Trypanosome Letm1 Protein Is Essential for Mitochondrial Potassium Homeostasis*

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Background: Letm1 is a mitochondrial protein attributed disparate roles, including cation/proton antiport and translation. Results: Letm1 RNAi silencing in Trypanosoma brucei triggers swelling mitochondria and translation arrest that is ameliorated by chemical potassium/proton exchangers. Conclusion: The ancestral function of Letm1, to maintain mitochondrial potassium homeostasis, shows remarkable conservation. Significance: Results from diverged T. brucei provide a better understanding of Letm1 function throughout eukaryotes.

Letm1 is a conserved protein in eukaryotes bearing energized mitochondria. Hemizygous deletion of its gene has been implicated in symptoms of the human disease Wolf-Hirschhorn syndrome. Studies almost exclusively performed in opisthokonts have attributed several roles to Letm1, including maintaining mitochondrial morphology, mediating either calcium or potassium/proton antiport, and facilitating mitochondrial translation. We address the ancestral function of Letm1 in the highly diverged protist and significant pathogen, Trypanosoma brucei. We demonstrate that Letm1 is involved in maintaining mitochondrial volume via potassium/proton exchange across the inner membrane. This role is essential in the vector-dwelling procyclic and mammal-infecting bloodstream stages as well as in Trypanosoma brucei evansi, a form of the latter stage lacking an organellar genome. In the pathogenic bloodstream stage, the mitochondrion consumes ATP to maintain an energized state, whereas that of T. brucei evansi also lacks a conventional proton-driven membrane potential. Thus, Letm1 performs its function in different physiological states, suggesting that ion homeostasis is among the few characterized essential pathways of the mitochondrion at this T. brucei life stage. Interestingly, Letm1 depletion in the procyclic stage can be complemented by exogenous expression of its human counterpart, highlighting the conservation of protein function between highly divergent species. Furthermore, although mitochondrial translation is affected upon Letm1 ablation, it is an indirect consequence of K⁺ accumulation in the matrix.

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The first hint of the role of Letm1 on the cellular level emerged from a deletion mutant screen for mt defects performed in Saccharomyces cerevisiae (9). The swollen appearance of the organelle in the Letm1 knock-out yeast strains prompted the authors to dub it MDM38, representing another alias for the protein, to reflect its effect on mitochondrial distribution and morphology. RNAi silencing of Letm1 in other opisthokont models like human cell cultures, Drosophila melanogaster, and Caenorhabditis elegans also resulted in swollen and fragmented mitochondria (3, 10–12), suggesting a conservation of function at least within this clade. This notion is further supported by the successful complementation of yeast Letm1 knockout by expression of the human ortholog (1). However, how Letm1 operates on the cellular level remains debated. Given its dramatic effect on mt morphology, it has been proposed to play an undefined structural role in the human organelle, particularly in maintaining the cristae that form inner membrane invaginations into the matrix (12). This morphological function was determined to operate indepen-

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dently of the fission and fusion machineries that maintain the mt network in these cells (3, 12).

Letm1 has also been hypothesized to take part in maintaining matrix volume as a cation/proton (H\(^+\)) antiporter. This function would also be consistent with the observed swollen mitochondria phenotype upon depletion of Letm1 because this treatment would negatively impact ion homeostasis and cause organellar osmotic stress. However, the identity of the cation that is translocated by Letm1 remains controversial. Several compelling studies in yeast, *Drosophila*, and human cell culture have shown that it has a central part in potassium/proton exchange (KHE), which maintains matrix volume by regulating potassium (K\(^+\)) extrusion (1, 3, 10, 13). In all of these model systems, treatment with the chemical K\(^+\)/H\(^+\) exchanger nigericin compensates for the loss of Letm1-mediated KHE. However, Letm1 was also identified as a calcium (Ca\(^2+\))/H\(^+\) antiporter in the genome-wide RNAi screen in *Drosophila* S2 cells (14), a finding corroborated in a later report (15).

Yet another role that has been attributed to Letm1 in *S. cerevisiae* is the anchoring of mt ribosomes to the inner membrane, into which it facilitates the incorporation of hydrophobic *de novo* translated subunits of the respiratory chain (4, 16, 17). This path of inquiry began with an observed reduction of the steady-state levels of a subset of mitochondrially encoded proteins in *Letm1* knockouts (4). A similar phenomenon was also reported in *Arabidopsis thaliana* bearing simultaneous homozygous and hemizygous knockouts of its two *Letm1* paralogs (18). Further support for this role, albeit indirect, was the report that Letm1 silencing in HeLa cells resulted in the disassembly of some respiratory chain complexes (12), which was nevertheless contradicted by another similar study on the same cell type (3).

To date, our understanding of Letm1 is rather convoluted. To shed light on this situation, we have undertaken functional analysis of *Letm1* (TriTrypDB genome database accession number Tb9273.4920 (19)) in the protozoan flagellate *Trypanosoma brucei*. As a member of the Kinetoplastea, it has a long and independent evolutionary history, perhaps due to its early branching from other eukaryotic lineages (20, 21). Kinetoplastids contain a single mitochondrion, with its organellar genome located in a discrete place as the giant kinetoplast DNA (kDNA) network (22). Most of the kDNA-encoded transcripts undergo elaborate post-transcriptional processing called RNA editing.

Research on kinetoplastids has proven to be invaluable to the field of mt comparative biochemistry. Notably, seminal work described Ca\(^2+\) influx into the trypanosome mitochondrion in a ruthenium red-sensitive fashion (23, 24). These data became definitive of the fission and fusion machineries that maintain the mt network in these cells (3, 12).

