BF CN or PF RNAi cells resulted in repression of growth and in vivo RNA editing in both life cycle stages (42). There was also a dramatic loss of editosomes and editosome components in BF upon knockdown of KREPA3, while PF contained editosomes that shifted somewhat to smaller S values (42). This resembles the result from loss of KREPB5 reported here (see Figure 1C), although the shift is greater in repressed PF KREPB5 CN cells. In order to determine whether the retention of the editosomes was due to incomplete knockdown of KREPA3 following PF RNAi, we generated PF KREPA3 CN cells in which both endogenous alleles were eliminated in cells that have a tet-regulatable WT KREPA3-myc allele inserted into the RRNA locus. Upon withdrawal of tet there was a dramatic reduction (~99%) in KREPA3 mRNA expression and repression of PF cell growth and in vivo editing (Figure 8A, B). Glycerol gradient analysis revealed that tet-inducible myc-tagged KREPA3 was barely detectable after two days, and was undetectable, after six days of growth in the absence of tet (Figure 8C). However, editosomes were still present and, similar to the RNAi studies, sedimented at a somewhat smaller S value when KREPA3 expression was repressed (Figure 8C). This indicates that the retention of editosomes upon PF KREPA3 RNAi knockdown, but loss of editosomes in BF CN knockdown is likely due to differences in the editosomes between these life cycle stages.

Earlier mutational analyses showed that the two ZnF motifs in KREPA3 are essential in BF for growth and editing progression in vivo without affecting editosome integrity or the incorporation of KREPA3 into editosomes (42,47). We tested the function of the ZnF motifs in PF by inserting WT, ZnF1 mutant (C53A/C56A; ZFm1), or ZnF2 mutant (C183A/C186A; ZFm2) alleles into the β-tubulin locus in PF KREPA3 CN cells. Western analysis was performed on total cell lysates from PF CN cells expressing these alleles and grown in the presence or absence of tet. Blots were probed using a monoclonal antibody for KREPA3 that recognized both the tet-inducible myc-tagged KREPA3 and the untagged WT or mutant KREPA3. The expression levels of untagged WT, ZFm1 and ZFm2 were similar to that of the regulatable myc-tagged KREPA3, and to each other (Figure 9A). Exclusive expression of WT KREPA3 or ZFm2 alleles resulted in normal growth of PF (Figure 9A). However, exclusive expression of the ZFm1 allele resulted in inhibition of PF cell growth. RT-qPCR analysis showed that in vivo editing was essentially unaffected in cells that exclusively express WT or ZFm2 KREPA3 while exclusive expression of ZFm1 resulted in inhibition of editing (Figure 9B). Nevertheless, both ZFm1 and ZFm2 KREPA3 proteins were incorporated into editosomes that displayed glycerol gradient sedimentation profiles indistinguishable from WT KREPA3 that was expressed either from the RRNA locus, or exclusively from the β-tubulin locus (Figure 9C). These results show that ZnF2 mutation does not detectably affect KREPA3 function in PF while ZnF1 is essential for editing in the PF. The dispensability of ZnF2 in PF is in contrast to BF where both ZnFs are required for KREPA3 function (42,47). The loss of editing in both BF and PF cells that exclusively express the ZFm1 allele is not due to loss of editosome integrity but rather some functional attribute of this domain. The loss of editing in BF but PF cells that exclusively express the ZFm2 allele suggests that the function of this domain is associated with the process that differentially regulates editing between life cycle stages, which notably is the reciprocal effect of KREPB5 PUF motif mutations.

**DISCUSSION**

We show here that editosome proteins KREPB5 and KREPA3 are essential for cell growth and RNA editing in both BF and PF cells. Surprisingly, we also find that specific, single amino acid changes in various domains of KREPB5 and KREPA3 affect cell growth and RNA editing differentially between the two life cycle stages (Table 5). These results are the first demonstration of functional differences within editosome proteins in different life cycle stages, even though BF and PF editosomes appear to
contain the same set of proteins. Indeed, these results more broadly show that specific functions of multi-protein RNA processing complexes can be altered without changing their protein composition. Mutations that exhibit this differential effect are in the degenerate RNase III domain and putative PUF motif of KREPB5, as well as the more C-terminal ZnF motif of KREPA3. These mutations differentially affect growth rate, RNA editing in vivo, and in the case of KREPB5, editosome integrity. An important consequence of these results is that experiments analyzing protein function in one life cycle stage are not necessarily predictive of function in another stage, and this phenomenon may not be restricted to RNA editing. Furthermore, our results strongly suggest that editosome proteins are involved in the processes that differentially regulate editing between life stages.

Mutational analyses show that a conserved aromatic residue in the PUF motif of KREPB5 is required for full KREPB5 function in PF, but not BF cells. Mutation of this residue to alanine in PF results in a modest inhibition of growth and in vivo editing, and affects the integrity of editosomes such that the association of KREPA3 with editosomes is compromised. Mutation to another aromatic residue does not have these effects, which is consistent with the predicted role in stacking for this residue in known PUF domains (66). Further mutation of the two other residues in the PUF motif (PUF-triple) has no additional effects in PF, but results in modest inhibition of COII editing in BF. Because these specific PUF motif mutations are in residues that are predicted to interact directly with RNA, these results suggest that KREPB5 directly interacts with the editing substrate or gRNA, potentially in a nucleotide specific or preferential fashion. PUF proteins typically bind RNA in a sequence-specific manner via multiple tandem RNA-binding domains (61-63,65,66). KREPB5 has a single PUF motif as does KREPB4, KREN1, KREN2, and KREN3 (36), and thus each may interact with editing substrates in a fashion that is preferential or specific for certain nucleotides. This also implies that these proteins may function in concert in aspects of substrate recognition and binding.

