LYSINE ACETYLTRANSFERASE GCN5-B REGULATES THE EXPRESSION OF CRUCIAL GENES IN *TOXOPLASMA* AND ITS FUNCTION IS REGULATED THROUGH LYSINE ACETYLATION

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ADA	alteration/deficiency in activation protein
AIDS	acquired immune deficiency syndrome
AP2	Apetala2
BSA	bovine serum albumin
CAM	chloramphenicol
CAT	chloramphenicol acetyltransferase
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation coupled to microarray
CNS	central nervous system
Co-IP	co-immunoprecipitation
DAPI	4',6'-diamino-2-phenylindole
DD	destabilization domain
ddH_2O	double distilled H_2O
DHFR	dihydrofolic acid reductase
DHFR-TS	dihydrofolic acid reductase-thymidylate synthase
DHPS	dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DN	dominant-negative
DNA	deoxyribonucleic acid
FITC	fluorescein isothiocyanate

GCN5	general control non-derepressible 5
gDNA	genomic DNA
HA	hemagluttin
KAT	lysine acetyltransferase
HAT	histone acetyltransferase
KDAC	lysine deacetylase
HFF	human foreskin fibroblast
IFA	immunofluorescent assay
IP	immunoprecipitation
kDa	kiloDalton
mRNA	messenger ribonucleic acid
MYST	MOZ, Ybf2/Sas3, Sas2, and Tip60 members
PCAF	p300/CBP associating factor
PCR	polymerase chain reaction
RNA	ribonucleic acid
SAGA	Spt-ADA-GCN5 acetyltransferase complex
SDS	sodium dodecyl sulfate

CHAPTER 1

Introduction

Toxoplasma gondii: a model apicomplexan

Toxoplasma is a protozoan parasite often used as a model system to study the biology of other protozoa in its phylum, the Apicomplexa [1]. There are almost 5,000 different species in the phylum Apicomplexa. Apicomplexan parasites are characterized by a polarized cell structure with two unique apical secretory organelles named micronemes and rhoptries. These organelles are responsible for parasite adhesion and invasion. Other members of the phylum include *Plasmodium*, the causative agent of malaria; *Eimeria*, which causes coccidiosis in poultry; and *Cryptosporidium*, which is another important opportunistic pathogen of AIDS patients. Unlike most other apicomplexans, *Toxoplasma* is easy to culture in the laboratory and is amenable to genetic manipulation. Experimental advantages include the ability to make transgenic parasites, including gene fusion, knockouts, or stable ectopic expression. The genome sequence is available from the *Toxoplasma* online database (http://toxodb.org/toxo/), facilitating molecular cloning and gene analyses. Additionally, there is a well-established mouse animal model to investigate *Toxoplasma* pathogenesis and host responses. Studies in *Toxoplasma* can facilitate our understanding of apicomplexan pathogenesis, so as to contribute to drug development against this phylum of parasites.

Life cycle of *Toxoplasma gondii*

Toxoplasma gondii is an obligate, intracellular parasite that causes toxoplasmosis. This parasite has the ability to infect almost all warm-blooded animals,

including humans and livestock. This protozoan parasite occurs worldwide and is estimated to infect approximately one-third of the world's population [2]. *Toxoplasma* has a complex life cycle consisting of a sexual cycle in its definitive host and an asexual cycle in its intermediate hosts. The sexual replication of the parasites can only take place in the intestine of felids, its definitive host, which excrete oocysts into the environment during infection [3]. Once exposed to the environment, sporulation of oocysts occurs, resulting in the formation of highly infectious sporozoites. Sporulated oocysts contaminate the environment, including soil and water, and can persist in the environment for up to 18 months [4]. An intermediate host can be infected by consuming undercooked meat of infected animals or water that contains oocysts [5]. If an animal consumes oocysts, sporozoites inside the oocysts will invade the epithelial cells of the intestine and then differentiate into tachyzoites, the rapidly replicating form of the parasites.

Tachyzoites are capable of infecting any nucleated cell and are responsible for tissue destruction. Tachyzoites divide asexually through a process called endodyogeny. The parasites keep replicating within the host cell vacuole with a generation time of 6-8 hours until they break the host cell and egress [6]. The parasites then invade and replicate within neighboring host cells. The replication of tachyzoites creates the acute phase of toxoplasmosis. The differentiation of tachyzoites to bradyzoites happens 7-10 days post-infection. Bradyzoites cause chronic infection by forming cysts in tissues, such as the central nervous system and muscle tissues. Tissue cysts can protect the parasites from the immune system, and as a consequence they can reside in host tissues for life. Humans can be infected by bradyzoites by ingesting undercooked meat. The cyst wall can be degraded by the digestive enzymes, causing bradyzoite release. The bradyzoites then invade the intestinal cells and differentiate back into tachyzoites. The reactivation of the parasites is normally prevented by the host immune response.

However, in immunocompromised individuals, reactivation of infection is continuous, therefore resulting in life-threatening damage to sensitive tissues.

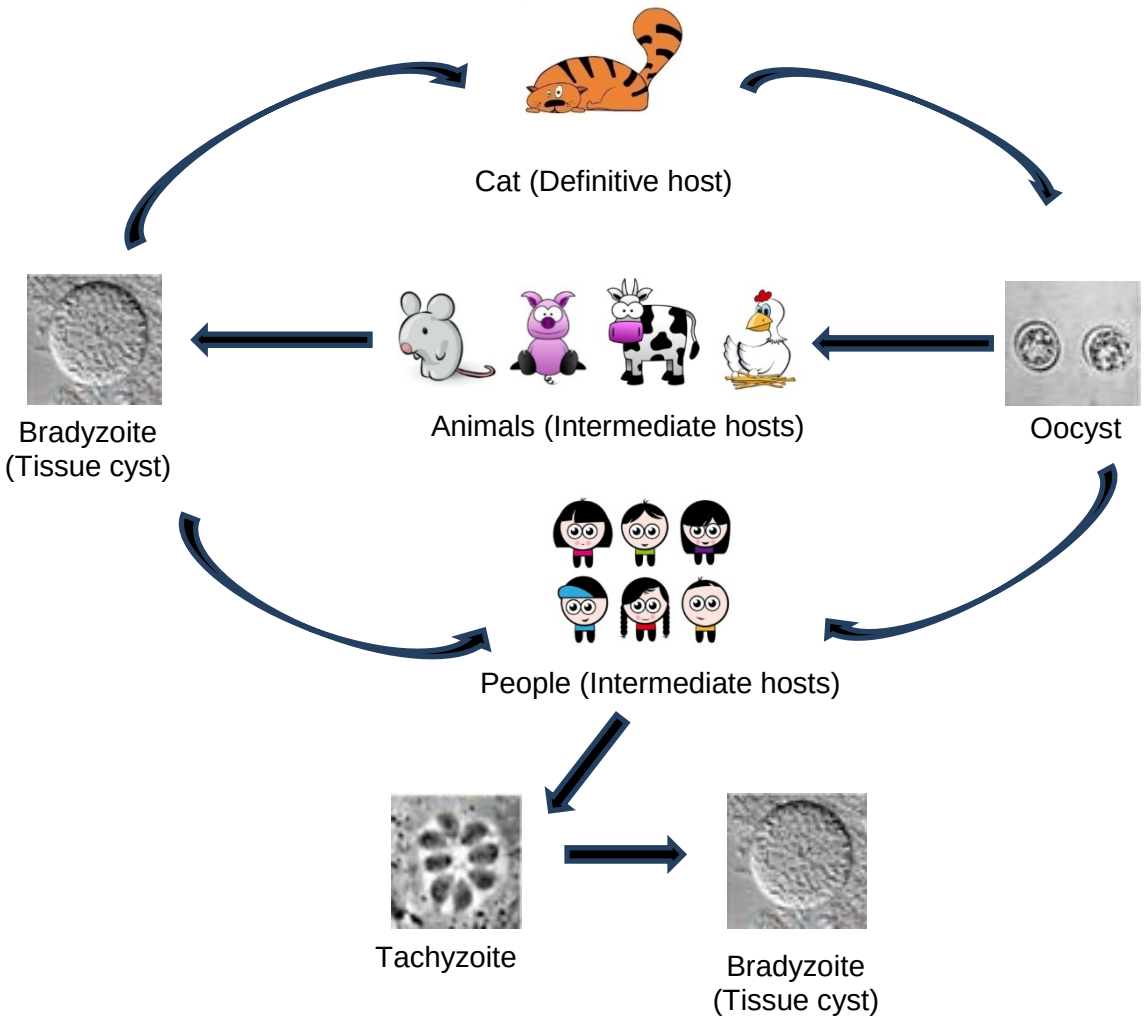


Figure 1. Life cycle and transmission of *Toxoplasma gondii*.

The sexual cycle of *Toxoplasma* starts with its definitive host, the cat. Sexual reproduction of the parasites results in the formation of oocysts, which will be shed into the environment through cat feces. Oocysts are highly infectious and can infect almost

all warm-blooded animals. Humans can become infected by consuming undercooked meat, contaminated water, or unwashed fruits and vegetables. After entering the host, oocysts will form tachyzoites, which will cause acute infection. Stress from the host immune response will induce tachyzoites to differentiate into bradyzoites. Bradyzoites form tissue cysts and can cause chronic infection.

Toxoplasmosis and treatments

Toxoplasmosis, caused by *Toxoplasma*, is a severe and life-threatening disease in immunocompromised patients and in developing fetuses. Under normal conditions, infection by *Toxoplasma* is asymptomatic. However, in immunocompromised individuals, such as AIDS patients, organ transplant recipients, and patients undergoing chemotherapy, reactivation of *Toxoplasma* can cause encephalitis, neurological disorders, and even death [7, 8]. Congenital toxoplasmosis is caused by vertical transmission of the parasites from a pregnant woman to her fetus, who may develop symptoms including deafness, chorioretinitis, and developmental delay [9]. Though this pathogen has developed a worldwide prevalence, there is still no vaccine available for humans. A combination therapy of pyrimethamine and sulfonamides is the standard treatment for toxoplasmosis. Current treatments have certain limitations, such as a narrow therapeutic window of pyrimethamine and allergic reactions caused by sulfonamides. Pyrimethamine may also cause bone marrow suppression and megaloblastic anemia [10]. Allergic reactions associated with sulfonamides include skin rashes and epidermal necrosis. More importantly, current available drugs can only eliminate tachyzoites but cannot act against bradyzoites. AIDS patients and immune-compromised patients have to be placed on long-term prophylaxis treatment to prevent the reactivation of bradyzoites to tachyzoites. Pyrimethamine is not a safe drug for

pregnant women due to its teratogenic effects. The preferred treatment is spiramycin, which is unable to cross the placenta, but is believed to block vertical transmission.

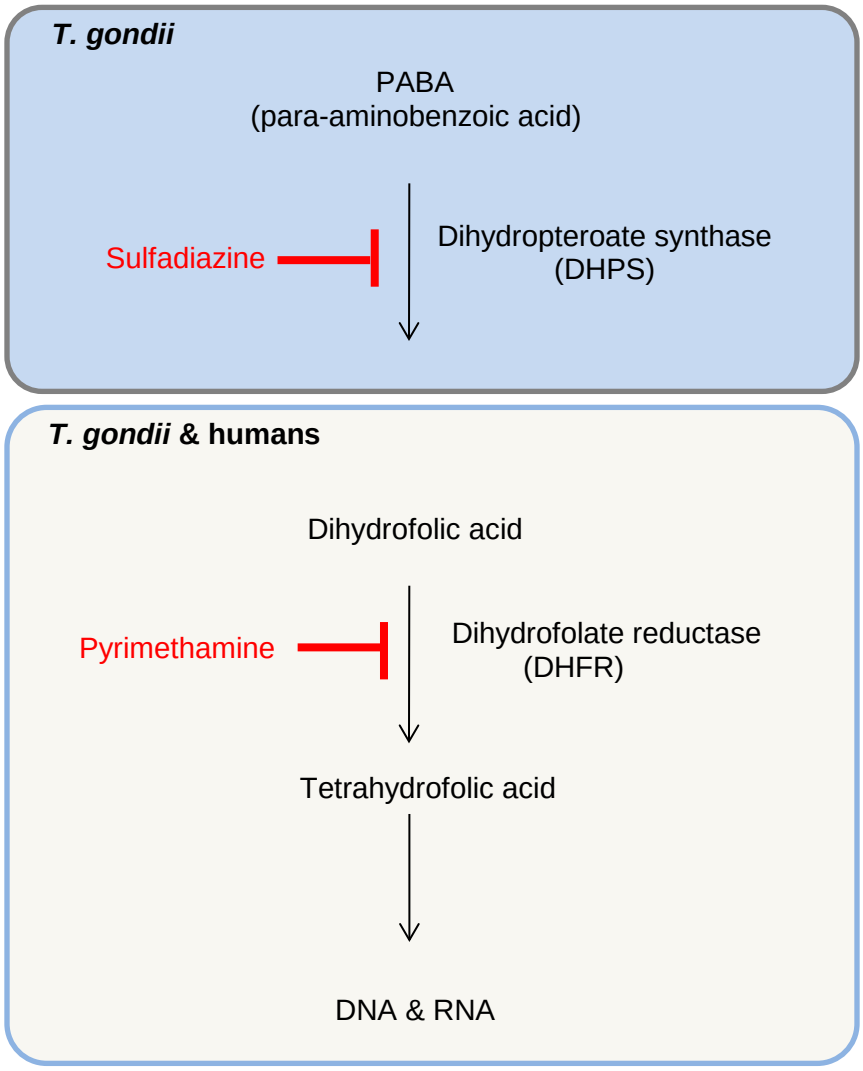


Figure 2. Treatment of toxoplasmosis.