In this study, we take advantage of RNAi permissibility and ease of transgenesis of PS, BS, and *T. brucei evansi* in *in vitro* cultures to generate conditional knockdown cell lines to test the effect of Letm1 silencing in three different physiological states of the mitochondrion. We also compare our results in this highly diverged organism with the previously enumerated results from opisthokont model systems in order to elucidate the basal function of the evolutionarily conserved Letm1. Although this study does not represent the first one performed outside of the opisthokont clade, as the aforementioned report in Plantae can attest (18), this is for the first time when almost complete silencing of Letm1 has been achieved, yielding a clear and robust phenotype. This study also reveals yet another essential function of the *T. brucei BS* mitochondrion: the maintenance of ion homeostasis.

**EXPERIMENTAL PROCEDURES**

Cloning, Cultivation, Transfection, Growth Curves, and 5'-End Mapping of *Letm1* mRNA—PS and BS *T. brucei* as well as *T. brucei evansi* were cultured, transfected, and selected for the relevant drug resistance for each of the given constructs and counted as described elsewhere (31, 34). A *Letm1* gene fragment amplified using forward primer GGATCCGTTCAAGCCTACCAGTACA (introduced BamHI site underlined) and reverse primer AGGCCCTGGTACATTCGCTACC (HindIII site underlined) was cloned into the p2T7-177 vector, bearing opposing T7 polymerase promoters/tetracycline oper-
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ators and targeted to a transcriptionally silent part of the T. brucei genome (41), via the indicated restriction sites. For in situ C-terminal tagging of Letm1 with YFP, the full open reading frame (ORF) excluding the stop codon was PCR-amplified with the forward primer GGTACCAGTTGG CAGCAACGGGGTT (Acc65I restriction site underlined) and reverse primer GATCCATTTTTGGCAATCACTCTGAGGCT (BamHI site underlined) and cloned into the p2937 vector, derived from the p2710 vector bearing the blasticidin resistance marker (42). The construct was linearized using the unique Ncol restriction site within the Letm1 ORF to yield homology flanks for integration into the endogenous locus. The full ORF of HsLetm1 was PCR-amplified from cDNA (clone FLBJ81297AAAF) supplied by the National Institute of Technology and Evaluation Biological Resource Center (Japan) using the forward primer TCAGATCTGCTCTTCACCTCTGCGA and reverse primer TCAGATCTTGTTCATGCGTGTGA and cloned into the pABPURO vector (43) via the underlined BglII restriction sites. We took advantage of every mRNA bearing a spliced leader RNA sequence by amplifying the 5’-end of Letm1 with the canonical spliced leader RNA forward primer and the reverse primer AGACATTAAACGGCCCTTCC. The relative abundance of Letm1 with the forward primer GGTACCAGTTGG CAGCAACGGGGTT (Acc65I restriction site underlined) and reverse primer GATCCATTTTTGGCAATCACTCTGAGGCT (BamHI site underlined) and cloned into the p2937 vector, derived from the p2710 vector bearing the blasticidin resistance marker (42). The construct was linearized using the unique Ncol restriction site within the Letm1 ORF to yield homology flanks for integration into the endogenous locus. The full ORF of HsLetm1 was PCR-amplified from cDNA (clone FLBJ81297AAAF) supplied by the National Institute of Technology and Evaluation Biological Resource Center (Japan) using the forward primer TCAGATCTGCTCTTCACCTCTGCGA and reverse primer TCAGATCTTGTTCATGCGTGTGA and cloned into the pABPURO vector (43) via the underlined BglII restriction sites. We took advantage of every mRNA bearing a spliced leader RNA sequence by amplifying the 5’-end of Letm1 with the canonical spliced leader RNA forward primer and the reverse primer AGACATTAAACGGCCCTTCC, as described previously (44).

Indirect Immunofluorescence and Confocal and Electron Microscopy—Indirect immunofluorescence was performed as described elsewhere (34) except that the fixed samples were permeabilized with 0.15% Triton X-100 in PBS (v/v). Samples were decorated with primary rabbit antibodies against either GFP or HAls, depending on the epitope and then subsequently with Alexa-488-conjugated anti-rabbit secondary antibody. Prior to this procedure, 2 × 10⁶ live PS cells were incubated for 20 min at 27 °C with 100 nM MitoTracker Red CMXRos. All antibodies and dyes were from Molecular Probes. Transmission electron microscopy was performed as before (40).

Digitonin Fractionation, Western Blot Analysis, and Triton X-114 Separation of Membrane Proteins—Digitonin fractionation of cells into cytosolic and mitochondrial compartments and Western blots were performed as previously described (36). Antibodies against the T. brucei mitochondrial heat shock protein, cytochrome c, TrCOIV, and the β-subunit of F₁-ATPase (31, 45) were used at 1:1000 dilutions, whereas enolase was used at 1:10,000 dilutions. The Triton X-114 isolation of membrane and soluble proteins was performed as described previously (46) on mitochondria hypotonically isolated from PS T. brucei by an established method (47). Acetone-precipitated proteins from the fractions were resuspended in equal volumes of ultrapure water.