While mutation of the PUF motif reveals defects in function in PF but not BF, the opposite result was observed after mutation of the highly conserved R77 at the boundary of the KREPB5 RNase III domain. Exclusive expression of R77L KREPB5 results in loss of growth, editing and editosomes in BF, but not PF. Mutation to R77L leads to complete loss of editosome integrity and editosome components in BF, in contrast to the partial defect observed following mutation of the PUF motif in PF. While the PUF motif likely interacts directly with RNA, R77 is in a location that suggests interaction with or proximity to other proteins, because homology modeling to other RNase III structures places this residue far from the catalytic active site (Figure 10). Indeed, mass spectrometry analysis of cross-linked purified editosomes (McDermott, et al. unpublished) revealed that lysine residues within 10 residues either side of R77 cross-linked to KREPA3, KREPA2, and KREPA6. This hypothesis is therefore consistent with the disruption of editosome integrity upon mutation of R77, and altered protein interactions within editosomes may be one aspect of how life cycle-dependent changes are manifest.

We also show that some amino acids of KREPB5 are essential in both life cycle stages. The G121R mutation in the RNase III domain results in loss of growth and editing in PF as it does in BF (36). G121 lies in a region that is predicted to function in the dimerization of RNase III domains (36) that creates the functional catalytic center in other RNase III endonucleases (71). While we cannot discount that G121R, or indeed any of the substitutions more generally affect protein folding, mutation of this glycine to arginine in KREPB5 may result in steric interference which prevents normal dimerization. This suggests that RNase III dimerization is an essential feature of KREPB5 function regardless of life cycle stage. G121 is essential for editosome integrity, perhaps via RNase III mediated interactions between KREPB5 and endonucleases and endonuclease associated proteins. The G121R mutation results in a shift in editosome sedimentation to smaller values in PF, which is distinct from the complete loss of editosomes and editosome components previously observed in BF. The shift caused by G121R in PF is greater than that caused by mutation of the PUF motif in PF, which may primarily be the result of dissociation of KREPA3. The dissociation of KREPA3 provides evidence that is complementary to earlier
yeast two-hybrid and co-expression analyses that showed KREPB5 interacts with KREPA3 via the OB-fold domain of KREPA3 (38). Cross-linking and mass spectrometry analysis (McDermott, et al. unpublished) also revealed cross-links between KREPB5 and KREPA3 that are consistent with these interactions.

Parallel analyses of KREPA3 in which ZnF1 and ZnF2 were mutated also revealed stage-specific effects. Mutation of ZnF2 in KREPA3 resulted in defects in growth and editing in BF (42,47) that were not observed in PF (this study). Mutation of ZnF1 resulted in a growth defect and loss of editing in both BF (42,47) and PF (this work). However, neither of these mutations affected editosome sedimentation in either BF or PF cells, indicating no major effect on editosome integrity. The effects of these mutations on BF and PF editing are therefore due to the loss of some functional attribute of the ZnFs, and not due to detectable editosome destabilization. Amplification and sequencing of partially edited A6 mRNAs in BF cells that were exclusively expressing ZFm1 and ZFm2 mutant KREPA3 (47) showed that these ZnF mutations had differential effects on the extent of A6 editing. Both ZnFs inhibited progression of editing in vivo, but exclusive expression of ZFm1 resulted in less canonical and non-canonical editing than exclusive expression of ZFm2. This indicates that the two ZnFs within KREPA3 function differently from each other in BF, and may be a reflection of differences in affinity for editosome proteins or RNA substrates. Our results now show that ZFm1 and ZFm2 not only function differently within BF, they also function differentially between BF and PF.

Several processes might be envisioned to explain how differential editing may be controlled between life cycle stages, and how KREPB5 and KREPA3 may participate in the regulation. Firstly, there appear to be fundamental differences between the BF and PF editosomes as illustrated by BF editosomes being more susceptible to loss upon knockdown of KREPB5 and KREPA3 than PF editosomes (Table 5). This could be due to life cycle stage differences in editosome protein conformation and/or post-translational modification with consequent effects on assembly and interactions among proteins within the editosomes. This would result in differences in the detailed architecture of the editosomes and their interactions with other proteins or complexes. This is consistent with different point mutations, even within the same protein, having opposite effects on editosome function. Within KREPB5 the R77L mutation results in loss of editosomes and editing in BF, but normal complexes and editing in PF, while the H233A mutation results in normal editosomes and editing in BF, but shifted editosomes and altered editing in PF. This implies that there are inherent differences in editosomes between the two life cycle stages despite them having the same set of proteins. How might these differences be involved in the differential editing? One possibility is that they might result in different mRNA/gRNA heteroduplex substrate (i.e. ES) recognition and cleavage specificity. This may affect the rate at which the editing sites are processed and hence the overall rate at which the RNA is edited. This might in turn be affected by different mRNA/gRNA structures in the different temperatures at which BF and PF live (20). It may also be linked to how these RNA structures affect changes in the associations among editosome components, and/or interactions between editosomes and other complexes that also function in mitochondrial mRNA processing/editing (72,73). This would not depend on gRNA abundance but it would affect their usage, consistent with previous studies that show that gRNAs that are required for differential editing are present in both life cycle stages (18).

