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Elongator Protein 3 (Elp3) Lysine Acetyltransferase Is a Tail-anchored Mitochondrial Protein in *Toxoplasma gondii*[*] [S]

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Background: Protein acetylation is prevalent in mitochondria, yet acetyltransferases mediating this activity are unknown.
Results: *Toxoplasma* Elongator protein 3 (Elp3) possesses a unique C-terminal transmembrane domain necessary and sufficient to target it to the mitochondria.
Conclusion: Elp3 is an essential tail-anchored mitochondrial acetyltransferase in *Toxoplasma*.
Significance: Elp3 has conserved functions involving mitochondria that may predate its established role in transcription.

Lysine acetylation has recently emerged as an important, widespread post-translational modification occurring on proteins that reside in multiple cellular compartments, including the mitochondria. However, no lysine acetyltransferase (KAT) has been definitively localized to this organelle to date. Here we describe the identification of an unusual homologue of Elp3 in early-branching protozoa in the phylum Apicomplexa. Elp3 is the catalytic subunit of the well-conserved transcription Elongator complex; however, Apicomplexa lack all other Elongator subunits, suggesting that the Elp3 in these organisms plays a role independent of transcription. Surprisingly, Elp3 in the parasites of this phylum, including *Toxoplasma gondii* (TgElp3), possesses a unique C-terminal transmembrane domain (TMD) that localizes the protein to the mitochondrion. As TgElp3 is devoid of known mitochondrial targeting signals, we used selective permeabilization studies to reveal that this KAT is oriented with its catalytic components facing the cytosol and its C-terminal TMD inserted into the outer mitochondrial membrane, consistent with a tail-anchored membrane protein. Elp3 trafficking to mitochondria is not exclusive to *Toxoplasma* as we also present evidence that a form of Elp3 localizes to these organelles in mammalian cells, supporting the idea that Elp3 performs novel functions across eukaryotes that are independent of transcriptional elongation. Importantly, we also present genetic studies that suggest TgElp3 is essential in *Toxoplasma* and must be positioned at the mitochondrial surface for parasite viability.

The functional diversity of a cell proteome is greatly expanded through post-translational modification (PTM).[2] Covalent addition of specific chemical moieties to a protein can have dramatic influences on its regulation and function, providing the cell with tremendous flexibility in responding to stimuli. Lysine acetylation has emerged as a prominent regulator of multiple cellular processes including transcription, metabolism, cell cycle, and apoptosis (1–4). Addition or removal of an acetyl group to lysine can affect protein localization, interactions, enzymatic activity, and stability. So-called "acetylomes" have revealed thousands of acetylated proteins in bacterial, plant, protozoan, and metazoan species, suggesting that acetylation may rival phosphorylation in regards to its universality and regulatory power (5). The current challenge is to identify the enzymes responsible for acetylating proteins involved in various pathways and determine the role of this PTM in regulating those pathways.

Lysine acetyltransferases (KATs) catalyze the transfer of an acetyl group from acetyl-CoA to the epsilon-amino group of a lysine residue. KATs have been extensively studied in the context of histone acetylation; however, the discovery of proteome-wide acetylation highlights the importance of investigating KATs beyond their roles in gene regulation. Extensive protein acetylation is found on non-histone proteins in the nucleus as well as the cytoplasm, and a number of KATs are present in these compartments (3, 4, 6). Abundant acetylation is also detected on mitochondrial proteins. While lysine deacetylases, such as SIRT3, have been found to reside in the mitochondria, the KAT-mediating acetylation of mitochondrial proteins has remained elusive (7, 8).

We recently performed acetylomic analyses of the human and veterinary pathogen *Toxoplasma gondii* (9, 10). *Toxoplasma* is an obligate, intracellular protozoan in the phylum Apicomplexa and causes life-threatening opportunistic infection in immunocompromised individuals (11). The parasite can also be transmitted vertically to the fetus of a woman infected for the first time during pregnancy, resulting in spontaneous abortion or birth defects. As seen for higher eukaryotic cells, the

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[S] This article contains supplemental Table S1.

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[2] The abbreviations used are: PTM, post-translational modification; KAT, lysine acetyltransferase; UTR, untranslated region; TMD, transmembrane domain; DHFR-TS, dihydrofolate reductase thymidylate synthase; HXGPRT, hypoxanthine-xanthine-guanine phosphoribosyl transferase; dd, destabilization domain; IFA, immunofluorescence assays; SAM, *S*-adenosylmethionine; IEM, immunoelectron microscopy; Elp3, elongator protein 3; OMM, outer mitochondrial membrane; TA, tail-anchored; YFP, yellow fluorescent protein.

Toxoplasma acetylome contains proteins residing in all cellular compartments, including the single parasite mitochondrion. Lysine acetylation is critical for parasite replication and survival, and enzymes modulating lysine acetylation have been validated as drug targets (12–14). We have characterized a number of KATs in the parasite, noting that TgGCN5 family KATs are exclusively nuclear and TgMYST family KATs are predominantly cytosolic (15–17).

Here we report the identification of the first KAT found on the mitochondrial surface in a eukaryotic cell. Surprisingly, this KAT is the *Toxoplasma* homologue of Elp3 (TgElp3), which is the catalytic component of the transcription Elongator complex in other eukaryotes. We validated this unexpected subcellular localization pattern for TgElp3 using multiple independent approaches and determined that the mechanism of trafficking to the outer mitochondrial membrane (OMM) involves a unique C-terminal transmembrane domain (TMD) present only on Elp3 homologues of apicomplexan protozoa and other select chromalveolates. We also show that Elp3 is targeted to mitochondria in mammalian cells, suggesting that Elp3 performs novel functions across eukaryotes. Additionally, we present evidence suggesting that TgElp3 is essential for parasite viability and must be positioned at the mitochondrial surface.

EXPERIMENTAL PROCEDURES

Parasite Culture and Transfection—*Toxoplasma* parasites were maintained by passage in human foreskin fibroblasts (HFFs) with DMEM supplemented with 1% heat-inactivated FBS at 37 °C and 5% CO_2 in a humidified incubator. For transfection of plasmids, 2×10^7 freshly lysed, purified tachyzoites of either the RHΔ*hx* or RHΔ*ku80*Δ*hx* strain were electroporated as previously described (18) with 50 μg of linearized plasmid. Twenty-four hours after electroporation, drug selection was applied and continued for three passages. Parasites transfected with a construct containing mutated dihydrofolate reductase thymidylate synthase (DHFR-TS) were treated with 1.0 μM pyrimethamine and those transfected with a construct containing hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) were treated with 25 μg/ml mycophenolic acid and 50 μg/ml xanthine. Parasites were cloned by limiting dilution in 96-well plates under continued drug selection (18).

Cloning and Tagging TgElp3—The TgElp3 (TGGT1_041990) coding sequence was amplified from RH strain cDNA using primers F1 and R1 (Table S1) and cloned into ZeroBlunt®TOPO® vector (Invitrogen) for sequencing. The GeneRacer™ kit (Invitrogen) was used to amplify the 5′- and 3′-UTRs with primer R2 and nested primer R3 for the 5′-UTR and primer F2 and nested primer F3 for the 3′-UTR. ClustalW (BioEdit software) was used to align amino acid sequences.