Isolation of Submitochondrial Particles and Proteinase K Protection Assay—Hypotonically isolated mitochondria were further processed to generate submitochondrial particles (SMPs) by adapting procedures described previously (13, 45). Briefly, a mitochondria suspension corresponding to 2.4 mg/ml protein was sonicated with three 10-s pulses (50% amplitude, 1 Hz) followed by 1-min pauses in ice water using a UP200S Ultrasonic Processor (Hielscher Ultrasound Technology). The SMPs were sedimented at 31,000 × g for 5 h at 4 °C. For the proteinase K protection assay, the SMPs were resuspended at a concentration of 1 mg/ml protein and incubated with or without 200 µg/ml proteinase K for 15 min on ice and then treated with 1 mM PMSF for another 15 min on ice.

Treatment of PS T. brucei with Nigericin, Monensin, and Valinomycin—For the nigericin (all ionophores used were from Sigma-Aldrich) rescue experiment described in the legend of Fig. 4A, PS T. brucei were grown for 2 days in SDM-79 medium supplemented with tetracycline. Cells were subsequently diluted to 2 × 10⁶ cells/ml into various media with a stepwise doubling of nigericin concentrations in the 0–100 nM range. The 0 nM medium was mock-treated with the nigericin solvent ethanol. This procedure was done in order to ensure that all cells had the same initial degree of Letm1 down-regulation before ionophore exposure. Cell density was then measured at each nigericin concentration every 24 h over a 3-day time course. The procedure for monensin treatment (Fig. 4B) was the same, except there was a stepwise 10-fold increase in this ionophore concentration at the 0–1000 ng/ml range. For the valinomycin treatment of PS cells described in the legend to Fig. 5, the PS cells were incubated with a 1 µM concentration of the compound for 2 h. A subset of the cells was pretreated with 2 µM of nigericin prior to valinomycin application.

Flow Cytometry for Membrane Potential—PS T. brucei incubated with MitoTracker Red CMXRos, as described above, were diluted 1:5 in PBS and placed into a FACSCanto II flow cytometer (BD Biosciences) for measurement of fluorescence. Twenty thousand cells were counted in each measurement. Controls in which membrane potential was collapsed by the simultaneous addition of 20 µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone to cells were also measured. Data were analyzed using the Flowing Software program (Turku Centre for Biotechnology, Finland).

Quantitative Real-time PCR and Northern Blot Analysis—Quantitative real-time PCR was performed as described previously (34), using primers homologous to mt mRNAs as designed by Carnes et al. (48). Letm1 mRNA was measured using specific primers CGGAATACCTGTCGTCCACT and AGACATTAAACGGCCCTTCC, as described previously (44). Letm1 mRNA was measured using specific primers CGGAATACCTGTCGTCCACT and AGACATTAAACGGCCCTTCC, as described previously (44). Letm1 mRNA was measured using specific primers CGGAATACCTGTCGTCCACT and AGACATTAAACGGCCCTTCC, as described previously (44). Letm1 mRNA was measured using specific primers CGGAATACCTGTCGTCCACT and AGACATTAAACGGCCCTTCC, as described previously (44). Letm1 mRNA was measured using specific primers CGGAATACCTGTCGTCCACT and AGACATTAAACGGCCCTTCC, as described previously (44).

Mitochondrial Translation Assay—The mitochondrial translation assay is discussed in detail elsewhere (49). Briefly, 4 × 10⁶ cells were incubated with [³⁵S]methionine for 1 h (Easy Tag Express Protein Labeling Kit, PerkinElmer Life Sciences) in the presence of 10 µCi/ml. The cells were lysed at 37 °C for 20 min in the loading buffer (2% SDS, 125 mM Tris-HCl, pH 6.8, 2% β-mercaptoethanol, 27% glycerol (v/v)) and then run on a 9% acrylamide SDS gel in the first dimension. Each lane was cut out and placed in a denaturing solution (1% SDS, 125 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol) at 37 °C for 1 h before being run in the...
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matrix from the IM (Fig. 1D), a topology kept by the human ortholog (3).

Because the in situ tagged protein is bigger than the predicted size of the tagged Letm1 protein (Fig. 1A), about 80 versus 68 kDa, respectively, we decided to map the 5’-end of the mature Letm1 mRNA to define its ORF. The start codon is actually further upstream than predicted (supplemental Fig. 1A), encoding a protein with the observed size and of a length comparable with that of the ortholog from the related Trypanosoma cruzi (supplemental Fig. 1B). The revised ORF contains the transmembrane domain that defines all Letm1 orthologs and other well conserved features, such as a putative protein kinase C phosphorylation site and C-terminal coiled-coil regions, but lacks the two Ca²⁺-binding EF-hand domains present in orthologs of some other eukaryotes.

RNAi Silencing of Letm1 in Procyclic Stage Results in Mitochondrial Swelling and Inhibited Growth—To test whether Letm1 is an essential protein for the PS cells, a conditional RNAi cell line was generated using an established system in which dsRNA overexpression is induced by the addition of tetracycline, as described elsewhere (34). To test whether the dsRNA successfully targets Letm1 mRNA for degradation, RNA was harvested from cells grown in the presence and absence of the antibiotic for 48 h for subsequent Northern analysis using a radioactively labeled antisense probe that anneals to the Letm1 sequence. The transcript is undetectable in the RNAi-induced cells as compared with the non-induced controls (Fig. 2A). Ethidium bromide-stained rRNA was used as a control for equal loading. Next, we measured the growth of RNAi-induced and uninduced PS cells every 24 h over a 10-day course. Fig. 2B depicts a representative line graph showing absolute cell density at each time point, including the dilution of cultures every other day to 2 × 10⁶ cells/ml. Reproducible growth inhibition is apparent 3 days after RNAi induction.