Overall, we show that specific domains of KREPB5 and KREPA3 both appear to function in stage-specific editing, although the mechanisms by which these effects are realized may differ. Similar differential effects between life cycle stages might also result from the mutation of other editosome proteins, but this has not been tested. Indeed, this may be true for non-editosome proteins as well. Both KREPB5 (36) and KREPA3 (41,42) may be required for both the activity and physical integration of the editing endonucleases with the common set of editosome proteins. Because endonuclease cleavage is the first step the catalytic cycle of editing, it is a likely point for editing regulation. The direct or indirect association of KREPB5 and KREPA3 with the endonucleases, along with their life-cycle dependent functionalities suggests that understanding the control of the endonuclease cleavage step may be
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FIGURE 1. Effects of KREPB5 knockdown in BF and PF cells. (A) Growth of BF and PF conditional null (CN) cells in which the tetracycline (tet)-regulatable WT KREPB5 allele was expressed (E) or repressed (R). (B) Western analysis of PF KREPB5 CN total cell lysates taken daily during growth analysis in the presence (E) or absence (R) of tet (equivalent of 2 x 10^6 PF cells/lane). Western blots were probed with Peroxidase Anti-Peroxidase (PAP) Antibody to allow detection of the TAP-tag on the tet-regulatable WT KREPB5 protein. WT KREPB5-TAP is not detected after four days of growth in the absence of tet. (C) RT-qPCR analysis of BF and PF CN cells in which tet-regulatable WT KREPB5 was expressed or repressed for 3 and 4 days, respectively. The abundances of pre-edited, edited and never-edited RNAs and KREPB5 mRNAs in repressed cells were calculated relative to the cells in which the WT tet-regulatable KREPB5 allele was expressed. For each target amplicon, relative abundance was determined using TERT as an internal control. 1 = no difference in relative RNA abundance, >1 an increase, and <1 a decrease (note the log scale). Bars represent mean relative abundances from three independent experiments, and standard deviations are presented in Table 3. Samples were assayed in quadruplicate in each experiment and the average standard deviations of measured Ct values were <0.09. (D) Western analysis of total cell lysates from BF and PF CN cells that were grown in the presence or absence of tet for 3 and 4 days respectively (equivalent of 1 x 10^7 BF cells/lane, and 5 x 10^6 PF cells/lane). Monoclonal antibodies against KREPA1, KREPA2, KREL1 and KREPA3 were used to probe for editosome components. A monoclonal antibody was also used to probe for mitochondrial Hsp70 (mtHsp70), which is not involved in editing. The asterisk on the PF input lysate blot indicates where the Protein A portion of the regulatable WT KREPB5-TAP protein is recognized by the primary antibodies in the Western analysis. (E) Western analysis of 5h 10-30% glycerol gradient fractions from lysates of enriched BF and PF CN mitochondria in which KREPB5 was expressed (E) or repressed (R) for 3 or 4 days, respectively. Western blots were probed as in (B) and purified ~20S editosomes (20S+) were used as a control to show sizes of assayed editosome proteins.

FIGURE 2. (A) Alignment of PUF motif sequences from T. brucei (Lister strain 427) KREPB4, KREPB5, KREN1, KREN2, KREN3, PUF2 and PUF9, and representative well studied PUF motifs from Drosophila melanogaster (DmPUM, accession P25822) and human (HsPUM1, accession Q14671) Pumilio proteins. Numbers in parentheses correspond to amino acid sequence position of PUF repeat motif used in alignment. R2 (repeat 2), R4 (repeat 4) and R6 (repeat 6) correspond to the specific PUF repeat from Pumilio proteins used in the sequence alignment. The amino acid position number in the PUF repeats is shown above the alignment. ‘Edge-on’ or ‘stacking’ residues at positions 12, 13 and 16 that are predicted to be involved in RNA binding are indicated by closed circles. Rectangles denote the α-helices in the structure of 1M8W, the Pumilio-homology domain from human Pumilio1 (63). (B) Western analysis with anti-V5 tag monoclonal antibody showing exclusive expression of V5-tagged mutant or WT KREPB5 proteins from the β-tubulin locus in BF and PF CN cells following repression (R) of the tet-regulatable WT KREPB5 allele for 3 or 4 days respectively (2 x 10^6 cells/lane). TAP-tagged WT KREPB5 is detected with anti-PAP antibody in PF cells in which the tet-regulatable allele is expressed (E).
FIGURE 3. Analysis of the growth of KREPB5 CN cells containing WT or PUF motif mutant versions of KREPB5. (A and B) Cumulative growth of cells expressing WT or PUF motif mutant versions of KREPB5 in (A) BF and in (B) PF CN cells in which the tet-regulatable WT KREPB5 allele was expressed (E) or repressed (R). Exclusive expression of WT and H233W mutant proteins (each indicated by (R)) permitted normal growth in BF and PF, while exclusive expression of H233A and PUF-triple mutant proteins (each indicated by (R)) produced a growth defect only in PF. (C) Mean BF and PF cell doubling times from three independent experiments, calculated over 144 (BF) or 192 (PF) hours using growth analysis described in (A and B). Error bars represent standard deviation. Statistical significance for differences in growth rates between cells in which the tet-regulatable WT KREPB5 allele was either expressed (E) or repressed (R) was calculated using Student’s t-test. Asterisks indicate where \( P < 0.05 \). (D) Same data as in (B) for WT, H233A, and PUF-triple mutant versions of KREPB5 in PF CN cells but presented as daily cell number as opposed to cumulative cell number. Exclusive expression of H233A and PUF-triple mutant proteins produced a growth defect in PF that was observable for up to 9 (H233A) or 7 (PUF-triple) days following tet withdrawal. (E) Western analysis of PF CN cells containing H233A and PUF-triple mutant KREPB5 alleles in which the tet-regulatable TAP-tagged WT KREPB5 allele was expressed (E) or repressed (R) for 10 days indicates loss of tet regulation and the re-expression of WT KREPB5 from the \( RRNA \) locus. The Western blot was probed with anti-PAP antibody to detect the TAP-tag of the tet-regulatable WT KREPB5.

