IDENTIFICATION OF TGELP3 AS AN ESSENTIAL, TAIL-ANCHORED
MITOCHONDRIAL LYSINE ACETYLTRANSFERASE IN THE PROTOZOAN
PATHOGEN \textit{TOXOPLASMA GONDI}

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Toxoplasma gondii, a single-celled eukaryotic pathogen, has infected one-third of the world’s population and is the causative agent of toxoplasmosis. The disease primarily affects immunocompromised individuals such as AIDS, cancer, and transplant patients. The parasites can infect any nucleated cell in warm-blooded vertebrates, but because they preferentially target CNS, heart, and ocular tissue, manifestations of infection often include encephalitis, myocarditis, and a host of neurological and ocular disorders. Toxoplasma can also be transmitted congenitally by a mother who becomes infected for the first time during pregnancy, which may result in spontaneous abortion or birth defects in the child. Unfortunately, the therapy currently available for treating toxoplasmosis exhibits serious side effects and can cause severe allergic reactions. Therefore, there is a desperate need to identify novel drug targets for developing more effective, less toxic treatments. The regulation of proteins via lysine acetylation, a reversible post-translational modification, has previously been validated as a promising avenue for drug development. Lysine acetyltransferases (KATs) are responsible for the acetylation of hundreds of proteins throughout prokaryotic and eukaryotic cells. In Toxoplasma, we identified a KAT that exhibits homology to Elongator protein 3 (TgElp3), the catalytic component of a transcriptional elongation complex. TgElp3 contains the highly conserved radical S-
adenosylmethionine and KAT domains but also possesses a unique C-terminal transmembrane domain (TMD). Interestingly, we found that the TMD anchors TgElp3 in the outer mitochondrial membrane (OMM) such that the catalytic domains are oriented towards the cytosol. Our results uncovered the first tail-anchored mitochondrial KAT reported for any species to date. We also discovered a shortened form of Elp3 present in mouse mitochondria, suggesting that Elp3 functions beyond transcriptional elongation across eukaryotes. Furthermore, we established that TgElp3 is essential for parasite viability and that its OMM localization is important for its function, highlighting its value as a potential target for future drug development.

William J. Sullivan, Jr., Ph.D., Chair
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LIST OF ABBREVIATIONS

αTAT1  alpha tubulin acetyltransferase 1
aa     amino acid
AcH3   acetylated histone H3
AcTubulin acetylated alpha-tubulin
AIDS   acquired immune deficiency syndrome
ALS    amyotrophic lateral sclerosis
ATP    adenosine triphosphate
BLAST  basic local alignment search tool
bp     base pairs
CBP    CRE binding protein
cDNA   complementary deoxyribonucleic acid
CoA    coenzyme A
COX IV cytochrome c oxidase subunit IV
CREB   cAMP-response-element-binding protein
Ct     C-terminal
DAPI   4',6-diamidino-2-phenylindole
dd     destabilization domain
DHFR   dihydrofolate reductase
DHFR-TS dihydrofolate reductase-thymidylate synthase
DHPS   dihydropteroate synthase
DMEM   Dulbecco’s Modified Eagle Medium
DNA    deoxyribonucleic acid
dNTP   deoxyribonucleotide triphosphate
DTT    dithiothreitol
EDTA   ethylenediaminetetraacetic acid
ELISA  enzyme-linked immunosorbent assay
Elp1   Elongator protein 1
Elp2   Elongator protein 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Elp3</td>
<td>Elongator protein 3</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FD</td>
<td>familial dysautonomia</td>
</tr>
<tr>
<td>FVB/NJ</td>
<td>Friend Virus B NIH Jackson</td>
</tr>
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<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCN5</td>
<td>general control non-derepressible 5</td>
</tr>
<tr>
<td>GCN5L1</td>
<td>general control of amino acid synthesis 5-like 1</td>
</tr>
<tr>
<td>GNAT</td>
<td>GCN5-related N-acetyltransferase</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
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<td>histone acetyltransferase 1</td>
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<td>histone deacetylase</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HepG2</td>
<td>human hepatocellular liver carcinoma</td>
</tr>
<tr>
<td>HFF</td>
<td>human foreskin fibroblasts</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HTERT</td>
<td>human telomerase reverse transcriptase</td>
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<tr>
<td>HXGPRT</td>
<td>hypoxanthine-xanthine-guanine phosphoribosyltransferase</td>
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<tr>
<td>IEM</td>
<td>immunoelectron microscopy</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assays</td>
</tr>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<td>IMPDH</td>
<td>inosine-monophosphate dehydrogenase</td>
</tr>
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<td>IMS</td>
<td>intermembrane space</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>KAT</td>
<td>lysine acetyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>KDAC</td>
<td>lysine deacetylase</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>mcm</td>
<td>methoxycarbonylmethyl</td>
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<tr>
<td>mcm$^5$s$^2$U</td>
<td>5-methoxycarbonylmethyl-2-thiouridine</td>
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<tr>
<td>MEC-17</td>
<td>mechanosensory abnormality protein 17</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholinopropane-1-sulfonic acid</td>
</tr>
<tr>
<td>MOZ</td>
<td>monocytic leukaemic zinc-finger protein</td>
</tr>
<tr>
<td>MPA</td>
<td>mycophenolic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MYST</td>
<td>MOZ, Ybf2/Sas3, Sas2, and Tip60 members</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ncm</td>
<td>carbamoylmethyl</td>
</tr>
<tr>
<td>ncm$^5$U</td>
<td>5-carbamoylmethyluridine</td>
</tr>
<tr>
<td>Nt</td>
<td>N-terminus</td>
</tr>
<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
</tr>
<tr>
<td>p300</td>
<td>E1A-associated protein of 300 kDa</td>
</tr>
<tr>
<td>PABA</td>
<td>para-aminobenzoic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
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<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PtK2</td>
<td><em>Potorous tridactylus</em> (kangaroo rat) kidney</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RIPA</td>
<td>radio immunoprecipitation assay</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAHA</td>
<td>suberyllanilide hydroxamic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Acronym or Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH</td>
<td>thiol</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<tr>
<td>Sir2</td>
<td>silent mating-type information regulation 2</td>
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<td>SIRT3</td>
<td>sirtuin 3</td>
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<tr>
<td>spp.</td>
<td>species</td>
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<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TAF1/250</td>
<td>TATA box binding protein-associated factor, 250kDa</td>
</tr>
<tr>
<td>TgIF2α</td>
<td>Toxoplasma gondii eukaryotic translation initiation factor 2-alpha</td>
</tr>
<tr>
<td>TIP60</td>
<td>Tat-interactive protein 60 kDa</td>
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<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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I. INTRODUCTION

A. Toxoplasma gondii, a eukaryotic pathogen

Toxoplasma gondii is a single-celled, eukaryotic protozoan belonging to the Phylum Apicomplexa. This hardy obligate, intracellular organism has the ability to infect any nucleated cell in warm-blooded animals, making it a powerful parasite that affects human health, and livestock production. Toxoplasma is also widespread, infecting about one-third of the world’s population all around the globe (1). As the causative agent of the disease toxoplasmosis, Toxoplasma is medically important in its own right, but its close relatives also cause devastating disease and have enormous socioeconomic consequences. Other members of the phylum include Plasmodium spp., Cryptosporidium spp., and Babesia spp. Malaria, caused by Plasmodium, kills over half a million people a year (2), and Cryptosporidium infections are one of the leading causes of waterborne illness in the United States (3). Many apicomplexan species can infect livestock in addition to humans and cause millions of dollars in economic loss. Toxoplasma and its close relatives are successful pathogens that must be investigated for the advancement of science and medicine.

While the genus Toxoplasma contains only one species, Toxoplasma gondii, distinct strains within the species have been characterized. Three major lineages, types I, II and III, have been identified that are proposed to have shared a common ancestor approximately 10,000 years ago (4). Several differences exist between the strains including virulence, growth rate, and cyst formation (5). Type I strains replicate quickly, have reduced cyst-forming capacity, and are hypervirulent. Type I strains such as RH are commonly used in the laboratory setting due to their fast doubling time and their amenability to genetic manipulation. RH parasites were named after the initials of the patient they were isolated from in 1938, and all Toxoplasma studies in this thesis used either RH parasites or a genetically modified form of RH. Type II and III strains are hypovirulent, grow slower than type I, and readily form bradyzoite cysts. These strains, particularly type II, are the most common found in humans and are often
used in animal and *in vitro* experiments to study tachyzoite and bradyzoite differentiation. Despite their phenotypic differences, the three strains differ by only about 1% genetically (5).

As an early-branching single, eukaryotic cell, *Toxoplasma*’s cellular content is made up of conserved eukaryotic features in addition to some that are parasite-specific (Figure 1). *Toxoplasma* cells contain a nucleus, endoplasmic reticulum (ER), golgi apparatus, and a single mitochondrion. Parasite-specific structures include the rhoptries, conoid, and micronemes, important for host cell detection, attachment, and invasion, as well as an apicoplast, a chloroplast-like remnant from an early symbiont form (6). Cellular functions can also be classified as well-conserved eukaryotic processes or parasite-specific processes. Several components of transcription, translation, metabolism, and signaling are similar to those in many eukaryotic cells, but the lack of homology and divergence of factors involved in many pathways suggests that these organisms are unique. For example, *Toxoplasma* contains the highly conserved RNA polymerase II for transcription but apparently lacks eukaryotic transcription factors such as p53. Instead, the parasites possess unique plant-like AP2 domain-containing factors thought to serve as transcription factors. As a result, by studying *Toxoplasma* we can both unearth biological secrets that are preserved in eukaryotes from parasites to humans and identify ways of specifically targeting the parasites for therapeutic benefit.

Powerful tools facilitate the study of *Toxoplasma*. The organism is easily maintained in culture and is amenable to genetic manipulation. The *Toxoplasma* genome has been sequenced and is available on the online database ToxoDB (www.toxodb.org). This database is also a repository for datasets from other “-omic” studies including expression data, mass spectrometry analyses, and post-translational modifications.
B. *Toxoplasma* life cycle

The complex life cycle of *Toxoplasma* contains both sexual and asexual stages with differentiation of the parasite into different forms - sporozoite, bradyzoite, and tachyzoite. When ingested by its definitive host, members of the Felidae family, parasites reproduce sexually within the cat's intestines and form sporozoite-containing oocysts that are excreted into the environment (Figure 2) (6). Oocysts can be ingested directly from contaminated soil and water by animals, including humans, birds, mice, and livestock, that serve as intermediate hosts. Once ingested, sporozoites are released from the oocysts and differentiate into tachyzoites.
Tachyzoites are the rapidly replicating form of the parasite, doubling approximately every 6-10 hours depending on the strain. *Toxoplasma* division occurs through a process termed endodyogeny. This form of asexual reproduction involves the formation of two daughter cells within the original mother cell. As tachyzoites invade tissues in the intermediate host, they are signaled by changes in the immune system, such as increases in interferon gamma, and potentially other unidentified components, to differentiate into bradyzoites (7,8). Bradyzoites are slow-growing and form cysts that reside permanently in tissues, particularly in neural, cardiac, and muscle tissue. Cysts embedded in the tissues of an intermediate host can then be consumed by other animals, thus passing on the infection. If a cat ingests bradyzoites from an infected mouse or bird, the life cycle of *Toxoplasma* has been completed (Figure 2).

![Diagram of the life cycle of Toxoplasma](image)

Figure 2: Diagram of the life cycle of *Toxoplasma*. Parasites reproduce sexually within the gut of cats, the definitive host. Cats then shed oocysts into the environment, which are subsequently ingested by animals including humans. Within the intermediate hosts, *Toxoplasma* differentiates into fast replicating tachyzoites and then slow-growing bradyzoites. Bradyzoites form cysts in tissue and can be transmitted via consumption of the infected animal. The life cycle is considered complete when a cat ingests bradyzoites from infected prey or oocysts from the environment. Humans can vertically transmit tachyzoites to the fetus from a mother infected for the first time during pregnancy.
C. The disease toxoplasmosis

The human disease toxoplasmosis is caused by *Toxoplasma* infection. As previously mentioned, bradyzoites form tissue cysts that remain in the host permanently. The immune system is able to keep the parasites dormant and prevent reactivation of the bradyzoites into the actively dividing tachyzoites. In healthy individuals with a functioning immune system, *Toxoplasma* infection can be asymptomatic or cause mild flu-like symptoms. However, when an infected individual becomes immunocompromised, parasites are triggered to differentiate into tachyzoites. As a result, tissue is massively destroyed as the parasites quickly replicate, killing host cells in the process.

Toxoplasmosis primarily affects those whose immune system has been compromised such as patients with AIDS, cancer, or organ transplants and is often classified as an opportunistic infection (9,10). Disease manifestations commonly include myocarditis, encephalitis, ocular disease, and neurological disorders (11). In extreme cases the disease can be fatal. *Toxoplasma* can also cause congenital toxoplasmosis, as tachyzoites can be vertically transmitted from a first-time infected mother to her fetus. Fetal infection can result in spontaneous abortion or congenital defects. Infants often suffer from hydrocephalus, vision and hearing impairments, and cognitive disorders.

*Toxoplasma* causes a chronic life-long infection and, with preference for neural tissue, can reside dormant in the brain. While acute symptoms may not be observed, researchers have examined the ability of these parasites to alter behavior in their hosts. Studies have shown that rodents infected with *Toxoplasma* have lost their aversion to cats and may actually be attracted to cat urine, thus making it easier for cats to ingest the infected rodent and complete the parasite's life cycle (12-14). In humans, *Toxoplasma* infection has been found to have an association with several neurological disorders including schizophrenia, suicide, obsessive-compulsive disorder, bipolar disorder, and psychosis (15-19). Currently, the data does not prove causation but increasing evidence suggests *Toxoplasma* may play a role in modifying human behavior. While mechanisms underlying these changes are not fully understood and are
likely attributed to several factors, *Toxoplasma*’s effect on dopamine may be involved. Several neurological disorders are a result of or treated by changes in dopamine. One study showed that dopamine levels are significantly increased in *Toxoplasma*-infected mouse brains (20), and further analysis revealed high levels of dopamine in tissue cysts (21). In addition, the *Toxoplasma* genome harbors a tyrosine hydroxylase gene, the rate-limiting enzyme in dopamine synthesis, which may contribute to the increase in dopamine (22). Another study found that the parasites functionally silence the neurons they infect, potentially altering function in the brain (23).