The impairment of Letm1-depleted cells is most likely due to the appearance of a swollen mitochondrion, as revealed by transmission electron microscopy (Fig. 2C, I). The identity of this massive, electron-lucent organelle, as compared with the unaltered mt electrodensity in the untreated controls (Fig. 2C, 3), is supported by the surrounding double membranes (Fig. 2C, 2, arrowheads). The discoidal cristae, which are characteristic for T. brucei and other members of the phylum Euglenozoa (21), remain upon Letm1 down-regulation in the periphery of the swollen organelle (Fig. 2C, 1 and 2, arrows).

Human Letm1 Complements the Endogenous Trypanosoma Ortholog—To exclude the possibility that the Letm1-silencing phenotype is due to off target effects and also to determine the protein’s functional homology across the enormous evolutionary distance separating kinetoplastids from opisthokonts, we examined whether a constitutively expressed human Letm1 (HsLetm1) could complement the depletion of its T. brucei ortholog (TBLetm1). First, we confirmed that the exogenous HsLetm1, bearing a 3X hemagglutinin (HA) epitope tag on its C terminus, is targeted to the flagellum mitochondrion. Indirect immunofluorescence shows that HsLetm1 indeed co-localizes with the MitoTracker Red dye (Fig. 3A). This result was confirmed by digitonin subfractionation of cells into the cytosolic and mitochondrial compartments, in which the α-HA antibody

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**RESULTS**

Letm1 Is a Mitochondrial Inner Membrane Protein—In order to confirm that the annotated T. brucei Letm1 ortholog is targeted to the mitochondrion, cell lines were generated in which one of the gene loci was in situ tagged with sequence encoding a C-terminal YFP extension. Indirect immunofluorescence confocal microscopy revealed that Letm1 is indeed localized throughout the organelle because its signal overlaps with the specific marker MitoTracker Red CMXRos dye (Fig. 1A). This result was confirmed by digitonin permeabilization of cells into mitochondrial and soluble fractions, which reside in the detergent (Det.) or aqueous (Aq.) phases, respectively. The 75 kDa marker is indicated on the right. C, Western blot analysis of Triton X-114 fractionation of mitochondrial proteins into membrane and soluble fractions, which reside in the detergent (Det.) or aqueous (Aq.) phases, respectively. D, Western blot analysis of SMPs treated with proteinase K (+) or untreated controls (−). Antibodies used are indicated on the left.

Second dimension on a 14% acrylamide-SDS gel. The gels were Coomassie-stained, incubated for 1 h in 1 M salicylate (Sigma-Aldrich), and then dried before exposure to BioMax Film (Eastman Kodak Co.).

- **A** YFP, Mito, Merge, Merge-DIC
- **B** YFP, mHSP70, enolase
- **C** Det., Aq.
- **D** Proteinase K

**FIGURE 1. Localization of C-terminally YFP-tagged Letm1 to T. brucei mitochondrial inner membrane.** A, indirect immunofluorescence of fixed PS T. brucei labeled with MitoTracker Red, which visualizes the single reticulated organelle. Labels above pictures indicate signal from YFP antibody (YFP), MitoTracker (Mito), and both (Merge) and signals overlaid with a differential contrast image (Merge-DIC). DAPI-stained nucleus (n) and kDNA (k) are indicated by an arrowhead and arrow, respectively. Scale bar, 2 μm. B, Western blot analysis of digitonin fractionation of cytoplasm (Cyto) and mitochondrial (Mito) compartments in comparison with an equivalent amount of lysate from whole cells (Total). The 75 kDa marker is indicated on the right. C, Western blot analysis of Triton X-114 fractionation of mitochondrial proteins into membrane and soluble fractions, which reside in the detergent (Det.) or aqueous (Aq.) phases, respectively. D, Western blot analysis of SMPs treated with proteinase K (+) or untreated controls (−). Antibodies used are indicated on the left.
Trypanosome Letm1 Maintains Mitochondrial K+ signal associates with that of the mitochondrial marker mHSP70 (Fig. 3B). The abundant upper band migrated at the expected ~90 kDa size with a less intense one just below, a pattern also observed when the protein was expressed in Escherichia coli (14).

After verifying that the endogenous TbLetm1 is still silenced in cells expressing HsLetm1 (Fig. 3C), the growth of cell lines with and without TbLetm1 down-regulation was compared. As shown in the line graph in Fig. 3D, both samples grew at the same rate. This result indicates that HsLetm1 can fully complement the ablation of TbLetm1 in T. brucei, suggesting functional homology of the two orthologs. Indeed, the two orthologs share sequence homology in the region of the predicted transmembrane domain (Fig. 3E).

The Nigericin Ionophore Restores Cell Viability and Mitochondrial Function in Letm1 Knockdowns—The manifested swollen mitochondria upon Letm1 ablation may be due to a consequent accumulation of ions. To test whether K+ is the cation in question, we attempted to treat RNAi-induced T. brucei with varying doses of nigericin, an ionophore that acts as an antiporter of K+ and H+ across membranes, as described under “Experimental Procedures.” Fig. 4A depicts a line graph in which average cell density among quadruplicates is plotted against nigericin concentration. Each line presents measurements made 1–3 days after ionophore treatment. Growth rates at each nigericin concentration are inferred from the difference between points relative to the y axis and also depicted in supplemental Fig. 2A.