The In-Fusion® HD cloning kit (Clontech) was used to clone fragments into *Toxoplasma* expression vectors for all constructs; primer design and cloning was conducted according to the manufacturer's protocol. Phusion® High Fidelity DNA Polymerase (Thermo Scientific) was used for all PCRs, unless stated otherwise. To stably express ectopic $_{HA}$TgElp3 in parasites, the open reading frame was amplified from RHΔ*hx* cDNA with primers F4 and R4 and inserted into a *Toxoplasma* expression vector at the BglII restriction site. This expression vector uses the *Toxoplasma* tubulin promoter and contains an HXGPRT selection marker (pHXGPRT:tub) (19). TgElp3$_{HA}$ was made in the same manner with primers F5 and R5. $_{HA}$TgElp3ΔTMD was amplified with primers F4 and R6 and inserted downstream of the tubulin promoter in the pHXGPRT:tub expression vector at restriction sites BglII and EcoRV. All three constructs were transfected into RHΔ*hx* parasites.

YFP TgElp3 Fusion Proteins—YFP was amplified from pYFP-LIC-HXG (kindly provided by Dr. Vern Carruthers) with primers F6 and R7 and inserted downstream of the tubulin promoter in pHXGPRT:tub using the BglII and AvrII restriction sites. The resulting pHXGPRT:tub-YFP construct contained an NdeI site just upstream and an EcoRV site just downstream of YFP to allow for installment of designated TgElp3 fragments. DNA encoding the N-terminal region of TgElp3 (amino acids 1–273) was amplified with primers F7 and R8 and fused to the N terminus of YFP using the NdeI restriction site. The C-terminal region (amino acids 726 –984), amplified by primers F8 and R9, was fused to the C terminus of YFP using the EcoRV restriction site. Removal of the TgElp3 TMD was achieved by amplifying the C-terminal region with primers F8 and R6 and inserting the product downstream of YFP using the EcoRV site. YFP fusion constructs were transiently transfected into RHΔ*hx* parasites.

Δ*TgElp3 and* $_{ddHA}$*TgElp3 Constructs*—A TgElp3 knock-out construct was designed to facilitate double homologous recombination to replace the endogenous locus with the pyrimethamine-resistant DHFR-TS selectable marker. The 5′ genomic fragment encompassing a portion of the TgElp3 5′-UTR and first exon was amplified from RHΔ*ku80*Δ*hx* DNA with primers F9 and R10, and inserted into the pDHFR-TS cassette (20) using restriction sites NotI and SpeI. The 3′ genomic fragment, consisting of a portion of the last exon of TgElp3 and 595 bp downstream of the stop codon, was amplified with primers F10 and R11 and inserted into this plasmid using restriction sites HindIII and ApaI. The construct was linearized with NotI and transfected into RHΔ*ku80*Δ*hx* parasites followed by selection with pyrimethamine as described above.

Recombinant $_{ddHA}$TgElp3 was engineered by first amplifying $_{HA}$TgElp3 (without the ATG start codon) with primers F11 and R12 and then fusing the destabilization domain (dd) by inserting $_{HA}$TgElp3 into the SacI restriction site downstream of the dd in a Zero Blunt® TOPO® vector. The dd sequence had previously been amplified from pLIC.2XHA-dd::DHFR (kindly provided by Dr. Michael White) and ligated into a Zero Blunt® TOPO® vector. $_{ddHA}$TgElp3 was then amplified with primers F12 and R4 and inserted into the pHXGPRT:tub expression vector using the BglII restriction site. After establishing a clonal parasite line expressing $_{ddHA}$TgElp3, the TgElp3 knock-out construct was transfected and parasite clones were screened by PCR for the presence of ectopic $_{ddHA}$TgElp3 and absence of the endogenous TgElp3 locus. To obtain genomic DNA from the parasite clones for screening, 1.0 ml of freshly lysed extracellular parasites was centrifuged, resuspended in lysis buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.1% SDS, 20 mg/ml proteinase K), incubated at 55 °C overnight, and heated at 95 °C for 10 min. Lysates were used directly in PCRs with GoTaq® Green Master Mix (Promega) following the manufacturer's protocol. Reverse transcription (RT)-PCR was per-

formed to monitor levels of TgElp3 mRNA. The RNeasy® Plus mini kit (Qiagen) and Omniscript RT kit (Qiagen) were used to isolate RNA and make cDNA, respectively. Primers used for screening are depicted in Fig. 7*A* and listed in supplemental Table S1.

Plasmid Construction to Test Requirement of TgElp3 TMD—A plasmid was generated to facilitate double homologous recombination to remove the DNA encoding the TMD from the genomic locus of TgElp3 (ΔTMD). In parallel, we made a similar construct that contained the wild-type sequence (WT TMD) to use as a control for recombination efficiency. The ~1,700 bp 5′ genomic fragment of each construct contained a portion of the last intron, the entire last exon with or without the TMD (encoding amino acids 958–980), and the 3′ UTR (Fig. 8*A*). The 3′ genomic fragment consisted of 1,460 bp downstream of the 3′-UTR. The WT TMD 5′ fragment was amplified from RHΔ*ku80*Δ*hx* genomic DNA with primers F15 and R16. The ΔTMD 5′ fragment was amplified using a series of PCRs to piece together sequences upstream and downstream of the TMD. PCR-1 and PCR-2 consisted of RHΔ*ku80*Δ*hx* genomic DNA with primers F15+R17 and F16+R16, respectively. PCR-3 used template from PCR-1 and primers F15 and R18. PCR-4 combined the pieces by using template from PCR-2 and PCR-3 with primers F15 and R16. The 3′ fragment used for both the WT TMD and ΔTMD constructs was amplified from RHΔ*ku80*Δ*hx* genomic DNA using primers F17 and R19. The 5′ and 3′ fragments were inserted into the pDHFR-TS pyrimethamine-resistance cassette using the restriction sites XbaI and HindIII, respectively. WT TMD and ΔTMD constructs were transfected into RHΔ*ku80*Δ*hx* parasites and 24 clones from each parasite line were screened by PCR as described above using the primers shown in Fig. 8*A*.

Western Blotting—*Toxoplasma* lysates were prepared from freshly lysed, filter-purified parasites. Parasites were lysed in RIPA buffer followed by sonication and the DC Protein Assay (Bio-Rad) was used to quantify protein. The Novex® NuPAGE® SDS-PAGE gel system (Invitrogen) was used for protein separation and transfer to nitrocellulose membranes. Membranes were blocked in 4% nonfat milk followed by incubation with primary and secondary antibodies that were diluted in blocking buffer. Primary and secondary antibodies included 1:1,000 rat anti-HA (Roche cat. 11867423001) and 1:2,000 anti-rat conjugated to HRP (GE Healthcare cat. NA935), respectively. Proteins were detected by chemiluminescence using the FluorChem E Imager and AlphaView® software (ProteinSimple).

Mouse brain mitochondrial and cytosolic lysates were prepared from FVB/NJ mice and kindly provided by Dr. Nickolay Brustovetsky (IUSM). The mitochondrial fraction was prepared as previously described (21) and the cytosolic fraction was prepared by centrifuging brain homogenate at 12,000 × *g* for 10 min followed by a centrifugation of the supernatant at 100,000 × *g* for 30 min. The obtained supernatant was used as the cytosolic fraction. Western blotting was done following SDS-PAGE separation of 50 μg of each lysate. Primary antibodies included 1:1,000 rabbit anti-GAPDH (Cell Signaling cat. 2118), 1:1,000 rabbit anti-COX IV (Cell Signaling cat. 4850), and 1:1,000 rabbit anti-human Elp3 (Active Motif cat. 39949). The secondary antibody was anti-rabbit conjugated to HRP (GE Healthcare cat. NA934).