FIGURE 4. Differential effects of KREPB5 PUF motif mutations on \textit{in vivo} editing in BF and PF. (A) RT-qPCR analysis of \textit{in vivo} RNA editing in BF and PF cells in which the tet-regulatable WT KREPB5 allele was repressed for 3 (BF) or 4 (PF) days, and that were exclusively expressing (EE) WT, H233A, PUF-triple, or H233W alleles. The abundances of transcripts from the regulatable allele (KREPB5 Reg), the exclusively expressed WT or mutant allele (KREPB5 EE), all KREPB5 alleles (KREPB5 ORF), and pre-edited, edited and never-edited mitochondrial transcripts were normalized to TERT and compared to the corresponding cells in which the tet-regulatable WT KREPB5 allele was expressed. Bars represent mean relative abundances from two independent experiments, and standard deviations are presented in Table 3. Samples were assayed in quadruplicate in each experiment and the average standard deviation of replicate \( \text{Ct} \) values was \( \leq 0.09 \) for all samples. (B) RT-PCR analysis of MURF2 mRNAs in BF and PF cells described in (A), in which the tet-regulatable WT KREPB5 allele was expressed (E) or repressed (R) for 3 (BF) or 4 (PF) days. The locations of edited and pre-edited products are indicated.

FIGURE 5. Effects of KREPB5 PUF motif mutations on BF and PF editosomes. (A) anti-V5 immunoprecipitation of exclusively expressed V5-tagged mutant or WT KREPB5 from PF and BF cells in which the tet-regulatable WT KREPB5 allele was repressed for 4 (PF) or 3 (BF) days. Left panel: anti-V5 immunoprecipitates from WT or mutant KREPB5 from PF and BF (6.25% and 12.5% of each immunoprecipitate, respectively) probed with monoclonal antibodies against KREPA1, KREPA2, KREL1 and KREPA3 and with anti-V5-tag antibody. No antibody (mock), and untagged CN cell lysates are controls. Middle and right panels: comparisons of 6.25% of cell lysate samples from which the immunoprecipitates were derived. (B) Western analysis of 5h 10-30% glycerol gradient fractions of mitochondrial lysates from BF and PF cells expressing WT, H233W, H233A or PUF-triple alleles, and in which the tet-regulatable WT KREPB5 allele was also either expressed (E) or repressed (R) for 3 (BF) or 4 (PF) days. The Western blots were probed as above and with PAP antibody to detect the TAP-tag of the tet-regulatable WT KREPB5 in PF. Bottom middle panels show that KREPA3 and KREL1 are increased in fractions 1-3 in PF cells that exclusively express H233A or PUF-triple mutant alleles compared to PF that express WT or H233A KREPB5. Data are representative of two independent experiments.

FIGURE 6. RNase III domain mutations in KREPB5 can have reciprocal life cycle stage effects on editing, or can disrupt editing in both life cycle stages. (A) Growth analysis of WT or mutant versions
Differential Editing in T. brucei