The current treatment for toxoplasmosis is a combination of pyrimethamine and sulfonamides. Pyrimethamine, as a competitive inhibitor of *Toxoplasma* dihydrofolate reductase (DHFR), can cause adverse side effects because it is only six times as selective for the protozoal form of the enzyme over the mammalian form [11]. Sulfonamides targets dihydropteroate synthetase, an enzyme not found in mammalian

cells, but the patients usually develop severe allergic reactions [10]. These two drugs directly affect parasite amino acid synthesis pathways.

Lysine acetylation and lysine acetyltransferase (KAT)

Acetylation transfers an acetyl group from acetyl coenzyme A to the epsilon-amino group of a lysine residue, thus neutralizing the positive charge of lysine. This process is catalyzed by lysine acetyltransferases (KATs) and reversed by lysine deacetylases (KDACs). In 1996, Allis et al discovered the first KAT, GCN5, and the first KDAC, HDAC1, both of which were suggested to control transcription through acetylating histones [12, 13, 38]. Since then, a great deal of progress has been made to understand the roles of acetylation in transcription regulation, nucleosome remodeling, and chromatin assembly. When GCN5 histone acetyltransferases were found to have non-histone substrates as well, they became referred to as lysine (K) acetyltransferases (KATs) [40]. Global lysine acetylation analysis has been applied to different species from human cells, plants, *Drosophila*, lower eukaryotes, and bacteria. Using a human acute myeloid leukemia cell line, the Mann group identified 3600 lysine acetylation sites on 1750 proteins [14]. In *E. coli*, 1070 acetylation sites on 349 acetylated proteins were identified [15]. The Choudhary group identified 1981 lysine acetylation sites in the proteome of *Drosophila*. In *Arabidopsis*, 91 lysine acetylation sites on 73 proteins of diverse functional classes were identified. Our lab identified over 700 lysine acetylation sites on a wide variety of parasite proteins in *Toxoplasma* [17]. These “acetylome” studies have expanded our understanding of cellular signaling, suggesting that acetylation plays key roles in cytoplasmic functions in addition to well-characterized nuclear functions. Acetylation preferentially targets large macromolecular complexes and is suggested to play conserved functional roles during evolution [14].

Acetylation regulates transcription through modifying the amino-terminal tails of histones. Histone proteins and DNA are major components of chromatin existing in eukaryotic cell nucleus. The nucleosome, the basic repeating unit of chromatin, consists of 146bp of DNA wrapped around a histone octamer with two copies of H2A, H2B, H3, and H4. Nucleosomal DNA is generally repressive to transcription. However, acetylation increases the decompaction of chromatin by loosening the histone tail - DNA binding. The dynamic acetylation state of histone tails enhances transcription factor access [18, 19]. Many KAT and KAT-containing complexes have been demonstrated to function in transcriptional activation. Nuclear KATs can be divided into five families, GCN5-related N-acetyltransferases (GNATs); the MYST (for 'MOZ, Ybf2/Sas3, Sas2 and Tip60')-related KATs; p300/CREB binding protein (CBP) KATs; the general transcription factor KATs including the TFIID subunit TBP-associated factor-1 (TAF1); and the nuclear hormone-related KATs, SRC1 and ACTR (SRC3) [20]. Other than transcription, recent data suggests acetylation is a common modification on proteins involved in other nuclear processes, such as splicing, cell cycle, chromatin remodeling, DNA replication, and nuclear transport [14].

Our laboratory first produced the proteome-wide analysis of acetylation for *Toxoplasma*. Over 700 novel acetylation sites were detected on a wide variety of proteins, mostly involved in metabolism, translation, chromatin biology, and stress response. The Sullivan laboratory discovered K23, K79 on histone H3 and K31 on histone H4, as novel acetylation marks in Apicomplexa. In addition to histones, we also discovered acetylation sites on many KATs in *Toxoplasma*. The two members of GCN5 family, TgGCN5-A and TgGCN5–B both are acetylated. TgGCN5-A is acetylated at its bromodomain, which is responsible for binding to acetylated lysine [16]. TgGCN5-B is highly acetylated at its unique C terminal-tail. MYST family KATs, TgMYST-A and -B, are also acetylated. They exist in long and short forms in the parasites. Both TgMYST-A and

TgMYST –B are acetylated within the N-terminal extension of the long form of the protein. TgMYST-A is also acetylated at its catalytic domain. Moreover, acetylation is detected on proteins unique to Apicomplexa, such as Aptela-2 (AP2) domain containing proteins and rhoptry proteins. Interestingly, 32% of the acetylome proteins are parasite specific, implying a potential role of lysine acetylation as novel drug target.

Histone acetylation and diseases

The competing actions of KAT and KDAC create a balance of acetylation and deacetylation capable of nimble responses to environmental factors. Acetylation by KATs neutralizes the positive charge of lysine on histones, decreasing the ionic interaction between the positively charged histones and negatively charged DNA; the action of KATs therefore leads to less compact chromatin. Loose chromatin is more accessible to transcription machinery. HDACs regulate chromatin structure in a reverse way. Any imbalance of this process may lead to a dysfunction of transcription, DNA repair, recombination, and replication. In recent years, there has been significant growth in our knowledge about the involvement of abnormal histone acetylation patterns in diseases.

Abnormal histone acetylation has been linked to tumor development due to the aberrant transcription of genes regulating important functions such as proliferation, cell cycle, and apoptosis. Several events can result in abnormal acetylation patterns: changes in KATs or KDACs activities; changes in expression levels of KATs or KDACs; altered interactions with other proteins such as transcription factors. Studies of human cancers, such as skin cancer and gastrointestinal cancers, suggest a decrease of global acetylation, especially on histone H3 and H4 [21, 22]. These changes can be caused by a decrease in KAT activity or an increase in HDAC activity. Changes of HDAC

expression levels occur in different cancers. For example, there is an increase in HDAC1 expression in gastric and prostate cancers [23, 24]. Overexpression of HDAC2 has been found in cervical and gastric cancers [25, 26]. There are studies indicating that HDACs can directly interact with transcription factors such as E2f, Stat3, p53, NF-kB, whose loss of functions have been observed in many tumors and have attracted attention as drug targets in treating cancers [27]. There are few reports directly implicating the roles of KATs in cancers. Some members of MYST KAT family have been suggested to exist in the complex with ING tumor suppressor family [28].

KATs and KDACs also play important roles in neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease [29]. During neurodegenerative conditions, acetylation homeostasis is disrupted. The best studied KAT in neurodegeneration diseases is CBP. Several observations indicate that neurons are losing CBP and p300 KATs during neurodegeneration. On the other hand, overexpression of CBP increases neuronal viability [30, 31]. Various mechanisms behind the loss of acetylation homeostasis are known. An increase of proteosomal degradation of CBP has been reported in several neurodegenerative diseases [31]. Additionally, caspase -6-dependent CBP proteolysis is another mechanism to compromise the normal function of CBP [30]. Also, CBP is seen to be relocated into Huntington aggregates, thus having trouble to function properly in the nucleus [32]. Studies also suggest that HDAC inhibitors, like TSA and suberoylanilide hydroxamic acid (SAHA), may prevent neuronal apoptosis during oxidative stress [33].

Autoacetylation of KATs

Recently, global acetylation data indicates most subunits of KAT complexes are acetylated. Interestingly, many known KATs are acetylated themselves [34, 35, 36, 37].

A possible mechanism is KAT autoacetylation, and this may be a means to regulate its own function. Recently, there are some reports about KAT autoacetylation. The catalytic activity of KAT can be regulated by autoacetylation at certain active site lysine residues. For example, mutation of K274 to Arg on MOF, a MYST KAT, almost completely abolishes its acetylation activity on H4 [34]. Autoacetylation of Rtt109, an ortholog to p300 KAT, happens at K290. Autoacetylation stimulates Rtt109 KAT activity by increasing acetyl-CoA binding affinity [35]. PCAF, a homolog of GCN5, is acetylated within the nuclear localization signal. Catalytically inactive forms of PCAF accumulate in the cytoplasm, indicating that autoacetylation may control PCAF nuclear translocation [36]. Tip60, a member of MYST family, regulates its dissociation of oligomer and interaction with substrates through an autoacetylation mechanism [37]. Our acetylome data revealed that TgGCN5-B is acetylated at K857, within the putative ADA2-binding domain, and at three lysines at C-terminus. Our lab has identified four acetylated lysines on TgGCN5-B, K857, K989, K997 and K1027.

Toxoplasma GCN5 KATs

Lysine acetylation on histones is a well-characterized post-translational modification linked to the activation of gene expression. GCN5 is a highly conserved catalytic component present in multiple protein complexes linked to the regulation of gene expression [39]. The number of GCN5 KATs and their impact on cells or organisms depends on the species. GCN5 generally appears to be required for stress responses [41, 42, 43]. Consistent with this idea, the single GCN5 is dispensable in *Saccharomyces cerevisiae*, but required for growth on minimal media [44]. In contrast, mammals possess two GCN5 family members, only one of which is required for mouse

embryogenesis [45]. Null mutants of PCAF, have no discernible phenotype in mice [45, 46].

The relevance of lysine acetylation in pathogenic protozoa is underscored by potent antiprotozoal activity of lysine deacetylation inhibitors like apicidin and FR235222 [47, 48]. Histone acetylation has also been linked to a number of processes that underlie pathogenesis of apicomplexan parasites, including antigenic variation in *Plasmodium* (the protozoan causing malaria) or developmental transitions in *Toxoplasma gondii*, which can cause congenital birth defects or life-threatening opportunistic infections in immunocompromised individuals [62, 49, 50]. An extensive repertoire of histone modification machinery is present in these parasites, suggesting that epigenetic-based regulation contributes to gene expression control [51]. A related oddity regarding regulation of gene expression in Apicomplexa is that these early-branching eukaryotes appear to use an expanded lineage of so-called Apetela-2 (AP2) proteins as transcription factors rather than the master regulators conserved throughout most of the eukaryotic kingdom [52, 53]. AP2 proteins harbor a plant-like DNA-binding domain and emerging evidence supports that at least some of them function as *bona fide* transcriptional regulators [54, 55].

Toxoplasma has a number of unusual features with respect to its GCN5 KATs. First, there are two GCN5-family members in *Toxoplasma* (TgGCN5-A and –B) whereas other invertebrates, including *Plasmodium*, possess only one [56, 57]. Second, both TgGCN5s have long N-terminal extensions devoid of known protein domains. These N-terminal extensions are not homologous to those seen in higher eukaryotes, nor are they homologous to each other or other apicomplexan GCN5s [57]. One function of the GCN5 N-terminal extensions is to translocate the KAT into the parasite nucleus via a basic-rich nuclear localization signal [58, 59]. Yeast two-hybrid studies have suggested that the N-terminal extension of *Plasmodium* GCN5 plays a major role in mediating

protein-protein interactions [60]. We previously generated a gene knockout of TgGCN5-A, but similar methods have not produced viable TgGCN5-B knockouts. TgGCN5-A was found to be dispensable for parasite proliferation *in vitro*, but required for the parasite to respond properly to alkaline stress [42]. These findings are consistent with the well-documented role of GCN5 KATs in the cellular stress response. The goal of this thesis was to devise a novel way to define the role of TgGCN5-B in *Toxoplasma* and begin to understand the role of acetylation of TgGCN5-B.