Several methods are employed to diagnose toxoplasmosis and detect the presence of *Toxoplasma*. Serological tests are common for detecting the presence of antibodies and some can be used to determine if the infection occurred recently or in the distant past. The Sabin-Feldman dye test served as the gold standard for many years after it was published in 1948 (24). This test relies on the lysis of parasites by antibodies in the patient’s serum in the presence of complement and is visualized by the addition of a dye that stains intact parasites. While the Sabin-Feldman dye test is still used today, a number of other serological tests have been developed to recognize *Toxoplasma* antibodies including the enzyme-linked immunosorbent assay (ELISA), the modified agglutination assay, the indirect fluorescent antibody assay, and the IgG avidity test (25). Comparative Western blots can also be conducted between serum from a mother and child to determine congenital infection. In addition to serological tests, polymerase chain reaction (PCR) and real-time PCR have become widely used to detect *Toxoplasma* infection. Other techniques, useful in certain circumstances, include immunohistochemistry, mouse inoculation, and electron microscopy. As a result of the varied specificity and reliability of these tests, combinations of several detection methods are used in the diagnosis of toxoplasmosis.
D. Toxoplasmosis treatment

Toxoplasmosis cannot be cured but therapies are available for treating acute infection. The primary treatment is combined therapy with sulfadiazine and pyrimethamine, which targets the folic acid synthesis pathway (Figure 3) (26). Pyrimethamine inhibits the enzyme dihydrofolate reductase (DHFR), present in both parasites and humans. Despite having a slightly higher affinity for the *Toxoplasma* enzyme, pyrimethamine causes toxicity in humans, and side effects of DHFR inhibition include bone marrow suppression. Therefore, this treatment is supplemented with folinic acid to reduce side effects. Since pyrimethamine is teratogenic, suppresses bone marrow, and can cross the placenta, this therapy is not recommended for pregnant women, especially during the first trimester. Instead, spiromycin, a non-toxic macrolide unable to cross the placenta, is used to treat infected pregnant women to prevent or reduce transmission to the fetus (25). Therefore, it is used to reduce parasite burden in the mother but cannot be used to treat the fetus. In cases where the fetus is suspected of being infected after the first trimester, the combinational therapy of pyrimethamine, sulfadiazine, and folinic acid is administered.
Figure 3: Illustration of the pathway targeted by drugs used to treat toxoplasmosis. A combination of sulfadiazine and pyrimethamine is used to treat toxoplasmosis by targeting the pathway for folic acid synthesis. Sulfadiazine inhibits dihydropteroate synthase (DHPS), an enzyme not present in humans, and pyrimethamine inhibits dihydrofolate reductase (DHFR). DHFR is present in both *Toxoplasma* and humans but pyrimethamine has a slightly stronger affinity for the parasite enzyme. This therapy kills parasites by inhibiting the synthesis of nucleic acids precursors.

Unfortunately, these therapies are limited and can cause serious side effects and allergic reactions. As already mentioned, pyrimethamine can have toxic side effects including bone marrow suppression, and patients can develop allergies to sulfadiazine. The antibiotic clindamycin, which poisons the parasite's unique apicoplast organelle, can be used in cases where the patient is unable to tolerate the traditional treatment regimen but very few other alternative treatments exist. All drugs currently used for treating toxoplasmosis target only the acute and not the chronic form of infection. Bradyzoite cysts that permanently reside in host tissue cannot be eradicated by the immune system or any of the
current therapies. Current research focuses on studying molecular parasitology in order to identify alternative therapies.

E. Targeting lysine acetylation

One area of parasite biology that has been validated as a potential therapeutic target is lysine acetylation. Acetylation is a dynamic and reversible post-translational modification found in nearly every species that can regulate a protein’s function by modifying its localization, interactions, enzymatic activity, or stability. Lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) are the enzymes responsible for the addition and removal of acetyl groups, respectively, to or from the ε-amino group of lysine residues (Figure 4).

Acetylation of a lysine residue neutralizes the positive charge, thereby altering the electrostatic properties of the protein. Targeted disruption of this form of protein regulation has been revealed to have therapeutic benefits. The natural product apicidin is a KDAC inhibitor that was first discovered as having anti-protozoal activity (27) and is now used in cancer therapies (28). Trichostatin A, another KDAC inhibitor, was also shown to have anti-parasitic effects (27). These KDAC inhibitors demonstrate the importance of acetylation in protozoan parasites and highlight the need for studying Toxoplasma KATs and KDACs. Our laboratory has characterized several KATs in Toxoplasma and shown them to be important for parasite gene expression, differentiation, DNA repair, and survival in the parasites (29-32).
Figure 4: Chemical depiction of protein acetylation. The acetyl group (blue) is transferred from acetyl-CoA to the amine of a lysine residue by a KAT enzyme. The acetyl group is removed from lysine residues by KDACs.

For many years, KATs and KDACs were only known to regulate histone acetylation and were therefore referred to as histone acetyltransferases (HATs) and histone deacetylases (HDACs). Today, these enzymes are more accurately referred to as KATs and KDACs since they are now known to modify lysine (K) residues on a wide range of proteins. KATs, the enzymes responsible for protein acetylation, were first characterized in 1995 when GCN5 (general control non-derepressible 5) was purified from the free-living protozoan *Tetrahymena thermophila* (33). GCN5 was found to be highly conserved from yeast to humans and was identified as a key regulator of transcription via acetylation of histone tails. Since the discovery of GCN5, novel KATs continue to be identified. They are generally classified as belonging to one of three families depending on the sequence of their catalytic domain, although additional categories have been established. The three major families include GNAT (GCN5-related N-acetyltransferase), p300 (E1A-associated protein of 300 kDa)/ CBP [CREB (cAMP-response-element-binding protein)-binding protein], and MYST, named
for the proteins originally identified - MOZ (monocytic leukaemic zinc-finger protein), Ybf2/Sas3, Sas2 and TIP60 (Tat-interactive protein 60 kDa) (34).

KDACs, enzymes that deacetylate lysine residues, are organized into four classes (I, II, III, and IV) based on sequence homology. Classes I, II, and IV are “classical” KDACs that have a similar mechanism of action, while class III KDACs are referred to as sirtuins based on homology to a yeast gene called silent mating-type information regulation 2 (Sir2). Sirtuins require the cofactor NAD$^+$ to catalyze the removal of an acetyl group. Recently, mitochondrial sirtuins, particularly SIRT3, have gained a lot of attention because of their role in regulating metabolism and other mitochondrial functions. While mitochondrial KDACs have been identified and found to be critical for function (35), a mitochondrial-localized KAT has remained elusive.

A number of natural and chemically engineered inhibitors of both KATs and KDACs have been used over the years but most still lack the ability to be therapeutically beneficial due to poor pharmacological properties and a lack of specificity. Anacardic acid, garcinol, and curcumin are all natural products found to inhibit KATs (36-38), but studies indicate that they also interfere with other pathways in the cell (39-41). Synthetic peptide-CoA bisubstrate inhibitors have also been designed as highly specific KAT inhibitors but have poor cell permeability (42). The aforementioned KDAC inhibitors apicidin and trichostatin A have anti-protozoal activity but are ineffective for treating humans. Additional KDAC inhibitors include suberyllanilide hydroxamic acid (SAHA), sodium butyrate, valproate, and tubacin (43-45). Nicotinamide has also been shown to be a specific inhibitor of sirtuins (46,47). Targeting the regulators of lysine acetylation would be beneficial for treating several types of diseases including *Toxoplasma* infection. However, current compounds have many shortcomings, and in order to design more specific KAT and KDAC inhibitors, we must learn more about the enzymes and their targets.
F. Global protein acetylation

The functional diversity of a cell’s proteome is greatly expanded through covalent post-translational modifications such as phosphorylation, SUMOylation, acetylation, ubiquitination, and methylation. Post-translational modifications are critical for identifying and responding to internal and external stimuli from changes in the environment. Phosphorylation has been well-studied and is understood to be a global player in regulating cellular functions. Only recently has lysine acetylation emerged as a prominent regulator of multiple cellular processes including transcription, metabolism, cell cycle, and apoptosis. While the core histones were known to be highly acetylated for more than 50 years, identification of the first non-histone protein to be acetylated did not occur until 1997 with the discovery that the acetylation of the transcription factor p53 affected its DNA binding activity (48). Then, with the advancement of technology, it became possible to identify acetylation sites of entire proteomes, termed “acetylomes”. The first proteome-wide analysis of lysine acetylation sites was conducted in 2006 in HeLa cells and mouse mitochondria and identified 388 lysines acetylated on 195 proteins; 277 of the acetylation sites were found on mitochondrial proteins (49). This study showed conclusively for the first time that lysine acetylation was widespread and also abundant in the mitochondria. Recent acetylomes, using more high-resolution mass spectrometry, have revealed over a thousand acetylated proteins in bacterial, plant, protozoan, and metazoan species, suggesting that acetylation may rival phosphorylation in regards to its universality and regulatory power (50-55).

G. The Toxoplasma acetylome

In order to determine the scope of lysine acetylation in Toxoplasma, our laboratory recently analyzed the Toxoplasma acetylome of intracellular and extracellular tachyzoites by using pan acetyl-lysine antibodies and mass spectrometry to enrich for and identify acetylated peptides, respectively. This study was the first acetylome analysis conducted in a single-celled eukaryotic organism and resulted in the identification of 718 acetylated residues on 482
proteins (54,56). It uncovered a diverse array of acetylated proteins, both conserved and parasite-specific. In *Toxoplasma*, much of the proteome consists of hypothetical proteins that have little or no homology to characterized proteins. Therefore, the majority of acetylated proteins were found to be hypothetical (Figure 5). However, the remaining acetylated proteins were annotated as belonging to nearly every cellular pathway and present in all organelles throughout the parasite (Figure 5). Twenty confirmed and predicted mitochondrial proteins were found to be acetylated including isocitrate dehydrogenase, heat shock protein 60, ATP synthase, cytochrome c, and the outer mitochondrial membrane protein porin. Consistent with other species, acetylation in *Toxoplasma* is widespread and suggests that lysine acetylation plays a critical role in cellular function.

Figure 5: Proteins involved in nearly every parasite cellular process are acetylated. The pie chart of proteins found to be acetylated in *Toxoplasma* is categorized by functional group. The majority of acetylated proteins are either hypothetical or involved in translation, metabolism, stress response, and chromatin biology (54, 56).
While studies have led to the identification of a large number of previously unknown acetylated residues, these acetylomes are still not comprehensive due to various limitations. For example, acetylome analyses are a snapshot of cellular acetylation during a single timepoint under one set of experimental conditions. Also, due to the lack of a consensus sequence surrounding acetylated residues, identification of acetylated residues is limited by the specificity of the antibodies used for affinity-enrichment of peptides. Large-scale acetylome analyses have proven useful but studies of individual acetylated proteins and the enzymes responsible for their acetylation are still required in order to fully understand the role of this post-translational modification in cellular biology.

H. Toxoplasma KATs

Based on homology searches, eight KATs and seven KDACs have been identified in the Toxoplasma genome to date. Our laboratory has previously identified and characterized two GCN5 KAT homologues, TgGCN5-A and TgGCN5-B, and two homologues in the MYST family, TgMYST-A and TgMYST-B. Both of the Toxoplasma GCN5s were found to be localized exclusively in the nucleus and to acetylate histone H3 (30). TgGCN5-B appears to be essential for tachyzoite survival, and TgGCN5-A, while dispensible in tachyzoites, regulates stress-induced gene expression during differentiation (32). Unlike the GCN5s, TgMYST-A and TgMYST-B are both nuclear and cytosolic, and TgMYST-A has been shown to preferentially acetylate histone H4 in vitro (31). Attempts to knockout or over-express TgMYST-A have been unsuccessful, suggesting that it is essential, and its expression levels are highly regulated. TgMYST-B has been implicated in regulating transcription and the response to DNA damage (29). Another laboratory has identified a homologue of TAF1/250 (TATA box binding protein-associated factor, 250 kDa) and found it to be essential in Toxoplasma (57). Toxoplasma also has predicted homologues of Hat1 (histone acetyltransferase 1), αTAT1 (alpha tubulin acetyltransferase 1), and Elp3 (Elongator protein 3). Characterization of these additional KATs will be vital to understanding acetylation in Toxoplasma and how it may be targeted.
therapeutically. This dissertation focuses on the characterization and unique properties of the *Toxoplasma* Elp3 homologue (TgElp3).

I. History of the Elongator protein 3 (Elp3) KAT

Before 2009, very little was known about Elp3 and its role in cellular biology. It was first identified ten years earlier in yeast when it was immunoprecipitated as part of a complex with RNA polymerase II during transcription elongation (58,59). Elp3 was found to be the catalytic component of a six-subunit complex (Elp1-6) termed the Elongator complex and is highly conserved from archaebacteria to humans. Elp3 contains two highly conserved domains, a KAT domain belonging to the GNAT family and a radical S-adenosylmethionine (radical SAM) domain.

The presence of a radical SAM domain is unique to the Elp3 family of KATs and its function is unclear. 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 (60). The function of this domain in Elp3 orthologues is not well understood, but the *Methanocaldococcus jannaschii* homologue was used to confirm that it can form iron-sulfur clusters and bind SAM, suggesting Elp3 has catalytic activity (61). By mutating the radical SAM domain, it has been shown to be important for DNA demethylation in mouse zygotes and critical for the function of the Elongator complex (62).

As radical SAM domains are poorly understood and can have multiple functions, Elp3 has primarily been studied for its role as an acetyltransferase. A combination of *in vitro* and *in vivo* studies shows that Elp3, as part of the Elongator complex, can acetylate histones H3 and H4, preferentially targeting H3 *in vivo* (63,64). While KATs such as GCN5 were thought to primarily acetylate histones near gene promoters to regulate transcription initiation, the Elongator complex was found to be located within gene bodies regulating transcription elongation (65,66). The proposed model describes Elp3 as acetylating H3 as part
of a transcription elongation complex in order to open chromatin during gene transcription. This role of Elp3 has been confirmed in yeast, plant, and mammalian species (66-69).