of KREPB5 in BF and PF CN cells where the tet-regulatable WT KREPB5 allele was expressed (E) or repressed (R). Exclusive expression of WT protein permitted normal growth in BF and PF, while exclusive expression of G121R mutant protein produced a strong growth defect in both BF and PF. Exclusive expression of R77L mutant protein permitted normal growth of PF, but led to a severe growth defect in BF. (B) Western analysis of BF and PF CN cell lysates (2 x 10⁶ cells/lane) containing V5-tagged WT or mutant KREPB5 alleles in which the tet-regulatable WT KREPB5 allele was expressed (E) or repressed (R) for 3 (BF) or 4 (PF) days. Western blots were probed for the V5-tag to detect the WT or mutant KREPB5 protein, and with PAP antibody to detect the TAP-tag of the tet-regulatable WT KREPB5 protein in PF. (C) RT-qPCR analysis of in vivo RNA editing in BF and PF cells in which the tet-regulatable WT KREPB5 allele was repressed for 3 (BF) or 4 (PF) days, and that were exclusively expressing (EE) WT, R77L, or G121R alleles. The abundances of transcripts from the regulatable allele (KREPB5 Reg), the exclusively expressed WT or mutant allele (KREPB5 EE), all KREPB5 alleles (KREPB5 ORF), and pre-edited, edited and never-edited mitochondrial transcripts were normalized to TERT and compared to the corresponding cells in which the tet-regulatable WT KREPB5 allele was expressed. The bars represent relative abundances calculated from quadruplicate assays. The average standard deviation of replicate Ct values was ≤ 0.09 for all samples.

FIGURE 7. RNase III domain mutations in KREPB5 can have reciprocal life cycle stage effects on editosomes, or can disrupt editosomes in both life cycle stages. (A) anti-V5 immunoprecipitation of exclusively expressed mutant or WT KREPB5 from PF and BF cells in which the tet-regulatable WT KREPB5 allele was repressed for 4 (PF) or 3 (BF) days. Western blots were simultaneously probed with monoclonal antibodies against KREPA1, KREPA2, KREL1 and KREPA3, and with anti-V5-tag antibody to detect the WT or mutant KREPB5 proteins. Left panel: anti-V5 immunoprecipitates from WT or mutant KREPB5 from PF and BF (6.25% and 12.5% of each immunoprecipitate, respectively) probed with monoclonal antibodies against KREPA1, KREPA2, KREL1 and KREPA3 and with anti-V5-tag antibody. No antibody (mock), and untagged CN cell lysates are controls. Middle panel: comparison of 6.25% of all PF anti-V5 and mock (no antibody) immunoprecipitates, with 2.5% of the PF input cell lysates. Right panel: comparison of 6.25% of all BF anti-V5 and mock (no antibody) immunoprecipitates, with 10% of the BF input cell lysates. (B) Western analysis of 5h 10-30% glycerol gradient fractions of mitochondrial lysates from BF and PF cells expressing R77L or G121R alleles, and in which the tet-regulatable WT KREPB5 allele was repressed for 3 (BF) or 4 (PF) days. Western blots were probed as above, and with PAP antibody to detect the TAP-tag of the tet-regulatable WT KREPB5 protein in PF. Data are representative of two independent experiments.

FIGURE 8. KREPA3 is essential for RNA editing in PF. (A) Growth analysis of PF CN cells in which the tet-regulatable myc-tagged WT KREPA3 allele was expressed (E) or repressed (R). Repression of KREPA3 leads to a large growth defect in both PF and BF. (B) RT-qPCR analysis of in vivo RNA editing in PF CN cells in which the tet-regulatable WT KREPA3 allele was repressed for 4 days. The abundances of transcripts from the regulatable allele (KREPA3 Reg), pre-edited, edited and never-edited mitochondrial transcripts were normalized to TERT and compared to the corresponding cells in which the tet-regulatable WT KREPA3 allele was expressed. Bars represent relative abundances calculated from quadruplicate assays. The average standard deviation of replicate Ct values was 0.08. (C) Western analysis of 9 h 10-30% glycerol gradient fractions of lysates from PF CN cells in which KREPA3 was expressed (E) or repressed (R) for 2, 6, and 8 days. Western blots were simultaneously probed with monoclonal antibodies against KREPA1, KREPA2, KREL1 and KREPA3. Myc-tagged KREPA3 is barely detectable after two days of growth in the absence of tet, and is undetectable after six days of growth in the absence of tet.

FIGURE 9. Functional analysis of KREPA3 zinc fingers (ZFs) in PF. (A) Growth analysis of exclusively expressed mutant versions of KREPA3 reveals that ZF1 is essential for function in PF, but that ZF2 is dispensable. Exclusive expression of WT and ZFm2 KREPA3 proteins permits normal
growth, whilst exclusive expression of ZFm1 KREPA3 demonstrates a strong growth defect when the tet-regulatable WT KREPA3 allele is repressed (R) compared to expressed (E). Insert shows Western analysis of PF CN cell lysates (2 x 10^6 cell equivalents/lane) containing untagged WT, ZFm1, or ZFm2 mutant KREPA3 proteins in which the tet-regulatable myc-tagged KREPA3 protein was expressed (E) or repressed (R) for 4 days. Western blots were probed using a monoclonal antibody for KREPA3. (B) RT-qPCR analysis of in vivo RNA editing in PF cells in which the tet-regulatable WT KREPA3 allele was repressed for 4 days, and that were exclusively expressing (EE) WT, ZFm1, or ZFm2 alleles. The abundances of transcripts from the regulatable allele (KREPA3 Reg), the exclusively expressed WT or mutant allele (KREPA3 EE), pre-edited, edited and never-edited mitochondrial transcripts were normalized to TERT and compared to the corresponding cells in which the tet-regulatable WT KREPA3 allele was expressed. Bars represent relative abundances calculated from quadruplicate assays. The average standard deviation of replicate Ct values was ≤ 0.09 for all samples. (C) Western analysis of 9 h 10-30% glycerol gradient fractions of lysates from PF cells expressing WT, ZFm1 or ZFm2 alleles and in which the tet-regulatable WT KREPA3 allele was expressed (E) or repressed (R) for 4 days. Western blots were simultaneously probed with monoclonal antibodies against KREPA1, KREPA2, KREL1 and KREPA3.