Hypothesis and aims

In *Toxoplasma*, precise control of gene expression is crucial for its complex life cycle and rapid proliferating stage, but this topic remains poorly understood. *Toxoplasma* lacks conventional transcription factors operating in other eukaryotes [3]. However, a rich but largely unexplored repertoire of chromatin remodelers and histone-modifying enzymes has been discovered in the parasites [4]. These findings led to the speculation that epigenetics and histone modification significantly contribute to the regulation of gene expression in *Toxoplasma*. Our preliminary data using a dominant-negative TgGCN5-B mutant parasite clone suggests that TgGCN5-B is important for parasite viability. Additionally, we have found that TgGCN5-B is acetylated *in vivo*, suggesting that its function may be regulated at a post-translational level. **We hypothesize that TgGCN5-B regulates the expression of crucial genes in *Toxoplasma* and its function is regulated through lysine acetylation.** This hypothesis will be examined by performing the following specific aims:

Aim 1: Determine how expression of an inactive form of TgGCN5-B affects parasite viability.

Aim 2: Study if lysine acetylation regulates the function of GCN5-B.

CHAPTER 2

Materials and Methods

Note: Biochemistry, proteomics, and ChIP-chip studies were performed in collaboration with Dr. Kami Kim's laboratory at Albert Einstein College of Medicine. Generation of tagged AP2 transgenic parasites and reciprocal co-immunoprecipitations were performed in collaboration with Dr. Kevin Liu (Sullivan lab, IUSM). Additional assistance with parasite replication assays was provided by Dr. Vicki Jeffers (Sullivan lab, IUSM). The dominant-negative TgGCN5-B clone was generated by Dr. Stacy Dixon (Sullivan lab, IUSM).

Parasite culture and methods

All *Toxoplasma* lines (RH strain) were propagated in monolayers of human foreskin fibroblasts (HFFs) in Dulbecco modified Eagle's medium (DMEM) supplemented with 1% heat-inactivated fetal bovine serum (Gibco/Invitrogen). Cultures were maintained in a humidified, 37°C incubator with 5% CO_2. To isolate parasites for experiments, intracellular tachyzoites were harvested through syringe passage of infected host cells followed by filtration through a 3 micron filter [79]. Where designated, Shield-1 (CheminPharma), dissolved in ethanol, was added to culture medium. Plasmids were introduced into *Toxoplasma* via electroporation, subjected to drug selection (20 µM chloramphenicol or 1 µM pyrimethamine), and cloned by limiting dilution as previously described [61].

Parasite assays

The *Toxoplasma* doubling assay was performed by counting individual tachyzoites in at least 50 randomly chosen parasite vacuoles as previously described [62]. Parasite proliferation was also evaluated using the PCR-based B1 assay [42]. For each treatment, 1000 parasites were allowed to infect an HFF monolayer for designated time course. Genomic DNA from these infected cultures was purified using DNeasy® Blood & Tissue Kit (Qiagen) and used as template in quantitative real-time PCR to amplify the *Toxoplasma* B1 gene. Parasite motility, adhesion/invasion to host cells, and egress assays were performed as outlined in [63].

Generation of transgenic parasite lines

Ectopic expression of inducible forms of TgGCN5-B. We constructed vectors that would express recombinant versions of TgGCN5-B fused to a destabilization domain (dd) and HA epitope tag at the N-terminal end (ddHA). Total RNA was purified from purified parasites using RNeasy® Plus Mini Kit (Qiagen) then made into cDNA with random primers using Omniscript® RT Kit (Qiagen). The TgGCN5-B coding sequence was amplified from cDNA, engineering an HA epitope (italics) and NsiI restriction site (underscored) at 5' end and an AvrII restriction site at 3' end (sense primer: 5'-ATGCAT*TACCCGTACGACGTCCCGGACTACGCG*GCGCCTTCAGAGTGTCCCAGCG ACGCG; anti-sense primer: 5'-CCTAGGCTAGAAAAATGTCGGATGCTTCGCGC CCACAAGCCCCTCGTCTCC). The dd fragment was amplified from plasmid pLIC.2×HA-DD::DHFR construct (kindly provided by Dr. Michael White [64]), engineering a NdeI restriction site (underscored) at the 5' end and NsiI and AvrII restriction sites (underscored) at the 3' end (sense primer: 5'-CATATGAAAATGGCGGG

AGTGCAGGTGGAAACCATCTCC; anti-sense primer: 3'-CCTAGGATCGATATGCAT TTCCGGTTTTAGAAGCTCCACATCGAAGACGAGAGTGGC). The amplified dd fragment was cloned into a *Toxoplasma* ptubXFLAG::CAT expression vector, which contains a tubulin promoter and CAT minigene for chloramphenicol selection, using the NdeI and AvrII restriction sites [58], followed by insertion of the HA-TgGCN5-B coding sequence at the NsiI and AvrII sites. The QuikChange™ Site-Directed Mutagenesis Kit (Agilent Technologies) was used to create a point mutation (E703G) in the TgGCN5-B coding region in this construct (sense primer: 5'-CAGCAGAAATTCGCCGGCATCGCTT TCCTCGCG;anti-sense primer: 5'-CGCGAGGAAAGCGATGCCGGCGAATTTCTGCTG). Plasmids were linearized using NotI prior to transfection into parasites by electroporation.

Endogenous tagging of AP2 factors. Genomic DNA from the 3' end of AP2X-8 (TGGT1_125480), AP2IX-7 (TGGT1_032510), or AP2X-5 (TGGT 1_068820) was amplified from parental strain RHΔ*Ku80* and cloned into the pLIC.HA3.DHFR vector (kindly provided by Michael White) at the PacI site using methods described by Huynh and Carruthers, 2009 [65]. Primers for the genomic fragments were as follows. AP2IX-7 (sense primer 5'-TACTTCCAATCCAATTTAATGCCGGCGGCATGAGCTTCAGTT; antisense 5'-TCCTCCACTTCCAATTTTAGCAAAGTCTTCGTCAACAACGAACTTGC). AP2X-5 (sense primer 5'-TACTTCCAATCCAATTTAATGCAGCTACACGACAGCGAC GGA; antisense 5'-TCCTCCACTTCCAATTTTAGCGGGCCAAAACGAGGAAGCGAG). AP2X-8 (sense 5'-TACTTCCAATCCAATTTAATGCAGCGCAGAAGCTGCAGAACC; antisense 5'-TCCTCCACTTCCAATTTTAGCTCCCCCCGCGCCTCTCAC). Fifty µg plasmid DNA of AP2IX-7_{HA}, AP2X-5_{HA}, and AP2X-8_{HA} were linearized by *Nco*I, *Bpu10*I, or *Nsi*I, respectively, and then electroporated into RHΔ*Ku80* parasites. Following three passages under 1.0 µM pyrimethamine selection, parasites were cloned by limiting dilution.

Immunofluorescence assays

Immunofluorescence assays (IFA) were performed as previously described [58]. Briefly, HFF monolayers grown on coverslips were inoculated with the designated parasite line, sometimes containing Shield-1 or EtOH vehicle. After removal of culture medium, infected HFFs were fixed in 3% paraformaldehyde for 10 min and then were permeabilized with 0.3% Triton X-100 for 10 min. For visualization of HA-fusion proteins, rat monoclonal anti-HA primary antibody (Roche #11867423001) was applied at 1:2000 followed by goat anti-rat Alexa Fluor 488 secondary antibody at 1:2000 (Invitrogen #A-11006). Nuclei were co-stained with 4',6-diamidino-2-phenylindole (DAPI). Samples were visualized using a Leica DMLB fluorescent microscope.

Western blotting

Analysis of Shield-based regulation of ddTgGCN5-B expressing parasites was performed by Western blot after resolving 50 µg parasite lysate on a 4-12% Tris-acetate polyacrylamide gradient gel (Invitrogen) and probing with 1:2000 rat anti-HA monoclonal antibody as the primary antibody (Roche #11867423001). Analysis of histones and tubulin used the following primary antibodies: rabbit polyclonal anti-H3 antibody (Abcam #ab1791, 1:2000), rabbit polyclonal antibodies against acetyl K9 (Millipore #06-942, 1:2000), acetyl K14 (Millipore #06-911, 1:2000), acetyl H3K18 (Abcam #ab1191, 1:2000) and rabbit polyclonal antibody against *Toxoplasma* β-tubulin (kindly provided by Dr. David Sibley, 1:5000). Anti-rat or anti-rabbit antibodies conjugated with horseradish peroxidase (GE Healthcare) were used as secondary antibodies at 1:5000. The blots were visualized using Chemiluminescence Western Blot Substrate (Pierce).

Purification of TgGCN5-B complex

The TgGCN5-B complex was purified from RH strain parasites stably transfected to ectopically express an Nt HAmyc-tagged form of full-length TgGCN5-B driven by the *Toxoplasma* tubulin (*TUB1*) promoter [58]. Large-scale tachyzoite cultures were grown in monolayers of HFF cells at 37°C for 42 hours post-infection. Prior to egress, culture medium was removed and the cell monolayers were washed once with PBS, scraped into cold PBS and then collected by centrifugation at 4°C for 10 min at 700*xg*. The cell pellets were resuspended in 25 ml cold PBS, and sequentially passed through 20/23/25-guage needles in a 30 ml syringe to release intracellular parasites from the host cells. To prepare parasite nuclear extracts, 3×10^9 parasites were incubated 5 min on ice in lysis buffer A (0.1% [v/v] NP-40, 10 mM HEPES pH 7.4, 10 mM KCl, 10% [v/v] glycerol, 20 mM sodium butyrate, plus protease inhibitors), and the nuclei were pelleted by centrifugation 6,000*xg* for 8 min at 4ºC. The parasite nuclei were then incubated 30 min at 4°C in lysis buffer B (0.1% [v/v] NP-40, 10 mM HEPES pH 7.4, 400 mM KCl, 10% [v/v] glycerol, 20 mM sodium butyrate, plus protease inhibitors) with rotation, and subjected to five freeze-thaw cycles followed by vortexing for 1 min at 4ºC before freezing. The nuclear extracts were clarified by centrifugation at 12,000*xg* for 30 min at 4ºC. The mixture of the clarified nuclear extracts (1 part) with lysis buffer A (2 parts) was used for co-immunoprecipitation. Nuclear extracts were incubated with mouse monoclonal anti-HA-tag magnetic beads (µMACS Anti-HA Microbeads; Miltenyi Biotec) overnight at 4ºC with rotation. After the beads were washed 4 times with cold wash buffer 1 (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) and once with wash buffer 2 (20 mM Tris-HCl pH 7.5) by using µ Column (pre-washed with buffer containing 0.1% [v/v] NP-40, 10 mM HEPES pH 7.4, 150 mM KCl, plus protease inhibitors) in the magnetic field of the separator, the bound proteins were eluted from the

magnetic beads by applying 50 µl of Laemmli's sample buffer (pre-heated to 95ºC) to the column. Eluted proteins were separated by SDS-PAGE (Any kD™ precast polyacrylamide gel; Bio-Rad), and stained with Coomassie blue (GelCode Blue Stain Reagent; Pierce). The entire length of each sample lane was systematically cut into 24 slices and the gel slices were maintained in MilliQ water until trypsin digestion.