Lysine Acetyltransferase Assay—Parasites expressing $_{HA}$TgElp3 and the parental line RHΔ*hx* were harvested and lysed by the same method described above, resulting in a total protein concentration of 1–2 mg each. Lysates were immunoprecipitated with 50 μl anti-HA conjugated beads (Roche cat. 11815016001). Beads were used directly in a 30 μl reaction including KAT assay buffer (250 mM Tris-HCL pH 8.0, 25% glycerol, 0.5 mM EDTA, 250 mM KCl, 5 mM DTT, 5 mM PMSF, and 50 mM sodium butyrate), 2 μg histone H3 (Millipore cat. 14-494), and 2 mM acetyl-CoA (Sigma cat. A2056). The negative control included all reagents except beads. Reactions were incubated at 30 °C for 1 h followed by the addition of 1× LDS NuPAGE® loading buffer supplemented with 5% beta-mercaptoethanol and heated at 95 °C for 10 min. Western blotting was performed and membranes were probed with 1:1,000 anti-AcH3 (Active Motif cat. 39139) to detect acetylated H3 followed by 1:2,000 anti-rabbit conjugated to HRP (GE Healthcare cat. NA934). The KAT assay was repeated for a total of three independent experiments.

Immunofluorescence and Immunoelectron Microscopy—Immunofluorescence assays (IFAs) were performed by inoculating parasites onto confluent HFF cell monolayers grown on coverslips. Infected HFF monolayers were fixed 16–24 h post-infection with 3% paraformaldehyde/PBS for 15 min then quenched with 0.1 M glycine/PBS for 5 min and permeabilized with 0.2% triton X-100/3% BSA for 10 min. Samples were blocked with 3% BSA and incubated with one or more of the following primary antibodies in 3% BSA overnight at 4 °C: 1:1,000 rat anti-HA (Roche cat. 11867423001) and 1:2,000 mouse anti-TgF$_1$B ATPase (22). Coverslips were then incubated with various secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen) and mounted to slides with VectaShield® Mounting Medium containing DAPI (Vector Laboratories). To examine the orientation of TgElp3 in the mitochondrial outer membrane, IFAs were conducted using digitonin to selectively permeabilize membranes. IFAs were performed in the same manner as described above with the exception that samples were incubated for 5 min in either 0, 0.004, or 0.1% digitonin to permeabilize cell membranes. In addition to the aforementioned antibodies, 1:10,000 rabbit anti-TgIF2α (23) was used as a cytoplasmic marker.

Immunoelectron microscopy processing and analysis was conducted by Wandy Beatty at Washington University, St. Louis. Clonal parasites stably expressing ectopic $_{HA}$TgElp3 or TgElp3$_{HA}$ along with the parental line (RHΔ*hx*) were grown overnight in a T-25 cm^2 flask containing confluent HFF monolayers. The infected monolayer was washed with PBS, scraped, and centrifuged for 10 min at 3,000 × *g*. Cells were resuspended in fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in PBS and incubated on ice for 1 h. Samples were then cryoprocessed and sectioned; 50-nm thick sections were used for immunolabeling. To immunolabel sections, they were blocked with 5% FCS/5% goat serum in PIPES buffer for 20 min at room temperature. Primary antibody, rat anti-HA (Roche cat. 11867423001), was used at a 1:25 dilution in blocking buffer and incubated for 1 h at room temperature. Samples were then

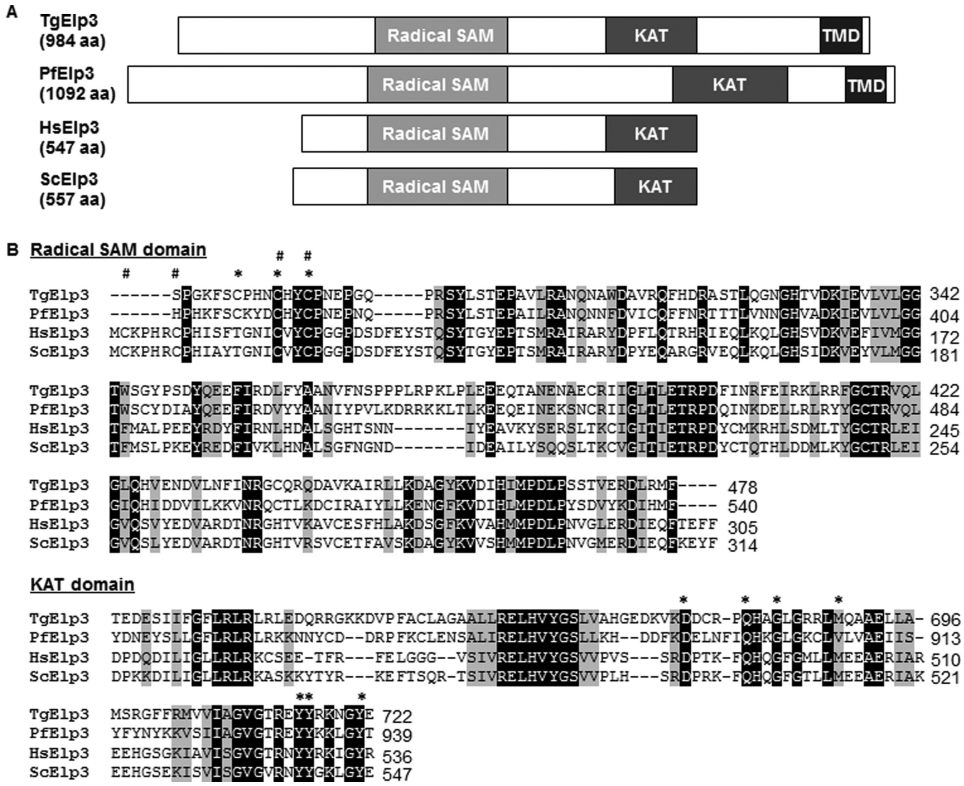

FIGURE 1. **Comparison of Elp3 homologues reveals unique and conserved features.** *A*, depiction of Elp3 protein sequences from *Toxoplasma gondii* (TgElp3), *Plasmodium falciparum* (PfElp3), *Homo sapiens* (HsElp3), and *Saccharomyces cerevisiae* (ScElp3). Protein diagrams are roughly to scale. Radical SAM, radical *S*-adenosylmethionine (SAM) domain; KAT, lysine acetyltransferase domain; TMD, transmembrane domain; aa, amino acids. *B*, amino acid alignments of Radical SAM and KAT domains with similar residues highlighted in *light gray* and identical residues highlighted in *black with white letters*. In the Radical SAM domain alignment, the "#" denotes conserved cysteine residues critical for iron-sulfur cluster formation in human and yeast homologues (29, 52, 53) while the *asterisks* indicate those in the *Toxoplasma* and *Plasmodium* homologues. In the KAT domain, *asterisks* denote residues previously shown to be important for KAT activity (25–28). Amino acid number is indicated on the *right*.

incubated for 1 h in a 1:30 dilution of goat anti-rat secondary antibody conjugated to 18 nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and stained with 5% uranyl acetate/2% methyl cellulose. Samples were analyzed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) with an AMT 8 megapixel digital camera and AMT version 602 software (Advanced Microscopy Techniques, Woburn, MA).