FIGURE 10. Ribbon representations of the RNase III domain of KREPB5 based on comparative modeling with template PDB structures (A) Aquifex aeolicus RNase III (D44N) complexed with product of double-stranded RNA processing (2EZ6) and (B) Campylobacter jejuni RNase III nuclease domain (3O2R). KREPB5 structure is shown in red, while template RNase III domain dimers and dsRBD domains (in 2EZ6) are shown in blue and yellow. RNA molecules are shown in green. The positions of R77 and G121 residues in KREPB5 are identified.

TABLES

TABLE 1. Antibodies used in this study. WB, Western Blot; IP, Immunoprecipitation.

| Antibody name                                      | Raised in     | Dilution                  | Source                                      |
|----------------------------------------------------|---------------|---------------------------|---------------------------------------------|
| V5 Epitope Tag Polyclonal Antibody                 | Rabbit        | 1 μg per IP with 1 x 10^8 cells | Rockland Immunochemicals 600-401-378        |
| V5 Epitope Tag Monoclonal Antibody                 | Mouse         | 1/5000 WB                 | Life Technologies R960-25                  |
| Peroxidase Anti-Peroxidase (PAP) Soluble Complex Antibody | Rabbit        | 1/2000 WB                 | Sigma P1291                                |
| KREPA1 Monoclonal Antibody                         | Mouse         | 1/25 WB                   | Panigrahi et al., 2001 (23) P4D8-F6         |
| KREPA2 Monoclonal Antibody                         | Mouse         | 1/12.5 WB                 | Panigrahi et al., 2001 (23) P1H3-D7         |
| KREL1 Monoclonal Antibody                          | Mouse         | 1/50 WB                   | Panigrahi et al., 2001 (23) P3C1-G2         |
| KREPA3 Monoclonal Antibody                         | Mouse         | 1/25 WB                   | Panigrahi et al., 2001 (23) P3C12-B6        |
| Mitochondrial Hsp70 (mtHsp70) Monoclonal Antibody  | Mouse         | 1/1000 WB                 | Panigrahi et al., 2001 (23) mAb78          |
TABLE 2. Effects of KREPB5 PUF motif mutations on BF and PF growth. Cell doubling times were monitored in vitro over 144 (BF) or 192 (PF) hours in the presence (tet-regulatable copy expressed; E) or absence (tet-regulatable copy repressed; R) of tet. The table reports mean values ± standard deviations (SD) from three independent experiments. CN denotes conditional null cell line. WT, PUF-triple, H233A, and H233W correspond to the different KREPB5 variants that are constitutively expressed from the β-tubulin locus.

| Cell line | BF mean doubling time ± SD (hours) | PF mean doubling time ± SD (hours) |
|-----------|-----------------------------|-----------------------------|
|           | + tet (E)                  | - tet (R)                  | + tet (E)                  | - tet (R)                  |
| CN        | 7.03 ± 0.10                | 13.09 ± 1.68               | 13.40 ± 0.25               | 21.02 ± 1.55               |
| WT        | 7.14 ± 0.09                | 7.11 ± 0.16                | 13.75 ± 0.21               | 13.62 ± 0.05               |
| PUF-triple| 7.07 ± 0.07                | 7.18 ± 0.06                | 13.49 ± 0.24               | 15.70 ± 0.22               |
| H233A     | 7.09 ± 0.10                | 7.11 ± 0.09                | 13.15 ± 0.21               | 16.13 ± 0.49               |
| H233W     | 7.05 ± 0.03                | 7.11 ± 0.06                | 13.54 ± 0.29               | 13.53 ± 0.40               |

TABLE 3. Means ± standard deviations of relative mRNA abundances in BF and PF KREPB5 CN cells that were exclusively expressing PUF motif mutants. The data summarize three independent RT-qPCR experiments for CN cells and two independent experiments for WT or mutant cells. Four technical replicates were carried out per experiment.