Liquid chromatography coupled to mass spectrometry (LC/MS-MS)

Proteins from a Coomassie-stained gel were reduced then alkylated with TCEP and iodoacetamide prior to digestion with trypsin. Trypsin (Sequencing grade, Promega) digestion was carried out for 1 hour at 50ºC using 10 ng/µl solution in 25 mM ammonium bicarbonate/0.1%ProteaseMax (Promega). The resulting digest was then diluted with 2%Acetonitrile/2%TFA prior to LC-MS/MS analysis. Nanospray LC-MS/MS was performed on a LTQ linear ion trap mass spectrometer (LTQ, Thermo, San Jose, CA) interfaced with a Rapid Separation LC3000 system (Dionex Corporation, Sunnyvale, CA). Thirty-five µl of the sample was loaded on an Acclaim PepMap C18 Nanotrap column (5 µm, 100Å,/ 100 µm i.d. x 2cm) from the autosampler with a 50 µl sample loop with the loading buffer (2% Acetonitrile/water + 0.1% trifluoroacetic acid) at a flow rate of 8 µl/min. After 15 minutes, the trap column was switched in line with the Acclaim PepMap RSLC C18 column (2 µm, 100 Å, 75 µm i.d. x 25 cm) (Dionex Corp). The peptides are eluted with gradient separation using mobile phase A (2% Acetonitrile/water +0.1% formic acid) and mobile phase B (80% acetonitrile/water + 0.1 % formic acid). Solvent B was increased from 2 to 35% over 40 min, increased to 90% over a 5-min period and held at 90% for 10 min at a flow rate of 300 nL/min. The 10 most intense ions with charge state from +2 to +4 determined from an initial survey scan from 300-1600 m/z, were selected for fragmentation (MS/MS). MS/MS was performed using an isolation

width of 2 m/z; normalized collision energy of 35%; activation time of 30ms and a minimum signal intensity of 10000 counts. The dynamic exclusion option is enabled. Once a certain ion is selected once for MS/MS in 7 sec, this ion is excluded from being selected again for a period of 15 sec.

Mgf files were created from the raw LTQ mass spectrometer LC-MS/MS data using Proteome Discoverer 1.2 (ThermoScientific). The created mgf files were used to search the Toxo_Human Combined database using the in-house Mascot Protein Search engine (Matrix Science) with the following parameters: trypsin 2 missed cleavages; fixed modification of carbamidomethylation (Cys); variable modifications of deamidation (Asn and Gln), pyro-glu (Glu and Gln) and oxidation (Met); monoisotopic masses; peptide mass tolerance of 2 Da; product ion mass tolerance of 0.6 Da. The final list of identified proteins was generated by Scaffold 3.5.1 (Proteome Software) with following filters: 99% minimum protein probability, minimum number peptides of 2 and 95% peptide probability.

Immunoprecipitation of TgGCN5-B complexes from parasites containing HA-tagged AP2 factors

Parasites from AP2IX-7_{HA}, AP2X-5_{HA}, AP2X-8_{HA}, and parental RHΔ*ku80* lines were harvested in lysis buffer (150mM NaCl, 50mM Tris-Cl pH7.4, 0.1% NP-40) with 1x protein inhibitor cocktail (Sigma) and 1mM PMSF. The lysates were then sonicated and centrifuged to remove the insoluble fraction. Immunoprecipitations were performed using anti-HA high affinity matrix (Roche) and 300 µg total parasite protein. After overnight incubation at 4°C, the beads were washed 3x in lysis buffer and treated at 95°C for 10 minutes to elute proteins. Eluted proteins were resolved by SDS-PAGE and analyzed by

western blot with anti-HA (1:2000), anti-GCN5B (1:500) and anti-β-tubulin (1:1000) antibodies.

Quantitative reverse-transcriptase PCR (qRT-PCR)

1.0 µg of total RNA purified from intracellular parasites was transcribed into cDNA using Omniscript® reverse transcriptase with oligo-dT primers according to the manufacturer's protocol (Qiagen). qRT-PCR was performed in 25 µl volume reactions containing SYBR Green PCR Master Mix (Applied Biosystems), 0.5 mM of each forward and reverse primer (Table 2), and 1.0 µl of a 1:10 dilution of cDNA. Target genes were amplified using the 7500 Real-time PCR system and analyzed with relative quantification software (7500 software v2.0.1, Applied Biosystems). The ratio of mRNA levels in Shield-treated parasites versus EtOH-treated parasites was calculated using *Toxoplasma* β-tubulin as an internal control for normalization. Reactions were performed in triplicate and Student's t-test was applied to RT-PCR data.

Primer sequence(5'-3')	**Primer name**
AAACGCCACCTGCAGAAAAT	TGME49_247690F
TTGAGGCCGATGCCGATAT	TGME49_247690R
TTGGCACAGCGCATCAGTT	TGME49_294550F
TCGATCCACTCTGCCAAAGTC	TGME49_294550R
GGCGAACCTGACGATGATG	TGME49_295760F
CTTTCGGCGACTTGCAAAAT	TGME49_295760R
CTCTTGGCAAGCCGTACGTT	TGME49_270840F

CCCCACACCGAACAGACTCT	TGME49_270840R
TACACGTCGCACGGAAAGG	TGME49_290960F
TGGCATCGTAGGACGTTGAA	TGME49_290960R
CCCCGCCAGGCAAGAG	TGME49_253170F
GAAATGGAGGAGACAGAGCAGAA	TGME49_253170R
GCATCAGCGCGTCATCCT	TGME49_319560R
CTCGGATCCCGATTCTCCTT	TGME49_319560R
GTCGGAGCAGCCAACACAGT	TGME49_280500F
CCCCCCACCTTTAGATTCAAG	TGME49_280500R
GGACGAGGCGGAATTGAAG	TGME49_297060F
TGTGCATGCGGCTTTCTTAT	TGME49_297060R
TTTTGCTTGGGATTCGAGGAT	TGME49_289690F
TGCAGGGTAACGATCAAAAAATG	TGME49_289690R
TCGACGAAGAGACAGGAAATGA	TGME49_294200F
CATGCGCTCCTGGATAAACA	TGME49_294200R
ACCTGTCGTGTGGTGTTTCTTCT	TGME49_236210F
GAAACCACACGCGAAACTGA	TGME49_236210R

Table 2. List of primers used in the RT-PCR.

CHAPTER 3

Results

Aim 1: Determine how expression of an inactive form of TgGCN5-B affects parasite viability.

TgGCN5-B interacts with a large number of novel proteins

Toxoplasma is unusual as an invertebrate, possessing two GCN5-family KATs. TgGCN5-A was shown to be dispensable for parasite replication in normal culture conditions, but TgGCN5-B appeared to be essential [57]. To gain insight into the role of TgGCN5-B in parasite physiology, we used parasites ectopically expressing recombinant TgGCN5-B with an N-terminal HA tag to define the KAT's interactome. By virtue of the epitope tag, we performed biochemical purifications of the TgGCN5-B complex from nuclear fractions of intracellular tachyzoites. Proteins immunopurifying with TgGCN5-B are listed in Table 1. The identification of TgGCN5-B itself as well as the known interacting co-activator protein ADA2-A [57], highlights the fidelity of the purification. It has been noted before that *Toxoplasma* lacks homologues of most proteins found in the GCN5 complexes in other species [57]. In support this *in silico* finding, the majority of proteins purified with TgGCN5-B are novel interacting partners. Nearly half of the proteins comprising the TgGCN5-B complex are hypothetical, parasite-specific proteins with unknown function.

Accession #	Product Description
TGME49_043440	TgGCN5-B*
TGME49_017050	transcriptional co-activator ADA2-A*
TGME49_120300	SNF2 family N-terminal domain-containing protein
TGME49_078440	bromodomain, transcription regulatory protein SNF2, putative
TGME49_058990	bromodomain-containing protein
TGME49_076180	bromodomain-containing KAT protein (TAF1/TAF250)*
TGME49_094350	DEAD/DEAH box helicase, putative
TGME49_118440	DEAH-box RNA/DNA helicase, putative
TGME49_003220	ATP-dependent RNA helicase, putative
TGME49_011690	ATP-dependent helicase, putative
TGME49_031970	pre-mRNA splicing factor PRP8, putative*
TGME49_023390	sec63 domain-containing DEAD/DEAH box helicase, putative
TGME49_030960	splicing factor 3B subunit 3, putative
TGME49_058240	chromodomain helicase DNA binding protein, putative
TGME49_109250	AT hook motif-containing protein*
TGME49_110900	AP2XI-2
TGME49_090630	AP2IX-7*
TGME49_047700	AP2XII-4*

TGME49_014960	AP2X-8*
TGME49_013900	regulator of chromosome condensation, putative
TGME49_065840	200 kDa antigen, SMC (structural maintenance of chromosomes)
TGME49_055340	TPR domain-containing protein
TGME49_091930	nucleolar phosphoprotein nucleolin, putative*
TGME49_064660	SRS domain-containing, N-acetylglucosamine-phosphate mutase, putative
TGME49_106400	hypothetical protein, conserved*
TGME49_108890	hypothetical protein, conserved (Spt6)
TGME49_071740	hypothetical protein (PHD domain)*
TGME49_011010	hypothetical protein, SANT domain myb-like
TGME49_074180	hypothetical protein
TGME49_114410	hypothetical protein
TGME49_025910	hypothetical protein
TGME49_053750	hypothetical protein*
TGME49_029790	hypothetical protein
TGME49_105340	hypothetical protein
TGME49_080590	hypothetical protein*
TGME49_065240	hypothetical protein*
TGME49_105790	hypothetical protein

TGME49_032680	hypothetical protein*
TGME49_026660	hypothetical protein
TGME49_039300	hypothetical protein
TGME49_053370	hypothetical protein
TGME49_070930	hypothetical protein
TGME49_041850	hypothetical protein*
TGME49_029750	hypothetical protein

Table 1. Proteins associating with TgGCN5-B in *Toxoplasma* tachyzoites.

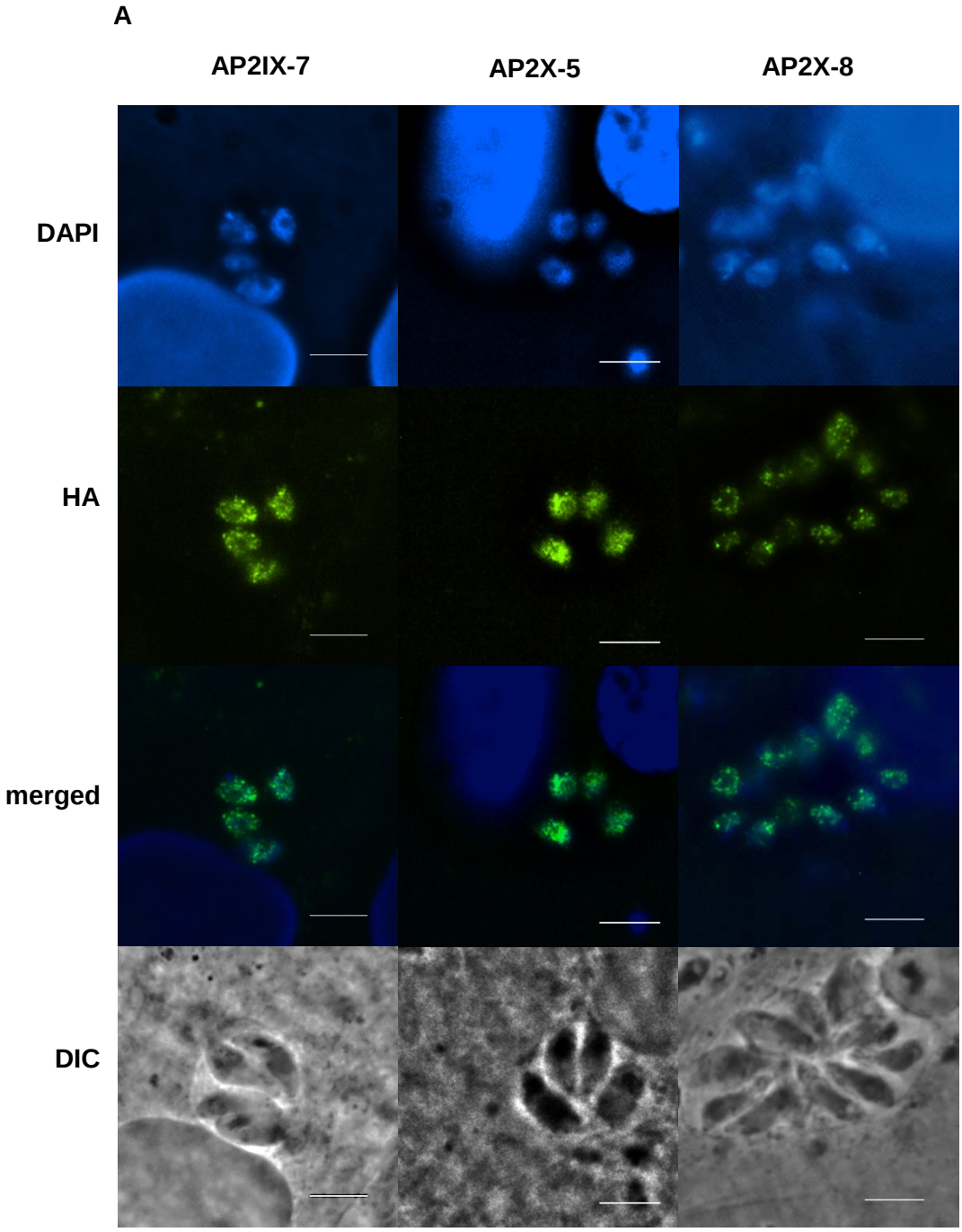
An asterisk denotes the protein was previously detected as acetylated. Shaded entries represent a core set of proteins commonly pulled down in independent purifications of TgGCN5-B, AP2IX-7, and AP2X-8.