Several studies have confirmed Elp3 as a KAT involved in transcription, yet Elp3 was revealed to be largely cytoplasmic. In 2009, Creppe et al provided exciting new evidence that Elp3 acetylates alpha-tubulin in the cytoplasm of human and mouse cells and that this function of Elp3 was necessary for cortical neuron growth and differentiation (70). This study not only identified a cytoplasmic substrate of Elp3 but also uncovered the previously unknown alpha-tubulin acetyltransferase that acetylates the highly conserved lysine 40 residue residing within the lumen of microtubules. These findings sparked a renewed interest in Elp3 and its functions outside of transcription. Subsequent studies showed Elp3-mediated acetylation of alpha-tubulin in other species and examined the effect of acetylation on tubulin (71). Evidence suggests that acetylation may stabilize microtubules and/or contribute to kinesin transport (72,73). Later, a second tubulin acetyltransferase was discovered, αTAT1 or MEC-17 (mechanosensory abnormality protein 17), which is now thought to be the primary tubulin acetyltransferase (74,75). Elp3 was shown to have weak tubulin acetylation activity compared to that of αTAT1, and while αTAT1 could acetylate tubulin in PtK2 [Potorous tridactylus (kangaroo rat) kidney] cells, Elp3 could not produce detectable levels of acetylation (75). In addition, C. elegans touch receptor neurons, which contain high levels of tubulin acetylation, require αTAT1 for tubulin acetylation and retain acetylated tubulin in the absence of Elp3, suggesting that αTAT1 is the primary tubulin acetyltransferase (74,75).

Another independent cytoplasmic function of Elp3 was uncovered when several groups found that the elongator complex modifies uridines at the wobble position of tRNAs (76,77). tRNA modifications, especially at the wobble position (position one of the anticodon), are important for proper translation efficiency and fidelity and can be used to regulate expression at the level of translation (78). The elongator complex was found to be responsible for the addition of a methoxycarbonylmethyl (mcm) and carbamoylmethyl (ncm) group to the C5
uridine nucleoside at the wobble position, resulting in the mcm\(^5\)s\(^2\)U (5-methoxycarbonylmethyl-2-thiouridine) and ncm\(^5\)U (5-carbamoylmethyluridine) modifications, respectively. Interestingly, over-expression of these modified tRNAs suppressed all Elongator/Elp3 mutant phenotypes, further confirming these tRNA modifications as an established role of Elp3 (79). The mechanism of tRNA modification by Elp3 is unknown and it is unclear if the function of the KAT and/or radical SAM domain is involved.

Elp3 deletions, knockdowns, and mutations have resulted in a number of different phenotypes depending on the cell type or organism. In yeast, Elp3 is dispensable but deletion causes growth defects when yeast are exposed to stresses such as changes in temperature and osmotic conditions (58,59,64). In addition, cells are hypersensitive to transcriptional inhibitors such as mycophenolic acid and 6-azauracil. Human Elp3 (hElp3) was shown to be functionally similar to the yeast homologue, as hElp3 was able to complement an Elp3 knockout in yeast (80). A similar complementation experiment was done with plants (77), suggesting the yeast, plant, and human Elp3 homologues are similar in structure and function. Elp3 mutations in plants resulted in changes in leaf morphology and polarity as well as a reduction in organ growth (81,82).

While Elp3 does not appear to be essential in certain cell lines, yeast, or plants, disruption in *Drosophila melanogaster* causes lethality during pupal development (83). A targeted knockdown of Elp3 in the *Drosophila* nervous system is not lethal but affects synaptic bouton expansion and axonal length, indicating a role of Elp3 in neurodevelopment (84). The importance of Elp3 in neuronal development was also seen in *Caenorhabditis elegans* when mutations resulted in impaired neuronal migration and axonal extension (71). Knockdown of Elp3 using shRNA also impairs the migration and differentiation of mouse cortical projection neurons by causing a reduction in alpha-tubulin acetylation (70). Few studies have examined the effect of mutating both catalytic domains (KAT and radical SAM) or made direct comparisons between mutations in either domain. However, one study found that both domains are essential for the function of Elp3 in basal and effector-triggered immunity in plants (85).
J. Elp3 and human disease

Modifications of Elp3 are associated primarily with neurological disorders in humans. Both Elp1 and Elp3 of the Elongator complex have been correlated with familial dysautonomia (FD), an autosomal recessive disease that affects the sensory and autonomic nervous system causing defects in neuronal development (86,87). The major cause of FD is a mutation in Elp1 that affects splicing, resulting in a truncated form of the protein. Low levels of Elp3 expression have also been found in FD patients. In addition, FD neuronal cells have reduced motility in vitro, a key phenotype often seen with disruption of Elp3. Another disease that causes the degeneration of motor neurons, amyotrophic lateral sclerosis (ALS), has been found to be associated with allelic variations of Elp3. Elp3 was identified in a genome-wide association study of 1,483 individuals from three human populations as being correlated with ALS (88). In addition, lower levels of Elp3 expression in the brain were associated with risk-associated alleles of the Elp3 gene in humans. Two loss-of-function mutations (R475K and R456K) were found to be important for neuronal survival in Drosophila, and knockdown of Elp3 in zebrafish resulted in abnormal branching of neurons (88), suggesting that Elp3 may play a role in neurological disorders.

K. Summary and hypotheses

*Toxoplasma* is a clinically relevant pathogen that causes both acute and chronic infection. Few drugs are available for treating acute infection, and they often have serious side effects and/or cause allergic reactions. In addition, the chronic form of infection is currently untreatable. Novel drug targets need to be identified for treating toxoplasmosis, and much of what is discovered in *Toxoplasma* can be readily translatable to close relatives such as *Plasmodium*.

Regulation of proteins by the acetylation of lysine residues has been established as a viable drug target for parasitic infections as well as other human diseases including cancer. *Toxoplasma*’s genome encodes several KATs responsible for regulating lysine acetylation, several of which have been previously characterized by our laboratory. Historically, our laboratory and others
have focused on the nuclear role of KATs in regulating transcription by acetylating histone tails. However, global acetylation analyses have been conducted in several species and revealed that hundreds of diverse proteins are acetylated throughout the entire prokaryotic and eukaryotic cell. The current challenge is to identify the enzymes responsible for acetylating these proteins and how this post-translational modification affects cellular processes.

In an attempt to uncover novel KATs in *Toxoplasma*, we identified a homologue of Elp3 (TgElp3) that contains predicted KAT and radical SAM domains. Based on evidence in the literature, we sought to characterize TgElp3 and hypothesized that it functions as a KAT, acetylating multiple substrates, and is essential for parasite viability. After examining the sequence of TgElp3 and performing several localization experiments, we discovered that TgElp3 contained several unique characteristics including the presence of a C-terminal transmembrane domain (TMD) and that it localized to the mitochondrion. Therefore, we subsequently hypothesized that the C-terminal TMD targets TgElp3 to the outer mitochondrial membrane. Our hypotheses were examined by testing the following specific aims: 1) identify the specific localization of TgElp3 and the role of the TMD in trafficking, 2) determine if TgElp3 has KAT activity and identify its substrates, and 3) establish if TgElp3 is essential for *Toxoplasma* tachyzoite survival.
II. MATERIALS AND METHODS

A. Tissue culture and parasite maintenance

*Toxoplasma* parasites, being obligate intracellular parasites, were maintained by serial passage in human foreskin fibroblasts (HFFs). HFFs were grown to confluency in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (host medium) in a humidified incubator at 37°C with 5% CO₂. HFFs were then inoculated with parasites and grown in DMEM supplemented with 1% FBS (parasite medium) until the parasites completely lysed the host cell monolayer, at which point the parasites were transferred to a new flask containing confluent HFFs. When necessary, parasite medium was supplemented with the compound Shield-1 (Cheminpharma cat# CIP-S1-0005), dissolved in 100% ethanol, in order to stabilize recombinant proteins fused to a destabilization domain (89). In addition to HFFs, an immortalized form of HFFs, HTER (human telomerase reverse transcriptase)-HFFs (ATCC #CRL-4001), were used for serial passage and to produce large numbers of parasites. Stocks of host cells and parasite lines were stored in liquid nitrogen.

There are several strains of *Toxoplasma*, and the parasites have multiple stages (tachyzoite, bradyzoite, and oocyst) in their full life cycle. All studies were done using a type I tachyzoite strain of *Toxoplasma* with an RH background. This parasite strain is hypervirulent and reproduces asexually by means of endodyogeny, two daughters forming within the mother cell. RHΔhx parasites have had the hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene disrupted for drug selection purposes (90). RHΔku80Δhx parasites, in addition to disruption of the HXGPRT gene, have a deletion in the Ku80 gene in order to promote homologous recombination for efficient plasmid integration (91,92).

*Mycoplasma* testing was conducted two to three times a year to check for *Mycoplasma* contamination of cells. The *Mycoplasma* Plus PCR Primer Set (Agilent #302008) was used and the manufacturer’s instructions followed. Briefly,
100 µl of medium from culture flasks was boiled, treated with StrataClean resin, and used for PCR with Taq. The provided primers can detect most species of *Mycoplasma*. If *Mycoplasma* was detected, the cells were discarded and new stocks thawed. If there was no stock available, the *Mycoplasma* Removal Agent (MP Biomedicals #093050044) was used according to instructions until the contamination was eliminated.

**B. *Toxoplasma* and HFF lysate preparation**

In order to harvest HFFs for downstream applications, they were trypsinized and centrifuged at 1,000 x g for 10 min. Pellets were washed in 1X phosphate buffered saline (PBS), centrifuged as before, and stored at -80°C until needed. Extracellular *Toxoplasma* parasites were harvested when the host cell monolayer had been completely lysed at which point the parasites were filtered through a 3 µm polycarbonate filter (Whatman) and centrifuged at 400 x g for 10 min. The pellet was washed in 1X PBS, centrifuged as before, and stored at -80°C until needed.

To prepare lysates from cells, pellets were resuspended in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.9, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) and incubated at 4°C with rocking for 30 min. Lysates were sonicated three times for 15 sec each with 30 sec on ice in between sonications. Large debris and insoluble material were discarded by centrifuging the lysate at 21,000 x g for 10 min at 4°C and discarding the pellet. Protein concentrations of cell lysates were determined using the *DC™* Protein Assay (Bio-Rad #500-0112) following the microplate assay protocol. Lysates were used for Western blotting, immunoprecipitation, and lysine acetyltransferase assays.

**C. *Toxoplasma* transfection**

Parasites were transfected with various plasmids in order to ectopically express recombinant DNA or to modify the endogenous genome. Because *Toxoplasma* tachyzoites are haploid, making endogenous alterations is
straightforward in that only a single locus requires modification. In general, the RHΔhx parasite line was used for expression of ectopic constructs whereas RHΔku80Δhx parasites were used for endogenous genomic manipulation.

Prior to transfection, plasmids were linearized using a restriction site downstream of the expression cassette in order to promote integration into the genome. Twenty-five to fifty micrograms of DNA was digested overnight with an appropriate restriction enzyme. DNA was purified by adding an equal volume of Phenol:Chloroform:Isoamyl Alcohol, mixing vigorously, and centrifuging at 21,000 x g for 2 min. The top layer containing DNA was transferred to a new Eppendorf tube and DNA was precipitated by adding 0.1 volumes of sodium acetate pH 5.2 and 2.5 volumes of ice cold 100% ethanol and kept at -20°C for several hours. The precipitated DNA was pelleted by centrifuging at 21,000 x g for 10 min and washed with 500 µl of 70% ethanol and centrifuged at 21,000 x g for 10 min. In a laminar flow hood the ethanol was removed and the DNA pellet left to dry. The DNA pellet was then suspended in 100 µl of sterile cytomix, a buffer that mimics intracellular conditions during transfection (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EDTA, 5 mM MgCl₂, 1.2 mg/ml ATP, and 1.44 mg/ml Glutathion) (93).

Parasites were prepared for transfection by filtering extracellular parasites through a 3 µm polycarbonate filter (Whatman) and centrifuging at 400 x g for 10 min. The parasite pellet was resuspended in 10 ml of cytomix and parasites were counted using a hemocytometer. After counting, the parasites were centrifuged as before and resuspended in cytomix to obtain 2 x 10⁷ parasites per 300 µl. Three hundred microliters of parasites was added to the 100 µl of DNA, mixed, and transferred to a 2 mm gap electroporation cuvette (Fisher #BTX620). For transfection, a BTX ECM 630 electroporator was used and the parasites were pulsed with 1.5 kV, 25 ohms, and 25 µF. After electroporation, parasites were left to recover for 15 min, split evenly between multiple T-25 cm² flasks of confluent HFFs, and incubated at 37°C with 5% CO₂.
D. Drug selection

Drugs used for selection were added 24 to 48 hrs post-transfection and were continued for several passages of the parasites. Different drugs were used for selection based on the selection cassette present in the transfected plasmid. Plasmids transfected into RHΔhx parasites for ectopic expression contained the HXGPRT minigene for selection. RHΔhx parasites, lacking HXGPRT, require inosine-monophosphate dehydrogenase (IMPDH) for the synthesis of guanine-based nucleotides (90,94). These parasites are susceptible to the drug mycophenolic acid (MPA) that inhibits IMPDH. However, parasites that have integrated a plasmid containing the HXGPRT minigene can now bypass the IMPDH pathway and survive in the presence of MPA with the addition of xanthine, a guanine nucleotide precursor. Therefore, 25 µg/ml of MPA and 50 µg/ml of xanthine were used to select for integration of HXGPRT containing plasmids in RHΔhx parasites.

Plasmids transfected into the RHΔku80Δhx parasite line contained a mutated dihydrofolate reductase-thymidylate synthase (DHFR-TS) minigene cassette that renders the recombinant protein resistant to pyrimethamine, a drug that kills parasites by inhibiting endogenous DHFR-TS (90). Parasites that have integrated the mutated DHFR-TS-containing plasmid are selected for by treating with 1 µM pyrimethamine.