| Transcript  | BF                  |                  |                  | PF                  |                  |
|-------------|---------------------|------------------|------------------|---------------------|------------------|
|             | CN                  | WT               | PUF-triple       | H233A              | H233W            |
|             | CN                  | WT               | PUF-triple       | H233A              | H233W            |
| KREPB5 Reg  | 0.05 ± 0.02         | 0.01 ± 0.01      | 0.01 ± 0.00      | 0.01 ± 0.00        | 0.00             |
| KREPB5 EE   | 1.17 ± 0.09         | 1.14 ± 0.09      | 1.16 ± 0.08      | 1.18 ± 0.04        | 0.08             |
| KREPB5 ORF  | 0.05 ± 0.02         | 0.43 ± 0.19      | 0.83 ± 0.09      | 0.32 ± 0.06        | 0.00             |
| COI         | 0.80 ± 0.13         | 1.06 ± 0.14      | 1.07 ± 0.21      | 0.86 ± 0.02        | 1.07 ± 0.03      |
| ND4         | 0.80 ± 0.11         | 1.28 ± 0.15      | 1.01 ± 0.14      | 1.08 ± 0.24        | 1.23 ± 0.30      |
| A6 pre      | 4.50 ± 1.16         | 1.67 ± 0.64      | 1.80 ± 0.83      | 1.17 ± 0.23        | 1.59 ± 0.12      |
| CYb pre     | 2.83 ± 0.97         | 1.39 ± 0.14      | 1.71 ± 0.58      | 1.17 ± 0.04        | 1.04 ± 0.29      |
| COII pre    | 0.97 ± 0.26         | 1.15 ± 0.51      | 0.94 ± 0.66      | 0.35 ± 0.15        | 0.58 ± 0.39      |
| COII pre    | 1.98 ± 0.06         | 0.77 ± 0.19      | 2.71 ± 0.27      | 0.93 ± 0.62        | 17.91 ± 7.08     |
| MURF2 pre   | 1.61 ± 0.20         | 2.26 ± 0.72      | 0.30 ± 0.23      | 0.71 ± 0.33        | 1.91 ± 0.75      |
| RPS12 pre   | 3.98 ± 1.38         | 1.03 ± 0.26      | 1.11 ± 0.02      | 1.49 ± 0.29        | 2.07 ± 0.28      |
| ND3 pre     | 1.18 ± 0.22         | 1.04 ± 0.18      | 1.37 ± 0.14      | 1.01 ± 0.07        | 3.15 ± 0.49      |
| ND7 pre     | 1.55 ± 0.52         | 1.19 ± 0.38      | 1.57 ± 0.17      | 1.27 ± 0.24        | 1.15 ± 0.49      |
| A6 edit     | 0.02 ± 0.02         | 1.14 ± 0.10      | 0.83 ± 0.20      | 0.88 ± 0.09        | 0.90 ± 0.02      |
| CYb edit    | 0.00                | 1.92 ± 0.51      | 0.51 ± 0.12      | 0.12 ± 0.09        | 0.01             |
| COII edit   | 0.12 ± 0.01         | 1.71 ± 0.03      | 0.87 ± 0.02      | 1.05 ± 0.10        | 0.18 ± 0.08      |
| COII edit   | 0.00                | 1.19 ± 0.33      | 0.94 ± 0.02      | 0.79 ± 0.07        | 0.79 ± 0.05      |
| MURF2 edit  | 0.02 ± 0.03         | 1.36 ± 0.56      | 0.79 ± 0.05      | 0.76 ± 0.04        | 0.00             |
| RPS12 edit  | 0.04 ± 1.13         | 0.95 ± 0.91      | 0.16 ± 0.11      | 1.08 ± 0.32        | 0.34 ± 0.81      |
TABLE 4. Effects of KREPB5 RNase III domain mutations on BF and PF growth. Cell doubling times were monitored in vitro over 144 (BF) or 192 (PF) hours in the presence (tet-regulatable copy expressed; E) or absence (tet-regulatable copy repressed; R) of tet. The table reports mean values ± standard deviations (SD) from three independent experiments. CN denotes conditional null cell line. WT, R77L, and G121R correspond to corresponding to the different mutated KREPB5s that are constitutively expressed from the β-tubulin locus and hence exclusively expressed in the absence of tet.

| Cell line | BF mean doubling time ± SD (hours) | PF mean doubling time ± SD (hours) |
|-----------|-----------------------------------|-----------------------------------|
|           | + tet (E)                         | - tet (R)                         |
|           | 7.03 ± 0.10                       | 13.09 ± 1.68                     |
| CN        | 7.14 ± 0.09                       | 13.75 ± 0.21                     |
| WT        | 7.01 ± 0.31                       | 14.41 ± 1.97                     |
| R77L      | 7.21 ± 0.09                       | 12.97 ± 1.13                     |
| G121R     | 7.03 ± 0.10                       | 13.40 ± 0.25                     |
|           | 13.11 ± 0.16                      | 13.75 ± 0.21                     |
|           | 14.41 ± 1.97                      | 13.71 ± 0.18                     |
|           | 12.97 ± 1.13                      | 13.54 ± 0.33                     |
|           | 21.02 ± 1.55                      | 21.67 ± 1.88                     |
|           | 21.02 ± 1.55                      | 21.67 ± 1.88                     |

TABLE 5. Summary of all cell lines described in this study and their phenotypes

| Protein | Cell line               | Growth | Editing | Complex | Growth | Editing | Complex |
|---------|-------------------------|--------|---------|---------|--------|---------|---------|
| KREPB5  | KD                      | I      | I       | L       | I      | I       | S       |
|         | PUF C232A EE            | WT     | WT      | ND      | WT     | WT      | ND      |
|         | PUF H233A EE            | WT     | WT      | WT      | I      | I       | S       |
|         | PUF E236A EE            | WT     | WT      | ND      | WT     | WT      | ND      |
|         | PUF C232A/H233A/E236A EE| WT     | WT (except COII) | WT     | I      | I       | S       |
|         | RNase III R77L EE       | I      | I       | L       | WT     | WT      | WT      |
|         | RNase III G121R EE      | I      | I       | L       | I      | I       | S       |
| KREPA3  | KD                      | I      | I       | L       | I      | I       | S       |
|         | ZFm1 C53A/C56A EE       | I      | I       | WT      | I      | I       | WT      |
|         | ZFm2 C183A/C186A EE     | I      | I       | WT      | WT     | WT      | WT      |