As GCN5 KATs lack DNA-binding domains, they are believed to be recruited by DNA-bound transcription factors. Consistent with this idea, TgGCN5-B was found in association with four putative AP2 transcription factors, AP2IX-7, AP2X-8, AP2XI-2, and AP2XII-4. Other TgGCN5-B-interacting proteins with potential DNA-binding domains include an AT-hook protein (TGME49_109250) and a myb-like protein (TGME49_011010). Two additional proteins, TGME49_013900 and TGME49_065840, have predicted roles in organizing and segregating chromosomes. GCN5 activities are typically coordinated with those of SWI/SNF complexes, and we detected two independent SWI/SNF ATPases associated with the TgGCN5-B complex (TGME49_120300 and TGME49_078440). A surprising number of ATP-dependent helicases and other components associated with pre-mRNA splicing were also found in association with TgGCN5-B (Table 1).

Four proteins, including TgGCN5-B itself, contain bromodomains, which recognize acetylated lysine residues [66]. Another bromodomain protein is TAF1/TAF250, a component of the TFIID complex that also possesses KAT activity [67]. The detection of TAF1/TAF250 is consistent with GCN5 complexes interacting with the transcriptional pre-initiation complex [68]. Sixteen of the proteins pulled down with TgGCN5-B were detected as acetylated in a previous study (denoted with asterisk in Table 1), including TgGCN5-B itself, the ADA2-A co-activating protein, TAF1/TAF250, and most of the AP2 factors. These proteins are likely to be acetylated by TgGCN5-B or TAF1/TAF250. The precise role of these acetylated lysines remains to be determined; however, the presence of four bromodomain modules in the complex supports the idea that they participate in intracomplex protein-protein interactions [69].

The finding that AP2 factors work in conjunction with KAT complexes to alter transcription is of particular relevance. Transcription factors such as GCN4 in yeast are known to recruit GCN5 [70], but it has not been established, even in plants, that AP2 factors can do the same. To validate the interactions between TgGCN5-B and AP2 factors, we endogenously tagged AP2IX-7 and AP2X-8 with a C-terminal 3xHA tag. Reciprocal co-immunoprecipitation of each AP2 factor pulled down TgGCN5-B and many of the other proteins in the TgGCN5-B complex. Proteins that were pulled down consistently in all three purifications are shaded in Table 1. To further confirm the interaction and specificity between TgGCN5-B and these AP2 factors, we performed Western blots for TgGCN5-B in AP2IX-7_{HA} and AP2X-8_{HA} immunoprecipitates. We also endogenously tagged AP2X-5, an AP2 factor that was not seen in the TgGCN5-B complex, to serve as a control. As shown in Figure. 3(A) and (B) top left panel, the three AP2s are properly expressed. Figure. 3(B) top right panel shows that the IPs were successful. Consistent with the reciprocal IP-MS analysis, TgGCN5-B was detected in a Western blot of HA-immunoprecipitated AP2IX-7_{HA} and AP2X-8_{HA}, but not AP2X-5_{HA}.

Collectively, these results demonstrate specific interactions between TgGCN5-B and AP2IX-7 and AP2X-8.



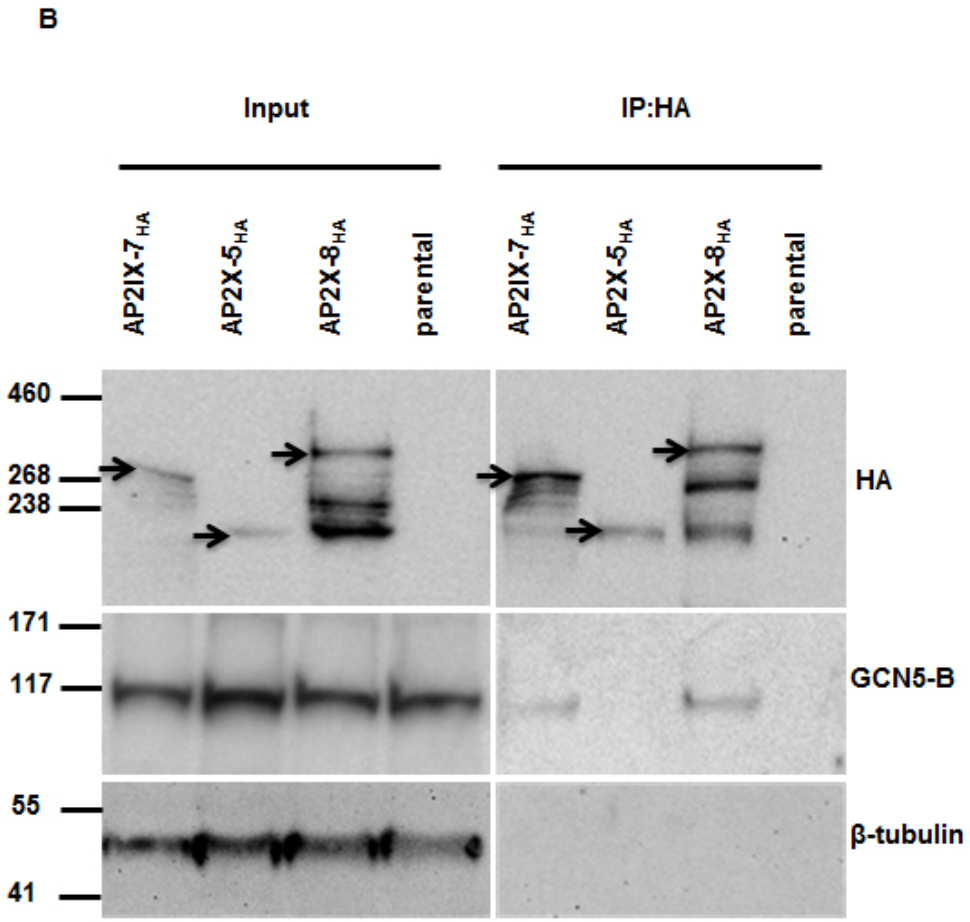


Figure 3. Independent confirmation of the in vivo interaction of TgGCN5-B with endogenously HA-tagged AP2IX-7 and AP2X-8.

(A) AP2IX-7_{HA}, AP2X-8_{HA}, and AP2X-5_{HA} parasites were subjected to immunofluorescence assays using an anti-HA antibody (Green), and the DNAs were stained by DAPI (blue). Scale bar = 2 µm. (B) Immunoprecipitations using an anti-HA antibody were performed on parasite lysates made from AP2IX-7_{HA}, AP2X-8_{HA}, and AP2X-5_{HA} parasites, as well as the parental RHΔ*ku80* line. The immunoprecipitated complexes were analyzed by Western blot using antibodies recognizing HA-tag, TgGCN5-B, or β-tubulin. Arrowheads mark the expected bands with predicted molecular

weights (in kDa) as follows: AP2IX-7: 268, AP2X-5: 220, AP2X-8: 399, GCN5-B: 110, and β-tubulin: 51.

Genome-wide localization of TgGCN5-B

We also used parasites expressing the HA-tagged TgGCN5-B to perform a genome-wide ChIP-chip analysis. Immunoprecipitated DNA associated with TgGCN5-B was identified following hybridization to custom Nimblegen microarrays that tile the entire *Toxoplasma* genome. TgGCN5-B was detected at ~14% of tachyzoite genes, nearly half of which are annotated as hypothetical genes of unknown function in the ToxoDB (Figure. 4). Most of the genes coinciding with TgGCN5-B localization are associated with signaling and gene expression (18%), followed closely by genes involved in translation and protein processing (15%). TgGCN5-B was also found at a moderate number of metabolic genes (11%).

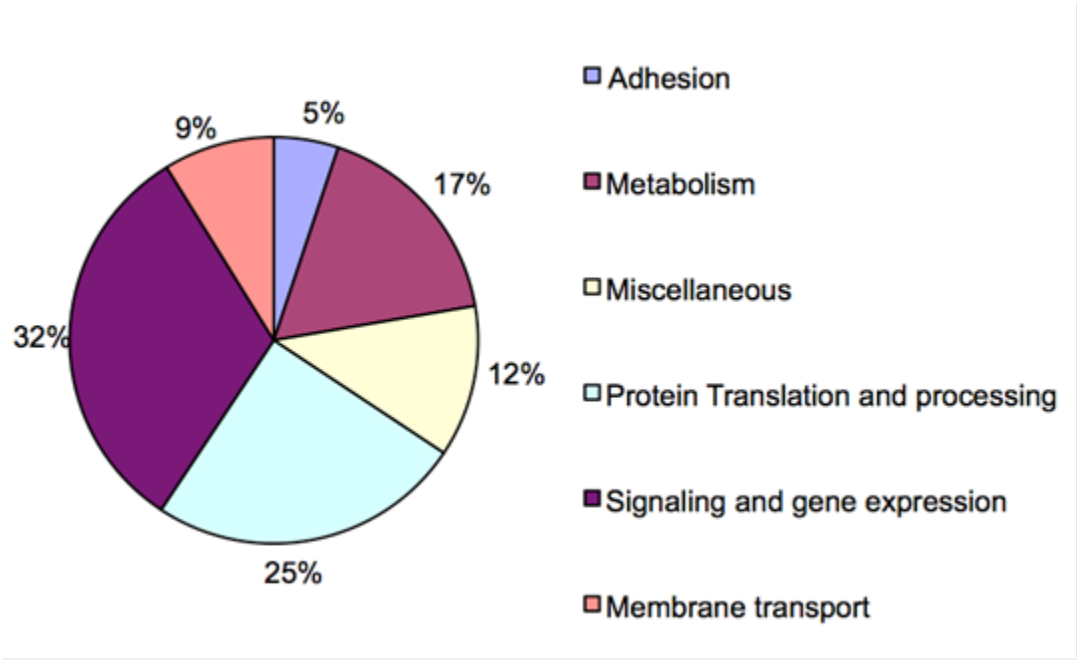


Figure 4. Genome-wide localization of TgGCN5-B.

TgGCN5-B was observed within the coding sequence of ~700 genes and the promoter region of ~30 genes. The functional breakdown of TgGCN5-B-associated genes displays a wide range of biological functions (241 hypothetical genes are not shown).

TgGCN5-B was detected in gene bodies as well as promoter regions, consistent with recent studies showing a role for GCN5 in transcriptional elongation by promoting nucleosome eviction [71]. Overall, the ChIP-chip results establish that TgGCN5-B is present within or near the loci of at least 14% of tachyzoite genes involved in a wide variety of cellular functions.