E. Cloning by limiting dilution

After several rounds of drug selection, limiting dilution was used to isolate individual clones from populations of transfected parasites if a clonal population was needed. Transfected parasites in each T-25 cm² flask were counted using a hemocytometer and the number of parasites calculated. Parasites were diluted 1:1,000 in medium and an appropriate volume was transferred to 20 ml of medium containing drug in order to obtain a concentration of one parasite per well in a 96-well plate containing confluent HFFs. Inoculated plates were left undisturbed for 5 to 7 days, at which point the wells were screened for those containing a single plaque formed by one original parasite. Several clones were
chosen, usually 24 to 48, and transferred to 24-well plates of HFFs. Clones were screened for correct construct integration by PCR of genomic DNA and/or immunofluorescence assays.

**F. Bioinformatics, alignments, and prediction algorithms**

The primary databases used for gene and protein information and BLASTp analyses included the *Toxoplasma* database (ToxoDB, http://toxodb.org) (95), National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) (96), and Eukaryotic Pathogen Database (EuPathDB, http://eupathdb.org) (97). In order to identify GNAT domain-containing proteins in *Toxoplasma*, the GNAT domain of TgGCN5-B was used as the query sequence for BLASTp analysis in ToxoDB. One of the hits, TGGT1_305480, we named TgElp3 because of its homology to yeast and human Elp3. Protein alignments of Elp3 homologues from *Toxoplasma gondii* (TGGT1_305480), *Plasmodium falciparum* (XP_001350675.1), *Homo sapiens* (NP_060561.3), and *Saccharomyces cerevisiae* (NP_015239.1) were performed using ClustalW (BioEdit software). Predicted domains of TgElp3 were identified using Pfam (http://pfam.sanger.ac.uk) (98) and SMART (http://smart.embl-heidelberg.de) (99) online algorithms.

Upon discovering TgElp3’s unique localization, we used several online predictive algorithms including TargetP (http://www.cbs.dtu.dk/services/TargetP/) (100), PSORT (http://psort.hgc.jp) (101), and Mitoprot (http://ihg.gsf.de/ihg/mitoprot.html) (102) to identify the presence of signal sequences and calculate the probability of localization to organelles throughout the parasite. We were not able to find a conclusive N-terminal or internal signal sequence in TgElp3. However, we did identify the presence of a C-terminal TMD using the online tools TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (103), DAS (http://www.sbc.su.se/~miklos/DAS/) (104), and TmPred (http://www.ch.embnet.org/software/TMPRED_form.html) (105). These algorithms were also used to search for TMDs in other Elp3 homologues.
G. Polymerase chain reaction (PCR)

All DNA sequences amplified for eventual expression in Toxoplasma were amplified using Phusion® High Fidelity DNA Polymerase (Thermo Scientific), producing blunt-end PCR products. Fifty microliter reactions were prepared using the supplied 5X GC buffer and 10 mM dNTP mix, 10 µM of each primer (forward and reverse), approximately 10-100 ng DNA template, and 0.5 µl of the Phusion® High Fidelity DNA Polymerase. All primers used for this thesis are listed in Table 1. PCRs were placed in an Eppendorf thermocycler and heated according to the following protocol:
1. 98°C for 30 sec
2. 98°C for 10 sec
3. 60-70°C for 30 sec
4. 72°C for 30 sec per 1 kb of amplicon
5. Repeat steps 2 through 4 for 30-35 cycles
6. 72°C for 10 min
7. Hold at 4°C

Toxoplasma genomic DNA sequences amplified for screening purposes were amplified using GoTaq® Green Master Mix (Promega). To obtain genomic DNA from parasite clones for screening, 1.0 ml of freshly lysed extracellular parasites was centrifuged at 6,000 rpm for 5 min, resuspended in lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl₂, 0.1% SDS, and 20 mg/ml ProteinaseK), incubated at 55°C overnight, and heated at 95°C for 10 min. One microliter of lysate was used in a 25.5 µl PCR reaction containing 12.5 µl of water, 0.25 µl of each primer, and 12.5 µl of GoTaq® Green Master Mix. The reactions were heated in a thermocycler according to the following protocol:
1. 95°C for 2 min
2. 95°C for 1 min
3. 60-65°C for 2 min
4. 72°C for 1 min per 1 kb of amplicon
5. Repeat steps 2 through 4 for 35 cycles
6. 72°C for 10 min
7. Hold at 4°C

PCR products were separated using gel electrophoresis with a 0.8-1.0% agarose gel containing 1 µg/ml ethidium bromide. When DNA needed to be used for downstream applications such as cloning, the band corresponding to the correct size of DNA desired was excised from the gel. The DNA was then purified from the gel slice using the NucleoSpin® gel and PCR clean-up kit (Clontech cat# 740609.250) according to the manufacturer’s protocol. DNA was eluted with water and stored at -20°C until needed.

Table 1: PCR primers

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<tr>
<th>Name</th>
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**Screen ΔTgElp3 and ΔTgElp3::ddHA TgElp3 clones**

**WT TMD 5' fragment**

**ΔTMD 5' fragment**

**WT TMD and ΔTMD 3' fragments**

**Screen TgElp3 WT TMD and ΔTMD clones**
H. Reverse transcription (RT)-PCR

RT-PCR was used to monitor the absence of endogenous TgElp3 mRNA transcript in the ΔTgElp3::ddHA TgElp3 parasite lines. Total RNA was isolated by harvesting a fully lysed T-25 cm$^2$ flask of each parasite line and filtering the parasites through a 3 µm polycarbonate filter followed by centrifugation at 400 x g for 10 min. The parasite pellets were washed in PBS, centrifuged as before, and stored at -80°C until needed. The RNeasy® Plus mini kit (Qiagen) was used to isolate total RNA, and an additional on-column DNA digestion step was performed. The Omniscript RT kit (Qiagen) was used to make cDNA twice, once using oligo dT primers and once using random primers. The resulting cDNA was used directly in PCRs using the GoTaq® Green Master Mix (Promega) as described above.

I. Cloning and sequencing TgElp3

The predicted TgElp3 gene in type I Toxoplasma tachyzoites is annotated as TGGT1_305480 in the online Toxoplasma database ToxoDB (toxodb.org). Primers were designed based on the predicted coding sequence (F1 and R1) and used to amplify TgElp3 from a RH strain cDNA library. The amplicon was ligated into ZeroBlunt®TOPO® vector (Invitrogen) for sequencing. Several vector- and gene-specific primers were used to sequence the entire 2,995 bp TgElp3 coding sequence, and they are listed in Table 1.

Rapid amplification of cDNA ends (RACE) was used to identify the 5’ and 3’ untranslated regions (UTRs) of TgElp3, and the GeneRacer™ kit (Invitrogen) was used according to the manufacturer’s protocol. Primer R4 and nested primer R5 were used for amplifying the 5’-UTR and primer F5 and nested primer F6 for the 3’-UTR.

J. Generation of TgElp3 antibody

In order to generate a TgElp3-specific antibody, we first produced a 120 amino acid antigen fused to glutathione S-transferase (GST) in bacteria. cDNA encoding TgElp3 amino acids 781-900 was amplified with primers F7 and R6 and
ligated into the pGEX-4T-3 vector (GE Healthcare cat# 28-9545-52) at restriction sites BamHI and NotI. This vector allows for the fusion of GST to the N-terminus of the TgElp3 antigen with a thrombin cleavage site for removal of the GST tag. The construct was transformed into Rosetta competent cells with ampicillin used for selection.

To produce antigen, bacteria were grown to an OD$_{600}$ of 0.6 in 500 ml of Luria broth containing ampicillin then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside for 3-4 hrs at 37°C. The bacteria were pelleted, resuspended in GST buffer (125 mM Tris-HCl pH 8.0 and 150 mM NaCl) supplemented with a protease inhibitor cocktail (Sigma cat# P8465), and sonicated 5 times for 15 seconds followed by 30 seconds on ice each time. Lysates were then centrifuged at 4°C for 20 mins and the insoluble pellet discarded. GST-tagged TgElp3 antigen was purified over glutathione resin (Clontech cat# 635607) for 4 hrs at 4°C. GST-bound resin was washed 3 times with GST buffer followed by either elution of GST-TgElp3 antigen or cleavage of the GST tag by thrombin. GST-tagged TgElp3 antigen was eluted by incubating the resin in GST elution buffer (50 mM Tris-HCl pH 8.0 and 10 mM reduced L-glutathione) for 5 min at 4°C and collecting the supernatant. In order to cleave off the GST tag for antibody production, resin was, instead, incubated with 10 units of thrombin (Sigma cat# T6884) overnight at 22°C. TgElp3 antigen, cleaved from GST, was present in the supernatant and quantified using the Bradford assay (Bio-Rad cat# 500-0002). The GST-removed antigen was injected into rabbits for polyclonal antibody production (Cocalico Biologicals). Rabbit pre-immune serum was collected prior to antigen injection, and serum containing antibody was collected after three antigen injections, resulting in the antibody termed anti-TgElp3. Western blotting was used to test the specificity of anti-TgElp3 and the lack of contaminants in the pre-immune serum.

**K. Endogenously tagging TgElp3**

The In-Fusion® HD cloning kit (Clontech) was used to clone fragments into *Toxoplasma* expression vectors for all constructs described in this thesis;
primer design and cloning were conducted according to the manufacturer’s protocol. To endogenously tag TgElp3 at the C-terminus with two hemagglutinin (HA) epitopes, TgElp3 genomic 5’ and 3’ fragments were amplified from RHΔku80Δhx DNA and inserted into the pDHFR-TS cassette (90) at restriction sites NotI and HindIII, respectively. The genomic fragments flanked a selectable marker that confers resistance to pyrimethamine to select for parasite clones that underwent homologous recombination. The 1,117 bp 5’ genomic fragment was constructed using primers F8 and R7 to fuse two fragments consisting of 1) 921 bp upstream the TgElp3 stop codon amplified by primers F9 and R8 and 2) 139 bp downstream of the stop codon with a 2X HA tag added upstream of the stop codon using primers F10 and R9. The 1,021 bp 3’ genomic fragment was amplified using primers F11 and R10. The construct was linearized with NotI and transfected into RHΔku80Δhx tachyzoites. The resulting parasite line was termed endoTgElp3HA.

L. Ectopically over-expressing tagged TgElp3

To stably express ectopic HA TgElp3 in parasites, the open reading frame was amplified from RHΔhx cDNA with primers F12 and R11 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) (106). TgElp3HA was made in the same manner with primers F13 and R12. HA TgElp3ΔTMD was amplified with primers F14 and R13 and inserted downstream of the tubulin promoter in the pHXGPRT:tub expression vector at restriction sites BglII and EcoRV. All three constructs were linearized with NotI and transfected into RHΔhx parasites.

M. YFP TgElp3 fusion proteins

YFP (yellow fluorescent protein) was amplified from pYFP-LIC-HXG (kindly provided by Dr. Vern Carruthers) with primers F15 and R14 and inserted downstream of the tubulin promoter in pHXGPRT:tub using the BglIII and AvrII restriction sites. The resulting pHXGPRT:tub-YFP construct contained an Ndel
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 F16 and R15 and fused to the N-terminus of YFP using the NdeI restriction site. The C-terminal region (amino acids 726-984), amplified by primers F17 and R16, 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 F18 and R17 and inserting the product downstream of YFP using the EcoRV site. YFP fusion constructs were transiently transfected into RHΔhx parasites.

**N. ΔTgElp3 and ddHA TgElp3 constructs**

A TgElp3 knockout construct was designed to facilitate a double crossover homologous recombination event 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 RHΔku80Δhx DNA with primers F19 and R18 and inserted into the pDHFR-TS cassette 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 F20 and R19 and inserted into this plasmid using restriction sites HindIII and ApaI. The construct was linearized with NotI and transfected into RHΔku80Δhx parasites.

Recombinant ddHA TgElp3 was engineered by first amplifying HA TgElp3 (without the ATG start codon) with primers F21 and R20 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 F22 and R21 and inserted into the pHXGPRT:tub expression vector using the BglII restriction site. After establishing a clonal parasite line expressing ddHA TgElp3, the TgElp3 knockout construct was transfected and parasite clones were screened by PCR for the presence of
ectopic $\Delta_{dHA} TgElp3$ and absence of the endogenous $TgElp3$ locus. Primers used for screening are depicted in Figure 16 and listed in Table 1.

**O. TgElp3 WT TMD and $\Delta$TMD constructs**

A plasmid was generated to facilitate homologous recombination to remove the DNA encoding the TMD from the genomic locus of $TgElp3$ ($\Delta$TMD). In parallel, we made a similar construct that contained the wild-type sequence (WT TMD) to use as a control for recombination efficiency. The $\sim$1,700 bp 5’ genomic fragment of each construct contained a portion of the last intron, the entire last exon with or without the TMD (encoding amino acids 958-980), and the 3’ UTR (Figure 19). The 3’ genomic fragment consisted of 1,460 bp downstream of the 3’-UTR. The WT TMD 5’ fragment was amplified from RH$\Delta$ku80$\Delta$hx genomic DNA with primers F27 and R26. The $\Delta$TMD 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 RH$\Delta$ku80$\Delta$hx genomic DNA with primers F28+R27 and F29+R28, respectively. PCR-3 used template from PCR-1 and primers F30 and R29. PCR-4 combined the pieces by using template from PCR-2 and PCR-3 with primers F31 and R30. The 3’ fragment used for both the WT TMD and $\Delta$TMD constructs was amplified from RH$\Delta$ku80$\Delta$hx genomic DNA using primers F32 and R31. 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 $\Delta$TMD constructs were transfected into RH$\Delta$ku80$\Delta$hx parasites, and 24 clones from each parasite line were screened by PCR using the primers shown in Figure 19.

**P. SDS-PAGE and Western blotting**

*Toxoplasma* lysates were prepared as described in Section II-B. The Novex® NuPAGE® SDS-PAGE gel system (Invitrogen) was used for protein separation and transfer to nitrocellulose membranes. NuPAGE® LDS sample buffer with 5% beta-mercaptoethanol was added to each sample and heated at 95°C for 10 min prior to loading on 4-12% Bis-Tris pre-cast gels. Gels were
electrophoresed in MOPS buffer at 200 V before transfer to a nitrocellulose membrane at 30 V for 1-2 hrs. Ponceau S stain was used to assess quality of the transfer and equal loading between samples. Membranes were blocked in 4% non-fat milk in tris-buffered saline and Tween followed by incubation with primary and secondary antibodies that were diluted in blocking buffer. Primary antibodies included 1:1,000 rat anti-HA (Roche cat# 11867423001), 1:1,000 rabbit anti-AcH3 (Active Motif cat# 39139) and 1:2,000 rabbit anti-H3 (Millipore cat#06-755). Secondary antibodies used were 1:2,000 goat anti-rat and 1:2,000 goat anti-rabbit, both conjugated to horseradish peroxidase (HRP) (GE Healthcare cat# NA935 and NA934, respectively). Proteins were detected by chemiluminescence using the FluorChem E Imager and AlphaView® software (ProteinSimple). Densitometry analysis was conducted using ImageJ software.