Key
BF, Bloodform; PF, Procyclic form; I, Inhibited (Growth/Editing); L, Lost (Complex); S, Shifted (Complex); WT, Wild type (Growth/Editing/Complex); ND, Not Done.
Figure 1

A

Day following tet withdrawal PF

KREPB5-TAP:

B

Relative mRNA abundance

C

Pre-edited mRNAs

Edited mRNAs

D

Input lysate

PF

mtHsp70

E

BF

PF

by guest on March 22, 2020
http://www.jbc.org/Downloaded from
Figure 2

A

| Protein          | Amino Acid Sequence |
|------------------|---------------------|
| TbKREPB4 (266-300) | ATRS-AAEQLVLNVLGTH | 161201030 |
| TbKREPB5 (217-252) | ALFP-FSDLILHVLCCCHV | 359402934 |
| TbKREN1 (376-412)  | FVEINGVGEARLAAALVQH | 412300000 |
| TbKREN2 (324-359)  | PLPP-TGEOFSLRALVHVM | 359402934 |
| TbKREN3 (294-329)  | PAFPP-THERVRA | 329000000 |
| TbPUF2 (347-384)   | EVFP-RTHLDVFGNY | 384000000 |
| TbPUF9 (290-325)   | EHAD-DLIVALDHGCHV | 325000000 |
| DmPUM R4 (1219-1254) | ELDG-HVLCVQDK | 125400000 |
| DmPUM R6 (1291-1326) | EHHE-HTEQLIODQ | 132600000 |
| HsPUM1 R4 (956-991) | EHHE-HTEQLIODQ | 991000000 |
| HsPUM1 R6 (1028-1063) | EHHE-HTEQLIODQ | 106300000 |

B

BF KREPB5:

| CN | WT | PUF-triple | H233A | H233W |
|----|----|------------|-------|-------|
| E  | R  | E R        | E R   | E R   |

PF KREPB5:

| CN | WT | PUF-triple | H233A | H233W |
|----|----|------------|-------|-------|
| E  | R  | E R        | V5    |       |
| TAP |
Figure 3

A

B

C

D

E

Figure 3

A

B

C

D

E
Figure 4

A

|                | Pre-edited mRNAs | Edited mRNAs |
|----------------|------------------|--------------|
| WT EE:         |                  |              |
| H233A EE:      |                  |              |
| H233W EE:      |                  |              |
| PUF-triple EE: |                  |              |

B

RT-PCR MURF2:

|       | CN | WT | H233A | H233W | PUF-triple |
|-------|----|----|-------|-------|------------|
|       | BF | E  | R     |       |            |
|       | PF | E  | R     |       |            |
Figure 5
### Figure 7

**A**

|                  | IP: anti-V5 | IP: Mock | Input |
|------------------|-------------|----------|-------|
| PF CN            |             |          |       |
| PF WT            |             |          |       |
| PF R77L         |             |          |       |
| PF G121R        |             |          |       |

**B**

|                  | IP: anti-V5 | IP: Mock | Input |
|------------------|-------------|----------|-------|
| BF CN            |             |          |       |
| BF WT            |             |          |       |
| BF R77L         |             |          |       |
| BF G121R        |             |          |       |

**PF:**

|                  | IP: anti-V5 | IP: Mock | Input |
|------------------|-------------|----------|-------|
| PF CN            |             |          |       |
| PF WT            |             |          |       |
| PF R77L         |             |          |       |
| PF G121R        |             |          |       |

**BF:**

|                  | IP: anti-V5 | IP: Mock | Input |
|------------------|-------------|----------|-------|
| BF CN            |             |          |       |
| BF WT            |             |          |       |
| BF R77L         |             |          |       |
| BF G121R        |             |          |       |

**R77L:**

|                  | IP: anti-V5 | IP: Mock | Input |
|------------------|-------------|----------|-------|
| PF CN            |             |          |       |
| PF WT            |             |          |       |
| PF R77L         |             |          |       |
| PF G121R        |             |          |       |

**G121R:**

|                  | IP: anti-V5 | IP: Mock | Input |
|------------------|-------------|----------|-------|
| PF CN            |             |          |       |
| PF WT            |             |          |       |
| PF R77L         |             |          |       |
| PF G121R        |             |          |       |

**20S+**
Figure 8

A

Cumulative cell number (log_{10} cells/ml)

Days

PF KREPA3 CN (E)

PF KREPA3 CN (R)

B

Relative mRNA abundance

Pre-edited mRNAs

Edited mRNAs

C

E

1 3 5 7 9 11 13 15 17 19 21 23 20S+

A1

A2

L1

A3

R

2 day

A1

A2

L1

A3

R

6 day

A1

A2

L1

A3

R

8 day

A1

A2

L1

A3
Differential Editosome Protein Function between Life Cycle Stages of *Trypanosoma brucei*

Suzanne M. McDermott, Xuemin Guo, Jason Carnes and Kenneth Stuart

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