In the cells depleted for Letm1 (RNAi+), there is a dose-dependent increase in growth from 0 to 50 nM nigericin, after which there is a decrease in growth. This latter trend is probably due to the intrinsic toxicity of the ionophore to T. brucei, as demonstrated by a line graph showing the dose-dependent decrease in growth of cells grown in the absence of tetracycline (RNAi–) throughout the whole concentration range. However, it should be mentioned that the Letm1-depleted cells grown in 100 nM nigericin still exhibit more rapid growth than those grown without the drug, indicating that this ionophore is able to partially restore cell viability upon loss of Letm1. The cells...
ablated for Letm1 exhibited comparable growth rates as compared with their RNAi-uninduced counterparts when treated with 25, 50, and 100 nM nigericin (supplemental Fig. 2A).

We next looked to see whether nigericin treatment restores mt morphology and physiology. Cells grown for 2 days in the presence of tetracycline to induce Letm1 silencing were subsequently diluted into media with or without 25 nM nigericin and grown for 24 h before being subjected to assays comparing RNAi-treated and untreated samples. Light microscopy reveals that about half of the cells grown in the absence of the ionophore (Fig. 4C, −) exhibit a rounded shape with lucent center, representing the swollen mitochondrion (arrows), whereas all of the treated cells exhibit normal gross morphology (Fig. 4C, +). The physiological state of the organelle in both samples was also determined using the MitoTracker Red dye, whose intercalation into the matrix correlates with membrane potential. According to the flow cytometry histogram (Fig. 4D, −), the membrane potential of Letm1-depleted cells (RNAi+) not treated with nigericin exhibits various degrees of membrane potential reduction compared with their non-induced counterparts (RNAi−), as represented by two broad fluorescence peaks. The broad range of membrane potential reduction also reflects the various morphological effects 3 days after Letm1 down-regulation. However, nigericin-treated cells with reduced or endogenous Letm1 levels exhibited the same membrane potential (Fig. 4D, +), suggesting that the ionophore mediates restoration of the physiological state of the organelle.

These results were further confirmed by treatment with monensin, a less specific ionophore that exchanges H+ and monovalent cations, such as K+ (Fig. 4B). Using the same previously described scheme, 100 ng/ml was determined to restore growth in the Letm1-depleted cells compared with the uninduced cells.
Depletion of Letm1 Phenocopies the K⁺ Ionophore Valinomycin—As further support for the notion that matrix K⁺ accumulation is behind the swelling of the PS *T. brucei* mitochondrion upon Letm1 silencing, the parental cell line used in the generation of the conditional RNAi knockdown was treated with the ionophore valinomycin. In contrast to nigericin, valinomycin acts in a Nerstian fashion by transporting K⁺ across the inner membrane. Consequently, these cells exhibit normal mt morphology and physiology.

We next asked whether nigericin pretreatment can prevent valinomycin-mediated swelling mitochondria. Cells were incubated with 2 μM nigericin for 15 min and then split into those exposed to or lacking valinomycin treatment as described above; cells without nigericin pretreatment prior to valinomycin exposure were included in this experiment. As visualized by light microscopy, *T. brucei* incubated only with this concentration of nigericin exhibit a shrunken appearance, possibly due to unspecified cell-wide effects (Fig. 5, B1). Nigericin pretreatment prevents the visible swelling of mitochondria caused by valinomycin (Fig. 5B, 2 and 3). It appears that Letm1 knockdown in PS phenocopies the swelling mitochondria effect of the K⁺ ionophore valinomycin in the parental cell line, including the susceptibility of this swelling to the K⁺/H⁺ antiporter nigericin.

**Letm1 Is Essential for *T. brucei evansi* and the Bloodstream Stage of *T. brucei*—To gain insight into the role of Letm1 in mitochondria bearing different physiological states, we generated conditional RNAi knockdowns in *T. brucei* BS and *T. brucei evansi*, a petite mutant of *T. brucei* lacking any kDNA and hence a canonical proton gradient across the inner mt membrane (31, 52). Efficient degradation of the *Letm1* transcript in either cell line was verified by Northern blot analysis (Fig. 6, A and C). Growth in the presence and absence of the RNAi induction agent was subsequently assayed as in PS, with a lower starting concentration of 5 × 10⁵ cells/ml. As shown in the line graph in Fig. 6B, Letm1 depletion in BS resulted in growth inhibition already 2 days after RNAi induction, after the first daily dilution. A recovery of growth often occurring when essential pro-
Teins are down-regulated (34) is also observed to begin at day 4 of the time course. Obvious growth inhibition is also observed on day 3 after the first cell passage in T. brucei evansi (Fig. 6D), which is performed every other day due to the slower growth rate of these trypanosomes as compared with BS. Both cell types exhibited swelling mitochondria at these time points, as shown in a representative picture from T. brucei evansi (Fig. 6E, 1 and 2), a very dramatic contrast to the thin morphology of the organelle in the untreated controls (Fig. 6E, 3). Thus, Letm1 plays the same role in mediating KHE in the mitochondrion of trypanosome stages that lack a respiratory chain and cristae, as exemplified by the results in BS T. brucei. Furthermore, this ion exchange functions even in the absence of a canonical proton gradient across the inner membrane, which is not a component of the mitochondrial membrane potential in T. brucei evansi (31).