The quality of the TgElp3-specific antibody was tested by Western blotting and probing 250 ng of GST-TgElp3 antigen with a 1:10,000 dilution of raw antibody-containing serum (anti-TgElp3) or the rabbit pre-immune serum followed by 1:2,000 anti-rabbit conjugated to HRP. In addition, 70 µg of parasite and HFF lysates were probed with a 1:2,000 dilution of anti-TgElp3 or the pre-immune serum to determine the specificity of the antibody.

Q. Preparation and Western blotting of mouse brain mitochondria

Mouse brain mitochondrial and cytosolic lysates were prepared from FVB/NJ (Friend Virus B NIH Jackson) mice and kindly provided by Dr. Nickolay Brustovetsky. The mitochondrial fraction was prepared as previously described (107) and the cytosolic fraction was prepared by centrifuging brain homogenate at 12,000 x g for 10 min followed by a centrifugation of the supernatant at 100,000 x g for 30 min. The 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-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling cat# 2118), 1:1,000 rabbit anti-cytochrome c oxidase subunit IV (COX IV) (Cell Signaling cat# 4850), and
1:1,000 rabbit anti-human Elp3 (Active Motif cat# 39949). The secondary antibody was goat anti-rabbit conjugated to HRP (GE Healthcare cat# NA934).

R. Immunoprecipitation

Parasites expressing HA-tagged forms of TgElp3 and their parental lines, typically RHΔhx, were harvested and lysed as described in Section II-B. A total protein concentration of 2-4 mg in a 500 µl volume of each parasite lysate was used for immunoprecipitation (IP). Lysates were first pre-cleared of materials that bind non-specifically to IP beads by incubating for 1-3 hrs at 4°C with 25 µl of Protein G beads (Roche cat# 11719416001). The beads had been washed three times in lysis buffer before being added to the lysates. Pre-cleared lysates were centrifuged at 8,000 rpm for 1 min and the beads discarded. Beads used for IP were conjugated to a high-affinity HA antibody (Roche cat# 11815016001) and were washed three times in 500 µl of lysis buffer. Fifty microliters of the washed anti-HA bead matrix was added to each pre-cleared lysate and incubated overnight at 4°C on a rocker. Samples were then centrifuged at 8,000 rpm for 1 min and the unbound lysate removed. The bead matrix was washed twice in 500 µl of lysis buffer and once in 500 µl of KAT assay buffer (see below) and used directly in in vitro KAT assays (Section II-S).

S. Lysine acetyltransferase (KAT) assays

IP 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 hr followed by the addition of 1X LDS NuPAGE® loading buffer supplemented with 5% beta-mercaptoethanol and heated at 95°C for 10 min. Western blotting was performed as previously described and membranes were probed with 1:1,000 anti-acetylated H3 (anti-AcH3) followed by 1:2,000 anti-rabbit conjugated to HRP.
They were also probed with rat anti-HA to detect TgElp3 that had been immunoprecipitated and used as substrate in the KAT assay.

In order to test tubulin as a potential substrate of TgElp3, IPs and KAT assays were conducted as before except using microtubules as substrate in place of histone H3. Tubulin purified from bovine brain was polymerized to form microtubules, and the microtubules were stabilized using paclitaxel. The reaction to form microtubules contained 1X general tubulin buffer (Cytoskeleton cat# BST01-001), 5% glycerol, 1 mM GTP (Cytoskeleton cat# BST06-001), 20 μM paclitaxel (Cytoskeleton cat# TXD01), and 3 mg/ml of tubulin (Cytoskeleton cat# TL238) and was incubated at 37°C for 45 min. The resulting stable microtubules were used directly in an in vitro KAT assay as substrate. Western blot membranes of the KAT assay reactions were probed with a 1:5,000 dilution of mouse monoclonal anti-acetylated alpha-tubulin (anti-AcTubulin) (Sigma cat# T7451) followed by a 1:5,000 dilution of anti-mouse conjugated to HRP (GE Healthcare cat# NA931).

T. Immunofluorescence assays

Immunofluorescence assays (IFAs) were performed by inoculating parasites onto confluent HFF cell monolayers grown on coverslips in 12-well plates. Infected HFF monolayers were fixed 16-24 hrs 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. Wells were washed with PBS three times between each step. Samples were blocked with 3% BSA for a minimum of 1 hr 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), 1:10,000 rabbit anti-TgIF2α (108), and 1:2,000 mouse anti-Tgf1B-ATPase (109). Coverslips were washed with PBS three times for 5 min each and then incubated with various secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen). Coverslips were mounted to slides with VectaShield® Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories cat# H-1200) and visualized on a Nikon Eclipse 80i.
fluorescent microscope. Image analysis was performed using the Nikon NIS Elements AR version 4.00.08 software.

MitoTracker® was used as an additional, independent mitochondrial marker. Before fixation for IFA analysis, the culture medium was replaced with fresh, warm medium containing 100 µM of MitoTracker® Red CMXRos (Invitrogen cat# M-7512) and allowed to incubate at 37°C with 5% CO₂ for 30 min. Cells were then washed with PBS and fixed and processed as described above.

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 in order to permeabilize cell membranes. Anti-TglF2α and anti-TgF₁B ATPase were used as cytoplasmic and mitochondrial matrix markers, respectively.

**U. Immunoelectron microscopy**

Immunoelectron microscopy processing and analysis were conducted with the help of Wandy Beatty at Washington University, St. Louis. Clonal parasites stably expressing ectopic HA-TgElp3 or TgElp3HA along with the parental line (RHΔhx) were grown overnight in a T-25 cm² flask containing confluent HFF monolayers. The infected monolayer was washed with PBS, scraped, and centrifuged for 10 min at 3,000 x g. Cells were resuspended in fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in PBS and incubated on ice for 1 hr. Samples were then cryo processed and sectioned; 50 nm thick sections were used for immunolabeling. To immunolabel sections, they were blocked with 5% fetal calf serum/5% goat serum in piperazine-N,N'-bis(2-ethanesulfonic acid) buffer for 20 min at room temperature. Primary antibody, rat anti-HA, was used at a 1:25 dilution in blocking buffer and incubated for 1 hr at room temperature. Samples were then incubated for 1 hr 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).

V. Toxoplasma plaque assay

Plaque assays were used to assess changes in parasite replication. Intracellular parasites were harvested by scraping the host cell monolayer infected with Toxoplasma and centrifuging at 400 x g for 10 min. The pellet was syringe lysed with a 25-gauge needle, and the parasites were filtered using a 3 µm polycarbonate filter (Whatman). After centrifuging at 400 x g for 10 min, the parasites were resuspended in 4 ml of parasite medium and counted using a hemocytometer. Each parasite line was diluted in 5 ml of parasite medium to a concentration of 5 parasites per microliter. Twelve-well plates containing confluent HFFs were inoculated with 100 µl (500 parasites) per well of each parasite line and grown in either regular parasite medium, medium supplemented with ethanol, or medium supplemented with 250 nM Shield-1. Each treatment was conducted in triplicate. Four hours after inoculation, medium and uninvaded parasites were removed and replaced with fresh medium supplemented with the same treatments as before, and the cultures were incubated under normal conditions (described in section II-A) for 5 days. Cells were then fixed with 100% ice-cold methanol for 10 min and stained with crystal violet in order to visualize the plaques. Pictures of the plaques in each well were taken using the FluorChem E Imager (ProteinSimple), and AlphaView® software (ProteinSimple) was used to calculate the area of host cells lysed by the parasites in each well. The average was calculated from the three wells for each treatment.
III. RESULTS

A. An Elp3 homologue is present in Toxoplasma

With evidence indicating the importance of KATs to Toxoplasma growth, differentiation, and survival, we searched the online Toxoplasma database for uncharacterized KATs and identified a predicted protein that had homology to the yeast KAT Elp3. The gene TGGT1_305480, which we have designated TgElp3, is located on chromosome IX and contains 9 exons and 8 introns. The database predicted the coding sequence of TgElp3 to be 2,955 bp in the type I GT1 strain, corresponding to 984 amino acids. We amplified and sequenced the TgElp3 coding sequence from a type I RH strain cDNA library, verifying the database prediction to be correct. In addition, we used rapid amplification of cDNA ends (RACE) to identify the 5' and 3'-UTRs of the TgElp3 transcript and found them to be 568 bp and 70 bp, respectively, making the entire TgElp3 mRNA transcript 1,622 bp in length.

TgElp3 is predicted to contain the highly conserved radical S-adenosylmethionine (SAM) and KAT domains found on all Elp3 proteins from archaebacteria to humans (Figure 6A). The critical residues for KAT activity are conserved (Figure 6B). Intriguingly, the radical SAM domain in the Toxoplasma and Plasmodium Elp3 homologues contains the canonical CxxxxCxxC motif important for iron-sulfur cluster formation while most other Elp3 homologues contain a non-canonical Cx4Cx2Cx2C motif (Figure 6B). Strikingly, TgElp3 also harbors a predicted transmembrane domain (TMD) at its extreme C-terminal (Ct) end followed by four arginine residues. Further analysis revealed that other members of the Apicomplexa phylum, including Plasmodium spp, Cryptosporidium spp, Neospora spp, Theileria spp, and Eimeria spp, 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.

Figure 6: 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). 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 (60,61,110) 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 (59,111-113). Amino acid number indicated on the right.
B. *Toxoplasma* lacks an Elongator complex

Elp3 homologues in most species studied to date have been shown to be the catalytic subunit in the six-subunit Elongator complex (Elp1-6). A thorough bioinformatics survey of the *Toxoplasma* database and those of other eukaryotic pathogens was unable to identify homology to any member of the Elongator complex aside from Elp3. The entire sequence of proteins Elp1-Elp6 from both yeast and human were used as input sequences for BLASTp analyses in the NCBI database, the eukaryotic pathogen database, and each individual database of several apicomplexans including *Toxoplasma*, *Plasmodium*, and *Cryptosporidium*. In all species examined, Elp3 homologues were identified with high bit scores and low E values. WD40 repeats present within Elp2 had homology to WD40 containing proteins but no definitive Elp2 homologues were identified in any of the apicomplexans. The remaining Elongator proteins had little to no homology to apicomplexan proteins. While the Elongator complex is highly conserved from yeast to humans, *Toxoplasma* and other apicomplexans appear to lack this complex but have retained the catalytic component, Elp3.

C. TgElp3 protein size and expression

After confirming the presence of TgElp3 mRNA and establishing the length of transcript, we next sought to confirm protein expression and size. This experiment was done by adding two HA epitopes (2X HA) just upstream of the stop codon of the endogenous TgElp3 genomic locus in order to express TgElp3 with a double HA tag at its C-terminus. Homologous recombination was used to add 2X HA to the 3’ end of the TgElp3 locus in RHΔku80Δhx parasites and introduce a mutated form of DHFR-TS to use for selection. A clonal parasite line was obtained with correct integration of the construct (endoTgElp3_{HA}), and Western blotting with an HA antibody was used to establish protein size and relative abundance. We found that TgElp3 is 100 kDa as expected but we also detected a band approximately 80 kDa in size, suggesting that TgElp3 may be cleaved or degraded (Figure 7). One possibility for the cleavage or degradation could be due to the addition of the HA tag near the TMD, resulting in a disruption
of protein folding or function. Our results also suggested that TgElp3 is expressed in very low abundance, which is in accordance with expression data provided in the Toxoplasma database and is consistent with expression of other KATs.

Since TgElp3 is naturally expressed at such a low level, it can be difficult to detect and difficult to characterize. Therefore, we also ectopically expressed HA-tagged TgElp3 under the tubulin promoter, a constitutively strong promoter. Clonal lines were obtained of TgElp3 tagged at both the N-terminus (HA-TgElp3) and C-terminus (TgElp3_HA) in RHΔhx parasites. Western blots of TgElp3_HA showed similar results as endoTgElp3_HA with the presence of two bands at sizes 100 kDa and 80 kDa (Figure 7). Interestingly, even with the tubulin promoter, expression levels of TgElp3_HA were similar to those seen for endoTgElp3_HA. Parasites expressing HA-TgElp3, on the other hand, expressed the ectopic protein at very high levels, and a single 100 kDa band was detected by Western blot (Figure 7). Results observed for both tagged lines were consistent among several aclonal and clonal parasite populations. Combined evidence suggests that the parasites do not tolerate the addition of an epitope tag in close proximity to the C-terminal TMD. Therefore, we used the HA-TgElp3 parasite line for the majority of our studies.
Figure 7: Size of TgElp3. Western blot of 30 µg of lysate from parasites expressing ectopic $\text{HA}_{\text{TgElp3}}$ and 80 µg from parasites expressing ectopic TgElp3$_{\text{HA}}$, endogenous TgElp3$_{\text{HA}}$, and the parental line RHΔhx. Blots were probed with an anti-HA antibody followed by a goat anti-rat antibody conjugated to HRP.