RESULTS

Toxoplasma Possesses a Unique Elp3 Homologue—We have previously characterized several KATs in *Toxoplasma* (TgGCN5-A, TgGCN5-B, TgMYST-A, and TgMYST-B) and found them to be important for parasite survival and differentiation (24). Bioinformatic surveys for additional members of GCN5-related *N*-acetyltransferases (GNATs) in the *Toxoplasma* genome identified TGGT1_041990 as having unequivocal homology to Elp3, the catalytic subunit of the Elongator complex. The gene, which we have designated TgElp3, is located on chromosome IX and contains 9 exons and 8 introns. Cloning and sequencing the open reading frame confirmed the *Toxoplasma* database annotation of 2,955 bp to be correct. Rapid amplification of cDNA ends (RACE) data identified the 5′- and 3′-UTRs to be 568 bp and 70 bp in length, respectively. TgElp3 is predicted to contain the hallmark Radical *S*-adenosylmethionine (SAM) and KAT domains found on all Elp3 proteins from archaebacteria to humans (Fig. 1, *A* and *B*). The critical residues for KAT activity are conserved (Fig. 1*B*) (25–28). Intriguingly, the Radical SAM domain in the *Toxoplasma* and *Plasmodium* Elp3 homologues contains the canonical C*XXX*C*XX*C motif important for iron-sulfur cluster formation while most other Elp3 homologues contain a non-canonical $CX_4CX_9CX_2C$ motif (29). Strikingly, TgElp3 also harbors a predicted transmembrane domain (TMD) at its extreme C-terminal (Ct) end followed by four amino acids (Fig. 1*A*). Further analysis revealed that other members of the Apicomplexa phylum, including *Plasmodium spp*, *Cryptosporidium*, *Neospora*, *Theileria*, and *Eimeria*, have this unusual Ct TMD on their Elp3 homologues. Another parasitic alveolate, *Perkinsus marinus*, has an Elp3 with a Ct TMD, but free-living alveolates like *Tetrahymena thermophila* do not. The presence of the Ct TMD is also found on a limited number of other chromalveolates, including brown algal and water mold species. We could not find any eukaryote outside of Chromalveolata in possession of an Elp3 with a Ct TMD, suggesting this feature is restricted to select members of this supergroup.

Elp3 is the catalytic subunit of the Elongator complex, which consists of six proteins (Elp1–6) that are well conserved from yeast to humans (30–32). Strikingly, bioinformatics surveys of apicomplexan sequence databases reveal no homologues of the other five Elongator subunits, suggesting that Elp3 may have functions pre-dating the evolution of the Elongator complex.

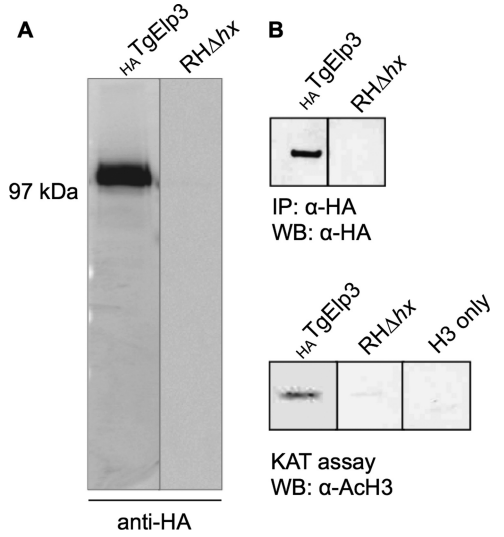

FIGURE 2. **TgElp3 has lysine acetyltransferase activity.** *A*, Western blot of lysates from parasites expressing ectopic $_{HA}$TgElp3 and the parental line RHΔ*hx*. Blots were probed with rat anti-HA. *B*, *top panel*: Western blot (*WB*) probed with anti-HA following immunoprecipitation (*IP*) of $_{HA}$TgElp3 and RHΔ*hx* lysates over anti-HA beads. IP samples were used in an *in vitro* KAT assay with histone H3 substrate and acetyl-coA. *Bottom panel*: Western blot of the KAT assay reaction probed with anti-acetylated H3 (α-AcH3). Acetylated H3 was detected in KAT assays containing $_{HA}$TgElp3 but not in the IP control (RHΔ*hx*) or H3 only control, which contained all KAT assay components except an IP sample.

To address if TgElp3 is a *bona fide* KAT, we expressed ectopic recombinant TgElp3 tagged with an HA epitope at the N terminus ($_{HA}$TgElp3) and performed a standard *in vitro* enzymatic assay using recombinant histone H3 as substrate. As shown in Fig. 2*A*, $_{HA}$TgElp3 migrates at the expected size of 100 kDa and does not appear to be subject to proteolytic cleavage. TgElp3 is able to acetylate H3 *in vitro* (Fig. 2*B*), but this does not demonstrate that H3 is a substrate of TgElp3 *in vivo*.

TgElp3 Localizes to the Mitochondrion—Given the unusual Ct TMD and lack of Elongator partners, we determined the subcellular localization of TgElp3 by performing IFAs with parasites expressing HA-tagged forms of TgElp3. IFA analysis of intracellular tachyzoites revealed an unexpected staining pattern that resembled the parasite's single mitochondrion organelle. HA-tagged TgElp3 co-localized with a mitochondrial marker TgF_1B ATPase (Fig. 3*A*) as well as MitoTracker. The same mitochondrial distribution was observed for both $_{HA}$TgElp3 and TgElp3$_{HA}$, indicating that full-length TgElp3 is present at the mitochondrion. For better resolution, immunoelectron microscopy (IEM) was also performed using an HA antibody with these parasites expressing $_{HA}$TgElp3 and TgElp3$_{HA}$. Virtually all gold particles were found in the mitochondrion with the vast majority located at the outer mitochondrial membrane (Fig. 3*B*). No gold particles were detected in the parental parasites probed with this HA antibody (not shown). Consistent with the idea that TgElp3 has a role independent of transcription, no TgElp3 was detected in the parasite nucleus by IFA or IEM.

Mitochondrial Targeting Is Mediated by the C-terminal TMD of TgElp3—TgElp3 localization to the mitochondrion was particularly surprising as online predictive algorithms including TargetP and PSORT failed to identify a canonical N-terminal or internal mitochondrial signal sequence in TgElp3. To illuminate the mechanism by which TgElp3 is targeted to the mitochondrion, we generated a series of YFP fusion proteins (Fig. 4*A*). As shown in Fig. 4*B*, YFP alone is located in the parasite cytosol, but its small size allows diffusion into the parasite nucleus as well. N-YFP consisted of the first 273 amino acids of TgElp3 (from the start codon to the Radical SAM domain) fused to the N terminus of YFP to test if the N-terminal region contained a novel type of mitochondrial targeting sequence, which does not appear to be the case (Fig. 4*B*). YFP-C consisted of the region downstream of the KAT domain (259 amino acids) fused to the C terminus of YFP and demonstrated that the C-terminal region and TMD are involved in mitochondrial localization. A third construct, N-YFP-C, included both N- and C-terminal TgElp3 fragments flanking YFP (lacking the Radical SAM and KAT domains) and also localized to the parasite mitochondrion. Together, these data show that the C-terminal portion of TgElp3 is necessary and sufficient for mitochondrial localization, with the N-terminal sequence having no role in protein targeting to this organelle (Fig. 4*B*). Since this C-terminal fragment contains the TMD, we made an additional YFP fusion protein with the TMD deleted. Additionally, we stably expressed an ectopic form of $_{HA}$TgElp3 lacking the TMD. The results consistently demonstrate that TgElp3 is unable to localize to the mitochondrion without the TMD (Fig. 4*B*).