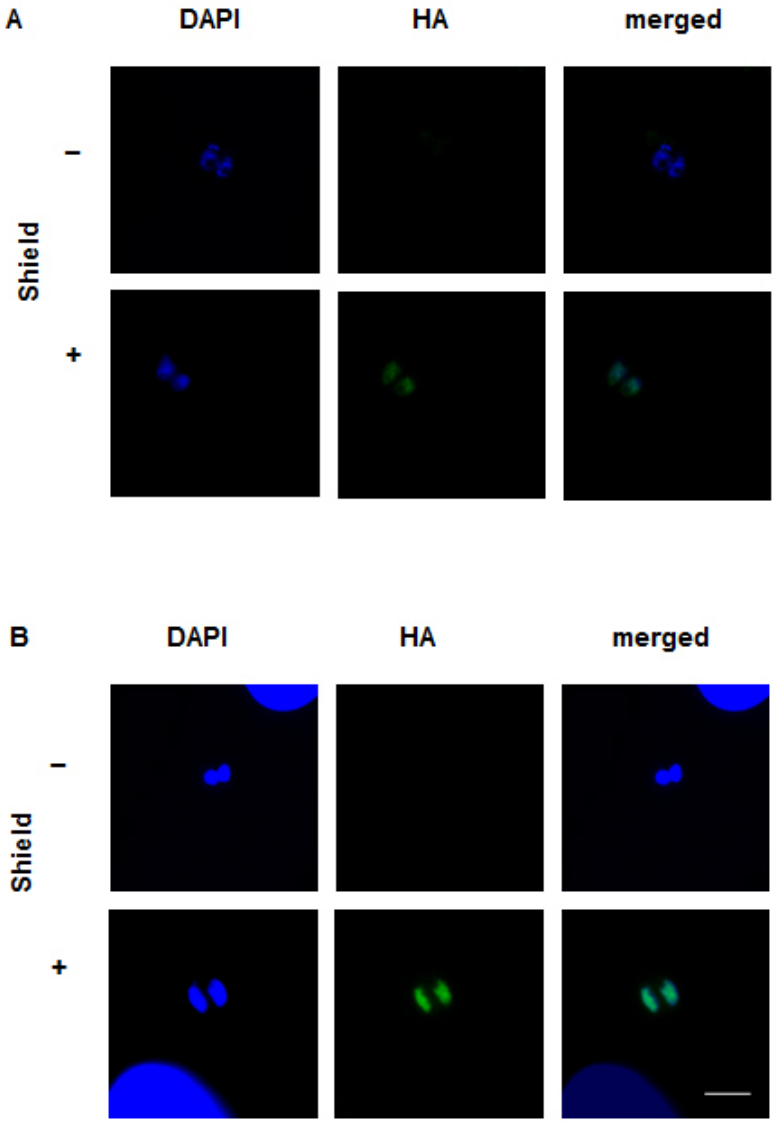
Expression of catalytically inactive TgGCN5-B arrests replication

In order to better define the role of TgGCN5-B in *Toxoplasma*, we attempted to generate a gene knockout. Repeated attempts to disrupt or replace TgGCN5-B using homologous recombination in haploid type I RH strain tachyzoites have not been successful, in contrast TgGCN5-A could be knocked out using the same approach. More recent attempts to knockout the TgGCN5-B locus in a Δ*ku80* background also failed to generate viable parasites. Considered together, these results suggest that TgGCN5-B may be essential in tachyzoites.

To address this hypothesis, we pursued an inducible dominant-negative strategy. As TgGCN5-B appears to work in a multi-subunit complex, this protein is a good candidate for a dominant-negative strategy whereby an enzymatically dead version would sequester essential interacting proteins from endogenous TgGCN5-B. Previous graduate student Stacy Dixon generated clonal parasites in an RH background expressing a catalytically inactive form of TgGCN5-B (mutated glutamic acid 703 to glycine, E703G [72] fused to a destabilization domain and HA tag (ddHA) at the N-terminus. As a control, we also generated a clone expressing wild-type (WT) TgGCN5-B in the same fashion. Expression of the $_{ddHA}$GCN5-B fusion proteins was driven by the *Toxoplasma* tubulin promoter. *In vitro* KAT assays using purified $_{ddHA}$GCN5-B proteins confirm the E703G mutation ablates enzymatic activity (data not shown).

The destabilization domain directs its fusion partner to the proteasome for rapid degradation, but this can be averted by adding Shield ligand to the culture medium. Fusion of ddHA to the N-terminus of TgGCN5-B or TgGCN5-B(E703G) allowed their ectopic expression to be regulated via Shield, as assessed in IFAs and immunoblots using anti-HA (Figure. 5). Addition of ddHA did not disrupt the expected nuclear localization of WT or mutant TgGCN5-B. No difference was observed between parental

wild-type parasites and those ectopically expressing $_{ddHA}$GCN5-B protein at any concentration of Shield (Figure. 6A). In contrast, parasites induced to express $_{ddHA}$GCN5-B(E703G) underwent rapid growth arrest in 48 hours with only 10 nM Shield (Figure. 6B). Similar results were obtained when we used a PCR-based assay (5) to measure parasite replication (data not shown).



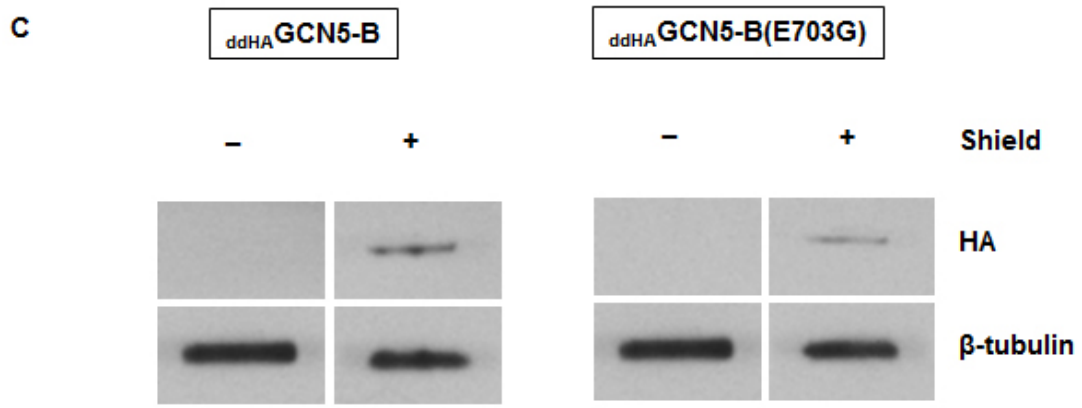


Figure 5. Inducible expression of ectopic $_{ddHA}$GCN5-B and $_{ddHA}$GCN5-B(E703G) in *Toxoplasma*.

Immunofluorescence assays using an anti-HA antibody on (A) $_{ddHA}$GCN5-B and (B) $_{ddHA}$GCN5-B(E703G) parasites, cultured in the presence or absence of 500nM Shield. Anti-HA signal is shown in green and nucleic acid staining with DAPI is depicted in blue. Scale bar = 2 µm. C. Western blots of $_{ddHA}$GCN5-B (left) and $_{ddHA}$GCN5-B(E703G) (right) parasite lysates using anti-HA antibody shows stabilization of the respective dd-fusion protein after 48 hr incubation with 500nM Shield.

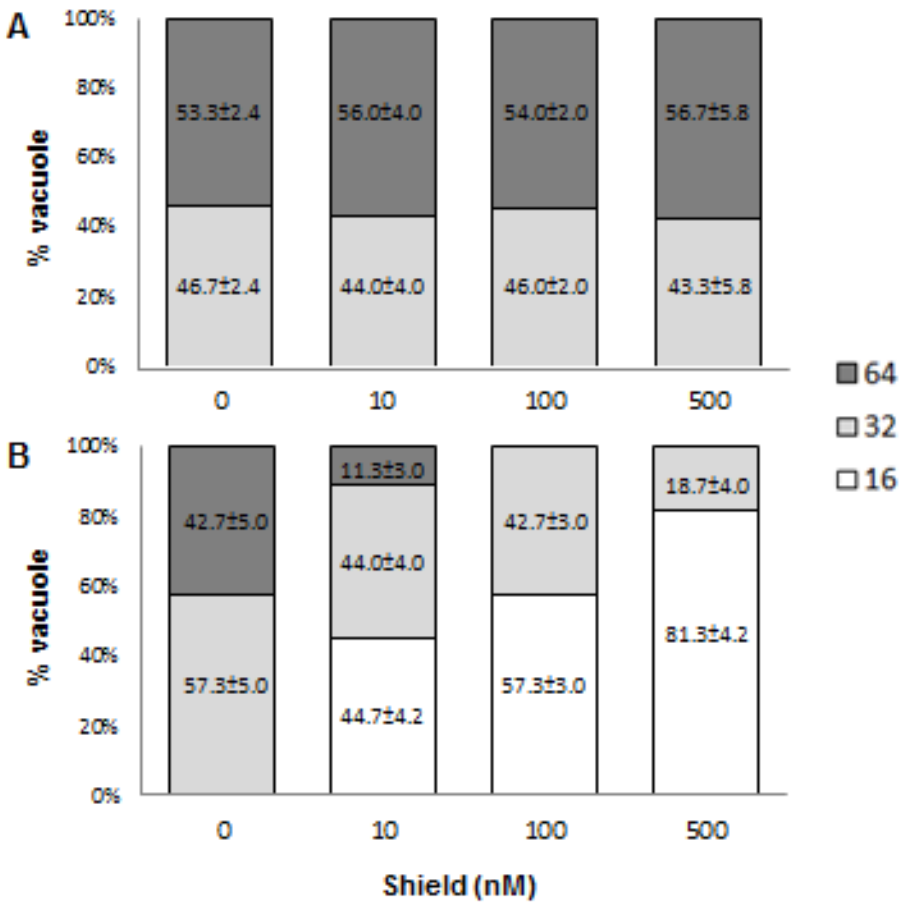


Figure 6. Induced expression of $_{ddHA}$GCN5-B(E703G) arrests parasite replication.

Doubling assays were performed to assess growth of (A) $_{ddHA}$GCN5-B and (B) $_{ddHA}$GCN5-B(E703G) parasites. Intracellular parasites were physically released from host cells and an equal number of parasites were allowed to infect fresh HFF monolayers. The infected cultures were subjected to the indicated Shield concentrations for 48 hr. Parasite proliferation was monitored by quantifying the numbers of parasites in 50 random vacuoles.

Reduced histone acetylation in TgGCN5-B DN parasites

We considered that the replication arrest observed in $_{ddHA}$GCN5-B(E703G) parasites may be due to a reduction in histone acetylation, which in turn would lead to dysregulation of the transcriptome. To assess this possibility, we analyzed the acetylation level of individual lysine residues in histone H3, the preferred substrate of GCN5 family KATs, purified from Shield- versus vehicle-treated $_{ddHA}$GCN5-B or $_{ddHA}$GCN5-B(E703G) expressing parasites. Acetylation levels of lysines K9 and K14 were decreased in only the parasites expressing $_{ddHA}$GCN5-B(E703G) protein (Figure. 7). The decreased acetylation was not an artifact of decreased H3 expression, and other acetylated proteins like tubulin, were unaffected by the induction of the $_{ddHA}$GCN5-B(E703G). Interestingly, the acetylation status of H3K18 was not affected, which may be explained by the fact that TgGCN5-A, which would not be attenuated by the expression of $_{ddHA}$GCN5-B(E703G), has an exquisite affinity for this particular lysine residue on H3 (20). These data indicate that the expression of $_{ddHA}$GCN5-B(E703G) protein diminishes acetylation on histone H3.

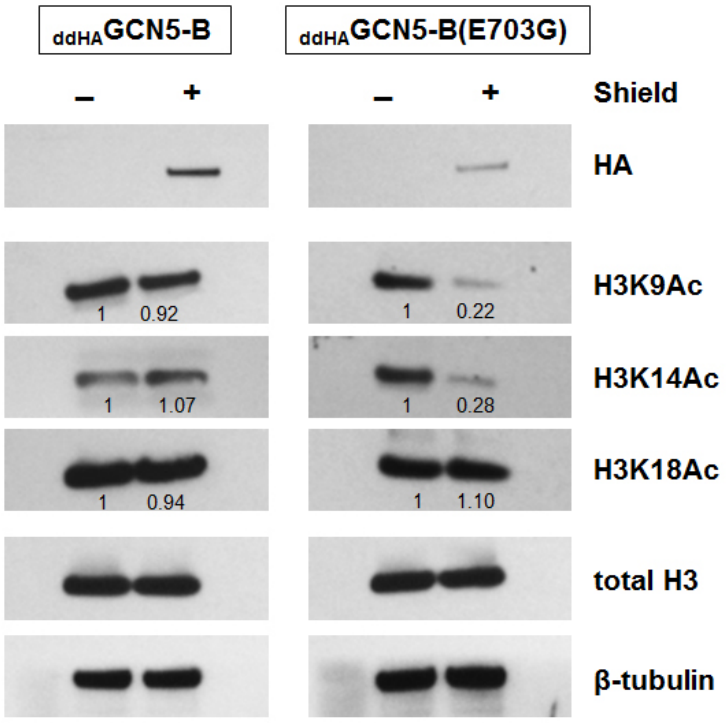


Figure 7. Reduced histone H3 acetylation in $_{ddHA}$GCN5-B(E703G) parasites.