D. Attempt to generate a TgElp3-specific antibody

In order to study native protein without the use of epitope tags, we generated a TgElp3-specific polyclonal antibody in rabbit. We chose a 120 amino acid sequence between the KAT domain and the TMD (amino acids 781-900) that would serve as a soluble antigen for antibody production. The resulting antibody clearly recognized antigen while rabbit pre-immune serum did not (Figure 8A). However, when the antibody was used to probe parasite lysates, it recognized many proteins as several bands were seen by Western blot (Figure 8B), and the bands were inconsistent between samples. Attempts to affinity-purify the antibody were also unsuccessful. Therefore, we concluded that the antibody recognizes non-specific products and is not reliable for use in our studies.
Figure 8: TgElp3-specific antibody. A) Western blot of 250 ng of GST-tagged TgElp3 antigen (arrow) probed with a 1:10,000 dilution of either the rabbit pre-immune serum or the final TgElp3 antibody followed by a 1:2,000 dilution of a goat anti-rabbit antibody. B) Western blot of 70 µg of parasite and host cell, HFF, lysates probed with a 1:2,000 dilution of either the anti-TgElp3 antibody or the rabbit pre-immune serum. Ponceau S stain shows the presence of protein in all lanes.
**E. TgElp3 localizes to the mitochondrion**

The most well characterized KATs are located in the nucleus and play a key role in regulating gene transcription. However, the increasing identification of non-nuclear KATs has prompted many to explore the function of these KATs outside of the nucleus. Elp3 homologues have been shown to have a role in transcription in the nucleus, but in most species, Elp3 is primarily found in the cytoplasm. We investigated the localization of TgElp3 by performing immunofluorescence assays (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, which forms a lasso-like structure around the nucleus. HA-TgElp3 and TgElp3_HA both co-localized with the mitochondrial matrix protein TgF₁β ATPase as well as with MitoTracker (Figure 9A-B). The TgF₁β ATPase-specific antibody was used to visualize the mitochondrion from parasites only whereas MitoTracker infiltrates both host cell (HFF) and *Toxoplasma* mitochondria.
Figure 9: TgElp3 localizes to the parasite mitochondrion. IFAs of HA-TgElp3, TgElp3-HA, and RHΔhx parasites probed with a rat anti-HA antibody (green) and a mouse anti-TgF1B ATPase antibody (red) (A) or MitoTracker (red) (B). Images were merged with the DNA stain DAPI (blue), and the white scale bar represents 5 µm.

For better resolution, immunoelectron microscopy (IEM) was also performed using an HA antibody with 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 (Figure 10). No gold particles were detected in the parental parasites probed with this HA antibody (not shown). Our IFA and IEM studies indicated that TgElp3 is targeted almost exclusively to the mitochondrion and may reside within the membrane.
Figure 10: IEM images of TgElp3 localization. Representative images of HA-TgElp3 and TgElp3-HA parasites probed with an anti-HA antibody and an anti-rat antibody conjugated to 18 nm gold particles. Gold particles were found almost exclusively at the parasite mitochondrial (M) membrane.

F. 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 identify the mechanism by which TgElp3 is targeted to the mitochondrion, we generated a series of YFP fusion proteins (Figure 11A). As shown in Figure 11B, 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 (Figure 11B). 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. 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 \( \text{HA} \)TgElp3 lacking the TMD (\( \text{HA} \)TgElp3\( \Delta \)TMD). With the TMD removed, TgElp3 is no longer targeted to the mitochondrion but instead appears to be localized at the parasite’s periphery for reasons that remain unknown (Figure 11B). The results consistently demonstrate that TgElp3 is unable to localize to the mitochondrion without the TMD.
### A.

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### B.

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Figure 11: 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; an anti-HA antibody was used to detect TgElp3ΔTMD (green); TgF1B 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.

G. 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. These proteins are referred to as tail-anchored 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 (114,115). Tail-anchored proteins are often inserted in the outer mitochondrial membrane 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 (116). To determine the orientation of TgElp3 in the mitochondrial membrane, we selectively permeabilized membranes of parasites expressing TgElp3 and TgElp3_HA 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 (TgF1B ATPase) was not (Figure 12). 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 TgF1B 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, TgElp3_HA 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 TgElp3 capable of enzymatically acting on cytosolic proteins, proteins associated with the mitochondrial surface, or proteins targeted for translocation into the mitochondrion.
Figure 12: Digitonin selective permeabilization determines TgElp3 orientation. 
HA-TgElp3 and TgElp3HA expressing parasites were used to visualize the N- and C-termini of TgElp3 (green), respectively, while parental RHΔhx parasites were used to establish the degree of membrane permeabilization. Detection of cytoplasmic TgIF2α (green) and mitochondrial matrix protein TgF1B 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 the results.

**H. Elp3 localization to the mitochondria of mammalian cells**

The localization of TgElp3 to the parasite mitochondrion is contingent upon the C-terminal TMD. Elp3 homologues in the vast majority of other species do not contain a TMD, but there has been a report showing human Elp3 co-localizing with the mitochondrial marker MitoTracker by IFA in HeLa cells (117). We examined whether mitochondrial Elp3 was unique to *Toxoplasma* by performing Western blots on fractions from mouse brain. As shown in Figure 13, 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.

![Figure 13: 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.](image-url)
I. TgElp3 KAT activity and potential substrates

Elp3 homologues have been shown to acetylate histones, with a preference for histone H3 \textit{in vivo}, and alpha-tubulin (63,70). We first tested if TgElp3 has KAT activity by performing an \textit{in vitro} KAT assay. This assay tests the ability of a protein to transfer an acetyl group from acetyl-CoA to a lysine residue on a substrate. Histone H3 was used as substrate because of the high number of lysine residues that are amenable to acetylation. Bona-fide KATs usually have the ability to acetylate H3 \textit{in vitro} even if H3 is not an \textit{in vivo} substrate. Recombinant HaTgElp3 was isolated from parasite lysate using IP with anti-HA conjugated beads (Figure 14A) and used directly in a KAT assay with acetyl-CoA and human histone H3. The same protocol was followed using RHΔhx lysate, containing no HA epitope, in order to confirm that the IP alone did not contribute to histone acetylation. A negative control was also included that contained all KAT assay reagents except IP beads. Western blot analysis with anti-acetylated H3 detected H3 acetylation only in the presence of HaTgElp3 (Figure 14A), indicating that TgElp3 has KAT activity.

Figure 14: TgElp3 acetylates H3 \textit{in vitro}. A) Left panel: Western blot (WB) probed with an anti-HA antibody following immunoprecipitation (IP) of HaTgElp3 and RHΔhx lysates over anti-HA conjugated beads. IP samples were used in an \textit{in vitro} KAT assay with histone H3 substrate and acetyl-CoA. Right panel: Western blot of the KAT assay reaction probed with an anti-acetylated H3 (α-AcH3) antibody. Acetylated H3 was detected in KAT assays containing HaTgElp3 but not in the IP control (RHΔhx) or KAT assay negative control that contained all KAT assay reagents except IP beads (Neg.). B) Western blot of 30 μg of HaTgElp3 and RHΔhx lysates probed with α-AcH3 and α-H3 antibodies. Numbers below the blot indicate the ratio of AcH3 compared to AcH3 in RHΔhx parasite lysate.
Our KAT assay results showed that TgElp3 can acetylate histone H3 \textit{in vitro}, so we next investigated H3 acetylation \textit{in vivo}. Using lysate from the parasite line over-expressing $\text{HA}_\text{TgElp3}$, we looked for changes in global histone H3 acetylation by probing for acetylated H3. The same level of acetylated H3 was detected in lysate from $\text{HA}_\text{TgElp3}$ and the parental line, suggesting that over-expression of TgElp3 does not increase H3 acetylation (Figure 14B).

In a surprising study published by Creppe \textit{et al} (70), Elp3 was shown for the first time to acetylate alpha-tubulin in mouse cortical neurons and human cell lines. This study, as well as others, also determined that microtubules serve as better substrates of Elp3 than monomeric or dimeric alpha-tubulin (71). Therefore, we used the \textit{in vitro} KAT assay to determine if TgElp3 acetylated tubulin. The KAT assay was conducted in the same manner as before except microtubules, polymerized from bovine brain tubulin, were used as the substrate. A caveat to using bovine brain tubulin is that it is already partially acetylated. In our study we would predict an increase in acetylated tubulin compared to the two controls, RHΔhx and the negative control, if tubulin serves as a substrate for TgElp3. However, no change in tubulin acetylation was detected when $\text{HA}_\text{TgElp3}$ was present (Figure 15A), suggesting that microtubules are not a primary target of TgElp3. We also examined \textit{in vivo} tubulin acetylation by Western blot in parasites over-expressing $\text{HA}_\text{TgElp3}$. Again, we saw no significant difference compared to the parental line, RHΔhx (Figure 15B).
Figure 15: Tubulin is likely not a primary substrate of TgElp3. A) Western blot of in vitro KAT assay probed with an α-acetylated tubulin (AcTubulin) antibody. Acetylated tubulin is detected in all samples but is not increased in the presence of HA{TgElp3 compared to the IP control, RHΔhx, or the KAT assay negative control containing all reagents except IP beads (Neg.). B) Western blot of 30 µg of HA{TgElp3 and RHΔhx lysates probed with an α-AcTubulin antibody and an α-H3 antibody as a loading control. Numbers below the blot indicate the ratio of acetylated tubulin compared to acetylated tubulin in RHΔhx parasites.

These results do not definitively prove that H3 and/or tubulin are not substrates of TgElp3 because of several caveats. For example, if TgElp3 requires co-factors or additional proteins in order to function, the stoichiometric ratio could prevent an increase in TgElp3 function as a result of an increase in TgElp3 protein. Also, because H3 and tubulin are naturally highly acetylated, minor changes in acetylation may not be detected by Western blot. However, our localization studies also suggest that H3 and tubulin are not primary substrates of TgElp3. After our discovery that TgElp3 localizes almost exclusively at the outer mitochondrial membrane, we hypothesized that targets of TgElp3 would reside near or within the mitochondrion. Our laboratory’s recent analysis of the Toxoplasma acetylome identified several acetylated mitochondrial proteins including porin, which is an integral outer membrane protein and highly acetylated (54, 56). Future studies will investigate porin and other mitochondrial or mitochondrial-associated proteins as potential substrates of TgElp3.

J. TgElp3 is essential for parasite survival

To provide insight into the role of TgElp3 in parasite physiology, we designed experiments to knockout the genomic locus. Despite several attempts to generate a TgElp3 knockout by replacing the genomic locus with a selectable marker in RHΔku80Δhx parasites (Figure 16A), we were unable to obtain viable clones, suggesting that TgElp3 is essential for parasite survival (Figure 16B). We were, however, able to knockout 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
We were unable to obtain a TgElp3 knockout in RHΔku80Δhx parasites after screening 60 clones from 11 independent populations. However, we were able to knockout the TgElp3 genomic locus at a high frequency (58%) in parasites expressing ectopic \( \text{ddHA}_\text{TgElp3} \). Both PCR of genomic DNA and RT-PCR verified the absence of endogenous TgElp3 in three independent knockout clones (Figure 16B-C). 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.
Figure 16: Knockout 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 knockout construct uses 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 diagram depicts the construct used to express an ectopic copy of TgElp3 tagged at the N-terminus with the destabilization domain (dd) and HA epitope containing the tubulin 5’-UTR and DHFR-TS 3’-UTR. HXGPRT was used for selection. The bottom panel shows the TgElp3 endogenous and ectopic mRNA transcripts with white lines representing removed introns. B) Genomic PCR of TgElp3 knockout attempts before and after introduction of ectopic ddHATgElp3. The first panel shows PCR amplifications from genomic DNA of 8 representative clones (out of 60 total), confirming that the TgElp3 locus was not replaced when a TgElp3 knockout was attempted. The second panel shows that several knockout clones were obtained when ectopic ddHATgElp3 was present, which is shown as the ~3.0 kb band in the bottom gel. The “+” denotes TgElp3 knockout 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::ddHATgElp3 clones and the presence of ectopic ddHATgElp3 mRNA. RHΔku80Δhx served as the parental line for ddHATgElp3, and ΔTgElp3::ddHATgElp3 served as the parental line for ΔTgElp3::ddHATgElp3. Primer pairs used for PCR screening are located to the right of the gel images. Markers are present in the first lane of each gel image and negative controls (Neg. control) contain all PCR reagents except DNA template.

K. Regulation of TgElp3 by an inducible destabilization domain

We next wanted to use the ΔTgElp3::ddHATgElp3 parasite line (clone 3A12) in order to study the effects of TgElp3 down-regulation. This parasite line is normally grown in the presence of Shield-1 in order to express ectopic ddHATgElp3 in the absence of endogenous TgElp3. The destabilization domain renders the ectopic copy of TgElp3 highly unstable, and it is quickly degraded. However, when the destabilization domain ligand Shield-1 is added, it binds and stabilizes the protein, allowing ddHATgElp3 to accumulate in the cell. Removing Shield-1 significantly reduces the amount of ectopic protein in ΔTgElp3::ddHATgElp3 parasites and the parental line, ddHATgElp3 (Figure 17A-B), and would be expected to result in a phenotype consistent with the down-regulation of TgElp3. We assessed the phenotype of ΔTgElp3::ddHATgElp3 parasites in the
presence and absence of 250 nM Shield-1 using the plaque assay as a measure of parasite replication. In addition, we examined the parental line \textit{ddHA}TgElp3 and the original parental “wild-type” parasites (RH\textit{Δku80Δhx}). Unexpectedly, all three parasite lines grew similarly in the presence of Shield-1 and those expressing recombinant TgElp3 appeared to grow better than “wild-type” parasites in the absence of Shield-1 (Figure 18).
Figure 17: Regulation of TgElp3 using a destabilization domain. A) Western blot of lysates from ΔTgElp3::ddHA TgElp3 parasites and the parental lines ddHA TgElp3 and RHΔku80Δhx that were grown in the absence (-) or presence (+) of 250 nM Shield-1. Blots were probed with a rat anti-HA antibody to detect ectopic TgElp3 or a rabbit anti-TgIF2α antibody. B) IFA images depicting expression of ectopic TgElp3 in the absence (-) or presence (+) of 250 nM Shield-1. An anti-HA antibody (green) was used to detect ectopic TgElp3 and was merged with DAPI (blue).

Figure 18: ΔTgElp3::ddHA TgElp3 parasites do not show a growth defect. Plaque assays of ΔTgElp3::ddHA TgElp3 parasites and the parental lines, ddHA TgElp3 and RHΔku80Δhx, were conducted using the following treatments: none, vehicle, or 250 nM Shield-1. Parasites were grown in 12-well plates of HFFs for 5 days with the respective treatment and the average area of lysed cells was calculated (a.u. = arbitrary units). Plaques can be seen in the bottom panels showing representative wells for each treatment. The graph shows data from one independent experiment (n=1).
The observed results are likely based on the fact that *Toxoplasma* only needs a small amount of TgElp3 in order to function normally. As seen in Figure 17, residual \( ddHA \) TgElp3 is present even in the absence of Shield-1. These results may, in part, be due to an inherent leakiness in the destabilization domain approach. However, over-expression of \( ddHA \) TgElp3 using the strong tubulin promoter may also result in an abundance of protein that is not fully degraded in the absence of Shield-1. Our current strategy for studying TgElp3 down-regulation focuses on a similar approach, using the endogenous promoter and UTRs of TgElp3 and targeting the construct to a specific locus in the genome. This approach should result in a more tightly regulated system and provide a more accurate TgElp3 down-regulated phenotype.