TgElp3 Is a Tail-anchored Outer Mitochondrial Membrane Protein—Immediately downstream of the TgElp3 TMD is a string of four arginine residues. In other eukaryotic species, a C-terminal TMD followed by several positively charged residues is sufficient for targeting a protein and inserting it into the outer mitochondrial membrane (OMM). These proteins are referred to as tail-anchored (TA) and, while the precise mechanism of mitochondrial membrane insertion is still unclear, mutational analyses have shown that the TMD sequence serves as a mitochondrial targeting signal (33, 34). TA proteins are often inserted in the OMM by the C-terminal TMD such that a short C-terminal tail faces the inner membrane space and everything upstream of the TMD resides in the cytosol (35). To determine the orientation of TgElp3 in the mitochondrial membrane, we selectively permeabilized membranes of parasites expressing N- or C-terminally HA-tagged TgElp3 with digitonin prior to IFA analyses. Treatment with 0.004% digitonin permeabilized only the plasma membrane of *Toxoplasma*; while a cytoplasmic marker (TgIF2α) was detectable, the mitochondrial matrix protein (TgF_1B ATPase) was not (Fig. 5). Exposure to 0.1% digitonin permeabilized the plasma membrane as well as the outer mitochondrial membrane, but the inner mitochondrial membrane remained largely intact. As a result, the mitochondrial matrix marker TgF_1B ATPase was only partially detectable with 0.1% digitonin but was fully visible when 0.2% Triton X-100 was used to permeabilize all membranes. When only the plasma membrane was permeabilized, $_{HA}$TgElp3 was recognized but TgElp3$_{HA}$ was not. However, TgElp3$_{HA}$ was visible once the mitochondrial outer membrane had been permeabilized. These results indicate that TgElp3 is anchored in the outer mitochondrial membrane with the N terminus facing the cytoplasm and the short C terminus located within the inner membrane space. Such an orientation leaves

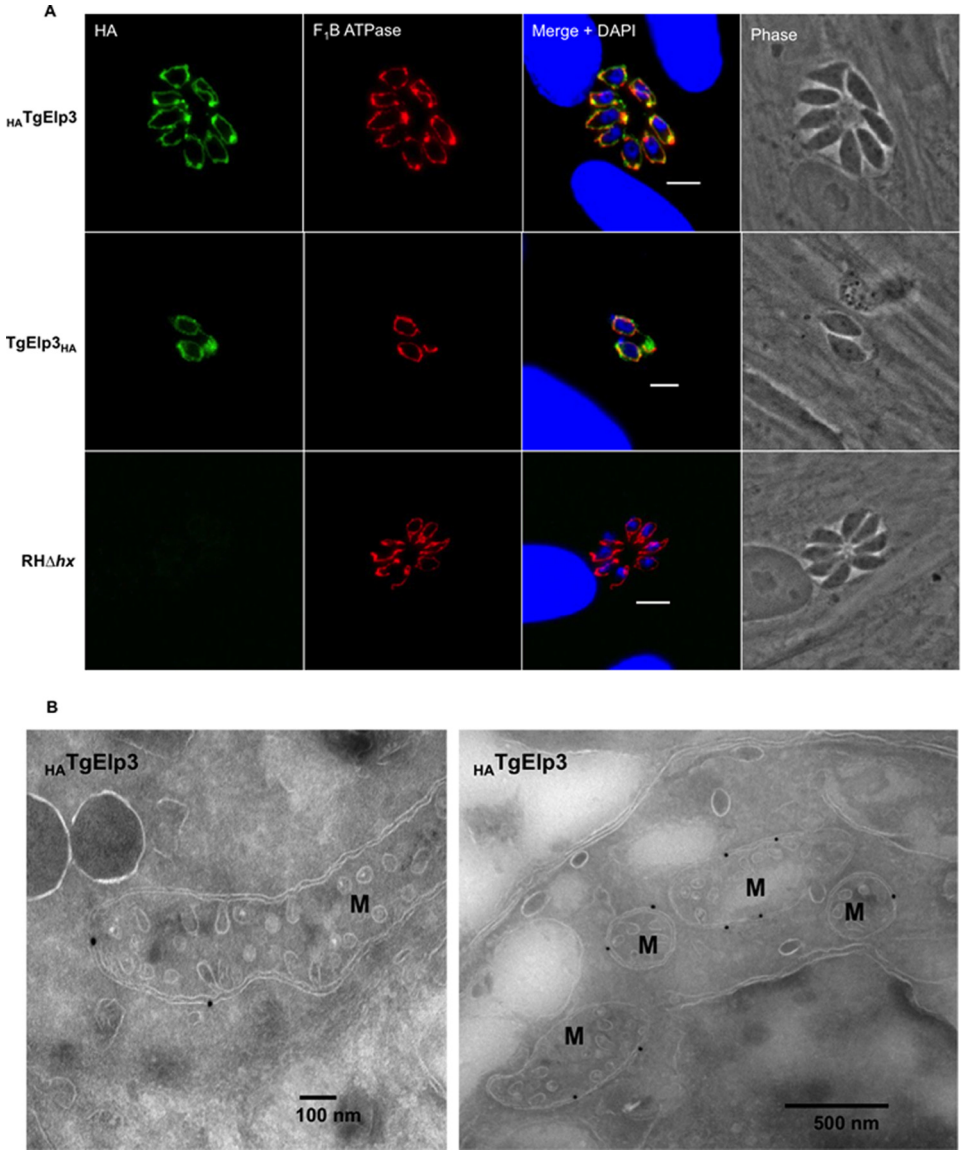

FIGURE 3. **TgElp3 localizes to parasite mitochondrion.** *A*, IFAs of RHΔ*hx*, $_{HA}$TgElp3, and TgElp3$_{HA}$ parasites with rat anti-HA (*green*) and mouse anti-TgF_1B ATPase, a mitochondrial marker (*red*). Images were merged with the DNA stain DAPI (*blue*), and the *white scale bar* represents 5 μm. *B*, representative IEM images of $_{HA}$TgElp3 parasites probed with anti-HA and anti-rat conjugated to 18-nm gold particles. Gold particles were found almost exclusively at the parasite mitochondrial (*M*) membrane. IEM of TgElp3$_{HA}$ parasites showed identical results (not shown).

TgElp3 capable of enzymatically acting on cytosolic proteins, proteins associated with the mitochondrial surface, or proteins targeted for translocation into the mitochondrion.

Elp3 Localization to the Mitochondria of Mammalian Cells—The localization of TgElp3 to the parasite mitochondria is contingent upon the Ct TMD. Elp3 homologues in the vast majority of other species do not contain a TMD, but there has been a report suggesting human Elp3 can traffic to the mitochondria in HeLa cells (36). We examined whether mitochondrial Elp3 was unique to *Toxoplasma* by performing Western blots of fractionations from mouse brain. As shown in Fig. 6, a shortened form of Elp3 (~49 kDa) is present in the mitochondrial fraction from mouse brain while only the full-length form of Elp3 (62 kDa) is in the cytosolic fraction. These results suggest that localization of Elp3 to mitochondria has been conserved throughout evolution.

TgElp3 Is Essential and Must Localize to Mitochondria for Parasite Viability—To provide insight into the role of TgElp3 in parasite physiology, we designed experiments to knock-out the genomic locus. Despite several attempts to generate a TgElp3 knock-out by replacing the genomic locus with a selectable marker in RHΔ*ku80*Δ*hx* parasites (Fig. 7*A*), we were unable to obtain viable clones, suggesting that TgElp3 is essential for parasite survival (Fig. 7*B*). We were, however, able to knock-out the locus in transgenic parasites expressing ectopic TgElp3 fused to a destabilization domain ($_{ddHA}$TgElp3), which targets the fusion protein for degradation unless stabilized by adding Shield-1 to the culture (37, 38). We were unable to obtain a TgElp3 knock-out in RHΔ*ku80*Δ*hx* parasites after screening 60 clones from 11 independent populations. However, we were able to knock-out the TgElp3 genomic locus at a high frequency (58%) in parasites expressing ectopic