**Letm1 Is Disposable for Mitochondrial Translation**—Because T. brucei evansi has lost its mt genome, components responsible for gene expression in the organelle have been rendered redundant (31, 36). Thus, the essential nature of Letm1 in these ρ0 trypanosomes argues ostensibly against a primary function in mt translation via its interaction with ribosomes. To further investigate, we decided to assay de novo translated apocytochrome b (CytB) and cytochrome c oxidase subunit 1 (Cox1) in PS (49), the T. brucei life cycle stage that assembles the respiratory complexes into which these proteins are incorporated (22).

Letm1 RNAi knockdowns grown in the presence of tetracycline for 2–4 days plus a non-induced control were subjected to the [35S]methionine labeling of de novo synthesized mt proteins, which were subsequently resolved on a 9%/14% acrylamide two-dimensional denaturing gel. A steady decrease in the labeled Cox1 and CytB, as well as still unidentified products, was observed over the time course (Fig. 7A). This decrease in 35S signal is compared with the Coomassie-stained cytosolic proteins (Fig. 7A, insets), which remain at a constant level in all samples. To ensure that this decrease was due to translational rather than transcriptional defects, steady-state levels of these transcripts were also determined by real-time quantitative PCR. Cox1 mRNA, which does not undergo RNA editing, and CytB mRNA, which is processed by moderate RNA editing, complemented by the massively edited Cox3 transcript, were virtually unaffected 3 days after RNAi induction (Fig. 7B). Therefore, it appears that mt translation is indeed compromised in Letm1-depleted T. brucei.

To resolve whether this phenomenon is directly due to the depletion of Letm1 or a downstream effect, mt translation was assayed in RNAi cell lines induced by tetracycline for 4 days with or without 25 nM nigericin treatment for the last 2 days plus the non-induced controls. As shown in Fig. 8A, translation proceeds in the Letm1-silenced cells, in which KHE is restored by nigericin. Letm1 mRNA was measured in the nigericin-treated cells to confirm that it was degraded upon RNAi induction (Fig. 8B). We conclude from these experiments that the observed hindering of mt translation in Letm1-depleted T. brucei is a secondary consequence of the disrupted ion homeostasis.

**DISCUSSION**

Mitochondria are ancient organelles of an endosymbiotic origin that are, in one form or another, maintained in all extant eukaryotes living in very different ecological niches. As such, although mitochondria have undergone divergent evolution in these various organisms, they still retain basal characters that are common to them, such as being bound by double membranes (53). Using the highly diverged T. brucei as a study model, we have exploited its amenable properties and its evolutionary divergence to determine the ancestral function of the ubiquitous Letm1 protein, which exhibits remarkable conservation of
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The T. brucei organelle is present in a single copy and carries discoidal cristae (21), and its energy metabolism undergoes dramatic alterations during the life cycle (22). However, despite such differences, our studies reproduced a phenotype seen in some of those previously carried out in other eukaryotes, namely that ablation of the Letm1 protein results in swelling mitochondria (1, 3, 10–12). This condition is alleviated by some of those previously carried out in other eukaryotes, such as differences, our studies reproduced a phenotype seen in although it should be noted that this study described a significantly more limited effect on mt morphology than previous reports. However, in our study, the aforementioned ability of the K^+/H^+-exchanging ionophores nigericin and monensin to rescue the Letm1-depleted T. brucei presents convincing evidence that it is indeed K^+ homeostasis that is disrupted. This in contrast to K^+, which represents the most concentrated intracellular cation (58, 59), Ca^{2+} is a carefully regulated secondary messenger whose intracellular concentration is in the micromolar range (60), making its accumulation in the matrix a less likely cause of the swelling mitochondria phenotype. Finally, yeast still retain an ortholog of the Letm1 gene despite lacking an active Ca^{2+} uptake mechanism (25–27, 60), presumably removing the evolutionary pressure to maintain the protein in this organelar context.

In T. brucei, Letm1 does not appear to play a direct role in mitochondrial translation as has been suggested for the yeast ortholog (4, 16, 17). Although this process is indeed compromised in the Letm1 knockdowns, it persists when these cells are treated with nigericin. Because mitochondrial ribosomes disassociate in high K^+ concentration environments (61, 62), we propose that the apparent effect on mitochondrial translation represents an epiphenomenon to the accumulation of the cation in the matrix upon Letm1 ablation. Furthermore, the similar growth rates of the Letm1-depleted and uninduced controls in the optimal doses of the ionophores nigericin and monensin suggest that additional processes are not affected besides mt K^+/H^+ antiport.

Taking all into account, we conclude that the ancestral function of Letm1 is KHE to maintain and/or regulate K^+ homeostasis and consequently mt volume. This conservation of function throughout eukaryotes is underscored by the ability of the human Letm1 ortholog to complement the down-regulation of the T. brucei ortholog. This evidence of common function in trypanosomes and humans is also interesting, given the wide structural variation between the two orthologs; although they share sequence homology in the defining trans-membrane domain, only human Letm1 bears two EF hands, indicating that they are superfluous to Letm1 function in the parasite. This result suggests that the motif does not directly contribute to cation extrusion from the organelle but perhaps serves a regulatory role for the human protein.
Our model system provided additional insight into conserved Letm1 function in different mt physiological states, as represented by the BS *T. brucei* and the ρ0 *T. brucei evansi*, both of which we show require function of this protein. In the BS stage, mt membrane potential is exclusively generated by the F0-F1-ATP synthase via ATP hydrolysis, whereas *T. brucei evansi* is quite extreme in that only the electrogenic component of mt membrane potential is responsible for maintaining the energized mitochondrion of the parasite. Interestingly, this component of the proton motive force appears to be sufficient to drive Letm1-mediated KHE.