$_{ddHA}$GCN5-B parasites and $_{ddHA}$GCN5-B(E703G) parasites were cultured in the presence of 500 nM Shield for 48 hr. Equivalent amounts of parasite lysate obtained from Shield- vs. vehicle-treated parasites was analyzed by Western blotting using antibodies recognizing various acetylated lysine residues of histone H3 (acetylated lysine 9, 14, and 18). Anti-HA was used to monitor protein stabilization. Antibodies to total H3 show that induced stabilization of each GCN5-B protein did not alter overall H3 protein levels. Western analysis of β-tubulin levels served as an additional loading control. Densitometry was used to compare relative levels of each histone acetylation mark between the Shield- and vehicle-treated parasites. Data is normalized to β-tubulin.

Expression of TgGCN5-B associated genes in $_{ddHA}$GCN5-B(E703G) parasites

Acetylation of H3K9 and K14 is required for recruitment of TFIID[73], therefore reduced histone acetylation levels should lead to inactivation of gene expression. Attempts were made to perform microarray analysis of $_{ddHA}$GCN5-B and $_{ddHA}$GCN5-B(E703G) under Shield versus vehicle conditions, but it proved challenging to obtain sufficient parasite RNA from the arrested $_{ddHA}$GCN5-B(E703G) parasites. We then attempted a more targeted approach to analyze genes that were strongly associated with TgGCN5-B in the ChIP-chip dataset. Primers were designed to amplify selected mRNAs in $_{ddHA}$GCN5-B or $_{ddHA}$GCN5-B(E703G) parasites in the presence or absence of Shield (Table 2). Another set of mRNAs from genes that did not associate with TgGCN5-B was included as a control. Virtually no changes in mRNA levels were detected in $_{ddHA}$GCN5-B parasites regardless of whether TgGCN5-B was detected at the gene locus (Table 2). However, expression levels of mRNAs in $_{ddHA}$GCN5-B(E703G) parasites were clearly altered. Four out of 6 genes associated with TgGCN5-B had lowered mRNA levels in the $_{ddHA}$GCN5-B(E703G) parasites following Shield treatment (Table 2). Two other genes tested showed increased mRNA levels. Genes that were not associated with TgGCN5-B generally exhibited no significant difference in mRNA levels in the $_{ddHA}$GCN5-B(E703G) parasites following Shield treatment, however one gene tested showed higher mRNA levels and another showed lower mRNA levels (Table 3). While there are exceptions, the results show a general trend that is consistent with the idea that the decreased acetylation observed in $_{ddHA}$GCN5-B(E703G) parasites leads to decreased transcription of TgGCN5-B-associated genes. The dysregulation of gene expression induced by the accumulation of $_{ddHA}$GCN5-B(E703G) protein may contribute to the arrest in parasite replication.

Gene ID	Product Description	ddHAGCN5-B	ddHAGCN5-B(E703G)
GCN5-B localizes on coding region			
TGME49_247690	phospholipid-translocating P-type ATPase	1.2±0.2	1.4±0.1
TGME49_294550	dynein heavy chain	1.1±0.2	0.4±0.1
TGME49_295760	calcium-dependent protein kinase CDPK4A	0.9±0.3	0.5±0.0
TGME49_270840	poly(ADP-ribose) polymerase catalytic domain-containing protein	1.0±0.2	0.6±0.0
TGME49_290960	pyruvate phosphate dikinase	0.8±0.1	1.3±0.1
TGME49_253170	zinc carboxypeptidase, putative	1.1±0.1	0.3±0.1
GCN5-B does not localize on gene locus			
TGME49_319560	microneme protein MIC3	1.1±0.2	1.3±0.3
TGME49_280500	inorganic anion transporter	0.9±0.2	0.5±0.3
TGME49_297060	phosphoglycerate mutase PGMII	1.0±0.3	1.4±0.4
TGME49_289690	glyceraldehyde-3-phosphate dehydrogenase GAPDH1	1.0±0.3	0.8±0.3
TGME49_294200	glucose-6-phosphate 1-dehydrogenase	1.2±0.3	1.3±0.5
TGME49_236210	mitochondrial processing peptidase	1.3±0.3	2.3±0.4

Table 3. Expression levels of select mRNAs in ddHAGCN5-B and ddHAGCN5-B(E703G) parasites.

Values represent fold-change of mRNA levels in parasites treated with Shield for 48 hours relative to vehicle control. The fold change normalized to tubulin and the expression level of each gene in EtOH control was set as 1. Student's t-test was performed and p values were <0.05.

Aim 2: Study if lysine acetylation regulates the function of GCN5-B.

GCN5-B is acetylated at K857 of ADA2 binding domain

To establish whether GCN5-B is acetylated *in vivo*, GCN5-B acetylation status in Shield-treated $_{ddHA}$GCN5-B or $_{ddHA}$GCN5-B(E703G) expressing parasites was analyzed by Western blotting using antibody anti-acetyl-Lys. $_{ddHA}$GCN5-B or $_{ddHA}$GCN5-B(E703G) was immunoprecipitated from parasites using specific anti-HA high affinity matrix and probed by Western blotting. $_{ddHA}$GCN5-B is recognized by the anti-acetyl-Lys antibody, suggesting that TgGCN5-B is acetylated *in vivo* (Figure. 8). However, $_{ddHA}$GCN5-B(E703G) is not recognized by the anti-acetyl-Lys antibody, indicating that TgGCN5-B may be subject to autoacetylation. It is possible, however, that endogenous TgGCN5-B in the $_{ddHA}$GCN5-B(E703G) expressing parasites may acetylate $_{ddHA}$GCN5-B(E703G). Acetylome data from our lab reveals four lysine acetylation sites on TgGCN5-B (Figure. 9) [16]. These four acetylation sites are unique to *Toxoplasma*. Interestingly, one of the acetylation sites, K857, is within the putative ADA2 binding domain.

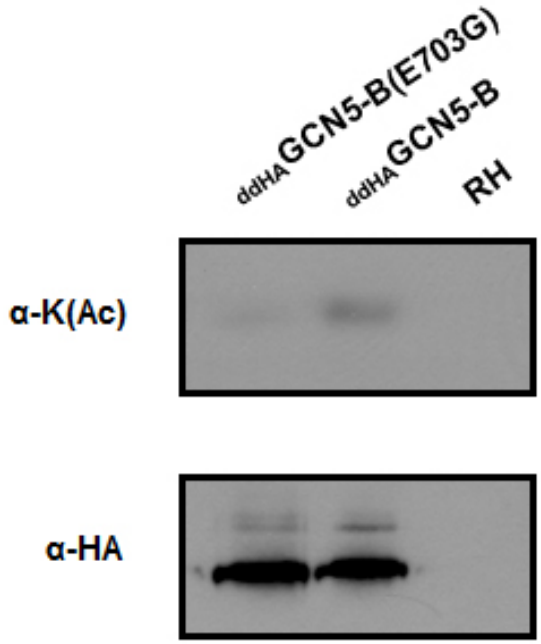


Figure 8. GCN5-B is acetylated in vivo and the catalytic inactive version of protein is barely acetylated.

$_{ddHA}$GCN5-B parasites and $_{ddHA}$GCN5-B(E703G) parasites were cultured in the presence of 500 nM Shield for 48 hr. Equivalent amounts of parasite lysate obtained from Shield- vs. vehicle-treated parasites was used for immunoprecipitation with anti-HA high affinity matrix. Protein from parental wild-type RH parasites was included as a negative control. The acetylation level of the products was analyzed by Western blotting using antibody that recognizes acetylated lysines (α-K(Ac)). The lower panel shows that the same amount of immunoprecipitated proteins were used in the immunoblots.

Figure 9. Acetylation sites on TgGCN5-B.

The acetylated lysine sites identified in our *Toxoplasma* acetylome study are marked in yellow [16]. K857 is within the putative ADA2 binding domain of TgGCN5-B. K989, K997, K1027 at the C-terminus of TgGCN5-B are also acetylated. PCAF, a homolog of TgGCN5-B in humans, is also suggested to be acetylated at the ADA2 binding domain (highlighted green) [14].

Acetylation of K857 is required for proper TgGCN5-B complex formation

To study if acetylation on K857 is required for protein-protein interactions, we mutated K857 to arginine (K857R) and assessed the impact on the TgGCN5-B interactome. Arginine conserves the charge of lysine, but cannot be acetylated. $_{HA}$GCN5-B parasites and $_{HA}$GCN5-B(K857R) parasites, each ectopically expressing the recombinant protein, were used to purify the TgGCN5-B complex. The immunopurified proteins for each are listed in Table 4. The complex for $_{HA}$GCN5-B was analyzed in aim 1. The data shows that $_{HA}$GCN5-B(K857R) still binds with ADA2-A, as observed for $_{HA}$GCN5-B. This data indicates that K857 acetylation does not affect the TgGCN5-B association with ADA2-A, although it should be noted that the minimal ADA2-A interaction domain has not been mapped. Interestingly, the K857R mutation had a profound impact on the recruitment of other subunits to the complex. For example, $_{HA}$GCN5-B was found in association with four putative AP2 transcription factors, AP2IX-7, AP2X-8, AP2XI-2, and AP2XII-4, which are suggested to recruit TgGCN5-B to the transcriptional machinery. In contrast, $_{HA}$GCN5-B(K857R) fails to associate with the AP2 proteins. In fact, only two proteins in addition to ADA2-A continued to be pulled down with $_{HA}$GCN5-B(K857R): the AT hook protein and a PHD finger protein. These results show that acetylation at K857 plays an important role in TgGCN5-B complex formation.

Accession #	Product Description
TGME49_043440	TgGCN5-B
TGME49_017050	transcriptional co-activator ADA2-A
TGME49_109250	AT hook motif-containing protein
TGME49_024260	PHD-finger domain-containing protein

Table 4. $_{HA}$GCN5-B(K857R) complex in *Toxoplasma*.

Please refer Table 1 from Aim1 for $_{HA}$GCN5-B complex.

Acetylation of K857 is not required for nuclear localization and catalytic activity

We also determined whether acetylation of K857 could affect acetyltransferase activity or protein localization of TgGCN5-B. To test the acetyltransferase activity of TgGCN5-B, a standard *in vitro* HAT assay was performed using recombinant histone H3 as substrate. Either immune purified $_{HA}$GCN5-B or $_{HA}$GCN5B(K857R) was reacted with acetyl-CoA and histone H3 substrate. Following the incubation time, the KAT assay reaction was resolved on SDS-PAGE for immunoblotting with anti-acetyl-H3 (Figure. 10). The results show that the same amount of $_{HA}$GCN5-B and $_{HA}$GCN5B(K857R) was applied for each KAT assay and that they both acetylate H3. This data suggests K857 acetylation is not required for TgGCN5-B KAT activity. Immunofluorescence assays was used to reveal the localization of $_{HA}$GCN5-B and $_{HA}$GCN5B(K857R) (Figure. 11). The results show that $_{HA}$GCN5-B and $_{HA}$GCN5B(K857R) have the same nuclear localization. These experiments suggest that K857 acetylation does not affect nuclear localization of TgGCN5-B.

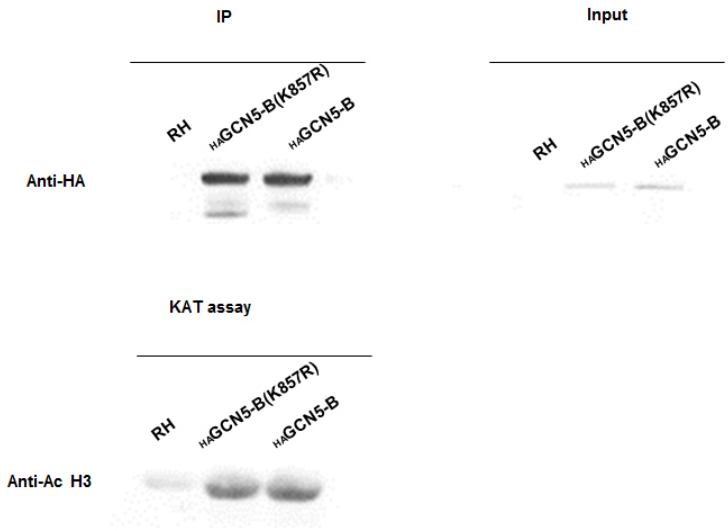


Figure 10. KAT assay of $_{HA}$GCN5-B and $_{HA}$GCN5-B(K857R).