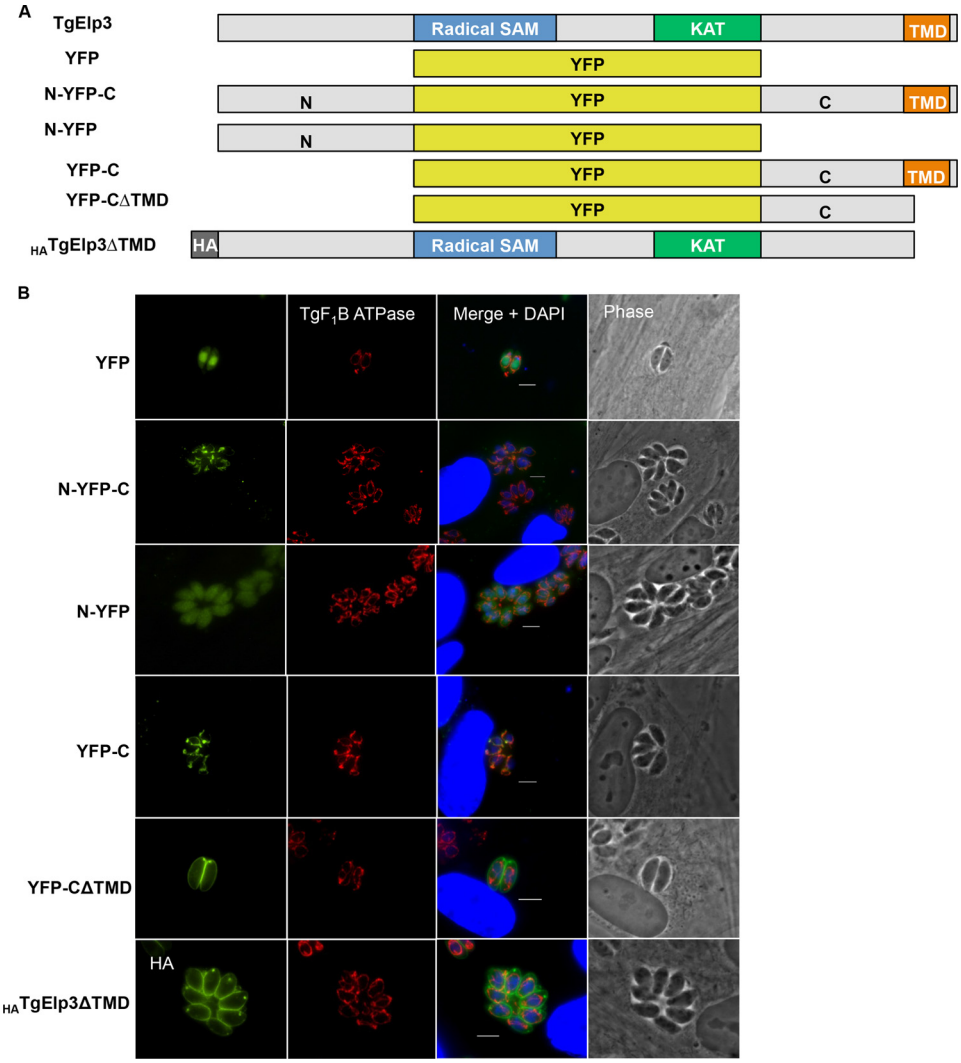

FIGURE 4. **The unique C-terminal TMD of TgElp3 targets the protein to the mitochondrion.** *A*, diagram of constructs used to map the sequence required for TgElp3 localization. *YFP*, yellow fluorescent protein; *N*, portion of TgElp3 N-terminal of the Radical SAM domain (amino acids 1–273); *C*, portion of TgElp3 C-terminal of the KAT domain (amino acids 726–984). *B*, IFAs of parasites expressing the proteins shown in *A*. YFP fusion proteins are shown in green; anti-HA was used to detect $_{HA}$TgElp3ΔTMD (*green*); anti-TgF$_1$B ATPase was used as a mitochondrion marker (*red*). Images were merged with the DNA stain DAPI (*blue*), and the *white scale bar* represents 5 μm.

$_{ddHA}$TgElp3. Both PCR of genomic DNA and reverse-transcription PCR (RT-PCR) verified the absence of endogenous TgElp3 in three independent knock-out clones (Fig. 6, *A*–*C*). Removal of Shield-1 from the ΔTgElp3::$_{ddHA}$TgElp3 clones did not fully reduce ectopic TgElp3, complicating phenotypic analysis. We conclude that the TgElp3 genomic locus is amenable to homologous recombination, but cannot be displaced unless a second copy is present, supporting the idea that TgElp3 is essential in tachyzoites.

As an alternative approach to address the importance of TgElp3, we investigated if parasites could survive when TgElp3 is expressed but not able to localize to the OMM by deleting the TMD (ΔTMD) from the endogenous locus (Fig. 8*A*). As a control, we replaced the endogenous TMD with a wild-type TMD sequence (WT TMD) to confirm that integration of our construct did not produce an artifact. Recombination frequency was ~70% (17/24) when the WT TMD construct was used, but we were not able to isolate any viable parasites when the ΔTMD construct was used. Fig. 8*B* shows five representative clones from each parasite line: five positive WT TMD clones and five negative ΔTMD clones. These results suggest that localization of TgElp3 to the OMM is essential for parasite viability.

DISCUSSION

In this report, we describe the first Elp3 homologue from an apicomplexan parasite, which exhibits unusual features suggestive of new functions across species. Most striking is the presence of a unique C-terminal TMD that targets TgElp3 to the mitochondrial surface with an orientation consistent with that of a tail-anchored membrane protein. Such an arrangement provides TgElp3 with great flexibility to acetylate a wide variety of substrates, both cytosolic as well as proteins associated with the mitochondrion. Acetylome analyses conducted by our laboratory have shown that ~500 *Toxoplasma* proteins of diverse function and localization are acetylated, including mitochondrial proteins (9, 10). Interestingly, the OMM porin was among the most heavily acetylated proteins detected. It is tempting to speculate TgElp3 may acetylate such proteins, or others that are