The essentiality of Letm1 offers new insight into the functions of the BS mitochondrion, known to be massively reduced in function and morphology (22) yet still indispensable. Among the few active pathways in the BS organelle requiring the preservation of mt membrane potential are the maintenance of the glycosomal redox balance via the glycerol-3-phosphatase/dihydroxyacetone phosphate shuttle, fatty acid synthesis, and thymidylate production for DNA synthesis (31, 32, 37–39). Although Ca2+ uptake activity has previously been described in the BS mitochondrion, the essentiality of this pathway was not explored, and we are now therefore able to add ion homeostasis to the list of indispensable functions of the BS organelle. The requirement for Letm1 is perhaps more surprising still in the even further reduced mitochondrion of *T. brucei evansi*, because it suggests that one reason why this petite mutant undergoes extraordinary lengths to maintain an energized mitochondrion (31, 40) is to maintain mt matrix K+, an irony, considering that it is this very feature that predisposes the organelle to cation accumulation.

This study exploited many features of the *T. brucei* subspecies complex that makes it a suitable model for studying mitochondrial function that extends from its role in this neglected pathogen to other eukaryotes. Its high evolutionary divergence allows it to serve as a valuable outgroup for establishing a potential role in the Wolf-Hirschhorn syndrome. *J. Biol. Chem.* 279, 30307–30315

1. Nowikovsky, K., Froschauer, E. M., Zsurka, G., Samaj, J., Reipert, S., Kolisek, M., Wiesenberger, G., and Schwyen, R. J. (2004) The LETM1/Y0L027 gene family encodes a factor of the mitochondrial K+ homeostasis with a potential role in the Wolf-Hirschhorn syndrome. *J. Biol. Chem.* 279, 30307–30315

2. Schlickum, S., Moghekar, A., Simpson, J. C., Steglich, C., O’Brien, R. J., Winterpacht, A., and Endele, S. U. (2004) LETM1, a gene deleted in Wolf-Hirschhorn syndrome, encodes an evolutionarily conserved mitochondrial protein. *Genomics* 83, 254–261

3. Dimmer, K. S., Navoni, F., Casarin, A., Trevisson, E., Endele, S., Winterpacht, A., Salvati, L., and Scorrano, L. (2008) LETM1, deleted in Wolf-Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability. *Hum. Mol. Genet.* 17, 201–214

4. Frazier, A. E., Taylor, R. D., Mick, D. U., Warscheid, B., Stoeapel, N., Meyer, H. E., Ryan, M. T., Guiard, B., and Rehling, P. (2006) Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery. *J. Cell Biol.* 172, 553–564

5. Endele, S., Fuhrly, M., Pak, S. J., Zabel, B. U., and Winterpacht, A. (1999) LETM1, a novel gene encoding a putative EF-hand Ca2+-binding protein, flanks the Wolf-Hirschhorn syndrome (WHS) critical region and is deleted in most WHS patients. *Genomics* 60, 218–225

6. Battaglia, A., Filippi, T., and Carey, J. C. (2008) Update on the clinical features and natural history of Wolf-Hirschhorn syndrome (4p-) syndrome: Experience with 87 patients and recommendations for routine health supervision. *Am. J. Med. Genet. C Semin. Med. Genet.* 148C, 246–251

7. South, S. T., Bleyl, S. B., and Carey, J. C. (2007) Two unique patients with novel microdeletions in 4p16.3 that exclude the WHS critical regions: Implications for critical region designation. *Am. J. Med. Genet. A* 143A, 2137–2142

8. Zollino, M., Lecce, R., Fischetto, R., Murdolo, M., Faravelli, F., Selicorni, A., Butté, C., Memò, L., Capovilla, G., and Neri, G. (2003) Mapping the Wolf-Hirschhorn syndrome phenotype outside the currently accepted WHS critical region and defining a new critical region, WHSCR-2. *Am. J. Hum. Genet.* 72, 590–597

9. Dimmer, K. S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W., and Westermann, B. (2002) Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 13, 847–853

10. McQuibban, A. G., Joza, N., Megighian, A., Scorzeto, M., Zanini, D., Reipert, S., Richter, C., Schwyen, R. J., and Nowikovsky, K. (2010) A *Drosophila* mutant of LETM1, a candidate gene for seizures in Wolf-Hirschhorn syndrome. *Hum. Mol. Genet.* 19, 987–1000

11. Hasegawa, A., and van der Blik, A. M. (2007) Inverse correlation between expression of the Wolf-Hirschhorn candidate gene Letm1 and mitochondrial volume in *C. elegans* and in mammalian cells. *Hum. Mol. Genet.* 16, 2061–2071

12. Tamai, S., Iida, H., Yokota, S., Sayano, T., Kiguchiya, S., Ishihara, N., Hayashi, J., Mihaa, K., and Oka, T. (2008) Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. *J. Cell Sci.* 121, 2588–2600

13. Froschauer, E., Nowikovsky, K., and Schwyen, R. J. (2005) Electroneutral K+/H+ exchange in mitochondrial membrane vesicles involves Y0L027/Letm1 proteins. *Biochim. Biophys. Acta* 1711, 41–48

14. Jiang, D., Zhao, L., and Clapham, D. E. (2009) Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca2+/H+ antiporter. *Science* 326, 144–147