**L. Mitochondrial localization of TgElp3 is essential**

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 outer mitochondrial membrane by deleting the TMD (\( \Delta TMD \)) from the endogenous locus (Figure 19A). 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 \( \sim 70\% \) (17/24) when the WT TMD construct was used, but we were not able to isolate any viable parasites when the \( \Delta TMD \) construct was used. Figure 19B shows five representative clones from each parasite line – five positive WT TMD clones and five negative \( \Delta TMD \) clones. These results suggest that localization of TgElp3 to the outer mitochondrial membrane is essential for parasite viability.
Figure 19: 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 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. RHΔku80Δhx was the parental line. The primer pair and expected sizes are located to the right of the gel image.
IV. DISCUSSION

This thesis describes the first characterization of the Elongator complex subunit Elp3 in an apicomplexan species. TgElp3 contains unique characteristics including the presence of a C-terminal TMD and a mitochondrial localization. We determined that the TMD is necessary and sufficient for targeting TgElp3 to the outer mitochondrial membrane. Importantly, TgElp3 is the first mitochondrial-localized KAT identified and we found this localization to be conserved in a higher eukaryotic species. We also identified TgElp3 as a functional KAT that can acetylate histone H3 in vitro. While the substrates and functions of TgElp3 have yet to be identified, we established TgElp3 is essential for parasite viability and that its critical function requires the presence of the C-terminal TMD.

A. Toxoplasma lacks an Elongator complex but possesses a unique Elp3 homologue

The six-subunit Elongator complex is conserved in yeast, plants, and animals, and it appears that the assembly and function of the complex relies on the presence of all six subunits. Nearly identical phenotypes are observed in yeast, C. elegans and plants when any of the six proteins is deleted (58,76,77,119-122); for example, all Elongator mutants in yeast have a delayed growth phenotype when introduced to stresses such as changes in carbon source or temperature. Elp3 is considered the catalytic subunit of the complex while the other proteins are suggested to be critical for assembly, integrity, scaffolding, and docking for interacting proteins (123). Surprisingly, Elp3 is the only component of the Elongator complex to exhibit convincing homology to a protein found in Toxoplasma and other apicomplexans. The presence of Elp3 in protists and other ancestral eukaryotes demonstrates the evolutionary importance of this protein but the lack of homology to the other Elongator complex members suggests that Elp3 may have acquired additional functions in other species.

In addition to the apparent lack of an Elongator complex in Toxoplasma, the TgElp3 enzyme has unique characteristics. The protein is much larger than
its yeast, plant, and human counterparts and contains little to no homology outside of the two conserved radical SAM and KAT domains, which have 53-56% and 53-54% similarities, respectively. In addition, TgElp3 contains a unique C-terminal TMD not present in any Elp3 homologue previously characterized. TgElp3 is most closely related to other apicomplexan homologues, which also possess a C-terminal TMD. However, the close relative *Tetrahymena*, a free-living protist, does not have this domain. Interestingly, outside of the Apicomplexa phylum, only a small number of other chromalveolates appear to have the C-terminal TMD. Why only a select few species contain this TMD in a protein that is conserved throughout every species is unclear.

A key motif within the radical SAM domain is also different in TgElp3 compared to homologues previously studied. 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 Cx₄Cx₉Cx₂Cx motif, and mutation of these cysteines to alanines recapitulates an Elp3 knockout phenotype in yeast (60). Interestingly, TgElp3 and several other predicted TMD-containing Elp3 homologues, including those in apicomplexan species, possess the more traditional radical SAM motif (CxxxCxxC) while Elp3 homologues lacking the C-terminal TMD have a non-canonical motif. Radical SAM domains can participate in a number of catalytic reactions and while the function of this domain in Elp3 orthologues is not clear, it has been implicated in DNA demethylation in zygotes (62). However, this function seems unlikely in *Toxoplasma* given that the parasites apparently lack or have little DNA methylation (124). Further investigation is necessary to determine if a connection exists between the presence of a C-terminal TMD and the function of the radical SAM domain based on its cysteine motif.
B. TgElp3 is the first KAT identified within the outer mitochondrial membrane

Our studies uncovered the first known acetyltransferase localized to the outer mitochondrial membrane in any species. Using IFAs with mitochondrial markers, we discovered the unique mitochondrial localization of TgElp3. These results were confirmed by IEM, its higher resolution able to pinpoint the localization to the mitochondrial membrane. Additional imaging experiments using selective membrane permeabilization revealed that TgElp3 is anchored in the outer mitochondrial membrane with its catalytic domains oriented towards the cytosol (Figure 20). Elp3 homologues examined to date have been portrayed as primarily located in the cytoplasm although one study did show a co-localization of Elp3 with mitochondria in HeLa cancer cells (117). In addition, several other studies showing cytoplasmic localization of Elp3 did not perform co-localization experiments or use high-resolution imaging. Elp3 often shows a somewhat punctate localization that, if examined more closely, may partially co-localize with mitochondria. To determine if Elp3 could be found in the mitochondria of other cells, we examined mitochondria purified from mouse brain by Western blot and found a shorter form of Elp3 (~49 kDa compared to the normal 62 kDa mouse Elp3) present in the mitochondria. Based on our results, we propose that we have uncovered a novel localization of Elp3 that is conserved in other species and should be examined more closely in other cell types.
Figure 20: Depiction of TgElp3 anchored in the outer mitochondrial membrane. TgElp3 contains a C-terminal TMD (orange) that anchors TgElp3 in the outer mitochondrial membrane such that its catalytic domains, radical SAM (blue) and KAT (green), reside in the cytosol and the C-terminal tail is in the intermembrane space (IMS).

The identification of TgElp3 as a mitochondrial KAT is a crucial discovery in the field of protein lysine acetylation. For years, it has been evident that mitochondrial proteins are acetylated, and acetylome studies revealed that a large proportion of acetylated proteins are in the mitochondria (49,55). In search of the enzymes regulating this acetylation, several mitochondrial KDACs were identified (SIRT3, SIRT4, and SIRT5). SIRT3, in particular, was found to be essential for mitochondrial protein deacetylation and critical for metabolism and stress response (125-128). However, a mitochondrial KAT remained elusive. Prior to our studies, only one report suggested the presence of a KAT-like protein in this organelle. The prokaryotic-like 125 amino acid protein named GCN5L1 [GCN5 (general control of amino acid synthesis 5)-like 1] was enriched in the mitochondria of HepG2 (Human hepatocellular liver carcinoma) cells and mouse brain (129). While GCN5L1 possesses a conserved acetyl-CoA binding region, it does not contain a KAT domain. Data did not show a direct acetylation of mitochondrial proteins by GCN5L1 in vivo but indicated that it does affect overall mitochondrial acetylation. In addition, in vitro enzymatic assays required the
presence of mitochondrial extract with GCN5L1 in order to achieve significant acetylation of the substrate. Therefore, GCN5L1 may serve as a cofactor, instead of a true KAT, in a mitochondrial acetylation mechanism. We have shown that TgElp3 is a functional KAT that is targeted to the mitochondrion, albeit its catalytic domains face the cytosol. Our studies place Elp3 as an attractive candidate KAT involved in regulating mitochondrial functions.

C. The C-terminal TMD is necessary and sufficient for targeting TgElp3 to the mitochondrion

The mitochondrial localization of TgElp3 was particularly surprising since we failed to identify a classic N-terminal or internal mitochondrial signal sequence. After investigating the targeting mechanism of TgElp3 using a series of YFP fusion proteins, we determined that the C-terminus (downstream of the radical SAM and KAT domains) was critical for its mitochondrial localization while the N-terminal region of TgElp3 (upstream of the domains) was not. In fact, the 23 amino acid TMD located at the extreme C-terminus was both necessary and sufficient to target TgElp3 to the outer mitochondrial membrane.

Whereas mitochondrial matrix proteins are targeted and inserted in the mitochondria via their N-terminal signal sequence, outer mitochondrial membrane proteins are targeted to the mitochondria based on their TMD and residues surrounding this domain (114,115). Four types of proteins are generally inserted into the outer mitochondrial membrane, those containing a single TMD at their N-terminus, those with a C-terminal TMD, those with two TMDs and those containing multiple β-strands, forming pores or channels through the membrane (130,131). Proteins such as TgElp3 that have a C-terminal TMD inserted into the outer mitochondrial membrane are often referred to as tail-anchored proteins. The mechanism for how these proteins are inserted into the membrane remains poorly understood but the sequences necessary for targeting have been studied in depth.

Tail-anchored proteins are targeted to cellular membranes based on the length and hydrophobicity of the C-terminal TMD as well as the presence of
positively charged residues surrounding the TMD. Several studies have investigated how changes in these properties can redirect a protein to target different membranes such as those of the ER or mitochondria. Tail-anchored proteins are generally targeted to the mitochondria when the TMD is relatively short (approximately 20 amino acids) with a moderate hydrophobicity followed immeasurably by several positively charged residues (115). The C-terminus of TgElp3 contains these characteristic features including the presence of four arginines directly downstream of the TMD.

Another characteristic thought to influence the targeting of a tail-anchored protein is the lipid composition of the membrane. Membranes are made up of different proportions of the varying kinds of lipids, giving each membrane a “lipid signature”. For example, ergosterol, which increases a membrane’s rigidity, is more abundant in the ER membrane than the outer mitochondrial membrane. It has been demonstrated that when the ergosterol content is lowered in the ER, mitochondrial tail-anchored proteins can be targeted to the ER (132). Loss of the TMD in TgElp3 prevents its mitochondrial localization, but with the four arginine residues remaining, the protein can be found at the parasite periphery (Figure 11B). The presence of these positively charged residues likely tethers TgElp3 to the plasma membrane, a membrane whose lipid content may be favored by TgElp3 when the TMD is absent.

D. Elp3 likely has novel substrates in Toxoplasma

Originally, Elp3 was identified as a KAT that acetylated histones, preferentially targeting histone H3, and several studies have confirmed histones as in vitro and in vivo substrates (59,63,66,69). However, with the realization that Elp3 was localized predominantly outside of the nuclear compartment, it was proposed that Elp3 targets additional substrates. In support of this idea, alpha-tubulin was discovered to be acetylated by Elp3 in mouse and human cells (70). Several years later, another study found bruchpilot, a cytoskeleton-like protein present in the active zone of neurons in Drosophila, to be acetylated by Elp3 (133). Since Toxoplasma does not have a bruchpilot homologue, we tested both
histone H3 and alpha-tubulin as potential substrates of TgElp3. As expected, TgElp3 was able to acetylate histone H3 in an in vitro assay (Figure 14A). Containing a large number of acetylatable lysines, H3 can be acetylated by most functional KATs in vitro regardless of whether it serves as a natural substrate in vivo. However, we did not find evidence that TgElp3 acetylated H3 in vivo. Nor did our data suggest alpha-tubulin was a primary target of TgElp3. TgElp3 did not co-localize with either target, and the potential substrates did not show an increase in acetylation when TgElp3 was over-expressed.

Unfortunately, the experiments used to test if H3 and/or alpha-tubulin are substrates of TgElp3 had several caveats and could not definitively prove that TgElp3 does not acetylate them in vivo. Additional experiments could be performed to obtain more conclusive results. However, our localization data is consistent with these findings and suggests that TgElp3 may acetylate novel targets. Hundreds of cytosolic and mitochondrial proteins have been detected as lysine acetylated in many species including Toxoplasma. The positioning of TgElp3 on the cytosolic side of the mitochondrion makes it accessible to both cytosolic substrates and mitochondrial substrates, including outer mitochondrial membrane proteins or those destined for transport into the organelle. Acetylomic analyses of tachyzoites identified both outer mitochondrial membrane proteins, such as the heavily acetylated porin, as well as mitochondrial matrix proteins as being targets of lysine acetylation (54,56). Therefore, TgElp3 likely acetylates novel substrates located near or within the mitochondrion.

E. TgElp3 performs an essential function at the mitochondrion

The function of Elp3 in protozoans is unknown and widely debated in higher eukaryotes. However, disruption of this KAT in several species has been shown to cause significant defects, underscoring the importance of Elp3 in cellular physiology. Deletion of Elp3 in Arabidopsis impaired the mitotic cell cycle as well as leaf polarity (81,82,134). Migration and differentiation of mouse cortical neurons was significantly altered when Elp3 was decreased (70), and deletion in Drosophila caused larval lethality (83,84). Our results strongly support our
hypothesis that TgElp3 is essential for parasite viability. The genomic locus of TgElp3 could only be disrupted when an ectopic copy was present. The parasites apparently require TgElp3 but only a low level of protein is necessary for survival. mRNA and protein expression data provided on the Toxoplasma online database (toxodb.org) indicate that native TgElp3 is expressed at a low level, making conditional knockout or knockdown studies difficult. Using our ΔTgElp3::ddHA TgElp3 clone, we attempted to reduce TgElp3 protein levels by growing parasites in the absence of Shield-1. However, using the destabilization domain approach often results in residual protein present in the cell. We found that even in the absence of Shield-1, ectopic TgElp3 was present at a high enough level for the parasites to function normally. Alternative approaches will be needed to study the role of TgElp3 and why it is essential. In addition to showing TgElp3 is essential, our data indicates that its mitochondrial localization is important for its vital function. We were unable to obtain viable parasite clones when the region encoding the TMD was removed from the genomic locus encoding TgElp3. The TMD of TgElp3 is necessary for its mitochondrial localization and required for parasite survival.