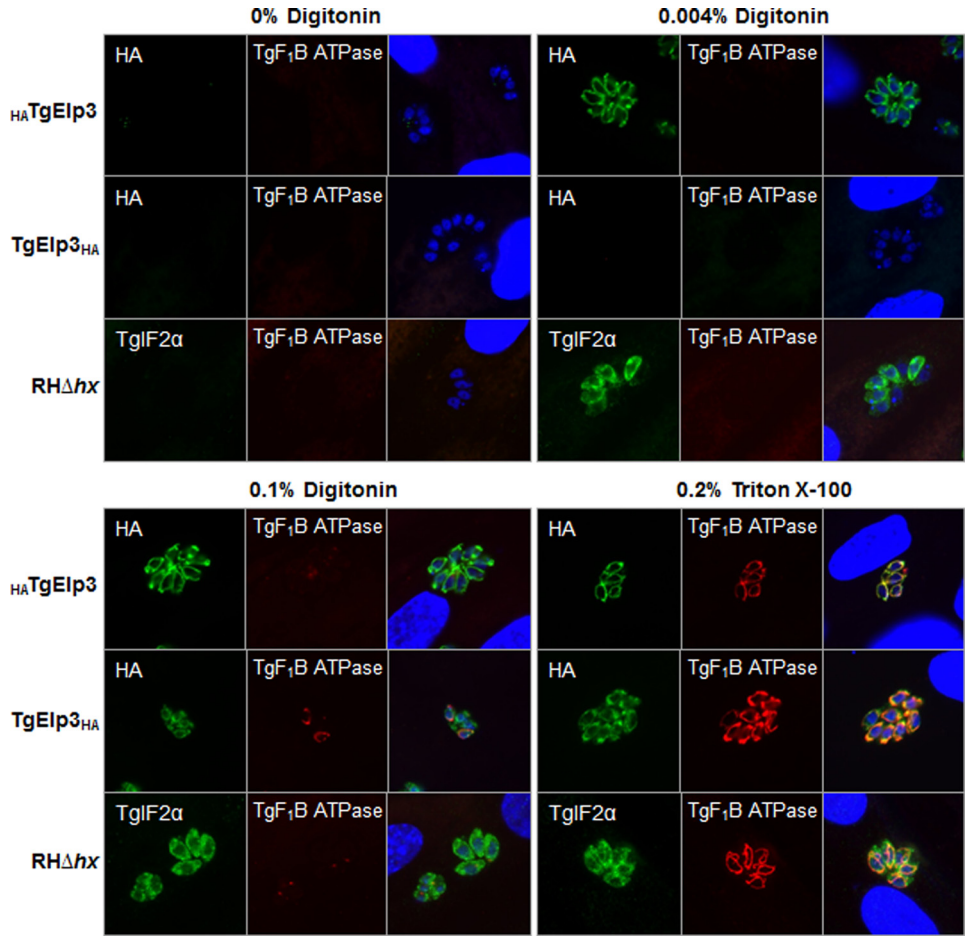

FIGURE 5. **Digitonin selective permeabilization determines TgElp3 orientation.** $_{HA}$TgElp3 and TgElp3$_{HA}$ expressing parasites were used to visualize the N- and C termini of TgElp3 (*green*), respectively, while parental RHΔ*hx* parasites were used to establish the degree of membrane permeabilization. Detection of cytoplasmic TgIF2α (*green*) and mitochondrial matrix protein TgF$_1$B ATPase (*red*) confirmed permeabilization of *Toxoplasma* plasma membrane and both mitochondrial membranes, respectively. IFAs were performed using indicated concentrations of digitonin or Triton X-100 for permeabilization as described in "Results."

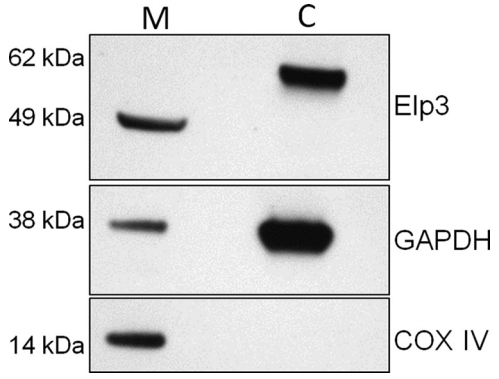

FIGURE 6. **Mouse Elp3 present in brain mitochondria.** Western blot of Elp3 in mitochondrial (*M*) and cytoplasmic (*C*) fractions purified from mouse brain. Mouse Elp3 is detected at its full-length form (62 kDa) in the cytoplasmic fraction and a shorter form (~49 kDa) in the mitochondrial fraction. GAPDH and COX IV were used as cytoplasmic and mitochondrial markers, respectively.

within proximity of the parasite mitochondrion. These unexpected findings prompted us to test if Elp3 localizes to the mitochondria in other species. To date, one report has been published showing that Elp3 localizes to mitochondria in HeLa cells (36). Our results suggest that a 49 kDa form of mouse Elp3 localizes to the mitochondria in neural cells. While its presence at this organelle is conserved, the lack of a C-terminal TMD on mammalian Elp3 suggests it may be targeted to the mitochondria in a different manner than TgElp3. The identification of a mitochondrial KAT potentially addresses the long-lingering question as to which KAT is responsible for acetylating mitochondrial proteins.

TgElp3 lacks conventional mitochondrial targeting sequences, but several parallel lines of investigation clearly demonstrate that the C-terminal TMD is necessary and sufficient to traffic proteins to the mitochondrion. All other apicomplexan parasites for which complete genome sequence data is available, and the marine parasite *Perkinsus marinus*, have an Elp3 with a C-terminal TMD. However, free-living alveolates like *Tetrahymena*, do not have a predicted TMD on their Elp3. Complicating the matter is the fact that other members of the Chromalveolata supergroup, namely brown algae and water molds, have an Elp3 with the Ct TMD. Further analysis into this potential dichotomy among the chromalveolates requires more genome sequencing of other species.

The six-subunit Elongator complex, of which Elp3 is the catalytic component, is highly conserved in yeast, plants, and animals, to facilitate transcriptional elongation through association with RNA polymerase II (39). In contrast to higher