Immunoprecipitations using an anti-HA antibody were performed on parasite lysates made from $_{HA}$GCN5-B and $_{HA}$GCN5-B(K857R) parasites. The KAT assay reaction includes either $_{HA}$GCN5-B or $_{HA}$GCN5-B(K857R), histone H3, and acetyl-CoA. An equivalent amount of parental strain RH was used as a negative control. Western blotting of KAT assays was performed with antibody to specific acetylated (Ac) H3 lysine residues.

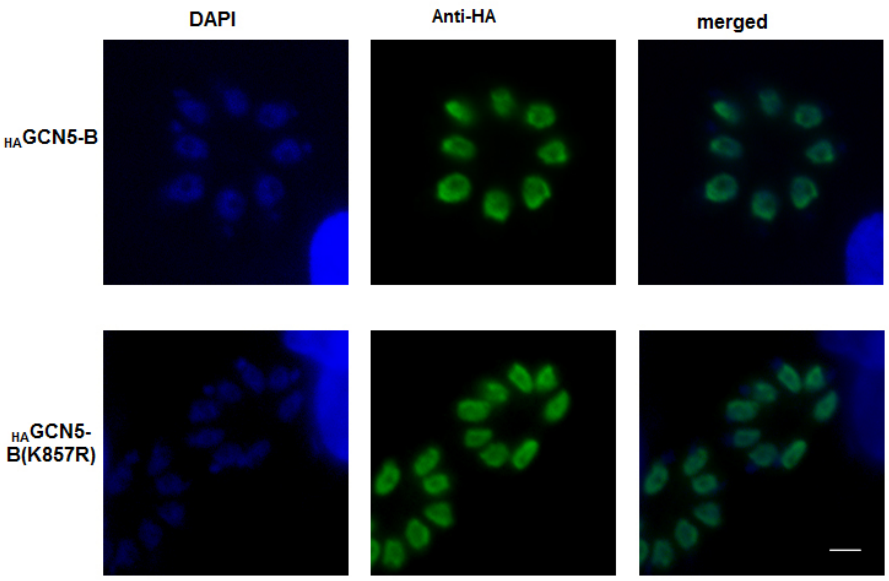


Figure 11. Nuclear localization of $_{HA}$GCN5-B and $_{HA}$GCN5-B(K857R).

Immunofluorescence assays using an anti-HA antibody on $_{HA}$GCN5-B and $_{HA}$GCN5-B(K857R) parasites. Anti-HA signal is shown in green and nucleic acid staining with DAPI is depicted in blue. scale bar = 2um.

C-truncated TgGCN5-B has altered localization

To explore if there is a functional role associated with the three acetylated lysines at C-terminus of TgGCN5-B (K989, K997, K1027) a parasite line was generated that expresses $_{HA}$GCN5-BΔ988-1033, which lacks the final 45 amino acid C-terminus fragment containing the three acetylated lysines. Immunofluorescence assays were performed to reveal the localization of $_{HA}$GCN5-BΔ988-1033, with $_{HA}$GCN5-B as control (Figure. 12). The results show an altered localization of $_{HA}$GCN5-BΔ988-1033 compared to $_{HA}$GCN5-B. $_{HA}$GCN5-B localizes clearly to the parasite nucleus. However, $_{HA}$GCN5-

BΔ988-1033 localizes in a distinct, punctate pattern that appears to extend throughout the whole parasite. Further study is needed to determine if this localization phenotype is associated with impaired function of acetylated lysines or some other element in the C-terminal tail of TgGCN5-B.

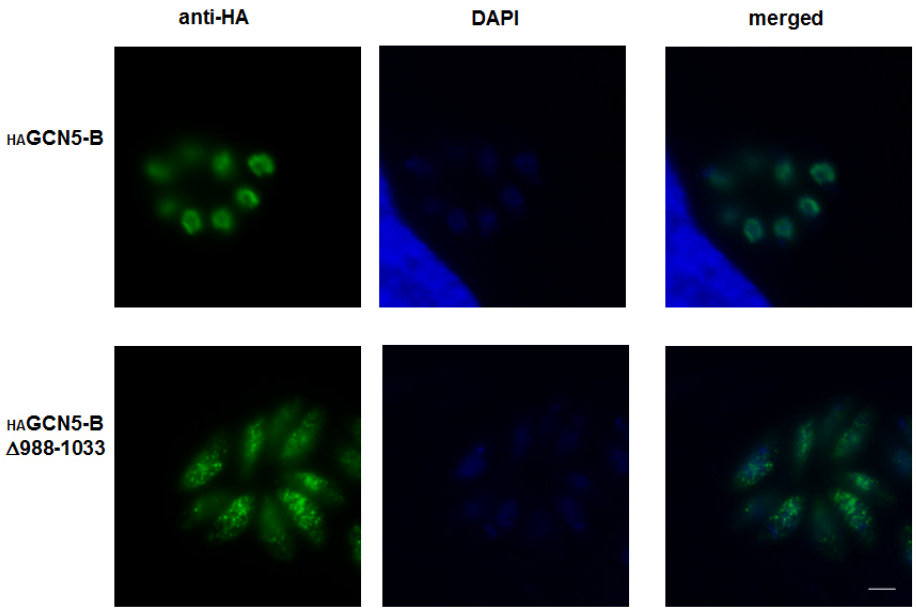


Figure 12. Altered localization of $_{HA}$GCN5-BΔ988-1033.

Immunofluorescence assays using an anti-HA antibody on $_{HA}$GCN5-B and $_{HA}$GCN5-BΔ988-1033 parasites. Anti-HA signal is shown in green and nucleic acid staining with DAPI is depicted in blue. Scale bar = 2um.

CHAPTER 4

Discussion and Future direction

Aim 1: Determine how expression of an inactive form of TgGCN5-B affects parasite viability.

The objective of this study was to gain a better understanding of the role played by the second GCN5 KAT in *Toxoplasma* parasites through biochemical purification of associating proteins, ChIP-chip analyses, and the generation of mutants. Our findings reveal that TgGCN5-B interacts with a large number of novel proteins and is enriched at genes involved in transcription, translation, and metabolism. Consistent with previous failures to knockout TgGCN5-B, inducible expression of a catalytically dead version acted like a dominant-negative mutant and displayed replicative arrest, supporting the conclusion that TgGCN5-B is essential in tachyzoites.

The TgGCN5-B interactome is remarkably unique and includes putative parasite-specific transcription factors. A high-throughput yeast two-hybrid approach previously identified the single *Plasmodium falciparum* GCN5 to be the most interconnected protein in the parasite integrating chromatin modification, transcriptional regulation, mRNA stability, and ubiquitination (23). The discovery of AP2 factors in the TgGCN5-B interactome prompted us to re-examine the PfGCN5 data, since this analysis was done prior to the identification of AP2 domains. PfGCN5 interacts with two predicted AP2 factors, PF3D7_1007700 and PF3D7_0802100. It is not possible at the present time to discern if the AP2s interacting with TgGCN5-B are orthologous. Interestingly, no other PfGCN5-interacting proteins cross-reference to the TgGCN5-B interacting proteins. This may be produced by the different techniques that were used (yeast two-hybrid using only the N-terminal extension of PfGCN5 as bait versus biochemical purification of TgGCN5-

B), or could indicate that GCN5 complexes between apicomplexan species have significantly diverged. In support of this conclusion, the lengthy N-terminal extensions between PfGCN5 and TgGCN5-B share no homology. Another possibility is that the PfGCN5 complex may be more analogous to TgGCN5-A, whose interactome has yet to be resolved.

It is well-established that histone acetylation complexes generally aggregate at gene promoters, but the considerable proportion of TgGCN5-B located within gene bodies is not without precedent. Govind et al reported that yeast GCN5 plays a role in transcriptional elongation by promoting histone eviction. Interestingly, a Spt6 homologue was purified with the TgGCN5-B complex, a protein that has been implicated in transcription elongation through binding of RNA polymerase II [74]. In other species, GCN5 has also been shown to play a role in co-transcriptional splicing [75]. The disproportionate number of pre-mRNA splicing components that we identified in the TgGCN5-B complex might be suggestive of additional roles for TgGCN5-B in splicing. It is possible that TgGCN5-B forms multiple complexes and contributes to an assortment of cell biological functions. While our data clearly shows that histone acetylation is decreased in the dominant-negative clone, we cannot rule out that the arrest in parasite replication is solely due to dysregulation of gene expression. Complicating matters is the recent observation that lysine acetylation is widespread on hundreds of non-histone proteins [69]. It is conceivable that TgGCN5-B has non-histone substrates and decreased efficiency in acetylation of those substrates contributes to the replication arrest in the dominant-negative parasites.

Toxoplasma is unique as a lower eukaryote to possess a pair of GCN5 KATs. Studies to date suggest that these two TgGCN5 KATs have non-redundant functions. TgGCN5-B was not sufficient to compensate for a lack of TgGCN5-A, which is required for adequate responses to alkaline stress [42]. Similarly, TgGCN5-A is not able to

compensate when the function of TgGCN5-B is attenuated through expression of a dominant-negative version. Our findings suggest that pharmacological inhibition of TgGCN5-B or disruption of the TgGCN5-B complex may be novel avenues for therapy against toxoplasmosis.

Aim 2: Study if lysine acetylation regulates the function of TgGCN5-B.

The objective for Aim 2 was to investigate the consequences of lysine acetylation on TgGCN5-B protein. Acetylation is an important post-translational modification that regulates biological activities of protein by affecting protein-protein interaction, enzyme activity, and protein localization. TgGCN5-B is acetylated *in vivo* and this modification occurs at four lysines on the protein, K857, K989, K997, and K1027. K857 is within the putative ADA2 binding domain, while the other three lysines are at C-terminus of TgGCN5-B. The K857 to R mutation conserves the charge of lysine but cannot be acetylated. Our results suggest that $_{HA}$GCN5-B associates with four putative AP2 transcription factors (AP2IX-7, AP2X-8, AP2XI-2, AP2XII-4) and other proteins with important roles in control of transcription (discussed in Aim 1). However, the K857 to R mutation remarkably caused TgGCN5-B to lose its ability to associate with AP2s and most other subunits in the complex. One possible scenario that explains this data is that this acetylated lysine is crucial for the binding of another protein that helps to nucleate the majority of the TgGCN5-B complex. Alternatively, acetylated K857 of TgGCN5-B might regulate complex formation through an interaction with its own bromodomain, thus resulting in a structural change that favors association of AP2s and other subunits. Interestingly, the K857R mutation does not affect the association between TgGCN5-B and its co-activator protein ADA2-A, even though K857 lies within the putative ADA2 binding domain. As the precise ADA2-binding domain has not been defined, we cannot

rule out that K857 is actually outside of the ADA2-binding domain. It is also possible that acetylation of K857 induces the disassociation of TgGCN5-B and ADA2-A, and deacetylation leads to their association. The IFA analysis showed that the effect on complex formation in the K857 mutant is not due to mislocalization of the mutant TgGCN5-B protein.

$_{HA}$GCN5-BΔ988-1033, lacking the final 45 amino acids of TgGCN5-B, has a localization phenotype. $_{HA}$GCN5-B localizes clearly to the nucleus, however, $_{HA}$GCN5-BΔ988-1033 has a more punctate and diffuse localization throughout the parasite body. This C-terminal region is lacking a cluster of three acetylated lysines, K989, K997 and K1027. To further determine of any of the acetylated lysines at C-terminus have a functional role of protein localization, each of them should be mutated and studied individually. Other elements within this 45 amino acid region may also account for the differential localization. For example, there might be a novel type of nuclear localization signal at C-terminus of TgGCN5-B or other PTMs might occur here that contribute to nuclear localization.

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