The mitochondrion has been a largely understudied organelle in Toxoplasma, and its functions in the parasite are poorly understood. Furthermore, several mitochondrial components and pathways appear to be lacking in Toxoplasma. For example, the Toxoplasma genome contains a few predicted orthologues of the mitochondrial import system such as Tim17, Tim22, Tim23, Tim44, Tom40 and heat shock protein 60 (135,136) but most of the import machinery has yet to be identified, and the mechanism of protein import is unknown. In general, the most well-known function of mitochondria is the involvement of the tricarboxylic acid cycle and electron transport chain in the production of ATP. Most components of the electron transport chain are encoded in the genome with the exception of some subunits of the ATP synthase complex but this pathway has yet to be characterized. While the Toxoplasma mitochondrion generates an electrochemical gradient, as indicated by the uptake of stains such as MitoTracker, the importance of mitochondrial respiration is not
known. Other important functions of the mitochondria that remain poorly understood include iron-sulfur cluster synthesis, heme biosynthesis, and acetyl-CoA production. One reason for the lack of information regarding these mitochondrial processes in *Toxoplasma* is because they also occur in the apicoplast, a more intensely studied organelle due to its unique and essential presence in apicomplexans.

The function of TgElp3 at the mitochondrion is unknown and may require the radical SAM domain, KAT domain, or a combination of the two. We have shown that TgElp3 can function as a KAT, and homology of the radical SAM domain suggests it may also have catalytic activity. As previously mentioned, the orientation of TgElp3 in the outer mitochondrial membrane allows for interaction with cytoplasmic and mitochondrial associated proteins. This position is ideal for detecting cellular stress and relaying the signal between the mitochondrion and the rest of the cell. TgElp3 may acetylate outer mitochondrial membrane proteins and/or proteins that are actively being translocated into the mitochondrion. For example, acetylation of the outer mitochondrial membrane pore-forming protein porin by TgElp3 may regulate the passage of proteins through the mitochondrial membrane. In addition to KAT activity, it is probable that TgElp3 performs other functions at the mitochondrial surface through its radical SAM domain. Radical SAM domains form iron-sulfur clusters that are used to catalyze reactions by binding and cleaving SAM, forming the 5’-deoxyadenosyl radical. Iron-sulfur clusters are critical components of the electron transport chain, several metabolic processes, and mitochondrial iron homeostasis (137-139). Perhaps the iron-sulfur cluster of TgElp3 is critical for iron maintenance at the mitochondrion. Alternatively, the two domains may work together, requiring both to perform the function of TgElp3. Further studies are necessary to pinpoint the vital role TgElp3 plays in parasite physiology and the contribution of its catalytic domains.
F. TgElp3 as a potential drug target

We have shown TgElp3 to be a unique KAT with an outer mitochondrial membrane localization that is essential for parasite survival. TgElp3 has several characteristics that warrant further investigation as a potential drug target for treating Toxoplasma infection. Since it is essential, inhibiting TgElp3 could kill tachyzoite parasites during acute infection and potentially help prevent transfer of parasites from mother to fetus. Current KAT inhibitors work by binding and inhibiting the highly conserved KAT domain but because the KAT domain is present in several different proteins, these inhibitors are often non-selective and cause off-target effects. In addition, KATs are often highly conserved between species, and compounds can inhibit both the parasite and human host enzymes, causing toxicity. Fortunately, TgElp3, which could still be targeted with KAT inhibitors, could also be targeted more specifically thanks to the presence of a unique TMD not present in the human Elp3 homologue. By disrupting the targeting of TgElp3 to the mitochondrion, which we have shown to be lethal, it may be possible to specifically inhibit a critical enzyme in a parasite-specific manner. TgElp3 also has N- and C-terminal extensions that have no homology to human proteins. By targeting a different region of the protein, we may also be able to specifically inhibit TgElp3.
V. FUTURE STUDIES

The work described in this thesis was the first characterization of an Elp3 homologue in *Toxoplasma*. Our exciting findings have justified the continued study of TgElp3 and have led to a number of new questions to be answered. A key question that remains unanswered is, “What is the function of TgElp3 at the mitochondrion?” Our data suggest that its mitochondrial localization is vital for parasite survival but we are unclear what role(s) it plays at the outer mitochondrial membrane.

A. Uncovering the role of the radical SAM and KAT domains

Several approaches can be taken towards addressing the above question. One approach would be to determine the effect of each domain, radical SAM and KAT, on the parasites. We have shown that TgElp3 possesses KAT activity and that its radical SAM domain contains the conserved sequence necessary for catalytic activity. Therefore, we would like to test if one or both of the domains is necessary for TgElp3’s function by mutating key residues within the domains that would eliminate activity. Previous studies have shown that mutating two tyrosine residues in the KAT domain to alanines (Y715A and Y716A) greatly reduces activity (63,140). Also, radical SAM domain function can be disrupted by mutating any of the key cysteine residues in the CxxxCxxC conserved motif to alanine (C280A, C284A, or C287A) (62,140). Ideally, mutations would be made in the genomic locus of TgElp3, but alternatively, an ectopic copy of mutated cDNA could be introduced into the genome followed by deletion of the endogenous locus. Resulting parasites could be studied for defects in growth, and subsequent analysis could be performed to determine what aspect of parasite biology is affected by mutation of each or both of the domains.

B. Using an inducible system to study TgElp3 function

Another global approach to pinpointing the function of TgElp3 would be to create an inducible knockdown or knockout of TgElp3. Since we were unable to knockout the endogenous locus, inducible systems will need to be employed in
order to study the role of TgElp3. It is important to note that RNAi techniques have largely been unsuccessful in Toxoplasma. However, there are currently two independent techniques that could be attempted. One involves the use of the destabilization domain as described in section III-K. We suspect our attempt using this system was unsuccessful because low levels of TgElp3 are sufficient for function and our clones, when knocked-down, still contained sufficient levels of enzyme. This result was most likely due to the introduction of multiple ectopic copies that were expressed using the strong Toxoplasma tubulin promoter. A more tightly regulated system could be obtained by introducing a single ectopic copy into the genome expressed under the native TgElp3 promoter. Therefore, when the endogenous locus is removed, the ectopic copy would be expressed similarly to endogenous. In the absence of Shield-1, very little or no TgElp3 should be present and a phenotype could be observed. The other inducible approach would be a modified version of the cre-lox system that was recently established in Toxoplasma (141). Cre recombinase, the enzyme responsible for recognizing loxP sites and excising the gene of interest, would be introduced into the Toxoplasma genome in two inactive fragments that requires rapamycin in order to dimerize and become functional (142). LoxP sites would then be introduced at the TgElp3 locus in parasites expressing the two Cre recombinase fragments. With the addition of rapamycin, the locus would be removed and the TgElp3 knockout phenotype could be studied. This technique was used by Andenmatten et al to create conditional knockouts of the following genes in Toxoplasma: gliding motor myosin A, the micronemal protein MIC2 and actin (141). All three proteins, previously determined to be essential, were able to be studied and were identified as part of a novel parasite invasion mechanism.

C. Identification of TgElp3 substrates

An additional tactic for uncovering the function of TgElp3 is to identify its substrates. As was mentioned in the discussion, TgElp3 is an outer mitochondrial membrane KAT with its domains facing the cytosol, allowing it access to a number of cytosolic and mitochondrial substrates. Our Toxoplasma acetylome
analysis identified the outer mitochondrial membrane porin to be highly acetylated. Based on proximity, porin would be an ideal protein to test as a potential substrate of TgElp3. However, testing potential substrates one-by-one is time consuming. Therefore, more global studies should be employed to identify proteins targeted by TgElp3. One such technique would be a parasite-wide acetylome analysis to compare acetylated residues in a wild-type strain versus a TgElp3 knockdown, with the expectation that substrates of TgElp3 would have lower levels of acetylation in the knockdown strain. Additional in vitro and in vivo experiments would be required to confirm that those found in the analysis were true substrates. An alternative strategy would be the application of click-chemistry in a global search for substrates. In this approach, purified TgElp3 would be added to total parasite lysate along with 4-pentynoyl-coenzyme A, an alkynyl chemical reporter. Therefore, substrates acetylated by TgElp3 will also contain the reporter, which can then be attached to biotin and isolated for identification by mass spectrometry. By identifying substrates of TgElp3 we will have a better understanding of the cellular pathways it regulates and how it functions.

D. Establishing TgElp3 as a drug target

Ultimately, we hope that future studies involve the identification of compounds to target TgElp3 for both therapeutic benefit and as useful reagents for the further study of this KAT. All of the studies described in this thesis used a type I, hypervirulent strain of Toxoplasma not normally found in human infections. Future studies should include the disruption of TgElp3 in a type II strain that can form bradyzoite cysts. If TgElp3 were found to be essential in this strain as well, it would further underscore its suitability as a target for therapeutic development. Another step in drug development would be a large-scale screen of compounds to identify those that inhibit TgElp3. By identifying or designing small molecule inhibitors, we could pharmacologically inhibit and study the function of TgElp in Toxoplasma and other apicomplexans. Currently, no TgElp3-specific inhibitor
exists. We would also be able to use those compounds in a mouse model of infection, bringing this research one step closer from the bench to the bedside.
VI. SUMMARY

Acetylation is a critical post-translational modification regulated by multiple KATs throughout the cell. Histone acetylation has been the best characterized to date, in part due to the nuclear localization of well-known KATs such as GCN5. However, with the increased availability and sensitivity of large-scale proteomics studies, global cellular acetylation has been characterized for several species, and the extent of protein acetylation is just now being understood. The challenge has now become to identify the enzymes responsible for protein acetylation and the role of this post-translational modification in regulating cellular pathways.

Several advances have been made in identifying novel substrates of well-known KATs and characterizing previously unidentified KATs.

This thesis describes the first Elp3 KAT 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. These unexpected findings prompted us to test if Elp3 localizes to the mitochondria in other species, and we found a processed form of mouse Elp3 localized to the mitochondria in neural cells. Since most Elp3 homologues, including mouse, do not contain the C-terminal TMD, the localization and perhaps function of Elp3 may be conserved but the targeting mechanism differs between Toxoplasma and other species.

We established that TgElp3 is essential for parasite survival and that this is dependent on its mitochondrial localization. The precise function of TgElp3 currently remains unclear but a number of approaches outlined above can be used to identify its substrates and the role(s) of TgElp3 in parasite physiology. Identifying the substrates and functions of Elp3 in ancient eukaryotes, particularly apicomplexan parasites, which possess homologues that are likely mitochondrial tail-anchored proteins, will provide a much better understanding of the
importance of this protein throughout evolution and may open avenues for targeted therapeutic development.
VII. REFERENCES


CURRICULUM VITAE

Krista L. Stilger

Education

2008-2013 Doctor of Philosophy in Pharmacology
Indiana University
Indianapolis, IN

2004-2008 Bachelor of Science in Biology
University of Evansville
Evansville, Indiana

Research Experience

2008-2013 Graduate research
Indiana University School of Medicine, Department of Pharmacology and Toxicology
Indianapolis, Indiana
Mentor – William J. Sullivan, Jr., Ph.D., Associate Professor Pharmacology & Toxicology, Microbiology and Immunology

For my thesis research, I studied the functions and therapeutic potential of a novel lysine acetyltransferase, Elp3, in the protozoan parasite Toxoplasma gondii. I used multiple genetic, molecular, biochemical, and parasite-specific techniques. My thesis work resulted in the identification of the first mitochondrial localized lysine acetyltransferase in any species.

Summer 2007 Undergraduate research
Indiana University School of Medicine – Evansville
Evansville, Indiana
Mentor – Cezary Wójcik, M.D., Ph.D., Assistant Professor Anatomy and Cell Biology

I worked on a project to elucidate the roles of Npl4 in the endoplasmic reticulum-associated degradation (ERAD) pathway. Npl4 mutants were generated and the effect of these mutants on ER stress was determined by monitoring ER stress markers such as BIP and XBP1 in HeLa cells. I was exposed to human cell culture, biochemical, and molecular techniques.
Summer 2006 Undergraduate research
University of Evansville
Evansville, Indiana
Mentor – Cris Hochwender, Ph.D., Assistant Professor
Department of Biology

I spent the summer collecting and organizing data on glucosinolates in *Brassica rapa* to determine the costs and benefits of these chemicals as a defense mechanism. While analyzing the data, I learned several statistical tests using JMP software.

**Peer-Reviewed Publications**


**Poster & Presentation Abstracts**

Stilger K and Sullivan Jr WJ. *Toxoplasma gondii* lysine acetyltransferase TgElp3 found in an unexpected place. Midwest Neglected Infectious Disease Meeting, Notre Dame, IN 2013.


**Fellowships & Grants**

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<tr>
<th>Date</th>
<th>Description</th>
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<tr>
<td>July 2013</td>
<td>Paradise Travel Fellowship to attend the Midwest Neglected Infectious Diseases Meeting in Notre Dame, IN</td>
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<tr>
<td>1 July 2012-30 June 2014</td>
<td>American Heart Association grant entitled “Elucidating the roles of a novel lysine acetyltransferase, TgElp3, in the heart pathogen <em>Toxoplasma gondii</em>” 12PRE11940015</td>
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<tr>
<td>May 2012</td>
<td>IUPUI Travel Fellowship to attend the 2011 Molecular Parasitology Meeting in Woods Hole, MA to present abstract entitled “Unusual properties of TgElp3, the <em>Toxoplasma gondii</em> elongator lysine acetyltransferase”</td>
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<tr>
<td>1 July 2010-30 June 2012</td>
<td>Indiana Clinical and Translational Sciences Institute (CTSI) Career Development Award PHS (NCCR) 5TL1RR025759-03</td>
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Honors & Awards

June 2013  Indiana University Dept. of Pharmacology & Toxicology K.K. Chen Award

Aug. 2011  Midwest Neglected Infectious Disease Meeting poster award

Aug. 2011  Sigma Xi Research Presentation Competition, Honorable Mention

May 2008  Graduated Cum Laude, B.S. Biology, University of Evansville

Teaching & Mentoring Experience

*Indiana University School of Medicine* - Indianapolis, IN

May-June 2012  Mentored MD/PhD student Sherri Huang  
*Indiana University School of Medicine* - Indianapolis, IN

June-Aug. 2010  Mentored undergraduate student Krista Podell  
*Indiana University School of Medicine* - Indianapolis, IN

2006-2007  Undergraduate Biology Writing Assistant  
*University of Evansville* - Evansville, Indiana

2005-2007  Undergraduate General Biology and Genetics Laboratory Assistant  
*University of Evansville* - Evansville, Indiana

Memberships

2012-2013  Sigma Xi – Indiana University Medical Center Chapter
2009-2012  American Society for Microbiology
2009-2012  American Association for the Advancement of Science