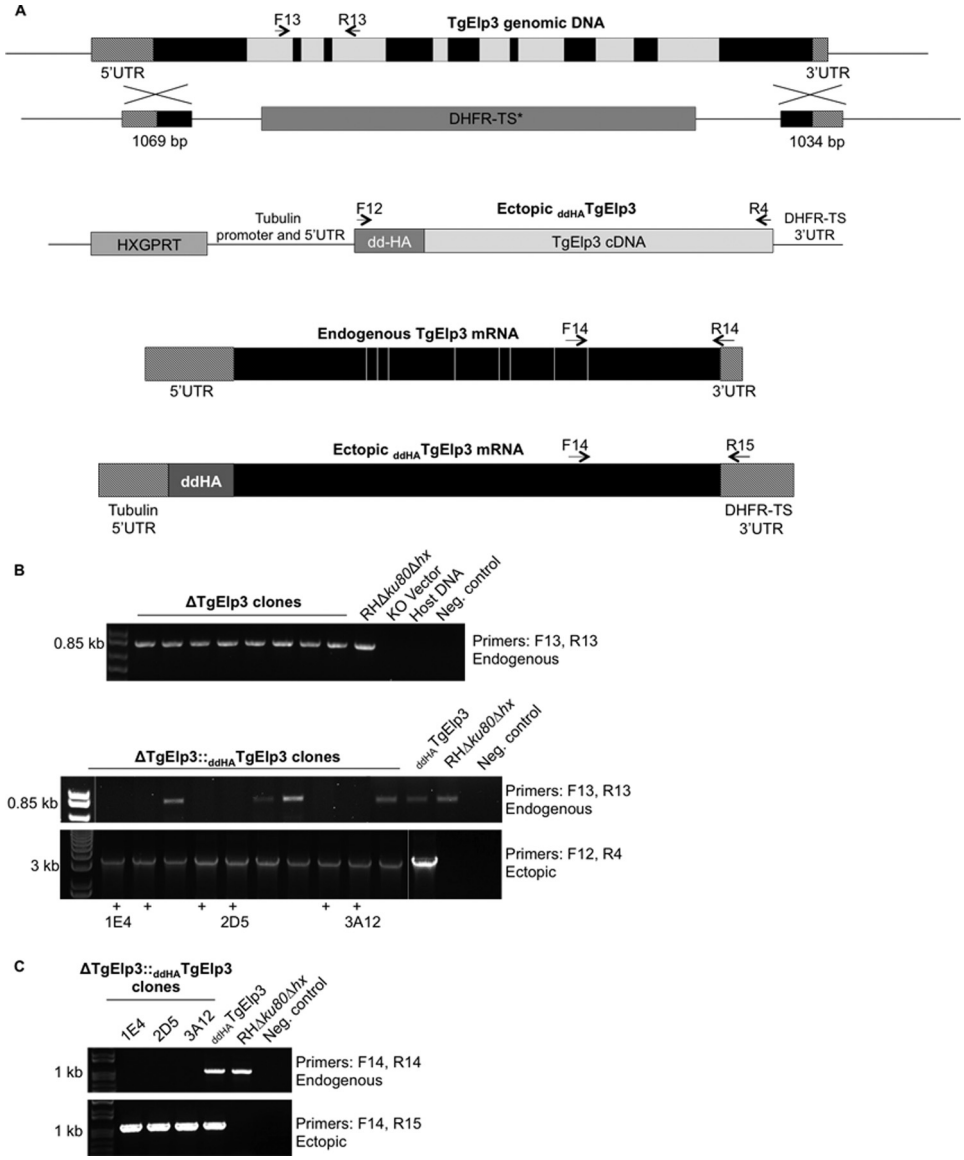

FIGURE 7. **Knock-out of TgElp3 genomic locus is only possible if ectopic TgElp3 is present.** *A*, diagrams of TgElp3 genomic locus, constructs, and mRNA including primers for screening clones. In the *top panel black*, *gray*, and *striped bars* represent exons, introns, and UTRs, respectively. The TgElp3 knock-out construct uses double homologous recombination to replace the genomic locus with a mutated form of dihydrofolate reductase-thymidylate synthase (DHFR-TS*) to confer pyrimethamine resistance for selection. The middle panel depicts the construct used to express an ectopic copy of TgElp3 tagged at the N terminus with the dd and HA epitope containing tubulin and DHFR 5′- and 3′-UTRs, respectively. HXGPRT was used for selection. The *bottom panel* shows the TgElp3 mRNA transcripts with *white lines* representing removed introns. *B*, genomic PCR and RT-PCR of TgElp3 knock-out attempts before and after introduction of ectopic $_{ddHA}$TgElp3. The *top panel* shows a PCR of genomic DNA from 8 representative clones (out of 60 total) confirming that the TgElp3 locus was not replaced when a TgElp3 knock-out was attempted. The *middle panel* shows that several knock-out clones were obtained when ectopic $_{ddHA}$TgElp3 was present, which is shown as the ~3.0 kb band in the *bottom panel*. The "+" denotes TgElp3 knock-out clones, three of which (1E4, 2D5, and 3A12) were selected for further analysis by RT-PCR. *C*, RT-PCR was used to confirm the absence of endogenous TgElp3 mRNA in the ΔTgElp3::$_{ddHA}$TgElp3 clones and the presence of ectopic $_{ddHA}$TgElp3 mRNA. RHΔ*ku80*Δ*hx* served as the parental line for $_{ddHA}$TgElp3, and $_{ddHA}$TgElp3 served as the parental line for ΔTgElp3::$_{ddHA}$TgElp3. Primer pairs used for PCR are located to the right of the gel images.

eukaryotes, Apicomplexa lack all Elongator components except Elp3, suggesting that histone acetylation is not the original function of this KAT. In further support of this idea, we were unable to detect TgElp3 in the parasite nucleus. Moreover, numerous reports in other species have ascribed additional functions to Elp3 including tRNA modification, DNA demethylase activity, and acetylation of α-tubulin (40, 41). Some of these additional functions may be due to the presence of the Radical SAM domain, which is unique to the Elp3 family KATs. In general, Radical SAM domains do not have a specific function but rather they can participate in a number of catalytic activities, such as oxidation-reduction, isomerization, methylation, and protein radical formation, which seem to be specific to the protein or its targets (42, 43). The function of this domain in Elp3 orthologues is not clear but it has been implicated in DNA demethylation in zygotes (44). The first step in all reactions involving the Radical SAM domain is reduction of the iron-sulfur cluster formed by three or four key cysteine residues in the domain. Yeast and human Elp3 homologues contain a non-canonical $CX_4CX_9CX_2C$ motif, and mutation of these cysteines to alanines recapitulates an Elp3 knock-out phenotype in yeast (29). Interestingly, the apicomplexan and several other TMD-

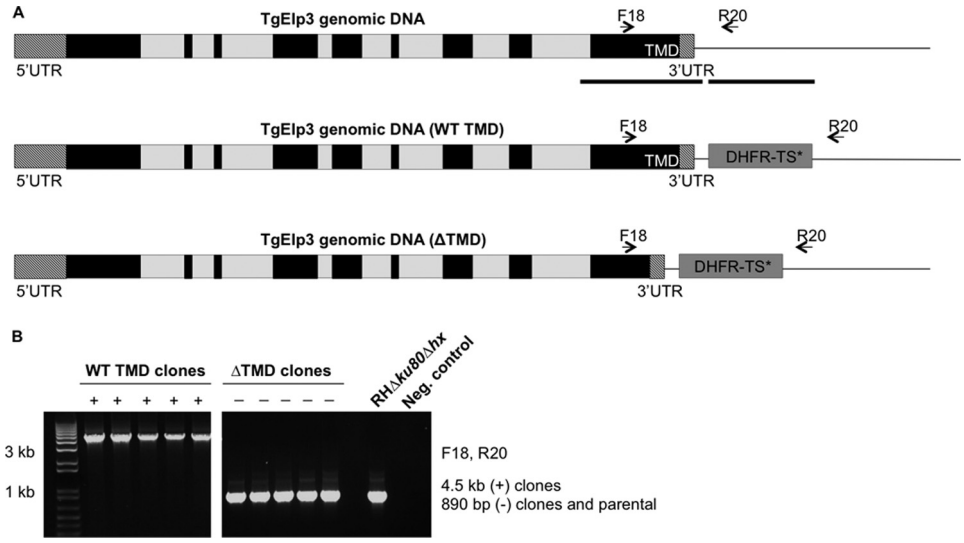

FIGURE 8. **Deletion of TgElp3 TMD is lethal.** *A*, schematic of endogenous TgElp3 genomic locus with integration of WT TMD and ΔTMD. Two *thick black lines* indicate the regions used for double homologous recombination. Primers used to screen for the correct integration event are shown. *B*, PCRs of genomic DNA of WT TMD and ΔTMD parasite clones. Five representative clones of each are shown with (+) indicating integration and (−) indicating no integration. RHΔ*ku80*Δ*hx* was the parental line. The primer pair and expected sizes are located to the *right* of the gel image.

containing Elp3 homologues possess the more traditional Radical SAM motif (C*XXX*C*XX*C), while Elp3 homologues lacking the C-terminal TMD have a non-canonical motif. Further investigation is necessary to determine if a connection exists between mitochondrial localization via the TMD and function of the Radical SAM domain based on its cysteine motif. The function of the Radical SAM domain of TgElp3 at the mitochondrion may be independent or co-dependent with that of the KAT domain. Understanding the functions of Elp3 and other members of the Elongator complex remains an important task as they have been associated with several human diseases, including familial dysautonomia and amyotrophic lateral sclerosis, a motor neuron degeneration disorder (45–48).

Underscoring the importance of Elp3 in cellular physiology, disruption of this KAT in several species has been shown to cause significant defects. Deletion of Elp3 in *Arabidopsis* impairs the mitotic cell cycle as well as leaf polarity (49). Migration and differentiation of mouse cortical neurons was significantly altered when Elp3 was decreased (50). In *Drosophila*, deletion of Elp3 results in larval lethality (51). In *Toxoplasma*, we found TgElp3 to be indispensable for parasite viability as the genomic locus could only be disrupted when an ectopic copy of the KAT was present. Additionally, parasites require that TgElp3 be positioned at the mitochondrion, indicating an essential function at this organelle. While TgElp3 is clearly important, the parasite evidently requires very little of the protein. Multiple lines of data provided on the *Toxoplasma* online database (toxodb.org) indicate that TgElp3 mRNA and protein are expressed at very low levels. This has complicated the use of knockdown technologies to further study the phenotypic consequences of TgElp3 depletion as minute amounts of residual TgElp3 are all that seem necessary for the parasites to survive.

In summary, the identification and characterization of Elp3 in *Toxoplasma*, in conjunction with fractionation studies performed on mouse brain, have revealed the strongest evidence to date that Elp3 plays important roles beyond transcription at the mitochondria. Curiously, no other subunits of the Elongator complex could be identified in early-branching eukaryotes. It is therefore possible that Elp3 may have a function involving the mitochondria that predates its well-established role in transcriptional elongation in higher eukaryotes. Alternatively, the other Elongator subunits have been lost in these organisms or are too divergent for *in silico* detection. Our studies also bolster the model that C-terminal TMDs can operate as a membrane targeting mechanism and reveal that this mechanism appeared very early in the course of eukaryotic cell evolution.

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