15. Waldeck-Weiermair, M., Jean-Quartier, C., Rost, R., Khan, M. J., Vishnu, N., Bondarenko, A. I., Imamura, H., Malli, R., and Graier, W. F. (2011) Leucine zipper EF-hand-containing transmembrane protein 1 (Letm1) and uncoupling proteins 2 and 3 (UCP2/3) contribute to two distinct mitochondrial Ca2+ uptake pathways. *J. Biol. Chem.* 286, 28444–28455

16. Bauschmann, H., Mick, D. U., Deckers, M., Vollmer, C., Funes, S., Kehren, K., Ott, M., Rehling, P., and Herrmann, J. M. (2010) Ribosome-binding proteins Mdm38 and Mba1 display overlapping functions for regulation of mitochondrial translation. *Mol. Biol. Cell* 21, 1937–1944

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REFERENCES
Trypanosome Letm1 Maintains Mitochondrial K⁺

17. Lupo, D., Vollmer, C., Decker, M., Mick, D. U., Tews, I., Sinning, L., and Rehling, P. (2011) Mdm38 is a 14-3-3-like receptor and associates with the protein synthesis machinery at the inner mitochondrial membrane. Traffic 12, 1457–1466

18. Zhang, B., Carrie, C., Ivanova, A., Narrsi, R., Murcha, M. W., Duncan, O., Wang, Y., Law, S. R., Albrecht, V., Pogson, B., Giraud, E., Van Aken, O., and Whelan, J. (2012) LETM proteins play a role in the accumulation of mitochondrially encoded proteins in Arabidopsis thaliana and AtLETM2 displays parent of origin effects. J. Biol. Chem. 287, 41757–41773

19. Aslett, M., Aurrecoechea, C., Berriman, M., Brestelli, J., Brunk, B. P., Carring-hton, M., Depledge, D. P., Fischer, S., Gajria, B., Gao, X., Gardiner, M. J., Gingle, A., Grant, G., Harb, O. S., Heiges, M., Hertz-Fowler, C., Houston, R., Innamorato, F., Iodice, J., Kissinger, J. C., Kraemer, E., Li, W., Logan, F. J., Miller, J. A., Mitra, S., Myler, P. J., Nayak, V., Pennington, C., Phan, I., Pinney, D. F., Ramasamy, G., Rogers, M. B., Ross, D. S., Ross, C., Sivam, D., Smith, D. F., Srinivasasamoorthy, G., Stoeckert, C. J., Jr., Subramanian, S., Thibodeau, R., Tivey, A., Treatman, C., Velarde, G., and Wang, H. (2010) TriTrypDB. A functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. 38, D457–D462

20. Philippe, H., Lopez, P., Brinkmann, H., Budin, K., Germot, A., Laurent, J., Moreira, D., Muller, M., and Le Guyader, H. (2000) Early-branching or fast-evolving eukaryotes? An answer based on slowly evolving positions. Proc. Biol. Sci. 267, 1213–1221

21. Cavalier-Smith, T. (2010) Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. Biol. Lett. 6, 342–345

22. Lukesˇ, J., Hashimi, H., Verner, Z., and Cicová, Z. (2010) The remarkable mitochondrial of trypanosomes and related flagellates. in Structures and Organelles in Pathogenic Protozoa (de Souza, W., ed) pp. 227–252, Springer, Berlin

23. Xiong, Z. H., Law, S. R., Albrecht, V., Girog, E., Van Aken, O., and Whelan, J. (2012) LETM proteins play a role in the accumulation of mitochondrially encoded proteins in Arabidopsis thaliana and AtLETM2 displays parent of origin effects. J. Biol. Chem. 287, 41757–41773

24. Vereci, A. E., Docampo, R., and Moreno, S. N. (1992) Energization-de-...
53. Vafai, S. B., and Mootha, V. K. (2012) Mitochondrial disorders as windows into an ancient organelle. *Nature* **491**, 374–383
54. Mitchell, P. (2011) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. 1966. *Biochim. Biophys. Acta* **1807**, 1507–1538
55. Paucek, P., Mironova, G., Mahdi, F., Beavis, A. D., Woldegiorgis, G., and Garlid, K. D. (1992) Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K⁺ channel from rat liver and beef heart mitochondria. *J. Biol. Chem.* **267**, 26062–26069
56. Costa, A. D., and Krieger, M. A. (2009) Evidence for an ATP-sensitive K⁺ channel in mitoplasts isolated from *Trypanosoma cruzi* and *Crithidia fasciculata*. *Int. J. Parasitol.* **39**, 955–961
57. Inoue, I., Nagase, H., Kishi, K., and Higuti, T. (1991) ATP-sensitive K⁺ channel in the mitochondrial inner membrane. *Nature* **352**, 244–247
58. Haddy, F. J., Vanhoutte, P. M., and Feletou, M. (2006) Role of potassium in regulating blood flow and blood pressure. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R546–R552
59. Rodríguez-Navarro, A. (2000) Potassium transport in fungi and plants. *Biochim. Biophys. Acta* **1469**, 1–30
60. Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012) Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 566–578
61. Spremulli, L., and Kraus, B. L. (1987) Bovine mitochondrial ribosomes. Effect of cations and heterologous dissociation factors on subunit interactions. *Biochem. Biophys. Res. Commun.* **147**, 1077–1081
62. Maslov, D., and Agrawal, R. (2012) Mitochondrial translation in trypanosomatids. in *RNA Metabolism in Trypanosomes* (Bindereif, A., ed) pp. 215–236, Springer, Berlin